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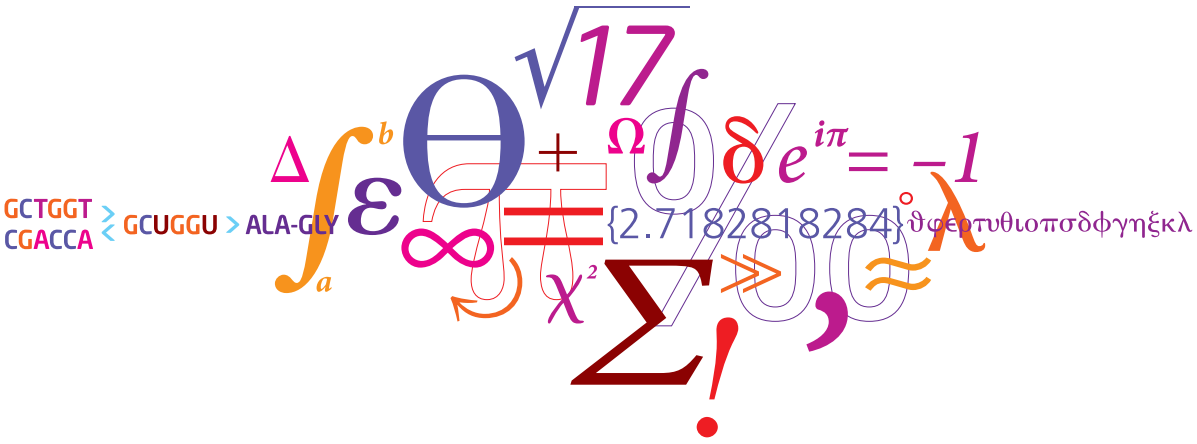

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A proteomics-based study of endogenous and microbial xylanases and xylanase inhibitors associated with barley grains used for liquid feed

Ph.D. Dissertation

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PREFACE

The present thesis represents the results of my Ph.D. study carried out at Enzyme and Protein Chemistry (EPC), Department of Systems Biology, Technical University of Denmark (DTU) for the period of September 2009 to February 2013 under supervision of Associate Professor Christine Finnie and Professor Birte Svensson. The Ph.D. project was performed in collaboration with the University of Aarhus (Department of Molecular Biology and Genetics, Faculty of Agricultural Sciencies, University of Aarhus) under supervision by Associate Professor Henrik Brinch-Pedersen and Dr. Giuseppe Dionisio. The Ph.D. stipend was founded by DTU and the work was supported by the Directorate for Food, Fisheries and Agri Business (DFFE) for the project "*Exploiting barley first wave enzyme activities for better feed*". Other partners in the project include Sejet Plantbreeding and Novozymes A/S.

The work of this Ph.D. project has resulted in the following manuscripts:

Sultan A, Andersen B, Christensen JB, Poulsen HD, Svensson B, Finnie C. Establishing the protein profile of liquid feed and analysis of application of protease inhibitors and NADPH-dependent thioredoxin reductase/thioredoxin (NTR/Trx) system. Manuscript to be submitted *to Journal of Proteomics* (Chapter 2).

Sultan A, Andersen B, Frisvad JC, Svensson B, Finnie C. Exploring the plant-microbe interface – Profiling and characterizing the indigenous fungal community, proteins and enzymes associated to surface of barley grains and xylanolytic activities. Manuscript to be submitted to *Journal of Proteome Research* (Chapter 3 and 4).

Sultan A, Dionisio G, Brinch-Pedersen H, Svensson B, Finnie C. Secretory expression of functional barley xylanase inhibitor protein (XIP-III) by yeast *Pichia pastoris*. In Prep. (Chapter 5).

Finnie C, **Sultan A**, Grasser KD. A review: From protein catalogues towards targeted proteomics approaches in cereal grains. *Phytochemistry* 2011;72:1145-53. (Appendix).

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Finally, no words would suffice to express my feelings for my family, especially my mom and dad, siblings Alia, Zaki, Shahina and Jasmin, whose endless love, encouragement and support have continued to enrich my life and made me into the person I am today.

Dedicated to the Sultans

ABSTRACT

The mature barley grain contains a complement of enzymes that are synthesized during seed development for degradation of seed storage reserves during germination. These enzyme activities (first wave enzymes) are considered important for maximizing nutrient digestibility in food and feed. Several strategies, such as liquid feed and supplementation of amino acids and microbial exogenous enzymes, are applied to improve protein absorption. A diverse commensal microbial community populates the cereal grains. The colonizing microflora constitute an integrated part of the seeds and interact/influence the plant and/or competitors via secretion of an array of enzymes and compounds/metabolites. The occurrence of these enzyme activities both of plant and fungal origin present a great potential for improvement of grain nutritional components for feed applications. Knowledge is lacking in the variation of these enzyme activities between barley cultivars, as well as the distribution and composition of the residing commensal fungal community and the grain surface-associated proteins. To obtain new insight into the interplay between barley grains and the colonizing fungi, we set out to study the activities of barley grains as well as the populating fungal community using different approaches.

The present thesis aimed at profiling the changes in proteome of liquid feed using gel-based proteomics. Comparative proteomics analysis was performed on liquid feed supplemented with protease inhibitors and/or the thioredoxin system (NTR/Trx). Addition of protease inhibitors to liquid feed seemed to some extent hamper protein degradation. With the NTR/Trx, the 2D-gels clearly showed an altered protein pattern and increased solubility of storage proteins, CM-proteins and protease inhibitors.

The variability of microbial and endogenous xylanase, as well as xylanase inhibition activities across a set of barley cultivars were determined using activity assays and gel-based proteomic analysis. Considerable inter-cultivar variability was found in the level of both microbial and endogenous xylanase, as well as xylanase inhibition activity levels. Harvest year and cultivar/genotype had a significant impact on the variability of all three parameters. Harvest year/weather conditions seem to largely affect xylanases and inhibition activity levels, thus indicating the instability of these parameters.

A Reference map over the surface-associated proteins (surfome) constitutively present on barley grains of two barley cultivars were established using 2-DE and mass spectrometry. The majority of the identified proteins was of plant origin and ascribed to play a role in defense and/or oxidative stress mechanisms. A metaproteomics approach was applied to profile and characterize the composition of the fungal community populating the surface of barley grains across different cultivars and their secretomes. It was revealed, the barley cultivars with high microbial xylanase activity levels were found to contain high numbers of storage fungi e.g. Aspergilli and Penicillia. A detailed analysis of the secretomes of two of the isolated fungi, i.e *Aspergillus niger* and *Fusarium poae,* grown on barley flour and wheat arabinoxylan enabled identification of a collection of amylopectic and xylanolytic enzymes involved in cell wall degradation and carbohydrate catabolism.

To gain a better understanding of the role of these xylanase inhibitors, the barley XIP-III was expressed in a secretory *Pichia pastoris* system. The expressed rXIP-III with a HIS_6-tag at the C-terminal was purified from the culture medium using metal affinity chromatography. Varying degree of inhibitory activity was found against fungal xylanases, xylanases recovered from the surface of barley grains, and secretomes of grains-associated fungi grown on WAX and barley flour. The secretomes of *Fusarium tricinctum* were found to be moderately resistant to the rXIP-III, with high residual xylanase activity levels.

SAMMENDRAG (SUMMARY IN DANISH)

Det modne bygkerne indeholder et komplement af enzymer, der syntetiseres under kerneudviklingen til nedbrydning af næringsstof-reserver i bygkernen under spirring for at supplere med carbon- og nitrogenkilder til syntesen af den nye kimplante. Disse enzymaktiviteter (førstebølge enzymer) er af vigtig betydning for maksimering af næringsstofudnyttelsen af fødevarer og foder. For at forbedre optagelse af proteiner fra bygkerner er adskillige strategier blevet anvendt, såsom vådfoder samt supplement af aminosyrer og mikrobielle enzymer til foder. En mangfoldig og diverse population of mikroorganismer findes på bygkernerne. Denne mikroflora udgører en integreret del af bygkernerne, der aktivt interagerer/påvirker planten og/eller andre konkurrenter via sekretion af en række enzymer og komponenter/metabolitter. Forekomst af disse enzym-aktiviteter af plante og microbiel oprindelse udgør et stort potentiale til forbedring af næringsværdi af byg som foder. Der er manglende viden om variation af disse enzym-aktiviteter mellem bygsorter, samt fordeling og sammensætning af de koloniserende svampe og de overflade-associerede proteiner. Denne Ph.D afhandling har til formål at studere samspillet mellem byg og svampe, samt karakterisering af deres proteom og enzym-aktiviteter ved hjælp af diverse teknikker.

Ændringer i proteinmønsteret af vådfoder blev fulgt ved anvendelse af gel-baserede proteomics teknikker. Komparativ protomeanalyse blev udført på vådfoder suppleret med enten proteaseinhibitorer eller thioredoxin-systemet (NTR/Trx). Iblanding af proteaseinhibitorer til vådfoder viste sig til en vis grad at hæmme nedbrydning af proteiner. 2D-geler af vådfoder tilsat NTR/Trx viste fremtrædende ændringer i proteinmønsteret samt tendens til øget opløselighed af en lang række proteiner herunder lagerproteiner, CM-proteiner og protease-inhibitorer.

Variationen i mikrobielle og endogene xylanase, samt xylanase-hæmmer aktivitet på tværs af forskellige bygkultivarer blev bestemt ved anvendelse af aktivitetstests og gel-baserede proteomanalyse. Betydelig inter-kultivar variation blev fundet i både mikrobiel og endogen xylanase, samt xylanase-hæmmer aktivitetsniveaue. Høståret og kultivaren/genotypen havde en betydelig påvirkning på variabiliteten af alle tre parametre. Høståret/vejrforholdet har en væsentlig indflydelse på xylanase og xylanase-hæmmer aktivitetsmålinger, hvilket indikerer ustabilitet af disse parametre.

Overflade-associerede proteiner (surfome) af to bygkultiverer blev etablereret ved brug af 2D-gel elektroforese (pH 3–10). De fleste af de identificerede proteiner var af planteoprindelse og tilskrevet at spille en rolle i forsvaret mod sygdomsrelaterede mekanismer eller oxidativ stress. Metaproteomanalyse blev anvendt for at profilere og karakterisere population af svampe på forskellige bygkultivarer, samt deres secernerede proteiner (secretome). Det blev afsløret, at byg kultivarer med høj mikrobielle xylanase aktivitet havde en forekomst af lagersvampe f.eks Aspergilli og Penicillia. En detaljeret analyse af secretomet af *Aspergillus niger* og *Fusarium poae* dyrkede på bygmel eller arabinoxylan fra hvede resulterede i identifikation af en samling af amylopektisk og xylanolytisk enzymer involveret i nedbrydning af cellevægge og kulhydrat katabolisme.

Endvidere blev xylanase hæmmer protein (XIP-III) fra byg udtrykt i *Pichia pastoris*. Det udtrykte rekombinant XIP-III med en His_6-tag ved C-terminalen blev oprenset fra dyrkningsmediet ved hjælp af metalaffinitetskromatografi. Inhibitor proteinet var aktiv mod diverse mikrobielle xylanaser, såsom prøver indeholdende xylanaser genvundet fra overfladen af bygkerner, og secretomer af isoleret cerealie-associerede svampe dyrket på bygmel eller arabinoxylan fra hvede. Endvidere viste det sig at secretomet af *Fusarium tricinctum* var relativ resistent over for rXIP-III med høje residual xylanase aktivitetsmålinger.

ABBREVIATIONS

1D	One-dimensional
2-DE	Two-dimensional gel electrophoresis
2D-DIGE	Two-dimensional difference gel electrophoresis
ABA	Abscisic acid
AMY	α-amylase
ANOVA	Analysis of variance
ASP	aspergine
Ara*f*	α- L-arabinofuranoside
AX	Arabinoxylan
AXOS	Arabino xylo-oligosaccharides
BASI	Barley-α-amylase/subtilisin inhibitor
BDAI	Barley dimeric a-amylase inhibitor
BSA	Bovine serum albumin
CAZy	Carbohydrate-Active EnZymes Database
CBB	Coomassie Brilliant Blue
CBM	Carbohydrate binding module
CM	Chloroform/methanol
COR	Cold-regulated protein
Cys	L-cysteine
DHAR	Dehydroascorbate reductase
DMSO	Dimethyl sulfoxide
DON	Deoxynivalenol
DTT	Dithiothreitol
ENO	Enolase
ESI	Electrospray ionization
EST	Expressed sequence tags
FAD	Flavin adenine dinucleotide
Fd	Ferredoxin
FPA	fructose-bisphosphate aldolase
FTR	Ferredoxin thioredoxin reductase
GA	Gbberellic acid
GH	Glycoside hydrolase
GlcA	α-D-glucuronic acid/glucuronopyranoside
GLX	Glyoxalase
IAA	Iodoacetamide
IEF	Isoelectric focusing
IMAC	Immobilized metal ion affinity chromatography
IPG	Immobilized pH gradient
iTRAQ	Isobaric tags for relative and absolute quantitation
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography-mass spectrometry
MALDI	Matrix assisted laser desorption/ionization
MDH	Malate dehydrogenase
Me-GlcUA	α-methylglucuronidase
MeOH	Methanol
MS	Mass spectrometry

Mw	Molecular weight
MudPit	Multi-dimensional protein identification technology
m/z	Mass to charge ratio
NADPH	Nicotinamide adenine dinucleotide phosphate
NCBI	National centre biological information
NIV	Nivalenol
NTR	NADPH dependent thioredoxin reductase
PCA	Principal component analysis
PCD	Programmed cell death
PCR	Ploymerase chain reaction
PDI	Protein disulfide isomerase
PGK	Phosphoglycerate kinase
PMF	Peptide mass fingerprint
PR	Pathogenesis-related
PTM	Post-translational modification
ROS	Reactive oxygen species
rXIP	Recombinant XIP
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
S/N	Signal to noise ratio
SPR	Surface plasmon resonance
TAXI	*Triticum aestivum* xylanase inhibitor
TCA	Tricarboxylic acid
TLP	Thaumatin like protein
TLXI	Thaumatin-like xylanase inhibitor
TOF	Time of flight
TPI	Triosephosphate isomerase
Trx	Thioredoxin
WAX	Wheat arabinoxylan
WE-AX	Water-extractable arabinoxylan
WU-AX	Water-unextractable arabinoxylan
XIP	Xylanase inhibitor protein
XOS	Xylooligosaccharides
Xyl	1,4-β-linked xylose residues

CONTENTS

CHAPTER 1

Introduction

1.1. BARLEY (*HORDEUM VULGARE)* – A MAJOR CEREAL CROP

In general, cereal crops are exposed to various biotic and abiotic factors under field conditions. Barley is a major cereal crop and a representative of the grass family (*Poaceae*), tribe Triticieae, which also includes wheat, rice, oat, and maize. Barley *(Hordeum vulgare*) is a highly adaptable crop and can grow in diverse environments, including longitude and extremes of latitude. Barley is the fourth most abundant cereal crop and commercially used for feed, malting industries and human consumption. It is estimated that approximately 85% (2007) of the global barley production is used for livestock (http://faostat.fao.org). Between various cereals, barley can grow in diverse climates ranging from temperate to tropical. In the northern hemisphere barley are grown either spring (sown in spring March) or winter barley (sown in fall mid-September). Barley has been used as a model organism for genetic experiments, due to its properties such as its diploid nature, self-fertility and high adaptability (Nilan, 1974).

Very recently, the International Barley Sequencing Consortium (IBSC) published an integrated physical, genetical and functional sequence assembly of the barley genome in the journal Nature (29th of November 2012), which will inevitably improve barley with determination of genes conferring resistance to diseases and other agronomically desirable traits related to stress and enhancement of the nutritional value e.g. better feed. In addition, improved understanding of the crops immune system will facilitate the development of new barley cultivars able to cope with the demands of diverse and adverse climate and the combat against cereal diseases, as well as meeting the climate changes faced globally.

Cereals are exposed to numerous pathogens and insect pests at different growth and development stages. The degree of their impact on quantity and quality of barley grains varies according to the climate, surrounding environment, post harvest storage and procedures, etc. In response plants have developed several strategies to defend themselves in order to survive. Common disease cycle comprises establishment of infection, invasion (colonization), growth and reproduction of the pathogen. Fungal mechanisms used to cause damage to the host involve production of cell wall degrading proteins, inhibitory proteins and enzymes involved in synthesis of toxins

(Agriose, 2005). In the case of an attack, plants synthesize pathogenesis related proteins, which are involved in plant protection.

1.2. STRUCTURE AND COMPOSITION

The morphology and structure of the barley grain is well described and the events taking place during seed germination have been studied extensively (Briggs, 1978; Evers et al., 2001). Several tissues of the seed play a distinct role during seed germination and upon pathogen attack. In general all plant seeds are comprised of three basic parts, i.e. a dormant embryo, the endosperm and the coat. A longitudinal cross section of a barley kernel is shown in Figure 1. The seed coat provides protection against mechanical injury, penetration by parasites and insects, water loss and extreme temperatures. The seed coat comprises 10−15% of the dry weight of the seed and embeds the embryo and endosperm. The embryo is a living tissue and contains the necessary components for growth of a new plant when supplied by nutrients from the endosperm. The embryo, i.e. germ, is situated at the proximal base of the barley kernel as shown in Figure 1 and accounts for 3−4% of the grain dry weight. The embryo is relatively rich in lipids (41−17%), protein (34%), sugars (27−37%), vitamins and minerals, respectively. The primary nutrient reserve of the grains is the endosperm (80% of the dry weight of the seed), which is composed mainly of starch and proteins, but also lipids and other nutrients, which are utilized by the embryo during germination. The endosperm consists of two tissues types: the starchy endosperm and the aleurone layer. At maturity, the majority of cells in the endosperm are nonliving, whilst the surrounding aleurone layer is a living tissue that releases enzymes required for mobilization of storage components of the endosperm to support the growing plantlet in the embryo (Bewley and Black, 1994). The endosperm cells are composed of thin-walled cells, which are packed with starch granules embedded in a protein matrix. The cell walls are composed of 71% β-glucan, 23% arabinoxylan (AX), 6% cellulose, and trace amounts of protein, glucomannan and phenolic acid (Fincher and Stone, 1986).

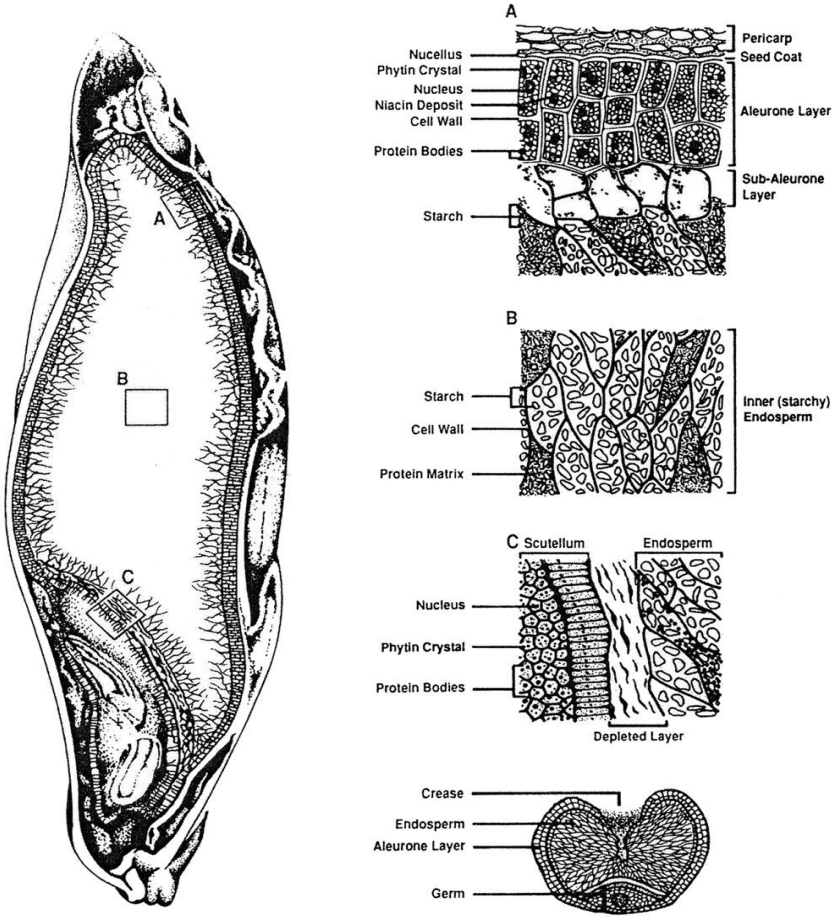

Figure 1: Enlarged vertical cross section of a barley kernel with further enlarged insets of the different regions. (A) Details of the pericarp, seed coat, aleurone layer and sub-aleurone layer, (B) Starchy endosperm, (C) Endosperm and scutellum. Right corner shows a cross section of a barley kernel (Sullivan et al., 2012).

The aleurone layer of barley seeds are multicellular consisting of 2−3 cell layers. The aleurone cells are filled with hundreds of small vacuoles, which contain inclusions of proteins, carbohydrates, minerals and phytins (a potassium, magnesium, and calcium salt of myo-inositol hexakisphosphate) (Jones, 1969; Olsen, 2001; Swanson et al., 1998; Stewart et al., 1988). The protein storage vacuoles are also known as protein bodies or aleurone grains. The aleurone layer plays an important part in producing hydrolytic enzyme upon stimulation by the phytohormone gibberellic acid (GA),

which is synthesized by the embryo triggered by uptake of water during germination. The hydrolytic enzymes degrade the cell walls, starch and proteins of the endosperm and thereby supply sugars and amino acids to the growth of the new plantlet (Bethke et al., 1998; Ritchie et al., 2000). The aleurone cell walls of barley are composed of the two major polysaccharides, arabinoxylan (85%) and cellulose (8%) (McNeil et al., 1975).

1.3. SEED GERMINATION

The mature grain is typically composed of approximately 10−15% protein and 70-80% carbohydrate. Seed germination has been defined to encompass the numerous hormonal, genetic and metabolic events that commence upon water uptake by the dry seed (imbibition) and terminate with the elongation of the embryonic axis, usually described as the radicle (Bewly and Black, 1997). Processes include subcellular structural changes, respiration, macromolecular syntheses and cell elongation. Upon imbibition, the embryo synthesizes GA, which induces *de novo* synthesis of hydrolases in the aleurone layer, such as α-amylases and proteases enabling the mobilization of storage reserves in the endosperm (Baulcombe and Buffard, 1983). Subsequently, the breakdown products are translocated from the embryo across the scutellum by a range of secondary carrier systems into the embryo, where they serve as carbon and nitrogen source for the growth of the seedling (Bewley, 1997). The scutellum is a single cell layer thick tissue located between the embryo and the endosperm. It has been reported that the scutellum also contributes with synthesis and secretion of hydrolases, however the aleurone layer is the main producer. A simplified illustration of the structure and processes taking place is shown in Figure 2. Once the storage reserves of the starchy endosperm are consumed the aleurone cells undergo programmed cell death (PCD) (Hägglin et al., 2002; Olsen, 2001; Ritchie et al., 2000). The phytohormone abscisic acid (ABA) is the antagonist of GA and is able to suppress the expression of GA-inducible genes in the aleurone layer used to support seedling growth. Besides suppressing precocious germination, ABA plays a main role in embryo maturation by inducing the expression of maturation-associated genes for storage product accumulation and desiccation tolerance (Ho et al., 2003).

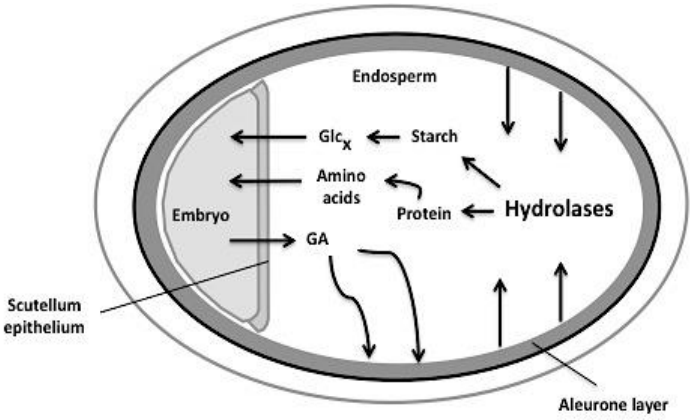

Figure 2: Illustration of the events taking place during seed germination. During imbibition, the increased concentrations of gibberellic acid of the embryo induces *de novo* synthesis of various hydrolases in the aleurone layer, which are secreted into the endosperm, where they catalyze the degradation of starch to maltose, glucose and oligosaccharides (Glc_x), and storage proteins to peptides and free amino acids. The released peptides and oligosaccharides are translocated across scutellum into the embro, where they serve as C and N source for the growing embryo.

1.4. BARLEY PROTEIN RESERVES

Barley is genetically diverse and the composition and properties of the grains vary with cultivar and environmental factors (Baik and Ullrich 2008). The protein content in mature barley grains varies in the range of 10−15% of dry weight (Gomez and De Datta, 1975; Finnie et al., 2006; Shewry and Halford, 2002). The variation is dependent on the variety/cultivar and environmental factors. Very early on, the cereal proteins were classified into four major groups based on their solubility and extraction in different solvents, i.e. the albumins (water), globulins (salt), glutelins (weak acid or alkali) and prolamins (alcohol), respectively (Osborne, 1907). Major storage proteins in barley endosperm are the hordeins, which belong to the prolamins. The prolamins have been further sub-classified into sulfur-rich, sulfur-poor and high molecular weight (HMW) on the basis of their amino acid sequence and composition. Moreover, the prolamins are deficient in essential amino acids, notably lysine, thus in the feeding industry barley is typically supplemented with other protein sources (Bewley and

Black, 1994; Shewry, 2007). The protein reserves are mobilized by a complement of proteases released from the aleurone layer as well as pre-formed proteases already present in the endosperm (Bewley and Black, 1994). Some of the proteases are also known to hydrolyze the protein reserves within the aleurone layer cells to provide amino acids for synthesis of other enzymes, such as α-amylase (Domingues and Cejudo, 1999; Ranki and Sopanen, 1984).

1.5. BARLEY CARBOHYDRATE RESERVES

The predominant component of barley grains is starch accounting for up to 65% of the dry weight (Aman et al., 1985; MacGregor and Fincher, 1993). Starch consists of two polysaccharides, i.e. a linear (1,4)-α-glucan (amylose) and a branched (1,4:1,6)-α-glucan (amylopectin). In barley seeds, the starch granules have been found to contain 25−30% amylose and 70−75% amylopectin, respectively (MacGregor and Fincher, 1993). A collection of several enzymes is required to degrade amylose and amylopectin. Initially, α- and β-amylases hydrolyze the 1,4-α-glucosidic linkages between the glucose units. However, they are unable to cleave 1,6-α-glucosidic linkages in the amylopectin, thus there is an accumulation of highly branched cores of glucose units, also termed limit dextrins (Briggs, 1998; Fincher and Stone, 1993). Debranching enzyme, such as limit dextrinase hydrolyzes 1,6-α-glucosidic linkages and produce glucose oligomers (maltooligosaccharides), which are further hydrolyzed by α- and β-amylase to yield glucose and maltose, which in turn is hydrolyzed by α-glucosidase to glucose (Ritchie et al., 2000; Sun and Henson, 1991). The majority of the starch degrading enzymes are synthesized and secreted by the aleurone layer during seed germination (Bewley and Black, 1994). However, β-amylase is synthesized during grain development in the endosperm (Lauriere et al., 1986).

1.6. PLANT CELL WALL – THE STRUCTURE AND COMPOSITION

The plant cell walls, a major dietary fiber source, are multi-component composites of non-starch polysaccharides, i.e. cellulose, hemicelluloses and pectins, structural proteins and in some cases polyphenolic polymer lignin. Although the different

compounds occur in different proportions dependent on the plant species, tissue and cell type, the walls provide rigidity to the cell and protection against mechanical stress (Sticklen, 2008; Taiz and Zeiger, 1991; Waldron and Faulds, 2007). Arabinoxylan (AX) is the predominant constituent of hemicellulose in the cell wall of monocotyledons, such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), accounting 5−10% of the dry weight of cereal grains (Fincher and Stone, 1986; Van Craeyveld et al., 2008). In barley, the cell walls of the aleurone layers are rich in AX (80%) with lesser amounts (1,3;1,4)-β-glucans (26%) of celluloses (8%) and proteins (6%), respectively (McNeil et al., 1975). While the endosperm cell walls contain 20−25% AX and 70% (1,3;1,4)-β-glucans (Fincher, 1975). The cell walls of aleurone and endosperm in wheat kernels also contain high levels of AX accounting for up to 70% and 65% AX and 29 and 20% (1,3;1,4)-β-glucans (Fincher and Stone, 1986; Mares and Stone, 1973).

1.7. ENZYMATIC ACTIVITIES INVOLVED IN CELL WALL DEGRADATION

Modulation and/or depolymerization of cell walls are important events necessary for the protein and starch degrading enzymes to reach their substrates. The synergistic interaction between these different cell wall degrading enzymes, including endo- and exo-β-glucanases, β-glucosidases, cellulases and β-xylanases, are known to participate in depolymerization or modulation of endosperm cell walls during seed germination. In the early stage of the germination process, endo-(1,3;1,4)-β-glucanases are prominent players, which catalyze the hydrolysis of 1,4-β-glucosyl linkages in (1,3;1,4)-β-glucans, a major cell wall component (Fincher and Stone, 1993). The released oligosaccharides are further degraded by exo-1,3-β-glucanase and β-glucosidase into its constituents, i.e. glucose, which are finally taken up by the developing seedling (Leah et al., 1995: Hrmova et al., 1996). Endo-1,4-β-glucanases hydrolyse the 1,4-β-glucosidic linkages in (1,3;1,4)-β-glucans. However, the levels of cellulase in germinating barley are typically low and variable, and most of the observed activity is attributed to the commensal microbiota populating the grain surface (Hoy et al., 1981).

1.8. ARABINOXYLANS

Despite the fact that AX represent a minor constituent of barley grains, they have a great impact in cereal based biotechnological processes and applications, such as bread-making, gluten-starch separation, malting, and processing of animal feed, due to their unique physicochemical properties (Countin and Delcour, 2002; Frederix et al., 2004). On the basis of their solubility, AXs are distinguished into water-extractable (WE-AX) and water-unextractable (WU-AX), respectively. The WU-AXs are retained in the cell walls by covalent (e.g. ester and ether bonds) and non-covalent (hydrogen bonds) interactions with the other cell wall constituents such as proteins and β-glucans (Cyran et al., 2004; Mares and Stone, 1973). While WE-AX is suggested to be loosely bound to the cell wall surface, probably due to incomplete cross-linking with the other constituents, structural differences or initial enzymatic degradation in the grains (Mares and Stone, 1973; Neukom, 1976).

Arabinoxylans are composed of a backbone of β-1,4-linked D-xylopyranosyl residues (xylose), which are to a varying degree substituted on the O2, O3 or at both positions with α-L-arabinofuranosyl (arabinose) residues. Ferulic acid and *p*-coumaric acid can be coupled to the O5 of arabinose via an ester linkage. Other less abundant substituents on xylose residues include acetic acid or (methyl)glucuronic acid, respectively, dependent on which tissue the AX is located in (Cleemput et al., 1993; Collins et al., 2005; Countin and Delcour, 2002; Cui and Wang, 2009). The different substituents are not regularly distributed on the AX backbone (Gruppen et al., 1993). The main constituents of AXs are shown in Figure 3, while the general structure of AX is shown in Figure 4. It has been proposed that the main difference between the overall structure of WU-AX and WE-AX is the degree of arabinose substitution, where WE-AX has the higher degree of substituents (Izydorczyk and Biliaderis, 1995). Arabinoxylans have raised a lot of interest, as the produced arabinoxylan-oligosaccharides (AXOS) have been shown to exert prebiotic properties (Van Craeyveld et al., 2008; Nyman et al., 1984). Arabinoxylan rich human diet can reach the colon in an intact form/undigested, where they are subject to degradation by enzymes like xylanases produced by microbial species belonging to the *Bacteroides* and *Roseburia* genera (Chassard et al., 2007). The released decorated arabino-

oligosaccharides (AXOS) and undecorated XOS can be utilized by other species, however growth on these substrates is limited to a few taxa, e.g. bifidobacteria (Flint et al., 2007; Lagaert et al., 2010; Lagaert et al., 2011). Several colonic commensals and potential pathogens, e.g. members of *Clostridium* and *Escherichia* genera, are unable to utilize XOS and AXOS (Moura et al., 2007). AXOS and XOS thus selectively stimulate growth of health beneficial bacteria of the microbiota, the so-called probiotic bacteria.

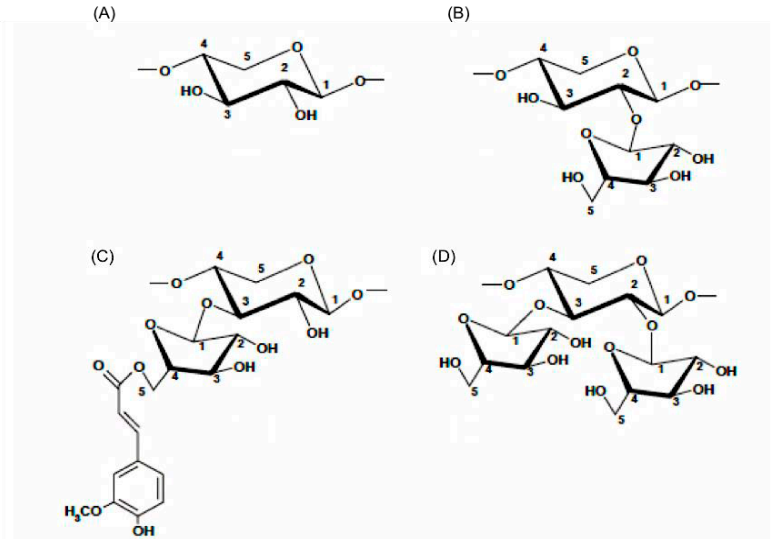

Figure 3: Structural constituents of arabinoxylan: (A) unsubstituted D-xylopyranosyl residue, (B) D-xylopyranosyl residue substituted on O2 with α-L-arabinofuranosyl residue, (C) D-xylopyranosyl residue substituted on O3 with α-L-arabinofuranosyl residue (showing the link of ferulic acid to O5 of α-L-arabinofuranosyl residue), (D) D-xylopyranosyl residue substituted on O2 and O3 with L-arabinofuranosyl residues (Courtin and Delcour, 2002).

1.9. ENZYMATIC DEGRADATION OF ARABINOXYLANS

Due to the heterogeneous and complex nature of AX, a consortium of xylanolytic enzymes is required for complete hydrolysis of AX to its constituent monosaccharides (Figure 4). Endo-1,4-β-xylanases (EC 3.2.1.8, referred to as xylanases) cleave glycosidic linkages in the AX backbone in a random manner, releasing xylo-oligosaccharides (XOS), arabino xylo-oligosaccharides (AXOS), xylobiose and

xylotriose. Beta-xylosidases (EC 3.2.1.37) cleave xylose units from the non-reducing end of XOS and xylobiose. Other accessory xylanolytic enzymes include debranching enzymes (α-L-arabinofuranosidases (EC 3.2.1.55) and α-D-glucuronidases (EC 3.2.1.139)), and esterases (acetylxylan (EC 3.1.1.72), ferulic acid (EC 3.1.1.73) and *p*-coumaric acid (EC 3.1.1.-)). These xylanolytic enzymes have been reported to work in synergy for efficient AX degradation (Biely, 1993; Collins et al., 2005; Jeffries, 1996; Subramaniyan and Prema, 1996).

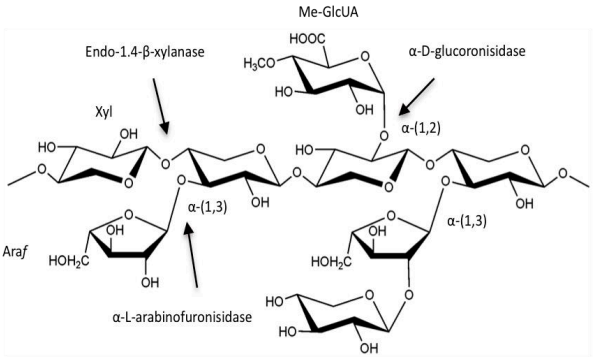

Figure 4: Structure of arabinoxylan and positions of its attack by some of the xylanolytic enzymes. The backbone is composed of 1,4-β-linked xylose residues (xyl). Ara*f*: α-L-arabinofuranosidase, Me-GlcUA: α-methylglucuronidase (adapted from Ebringerová, 2006).

Diverse collections of fungi, actinomycetes and bacteria have been found to produce a complement of these xylanolytic enzymes (Collins et al., 2005; Polizei et al., 2005). Some of the prevailing producers include Aspergilli, Trichodermi, Phanerochaetes (white rot), Chytridiomycetes, Streptomycetes, Ruminococci, Clostridia, Fibrobacteres and Bacilli (Matte and Forsberg, 1992; Subramaniyan and Prema, 1996; Sunna and Antranikian, 1997; Wubah et al., 1993). In addition, plants, algae, insects, molluscs and worms have also been reported to produce xylanolytic enzymes. These organisms are widespread and found to occupy diverse ecological niches. Some of which are known cereal pathogens, while others are found in the rumen of ruminants (Prade, 1995; Subramaniyan and Prema, 1996; Sunna and Antranikian, 1997).

1.10. INDUSTRIAL APPLICATION OF XYLANOLYTIC ENZYME PREPARATIONS

Microbial exogenous enzymes, in particular xylanases, which can significantly reduce the viscosity through relatively few cleavages in the main AX backbone, have been applied in various cereal-based processes. In cereal-based feed, the properties of AX such as solubility and susceptibility to enzymatic breakdown have a significant impact. A high content of AX has been reported to possess anti-nutritive effects. The intestinal tract of monogastric animals typically lacks the appropriate digestive enzymes, which results in inability to degrade/digest AX. The undigested AX causes an increase in the viscosity of the feed in the intestinal tract, which interfere with nutrient absorption (reduced), penetration of digestive enzymes, and unfavorable changes in the microbiota of the intestine, in particular in broiler chickens (Newkirk et al., 1993). The reduced rate of digestion of cereal-based feed consequently causes inefficient utilization of nutrients of the fodder, hence reduced growth rate of the livestock (Adeola and Bedford, 2005; Bedford and Classen, 1992; Scott, 2003). Application of commercial enzyme preparations containing a diversity of enzyme specificities to feed can facilitate reduction in viscosity and increase the nutritional value of feed (Courtin et al., 2008).

Application of xylanases in the baking industry has been studied for years. The main purpose of applying xylanases is to solubilize the AX fraction of the dough that involves changes in dough rheology, such as consistency, development time, extensibility and resistance to breakdown. The occurring changes are evident in the bread and seen as an increase in loaf volume as well as finer and uniform crumbs (Courtin et al., 2001; Courtin and Delcour, 2002). Overdosage of xylanases has been shown to result in highly sticky and slack dough due to the loss of water holding capacity (Grootaert et al., 2007; Rouau et al., 1994; Trogh et al., 2004; 2005). Common practices of xylanolytic enzymes in the industrial gluten-starch separation processes and brewing are based on reduction of dough or wort viscosity. Xylanases facilitate hydrolysis of AX and result in higher gluten and starch yields (Christophersen et al., 1997; Frederix et al., 2004; Weegels et al., 1992). In brewing, use of xylanases has lead to reduction in wort viscosity, haze formation of the beer and an increase in extract yield (Courtin et al., 2009; Debyser et al., 1997).

1.11. ENDO-XYLANASES - CLASSIFICATION

On the basis of amino acid sequence and structural similarities, endo-xylanases have been classified into 10 glycoside hydrolase (GH) families, i.e. 5, 7, 8, 10, 11, 16, 26, 43, 52 and 62, respectively (Coutinho and Henrissat, 1999, Carbohydrate-Active Enzyme database (CAZy). However, only the sequences classified in GH 5, 7, 8, 10, 11 and 43 contain truly distinct catalytic domains with a demonstrated xylanase activity (Collins et al., 2005). The majority of endo-xylanases falls into GH families 10 and 11.

Members of GH10 display a structure of a cylindrical eight-fold β/α-barrel resembling a bowl (Figure 5 a) and composed of catalytic and optionally also non-catalytic (substrate-binding) modules coupled with a flexible linker region (Henrissat et al., 1995; Kulkarni et al., 1999). The active site is situated in a shallow groove with a pair of glutamates as catalytic residues (Derewanda et al., 1994; Jeffries, 1996). GH10 xylanases have been characterized as having a relatively high molecular weight (>30 kDa) and low pI values (Simpson et al., 2003; Subramaniyan and Prema, 2002). As mentioned above, GH10 xylanases have been described to contain optionally non-catalytic domains, e.g. carbohydrate-binding modules (CBMs) and thermo-stabilizing modules (Connerton et al., 1999). A carbohydrate-binding module is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme with a discreet fold having carbohydrate-binding activity (Tomme et al., 1995). Xylanases of GH10 exhibit wider catalytic versatility, or broader substrate specificity, thus GH10 enzymes are able to attack a variety of polysaccharides and cleave within decorated regions of AXs (Biely et al., 1997). They have been reported to exhibit high activity on short XOS and low activity towards cellulosic substrates (Biely et al., 1997; Kolenová et al., 2006; Pastor et al., 2007).

(A) (B)

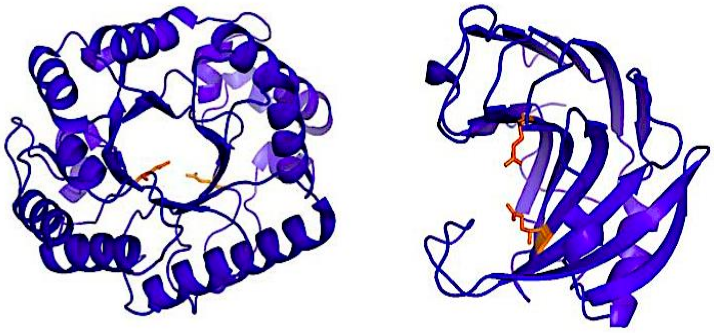

Figure 5: (A) Structure of GH family 10 xylanase of *Streptomyces lividans* (PDB code 1E0W) and (B) GH family 11 xylanase of *Aspergillus niger* (PDB code IUKR) with the catalytic residues highlighted in orange. Images adapted from Dornez (2007). Structures were generated with the PyMOL program (Delano et al., 2002).

Xylanases of GH11 are made up of a catalytic domain comprising two β-sheets and a single α-helix, which resemble a partially closed right hand with the active site (the palm) formed by the cleft between the fingers and the thumb, Figure 5 b). Analogue to GH10 xylanases, the active site of GH11 xylanases contains 2 glutamic acid residues (Berrin and Juge, 2008; Törrönen et al., 1994; Törrönen and Rouvinen, 1997). In general, this family have relatively low molecular mass (20 kDa) with high pI values, though xylanases with low pI 4−5 have also been discovered (Törrönen and Rouvinen, 1997). The GH11 xylanases have a lower catalytic versatility than GH10 xylanases and preferentially cleave unsubstituted regions of AX, where their end products can be further hydrolysed by GH10 xylanases (Berrin and Juge, 2008; Biely et al., 1997; Collins et al., 2005).

Xylanases have been found in bacteria, fungi, insects, snails and algae (Dekker and Richards, 1976). In general, xylanases of fungal origin are effective in the acidic pH range 3.5−5.5 and stable over a broad pH range 3.5−10.0. Bacterial xylanases display a higher pH optimum and a narrower activity range pH 5.0−7.5. The optimum temperature for bacterial and fungal xylanase action ranges between 35−60°C (Dekker and Richards, 1976). Discovery of extremophilic xylanases has been shown to exhibit activity up to 100°C or pH 2−11 (Beg et al., 2001).

1.12. OCCURRENCE OF ENDO-XYLANASES

1.12.1. BARLEY XYLANASES

Xylanases have been found in limited number of cereals, such as wheat, barley rye and maize, although the different cereal and tissue display low xylanase activity levels. These xylanases play an important physiological role in cereals and are involved in modulation and/or degradation of cell wall during seed development and germination (Simpson et al., 2003). In barley seeds, endo-1,4-β-xylanases are synthesized and accumulate as inactive precursors in the aleurone cells, and are released upon aleurone programmed cell death (PCD, apoptosis) during germination (Casper et al., 2001; Simpson et al., 2003). Subsequently, the released endo-1,4-β-xylanases undergo to proteolytic processing involving cleavage of amino- and carboxyl terminal regions of the precursor molecule into its active form, which assists in degradation of aleurone and endosperm cell walls (Nielsen et al., 2001). By contrast, according to Van Campenhaut et al. (2007), the precursor form of xylanase is an already active enzyme, where the N-terminal domain of the precursor xylanase is known to encode a putative CBM22, which promotes the binding toward xylans (Selvaraj et al., 2010). Thus far, all the identified plant xylanases belong to GH10 and not inhibited by the currently known proteinaceous xylanase inhibitors (Simpson et al., 2003).

1.12.2. MICROBIAL XYLANASE

Cereal grains are rarely free of microbial contamination. The level and types of microorganisms present is highly dependent on the cereal crop, stage of development and maturation, environmental as well as storage conditions. The usual suspects found comprise bacteria, actinomycetes and fungi. These microorganisms populating within and on the outer kernel layers are known to produce a complement of cell wall degrading enzymes, including xylanases, to invade, colonize and utilize the released nutrients (Walton, 1994; Williamson et al., 1998). Cereal pathogens, such as *Fusarium graminearum, Botrytis cinera, Magnaporthe grisea, Aspergillus fumigans,* produce xylanases and other cell wall degrading enzymes (Brutus et al., 2005; Degefu et al., 1995; Giesbert et al., 1998; Paper et al., 2007; Southerton et al., 1993; Wu et al.,

1997). Several microorganisms, e.g. *Aspergillus* sp. and *Trichoderma* sp. (c.f. paragraph 1.18 and Chapter 4), have been adopted for industrial production of xylanases and routinely used in diverse applications to improve end-product quality.

Until only recently, the surface-associated xylanases have been largely disregarded, though they contribute more then 90% of the determined xylanase activity levels in cereals and are likely the cause of seasonal quality variability (Dornez et al., 2008). In addition, several studies have shown that xylanases are mainly concentrated in the outer kernel layers (Bonnin et al., 1998; Croes et al., 2009). A study by Brito et al. (2006) reported that xylanases are essential for the virulence of the fungal plant pathogen *B. cinerea*. The majority of microbial xylanases are susceptible to the action of proteinaceous xylanase inhibitors with varying specificities, present in high amounts in seed endosperm and bran (Rouau and Surget, 1998). Meanwhile, plant xylanases belonging to GH10 are insensitive to inhibition by endogenous xylanase inhibitors.

1.13. XYLANASE INHIBITORS

Cereal grains have evolved groups of proteinaceous xylanase inhibitors (XIs), which have a strong impact on the functionality of microbial xylanases regularly used in cereal processing applications such as baking (Juge and Svensson, 2006). In cereals, three structurally distinct classes of xylanase inhibitors have been identified, namely xylanase-inhibiting protein (XIP), *Triticum aestivum* xylanase inhibitor (TAXI) and thaumatin-like xylanase inhibitor (TLXI), respectively (Debyser et al., 1997; Gebruers et al., 2004; Juge, 2006; McLauchlan et al., 1999; Rouau and Surget, 1998). XIP, TAXI, and TLXI-type inhibitors have been detected in whole meals by immunoblotting of various cereals, including wheat, durum wheat, barley, rye, as well as maize and oat (Beaugrand et al., 2006; Elliot et al., 2003; Goesaert et al., 2002). It has been reported that the xylanase inhibitor concentration in cereals are dependent on variety, milling fraction, and growing conditions (Bonnin et al., 2005).

The three types of inhibitors are basic proteins (with some exceptions) and exhibit different modes and specificities of inhibition (Pollet et al., 2009). Additionally, proteomic as well as transcriptomic studies have shown that xylanase inhibiting

proteins appear in cereal grains as polymorphic families (Takahashi-Ando et al., 2007). XIP-type inhibitors are specific for fungal xylanases belonging to GH10 and GH11 (Payan et al., 2004), whereas TAXI and TLXI-type exclusively inhibit xylanases of GH11 of bacterial and fungal origin (Fierens et al., 2007; Gebruers et al., 2004).

1.13.1. XYLANASE INHIBITOR PROTEIN (XIP)

The xylanase inhibitor protein has been found in seed endosperm and characterized to be monomeric glycosylated protein with molecular mass of 29 kDa and pI values of 8.7−8.9 (Flatman et al., 2002; Juge et al., 2004; McLauchlan et al., 1999). Based on kinetics studies, XIP competitively inhibits fungal xylanases of GH10 and GH11, but does not inhibit any bacterial xylanases of these families (Flatman et al., 2002). XIP exhibits high homology (63% identity) with plant chitinases of GH18, although, XIP does not possess chitinase activity. In addition, XIP has been reported to have two independent binding sites, allowing binding of two glycosyl hydrolases. One of these sites specifically interacts with xylanases of GH10 and the other with xylanases of GH11 (Payan et al., 2004). No inhibition activity has been detected towards endogenous plant xylanases. Notably, XIP has been found to inhibit α-amylases (AMY1 and AMY2) of GH13 (Juge et al., 2004; Sancho et al., 2003). The dual function of XIP suggests that xylanase inhibitors play a role in defense systems and provide protection against external action of phytopathogens. It also indicates that these inhibitors possibly reduce starch digestibility in the animal when used as feed. Moreover, barley α-amylase/subtilisin inhibitor (BASI) has been shown to partially inhibit GH11 xylanases (Sancho et al., 2003).

Studies on cereal flour demonstrate the presence of multiple XIP isoforms (28.5, 29, 30 kDa) (Elliot et al., 2003; Goesaert et al., 2003; Juge et al., 2003). Though it remains to be elucidated whether these multiple forms arise due to post-translational modifications, or are products of different XIP genes, or results of modifications during storage and purification.

1.13.2. *TRITICUM AESTIVUM* XYLANASE INHIBITOR PROTEIN (TAXI)

The TAXI-type inhibitors have been identified in whole meals of several cereals, including wheat, durum wheat, barley and rye (Goesaert et al., 2001; Goesaert et al., 2002; Goesaert et al., 2003). They are basic and low-glycosylated proteins with molecular mass of about 40 kDa. Potentially, several TAXI-type inhibitors have been discovered, however only the presence of two have been confirmed in wheat flour (TAXI-I and II) and one in barley (TAXI-I) (Goesaert et al., 2001). The two inhibitors, TAXI-I and II, have been shown to share 86% sequence identity, but differ in pI (8.9 and 9.3, respectively) and inhibition specificities (Gebruers et al., 2004). Both inhibitors occur in two molecular forms (A and B), where A constitutes of a single polypeptide chain (40 kDa) and B two peptide chains (10 and 30 kDa) held together by a disulphide bond, respectively. Form B is believed to be a result of proteolytic processing of form A. One potential *N*-glycosylation site and 12 cysteines forming 6 disulfides have been found in most of the cereal TAXI-type inhibitors. In general, TAXI-type inhibitors are active against both bacterial and fungal GH11 xylanases, but not against GH10 xylanases (Gebruers et al., 2001; Goesaert et al., 2001). The TAXI-I displays structural homology with the pepsin-like family of aspartic acid proteases, but is proteolytically non-functional (Sansen et al., 2004). In addition, Igawa et al. (2004) identified two more genes, i.e. *taxi-III* and *taxi-IV* in wheat, which share high sequence similarity (91.7% and 82.0%) identity with *taxi-I*. The detected transcripts were found to accumulate in leaves and roots of wheat plants and to be induced as seen at the expression level by infection with the pathogens *F. graminearum* and *Erisyphe graminis* (powdery mildew). By contrast wounding of leaves induced expression of *taxi-IA, taxi-III* and *taxi-IV*. The different responses upon infection and wounding emphasise the different roles of the different TAXI-type inhibitors.

1.13.3. THAUMATIN-LIKE XYLANASE INHIBITOR (TLXI)

The most recent discovery is the thaumatin-like xylanase inhibitor (TLXI) protein from wheat characterized as having high pI (> 9.3) and a molecular mass of 18 kDa (Fierens et al., 2007). It has been reported that TLXI occurs as multiple isoforms.

TLXI protein has been shown to inhibit both fungal and bacterial GH11 xylanases, but is inactive towards GH10 xylanases (similar to TAXI). By contrast to the other two classes of xylanase inhibitors, TLXI exerts its inhibition in a non-competitive mode (Fierens et al., 2007).

TLXI exhibits high sequence identity with plant thaumatin-like proteins belonging to the PR-5 family of pathogenesis-related (PR) proteins, thus they have been implicated in plant defence. The PR-5 family is one of 17 distinct PR protein families. These proteins have been shown to be induced in response to pathogen and insect invasion or wounding. Some TLPs are induced during flower development and fruit ripening (Arro et al., 1997). The antifungal activity is believed to be a result of permeabilizing the fungal membrane possibly by binding and hydrolyzing β-1,3-glucans, a major component of fungal cell walls (Skadsen et al., 2000; Trudal et al., 1998; Van Loon et al., 2006). Reiss et al. (2006) identified 8 different TLPs from barley, where TLP1−2 and 4−8 were found from infected leaves and TLP3 from developing grains. It was revealed that the barley TLPs fall into two groups, the short-chain proteins (TLP1−4) with 10 cysteine residues, and the long-chain proteins (TLP 5−8) with 16 cysteine residues. The cysteine residues participate in formation of 5 or 8 disulfide bonds, which give the properly folded TLPs a highly stabilized structure and form the basis for thermal and pH stability (Reiss et al., 2006). Immuno-localisation studies have shown that initially TLPs occur on the ovary wall and later is found in the aleurone and the endosperm during grain development (Fierens et al., 2007; Reiss et al., 2006; Skadsen et al., 2000).

1.14. Occurrence and variability of xylanase inhibitors

Various roles have been ascribed to these inhibitors including involvement in development, germination and defence against pathogens (Croes et al., 2008; Gebruers et al., 2004). Since they show almost exclusive activity towards microbial xylanases (in vitro) and are structurally homologous to many defence-related proteins, it is likely that xylanase inhibitors form part of the plant's defence mechanisms (Flatman et al., 2002; Gebruers et al., 2001; Goesaert et al., 2004).

In cereals, TAXI- and XIP-type xylanase inhibitors widely occur as multi-isoform families. Transciptomic studies revealed expression of multiple putative TAXI- and XIP-type genes and one TLXI gene in wheat grains (Igawa et al., 2004; 2005). The presence of TAXI and XIP proteins in wheat as polymorphic families was also demonstrated by comprehensive proteomics analyses using 2-DE and subsequent MALDI-TOF tandem MS analysis (Croes et al., 2008). In addition, a 2D-DIGE approach coupled with LC-ESI tandem MS in extracts from six different wheat cultivars enabled differentiation between TAXI, XIP and TLXI inhibitor isoforms and quantified their variation among the different cultivars. This study led to identification of a total 48 xylanase inhibitor protein forms, including 18 TAXI, 27 XIP, and 3 TLXI-type xylanase inhibitors (Croes et al., 2009a). The high heterogeneity was ascribed to both the presence of multiple *taxi* and *xip* genes (i.e. isoforms) and to post-translational modifications, e.g. glycosylation and other processing (Croes et al., 2009a), which often can easily be identified and characterized using gel-based proteomic approaches. Transcriptome analysis suggested that the expression of some *taxi* and *xip* gene family members was induced in spikelets and leaves infected with fungal pathogens, i.e. *Fusarium graminearum* and *Erysiphe graminis*, respectively, while others genes were unresponsive (Igawa et al., 2004; 2005). Thus suggesting that the different forms of these inhibitors might be induced by different pathogens or other stress conditions, or might be tissue specific. However, little is known about how and when the different xylanase inhibitor isoforms are regulated at various stages of seed growth and development.

Xylanase inhibitory activity assays combined with immunoblotting on extracts from wheat kernels demonstrate considerable variation in inhibitor levels among different wheat cultivars (Beaugrand et al., 2006; Croes et al., 2009b). Inhibition activity measurements of 19 different European wheat cultivars revealed that the inhibitor levels in wheat varied with a factor of 2−3, due to genetic and/or environmental effects (Gebruers et al., 2002b). All three types of inhibitors display high affinity for arabinoxylans (Fierens et al., 2009). This indicates that the inhibitors are found in or close proximity of the cell wall where they bind to the substrate, which they protect from cell wall degrading enzymes. Analysis of wheat milling fractions revealed that the three types of inhibitors are highly abundant in the outer layers of wheat kernels,

in particular in the caryopsis. Gebruers et al. (2002b) reported 2−3 folds higher inhibition activities in wheat shorts and bran fractions than in flour. A study by Croes et al. (2009b) detected that the highest concentrations of these inhibitors occur in the aleurone layer. Recently, the spatial distribution of TAXI and XIP in wheat grains was demonstrated to be predominantly found in nucellar tissue adjacent to the aleurone tissue by using proteomics and immunofluorescence microscopy (Jerkovic et al., 2010). Thus, proteomics in combination with classical protein chemistry, immunochemistry and activity measurements is providing a detailed picture of a complex protein family.

All the studies suggest that these inhibitors are predominantly located in the outer layers of wheat grains, and the presence of different types of these inhibitors with varying specificities, and the differential regulation of the expression of the corresponding inhibitor genes, may provide the plant with a convincing "toolbox" to adapt and ensure protection against different stress conditions and pathogenic attack.

1.15. First wave and second wave enzyme activities in barley

Barley is a major cereal crop and intensively used as feed. The mature barley grain contains a battery of hydrolytic enzymes that are synthesized during seed development and deposited in an inactive form (Bethke et al., 1998). During germination these enzymes are involved in degradation of seed storage reserves to support the growth of the new plantlet. These enzymes are referred to as the first wave enzymes, and are of fundamental biological importance for regular progression of germination, but they are also major quality determinants for feed, as they are activated during processing or in the digestive tract. However, knowledge is scarce regarding these enzyme activities. In parallel with imbibition, the embryo synthesizes and secretes the phytohormone GA. The aleurone and the scutellum layer of the embryo are thereby turned into secretory tissues where a wide range of hydrolytic enzymes are synthesized and secreted into the endosperm for degradation of cell walls, starch grains and storage proteins. These enzymes involved in this second wave of hydrolysis are referred to as the second wave enzymes.

Furthermore, uptake of water is accompanied by a extensive change in the redox state of proteins in cereals. Proteins of the endosperm and the embryo that mainly appear in the oxidized (SS) state in the dry seed are converted to the reduced or sulfhydryl (SH) state following imbibition (Marx et al., 2003). The regulatory disulfide protein thioredoxin (Trx) plays a central role in the redox conversion. When reduced by thioredoxin reductase (NTR) in the presence of NADPH, Trx can act as a signal in early germination to facilitate the mobilization of reserves by (i) inactivating and reducing disulfide proteins (e.g. amylase and protease inhibitors) that inhibit specific amylases and proteases, thereby facilitating the breakdown of stored protein and starch, (ii) reducing storage proteins and thereby enhancing their solubility and susceptibility to proteolysis, (iii) reductively activating individual enzymes functional in germination (Kobrehel et al., 1991; 1992; Lazona et al., 1993).

In addition, the diverse microbial community naturally colonizing the surface/outer layers of barley kernels also plays a significant role, as they are known to produce an array of enzymes, including proteases, amylolytic, xylanolytic and cell wall degrading enzymes. The colonizing microflora is an integrated part of the seeds and their enzyme activities have a greatly impact on the grain as well as the final quality of the product. Collectively, these enzyme activities present a great potential for improvement of grain nutritional components for feed applications.

1.16. CEREAL-BASED ANIMAL FEEDS

There is an increasing demand for a feed crop with optimal health effects and efficient release of the grain nutritional components in the animal digestive tract. Cereal grains constitute a large part of the diets used for animals. Cereals like barley provide various nutrients, but are also prime source of fiber/non-starch polysaccharides, in particular AX, which has been associated with decreased nutrient digestion and absorption. Supplementation of cereal-based feeds with microbial enzyme cocktails can improve nutrient uptake by reducing the viscosity of the digesta. Studies have shown that supplementation of xylanases can improve nutrient absorption of cereal based diets by depolymerizing the arabinoxylans (Bedford, 2003; Courtin et al., 2008). However, it is

likely, that the enzyme efficiency is highly variable due to many factors, including the presence of large amounts of xylanase inhibitors in cereals.

Monogastric animals are unable to digest and efficiently utilize various grain nutrients e.g. the nitrogen from protein or phosphorous from phytate, thus there is an excretion of large amounts in the environment, and consequently limited animal growth. It is estimated that livestock utilizes approximately half of the nitrogen of protein in cereal-based feed. In order to improve nutrient absorption, several strategies such as liquid feed and supplementation of microbial enzymes or amino acids have been employed for analysis. Pre-soaking of feedstuffs to increase their digestibility has been practiced for many years. As mentioned, upon imbibition, grain enzymes are activated for hydrolysis of grain nutritional components. Liquid feeding is based on pre-soaking feedstuff (e.g. mature barley grains) with water prior to delivery to the animals with monitoring of parameters such as pH value, incubation period and microbial growth (Scholten et al., 1999). Based on the length of the incubation time, the liquid feed has been differentiated into the non-fermented liquid feed, where there is almost no time between mixing and feeding, and the fermented liquid feed (where the mixture is kept long enough to achieve a steady state fermentation conditions. With initiation of the fermentation by lactic acid bacteria, the pH value drops from 6−7 to acidic 4−4.5, due to production of lactic acid, which inhibits the growth of pathogenic enterobacteria, e.g. coliforms, Figure 6 (Brooks et al., 2001; Canibe and Jensen, 2003; Plumed-Ferrer and von Wright, 2009; Missotten et al., 2010).

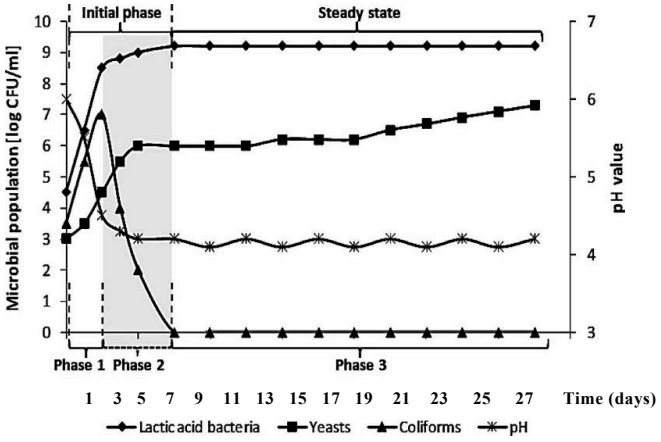

Figure 6: Graphical representation of the microbial population and pH of fermented liquid feed over time. The initial phase is divided in two phases, in phase 1 the high pH allows proliferation of coliform bacteria and in phase 2 the growth and fermentation by lactic acid bacteria (LAB) inhibits pathogenic and other organisms by the lowering the pH and production of organic acids (e.g. lactic acid), hydrogen peroxide and bacteriocins. In phase 3 (the steady state) the LAB population and pH are stabilized, although the yeast concentration of feed continue to increase over time. Modified from Missotten et al., 2010 and Brooks et al., 2001.

In addition to common practice of protein supplements e.g. soya bean and fish flour, in the present study the thioredoxin system was applied to liquid feed to investigate and profile the proteome changes.

1.17. THIOREDOXIN SYSTEM IN BARLEY

Numerous studies have established that germination of seeds is accompanied by a change in the redox state of proteins. In the dry seed, proteins are mainly in oxidized form (S-S) and converted to the reduced or sulfhydryl (SH) form following imbibition (Kobrehel et al., 1992). Thioredoxins (Trxs) are small proteins with a molecular mass of 12−14 kDa with disulphide reductase activity. Thioredoxins act as electron donors in various pathways through reduction of disulphide bonds in target proteins (Buchanan, 2001; Buchanan and Balmer, 2005).

Plants are characterized by a highly complex Trx system with a diverse selection of isozymes with temporal- and organelle-specific appearance (e.g. cytosol, chloroplast and mitochondria). In cereals, Trxs play a central role in redox regulation during seed development, germination and radicle elongation (Kobrehel et al., 2002; Lazano et al., 1996; Wong et al., 1995; 2001; 2004). In the NADPH-dependent thioredoxin reductase (NTR)/Trx system, the NADPH (e.g. the pentose phosphate pathway) serve as electron donor for the NTR which in turn reduce the disulphide bond in Trx (Figure 7). Subsequently, the reduced Trx catalyzes reduction of disulphide bond in other target proteins (Alkhalfioui et al., 2007; 2008; Hägglund et al., 2008; Kobrehel et al., 2002; Montrichard et al., 2003; Wong et al., 1995). In plants, specific types of Trxs are reduced by ferredoxin-thioredoxin reductase (FTR) and ferredoxin. In the ferredoxin/Trx system (FTS), the electrons from the photosynthetic electron transport reduce ferredoxin (Fd), which then serve as an electron donor for the ferrodoxin-thioredoxin reductase (FTR) that in turn reduce the disulphide bond in Trx. The reduced Trx can act as an electron donor to reduce target proteins. Furthermore, in some plants Trxs are also reduced by glutahionereductase (GR) and glutathione (de la Torre et al., 1970; Gelhaye et al., 2004). Application of proteomics approaches has lead to identification of a wide range of Trx target proteins, including BASI, Lipid transfer protein 1 (LTP), embryo-specific proteins and glyoxalase (Alkhalfioui et al., 2007; Hägglund et al., 2008; Maeda et al., 2004; Montrichard et al., 2003).

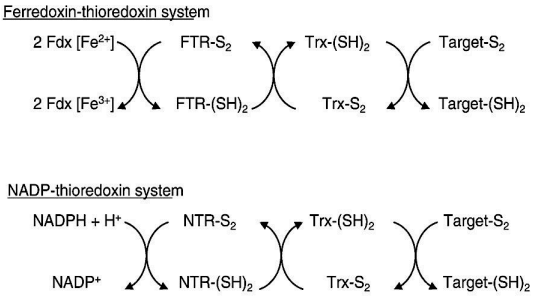

Figure 7: The ferredoxin- and NADP-thioredoxin system.

Disulfide bonds are prominent in seed storage proteins contributing in an increased stability, which also decrease their susceptibility to proteolysis and provide protection against denaturation. Upon imbibition, the oxidized disulfide state of storage proteins is converted to the reduced state, which increases their solubility and susceptibility to proteases (Buchanan and Balmer, 2005; Lazano et al., 1996). The cytosolic Trx h functions as an early signal in germination of cereals by facilitating the mobilization of reserves by reducing disulphide bonds in storage proteins and enhancing their solubility, thus making them more susceptibility to proteolytic degradation. Trxs are also involved in reducing disulphide bonds in inhibitors of proteases and amylolytic enzymes (e.g. BASI and α-amylase/trypsin inhibitor). Furthermore, Trxs have also been reported in activation of the calcium dependent substrate-specific protease, thiocalsin. Both features aim towards promotion of seed germination (Buchanan, 2001; Besse et al., 1996; Kobrehel et al., 2002; Montrichard et al., 2003; Wong et al., 2004). In barley seeds, two forms of Trx h and NTR have been identified with overlapping spatio-temporal appearance (Maeda et al., 2003; Shahpiri et al., 2008; 2009).

1.18. THE INDIGENIOUS MICROBIAL COMMUNITY OF BARLEY

Cereals continuously interact with widespread and countless microorganisms. Over 200 species of microorganisms have been found to populate the surface and/or outer layers of barley kernels (Briggs, 1978). The number and type of the microorganisms present is greatly influenced by the history of the grain during the development and maturation period, harvest, and storage. Microorganisms contaminating on cereal grains may derive from the air, dust, water, soil, insects, birds, humans and animals prior to harvest, and storage containers, handling and processing equipment, etc. (Noots et al., 1999; Flannigan, 1969). The diverse microbial community actively interacting with the grain has a great impact on quality of the grain and cereal products, as well as safety for consumption by humans and as livestock feed. The indigenous microbial community of barley kernels harbors a broad range of microorganisms including various species of bacteria, yeasts, actinomycetes and

filamentous fungi (Flannigan, 2003; Haikara et al., 1977; Noots et al., 1999; Petters et al., 1988).

Microbial contamination of barley starts at ear emergence and the number of populating microorganisms on the kernels increase immensely during development and ripening (Flannigan et al., 1982; Haikara et al., 1977; Noots et al., 1999). The microbial loads on the grains are highly influenced by multiple environmental factors, including rainfall, drought, temperature, humidity, sunlight, wind, soil conditions, fungicide treatment, insect, bird, and other organism activity. Thus the indigenous microbial community will differ with harvest year and geographical location (Lacey, 1991; Tuomi and Rosenqvist, 1995). Some of the organisms are recognized as harmful pathogens, resulting in the initiation of defense responses, whereas others are perceived as beneficial symbionts. The frequently occurring microbes on pre-harvest barley grains are summarized in Table 1. A more elaborate list can be found in Noots et al. (1999).

Table 1: Principal preharvest microbes of barley (Flannigan, 2003; Hill and Lacey, 1983; Haikara et al., 1977; Noots et al., 1999; Petters et al., 1988).

Bacteria	Yeasts	Filamentous fungi
Bacillus	*Candida*	*Alternaria*
Enterobacter	*Cryptococcus*	*Aureobasidium* (yeast-like)
Erwinia	*Pichia*	*Cephalosporium*
Flavobacterium	*Rhodotorula*	*Cladosporium*
Klebsiella	*Sporobolomyces*	*Dreachslera*
Micrococcus	*Trichosporon*	*Epicoccum*
Pseudomonas		*Fusarium*
Streptomyces		*Verticillium*
Xanthomonas		

The initial colonizers of cereal grains after ear emergence are bacteria, which are replaced by yeasts and eventually by filamentous fungi after anthesis of the grains (Flannigan, 2003; Hill and Lacey, 1983). During kernel development, the Gram-negative bacteria mainly dominate the populating microbial community, where *Erwinia herbicola* and *Xanthomonas campestris* are the abundant and prevalent bacterial species on barley (Clarke and Hill, 1981; Flannigan et al., 1982). As most seeds are stored dry, bacteria seldom cause deterioration of the grains, while at intermediate moisture content filamentous fungi and pests are of major concern.

Filamentous fungi greatly affects the quality of the grains by e.g. causing discoloration, degradation of nutrient constituents, reduced seed germination ability, production of volatile metabolites and mycotoxins, etc. (Lacey and Crook, 1988; Lacey et al., 1991). Mycotoxins are defined as natural products produced by fungi that evoke a toxic response when introduced in low concentrations to higher vertebrates and other animals by a natural route (Bennett, 1987). Fungi colonizing the grains are divided into two distinct groups, namely the field and the storage fungi, based on their ecological requirements. The most common field fungi in barley belong to the genera, *Alternaria, Cladosporium, Epicoccum, Fusarium* and *Drechslera,* respectively (Ackermann, 1998; Andersen et al., 1996; Flannigan, 2003; Haikara et al., 1977; Noots et al., 1999). The prevalent storage fungi colonizing grains comprise primarily *Aspergillus*, *Eurotium* and *Penicillium* (Hill and Lacey, 1983; Noots et al., 1999; Pitt and Hocking 1997; Samson et al., 2000).

Microbial growth and spoilage of barley grains are determined by water activity, temperature and aeration of grain mass (Justé et al., 2011). Microbes generally grow more rapidly at elevated temperatures and high grain moisture contents (Hoseney, 1994; Magan and Evans, 2000; Prange et al., 2005). Growth of field fungi is restricted during storage, due to the requirement of relatively high water availability ($a_w > 0.85$) corresponding to 20−25% moisture, while cultivars common during storage germinate at a_w 0.72−0.80 (18% moisture) or even 0.68 (14% moisture). In general, grain moisture contents below 14% are considered to be safe for storage (Fleurat-Lessard, 2002).

1.19. GRAIN QUALITY AND SPOILAGE

Barley grains represent a rich nutrient source of carbohydrates, proteins and lipids for colonizing fungi (Noots et al., 2003). The fungal community can have adverse impact on grain quality by e.g. decreased germination, loss of weight and nutritive value, discoloration, production of a range of hydrolytic enzymes leading to loss of dry matter, production of volatile compounds causing off-odors and mycotoxins (Lacey, 1989; Lacey et al., 1991). Decreased germination occurs when storage fungi invade the embryos of cereal grains and utilize its lipids and other nutrients, which usually

precedes discoloration of the grains. Both field and storage fungi can result in discoloration, e.g. species of *Alternaria, Aspergillus, Fusarium* and *Penicillum* are known to cause black point in barley and wheat, which is characterized by a dark discoloration of the embryo side of the grains (Christensen and Kaufmann, 1969; Mak et al., 2006; Toklu et al., 2008). Spoilage by some storage fungi such as *A. candidus and A. flavus* can, through their respiration, increase the temperature of stored grains (up to 55°C). If sufficient heating is available, the grain becomes dark and blackened (Christensen and Kaufmann, 1969; 1977). Fungi produce an array of hydrolytic enzymes to disintegrate the cell walls and degrade grain carbohydrates, protein and lipids energy source.

While grain spoilage can have adverse effects, these are less severe than the presence of mycotoxins. Numerous fungi are able to produce one or more mycotoxins and other metabolites as a result of the secondary metabolism of fungi during proliferation and adaptation to stressful situations, which confer toxic effects on other microorganisms, insects or plants (Bennett, 1987; Champeil et al., 2004; Frisvad, 1986). The diseases caused by mycotoxins, known as mycotoxicoses, are severe and can even be fatal for both humans and animals (Bennett, 1987; D'Mello et al., 1999; Peraica et al., 1999). *Fusaria* are one of the most ubiquitous genera of plant-pathogenic fungi. Species such as *F. graminearum* can cause head blight of barley and wheat, and ear rot in maize (Steffenson, 1998). Several species of *Fusarium* produce mycotoxins, i.e. trichothecenes, butenolide and zearalenone, on a variety of substrates, which are highly toxic and carcinogenic, and pose a critical hazard to human and animal health (Joffe, 1986; Marasas et al., 1984; Salleh and Sulaiman, 1984). The mycotoxins have been implicated in various animal disorders such as fescue foot, feed refusal, skin lesions and estrogenic symptoms (Mirocha and Christensen, 1976; Mirocha et al., 1977; Tanaka et al., 1986; Vesonder et al., 1978).

Albeit grain-associated microbes and their metabolites may confer adverse effects on barley gains and on subsequent processed products, the positive contribution of microbes on grain properties and characteristics is also of significance. Many grain-associated microbes produce hormones (e.g. gibberellic acid and abscisic acid) and various enzymes (Flannigan, 2003). Species of *Fusarium* have been reported to produce gibberellins enhancing barley growth (Flannigan, 2003; Haikara, 1983).

Several microbes are known to produce proteolytic, amylolytic and cell wall degrading enzymes, which confer positive effects on malt characteristics and represent great potential for improvement of the grain nutrients for feed (Hoy et al., 1981; Flannigan et al., 1982; Yin et al., 1989). Moreover, microbes may also contribute to the nutritional value of cereal grains by degrading anti-nutritive compounds (e.g. arabinoxylans) and consequently enhance the availability of nutrient components.

1.20. PROTEOMICS

Large-scale gene expression analysis has lead in generation of vast amount of gene sequences of unknown functions. The genome is the collective for all of the genetic material in a cell. However, in order to elucidate gene function, it is of necessity to investigate the gene product, the protein. The proteome is the collective term for the entire protein complement produced from the genetic material/genome in a given cell, tissue, or organism at a particular time under a particular set of conditions (Wilkins et al., 1996). Thus, proteomics implies a large-scale, systematic and comprehensive analysis of protein expression and regulation, together with their abundance and localization, post-translational modifications, structures and interactions, Figure 8 (Koomen et al., 2005; Rabilloud et al., 2008). Proteomics dates back to the 1970s with development of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and further advancement in the 90s with the advent of mass spectrometry (O'Farrell, 1974).

In general, proteomics comprehends two distinct areas, i.e. expression proteomics and functional proteomics. Expression proteomics entails establishment of quantitative maps of protein expression under specific physiological or developmental conditions, while functional proteomics focuses on the function, structure and activity of individual proteins and their interactions. Several proteomic approaches is available both gel-based and gel-free, all with inherent strengths and limitations. Implementations of the various strategies differ depending on the complexity of the sample and the objective of the analysis, and the various approaches are used complementary.

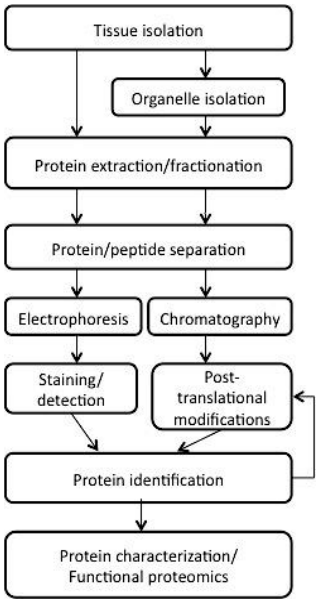

Figure 8: General overview over the experimental proteomics workflow. The classic proteomics workflow is adapted toward a targeted analysis of proteomes.

1.20.1. MASS SPECTROMETRY

With development of instrumentations and emerging technologies, mass spectrometry has become an indispensable technique in protein chemistry. Briefly, mass spectrometers (MS) consist of three distinct features: an ion source enabling conversion of analyte molecules into gas phase ions, a mass analyser which enable separation of ions based upon their mass-to-charge (*m/z*) values or ion velocity and a detector system to record the relative abundance of each ion type. The two most commonly applied ionization techniques are matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), which are used to volatize and ionize proteins or peptides. The mass analyzers measure the mass to charge ratio (*m/z*) of the ionized analytes. Different mass analyzers are available including time-of-flight (TOF), ion trap, orbitrap, quadruple and fourier transform ion cyclotron (Aebersold and Mann, 2003; Rabilloud, 2000). These analyzers can be used alone or together in tandem (MS/MS). In a single mass analyzer, peptide ions are

generated, and in tandem MS, specific precursor ions produced in the initial analyzer are selected for fragmentation into product/daughter ions.

1.20.2. GEL-BASED PROTEOMICS APPRAOCH

Proteomes are analyzed either by using gel-based or gel-free methods. The standard workflow of proteome analysis involves sample preparation, protein separation, enzymatic digestion, and protein identification by mass spectrometric analysis Figure 8. The classical proteomic approach is gel-based and dates back to 1975 with development of two-dimensional gel electrophoresis (2-DE) for separation of proteins according to their isoelectric point (pI) and molecular weight (Mw) (O'Farrel, 1975). With advent of mass spectrometry (MS) for determination of protein identity combined with enhancing amounts of sequence information databases, gel-based proteomics has established itself as an indispensable technology/tool for interpretation of information from the genome (Aebersold and Mann, 2003).

In gel-based proteomic approach, the extracted proteins are separated by 2DE, stained with dye, subjected to image-analysis, proteolytic digestion and protein identification by MS. The resolved protein pattern can be visualized by Coomassie Brilliant Blue or silver staining. Albeit, 2-DE technique enable establishment of high-resolution proteome maps and its uniqueness in displaying post translation modifications, the technique has some limitations including low reproducibility and poor detection of specific proteins e.g. low abundant proteins, highly acidic/basic proteins and hydrophobic proteins (Ong and Mann, 2005). In addition to the colorimetric dyes, application of the fluorescent Sypro Ruby staining method enables detection of the lower abundance proteins. As an alternative, protein samples can be labeled with fluorescent Cy-dyes (Cy2, Cy3 or Cy5) prior to separation by 2-DE. In two-dimensional differential in-gel electrophoresis (2D-DIGE) the samples are pre-labelled with one of the Cy-dyes (Cy2, Cy3 or Cy5) and multiplexed within the same gel. Main advantages are the improvement of accuracy of protein quantification and reproducibility due to elimination of gel-to-gel variability (Monteolivia and Albar, 2004). More targeted protein identification includes the use of 1D- and 2D-immunoblotting, in which the separated protein on the gel are transferred onto a membrane (e.g. nitrocellulose of polyvinylidene difloride, PVDF) and probed with the antibody

specific to the target protein. However, this technique is limited to the availability of the specific antibody. 1D- and 2D-immunoblotting has also been applied in the presented research work in chapter 3−4.

1.20.3. GEL-FREE PROTEOMICS APPROACH

With development of instrumentations, emergence of technologies and cumulative sequence information databases, proteome analysis has advanced to gel-free methods with application of liquid chromatography methods (LC) for separation of complex protein and peptide mixtures prior to introduction into the MS.

To circumvent some of the shortcomings of gel-based methods, various MS-based approaches have emerged. One approach is the application of multi-dimensional protein identification technology (MudPit), where digested protein mixture is separated by multi-dimensional chromatography prior to MS/MS (Link et al., 1999). The main drawback of this method is the lack of providing quantitative information. For comparative quantitative analysis, methods using labeling strategies, such as isotope-coded affinity tag (ICAT) or isobaric tags for relative and absolute quantification (iTRAQ) can be applied, where samples are differentially labeled with isotope-coded tags with varying mass prior to analysis by LC MS/MS. The tags label the cysteine residues within proteins. The relative protein abundance between samples is determined by the ratio of the signal intensities of the isotopically labelled peptide pairs revealed in the MS spectra (Gygi et al., 1999; Shadforth et al., 2005). Alternatively, label-free quantification strategies have emerged, in which relative quantitative information is inferred from peptide identification frequency or spectral counts (the number of acquired MS/MS spectra for a protein or peptide). Spectral counting is based on the assumption that the number of spectra is correlated linearly with protein abundance in a mixture. Thus, peptides from more abundant proteins will be more selected for fragmentation and accordingly produce a higher number of MS/MS spectra. The averaged spectral counts are used as protein abundance index for relative protein quantification (Liu et al., 2004; Ong and Mann, 2005). This method is reliable and biased toward large and abundant proteins, while the number of peptides from small protein and low abundant proteins are often insufficient for accurate quantification (Gstaiger and Aebersold, 2009).

1.20.4. PLANT PROTEOMICS

Gel-based proteomics has been the main approach applied in the field of plant proteomics, with successful use of 2DE and/or 2D-DIGE in the investigation pathogenesis-related proteins in Medicago truncatula in response to *Sinorhizobium meliloti* (Schenkluhn et al., 2010; Van Noorden et al., 2007) and the impact of abiotic stress, such as frost on Arabidopsis thaliana (Li et al., 2010), drought in oak (Sergeant et al., 2011) and heavy metals in poplar (Kieffer et al., 2008). Additionally, cereal grain proteins have also been extensively studies, in particular barley, wheat, rice and maize (Agrawal and Rakwal, 2006; Finnie and Svensson, 2009; Méchin et al., 2004; 2007; Skylas et al., 2005). Many studies have provided catalogues of proteins (Østergaard et al., 2004; Méchin et al., 2004), outlined temporal changes in protein abundances during seed development and germination (Østergaard et al., 2002; Bak-Jensen et al., 2004; Finnie et al., 2002; 2006; Méchin et al., 2007), a comparison of the proteomes of different seed tissues (Finnie and Svensson, 2003; Bønsager et al., 2007) or an analysis of changes induced by stresses such as heat (Skylas et al., 2002; Hurkman et al., 2009; Laino et al., 2010) salt (Witzel et al., 2010) or pathogen attack (Zhou et al., 2006; Yang et al., 2010).

Cereal grains pose challenges for extraction and analysis of proteins due to the high storage protein and starch content, which may have limited the progress towards more in-depth analyses. Many proteomic studies do not go beyond the mass spectrometric identification of proteins of interest, in which case no functional information about the identified proteins is obtained. Fractionation and enrichment techniques decrease sample complexity and enable analysis of lower-abundance proteins. To gain a deeper insight into cereal grain proteomes and functional information about the identified proteins, targeted proteomics approaches have been applied. The classical proteomics form the basis for targeted analyses, though specifically tailed for the sub-proteome of interest. Combining proteome analysis with protein chemical techniques such as activity assays, immunofluorescence microscopy, production of recombinant proteins and protein structural analysis can also be a powerful approach for analysis of selected proteins or groups of proteins. In this thesis, targeted proteomics and meta-proteomics approaches have been applied to provide a more in-depth view of barley grain proteomes, surface-associated proteomes and xylanase inhibitors.

1.20.5. TARGETED PROTEOMICS IN ANALYSIS OF XYLANASE INHIBITORS

The xylanase inhibitors in cereal grains provide a good illustration of the targeted use of proteomics for understanding these complex functional groups of proteins. All three types of xylanase inhibitors have been detected in whole meals of cereals, such as wheat, durum wheat, barley and rye (Elliot et al., 2003; Gebruers et al., 2002a) as well as maize and oat (Beaugrand et al., 2006). Xylanase inhibition activity assays combined with immunoblotting on extracts from wheat kernels demonstrate considerable variation in inhibitor levels among different wheat cultivars (Beaugrand et al., 2006; Croes et al., 2009b; Gebruers et al., 2002b). The three-dimensional structures of TAXI, XIP and TLXI-type inhibitors extracted from wheat have been determined with application of X-ray crystallography analysis and NMR (Payan et al., 2004; Sansen et al., 2004; Vandermarliere et al., 2010). In addition, the structures of TAXI-I and XIP-I in complex with fungal xylanases have also been resolved and key residues involved in the interaction between the inhibitors and target proteins determined (Juge, 2006).

Application of gel-based proteomics approach demonstrated the presence of TAXI and XIP proteins in wheat as polymorphic families (Croes et al., 2008). Moreover, 2D-DIGE approach coupled with LC-ESI tandem MS in extracts from six different wheat cultivars enabled differentiation between TAXI, XIP and TLXI inhibitor isoforms and quantified their variation among the different cultivars (Croes et al., 2009a).

Analysis of wheat milling fractions revealed that all three inhibitors were highly abundant in the outer layers of wheat kernels, in particular in the caryopsis. Gebruers et al. (2002b) reported 2-3 folds higher inhibition activities in wheat shorts and bran fractions than in flour. A study by Croes et al. (2009b) detected that the highest concentrations of these inhibitors occur in the aleurone layer. A study Jerkovic et al. (2010) revealed that the spatial distribution of TAXI and XIP in wheat grains was predominantly found in nucellar tissue adjacent to the aleurone tissue by using proteomics combined with immunofluorescence microscopy (Jerkovic et al., 2010). Proteomics in combination with classical protein chemistry, immunochemistry and activity measurements provide a detailed picture of a complex protein family.

1.20.6. METAPROTEOMICS

In the past year, studies of various microbial communities in their environment/habitats have provided increased knowledge of the microbial composition and diversity. Metagenomics is an approach to analyze the genomic DNA (metagenomes) of microbial communities recovered from their environmental habitats. Application of metagenomics circumvents the need to isolate and culture individual microbial species, as well as unravel the hitherto uncultured microbial species and potentially discovery of new genes. In order to enhance the understanding of how the microbial community operates and interacts with its habitat, and to link the composition of the microbial community to their functions, the post-genomic era have been extended to the emergence of metaproteomics.

Metaproteomics (microbial community proteomics) has been defined as the large-scale characterization of the entire protein complement of environmental microbiota at a given point in time (Wilmes and Bond, 2004). It basically implicates proteome analysis of complex biological systems in co-existence and being functionally interconnected. The microbial populations comprise a diverse, variable and highly dynamic mixture of microbes at a given point of time. Thus, the metaproteome simply portrays “snap-shots” of the highly dynamic microbial community. In the plant microbe-pathology field, proteomics has been applied to study microbial interactions. One of the limitations of metaproteomics is the insufficiently availability of sequence data (complete genomes and metagenomes) (Wilmes and Bond, 2006).

Integration of proteomics data with information generated from classical approaches has become useful in understanding the mechanisms involved in the plant-microbe interaction. Proteomics of microbial communities have employed both gel-based (1D and 2D) and gel-free (liquid chromatography) proteomics (O'Farrell, 1974; Klose, 1975; George et al., 2010). Today, a proteomics approach is far more complex than simple cataloguing of the present proteins. Combined with molecular techniques, proteomics also covers inquiring ranging from quantification of protein expression between different conditions, and post-translational modifications to structural information of the protein.

To date, a small number of studies have employed a metaproteomics approach to investigate the structural and physiological properties of microbial consortia present in a specific environment or ecosystem such as leaf phyllosphere, activated sludge, contaminated soil and ground water, ocean water, microbial biofilm (underground sites in Richmond mine at iron mountain, California) and human gastrointestinal tract, (Dill et al., 2010; Klaassens et al., 2007; Klose, 1975; Ram et al., 2005; Wilmes and Bond, 2004; 2006). In the present thesis, a metaproteomic approach is implicated to investigate the populating fungal community present on different barley cultivars and portray the surface-associated proteome of barley kernels.

1.21. SCIENTIFIC AIMS OF THE WORK

Barley is a major cereal crop with diverse applications ranging from feed to malt, thus there is a great interest in unraveling grain enzyme activities, which are determinants of grain nutritional quality, and exploiting these for improvement of grain nutritional components for feed. The mature barley grain contains a complement of enzymes that are synthesized during seed development for degradation of seed storage reserves during germination. These enzymes (first wave enzymes) are of fundamental biological importance as they ensure the regular progression of germination, but they are also major quality determinants for feed as well as food, as they will be activated during processing or in the digestive tract. The present thesis is composed of four main objectives/stories, which to some extent are interconnected, and targeted towards gaining more knowledge about the proteome and enzymatic activities of barley grains, as well as portraying the surface associated proteins and the indigenous fungal community of the grains, in order to explore their potential and exploit these for improvement of nutritional properties of barley for better feed.

In this thesis, several aspects of elucidating grain and fungal enzyme activities are addressed with application of proteomics and metaproteomics approaches coupled with molecular biochemical characterization. Firstly, a gel-based proteomics approach was applied to profile the proteome of the liquid feed and study the changes during the incubation period. Next, a comparative analysis was performed to study the changes in proteome profiles upon application of protease inhibitors and the thioredoxin

(Trx/NTR) system. Secondly, 2-DE based proteomics approach coupled with enzymatic assays was performed to investigate xylanase (plant and microbial) and xylanase inhibition activities and their variation among different barley cultivars of two different harvest years at four different growing sites in Denmark. Thirdly, a metaproteomics approach was applied to portray the composition of the indigenous fungal community populating different barley cultivars and their correlation with 1D-protein profiles and xylanolytic activities. The secretomes of two frequently occurring cereal fungi were mapped. 2-DE was also carried out to profile and identify the surface-associated proteins of two different barley kernels. Lastly, molecular and biochemical characterization was performed on recombinant xylanase inhibiting protein (XIP-III) from barley to assess inhibition activity against microbial xylanases and cereal associated fungal supernatants. The performed work has been described in the following chapters serving as manuscripts to be submitted.

Chapter 2 - describes the proteome (pH 4−7) of the liquid feed over a steeping period of 48 h using 2-DE. With establishment of a reference proteome, a comparative analysis was performed to study the changes in proteome profiles upon application of protease inhibitors and the thioredoxin system (HvTrx/NTR). Protease inhibitors to some extent inhibited degradation of storage protein during the steeping period. Application of the thioredoxin system resulted in increased solubility of enzyme inhibitors (chloroform/methanol inhibitor proteins), storage proteins of the endosperm and embryo, as well as amylases and protease inhibitors, which are known targets of thioredoxin. We also identified the outer membrane protein X, which could be an addition to the already known Trx targets.

Chapter 3 - explores the impact of harvest year and growing site on the variability of xylanases and xylanase inhibition activities in different barley cultivars (genotype vs. environmental factors) using activity assays, 1D- and 2D-immunoblots coupled with MALDI-TOF MS. Considerable inter-cultivar variation was found at microbial and endogenous xylanase and xylanase inhibition activity levels. It was reported that the harvest year/environment had a significant effect on both the endogenous and microbial xylanases, as well as xylanase inhibition activities. 1D and 2D-western blots

probed with anti-XIP, -TAXI and -TLXI polyclonal antibodies enabled identification of the various xylanase inhibitors in the barley cultivars (Barke, Cabaret, Prolog, Power, Simba, 1978). We found that the microbial xylanase activity and xylanase inhibition activity levels seemed to be correlated for two of the screened barley cultivars, namely 1978 and Power, which had high xylanase inhibition activity levels and low microbial xylanase activity levels. These two cultivars were also found to display an additional band in the 1D-immunoblots probed with anti-XIP.

Chapter 4 - describes application of a metaproteomics approach to characterize the composition of the indigenous fungal community populating different barley cultivars and their correlation with 1D-protein profiles and xylanolytic activities. The secretomes of two frequently occurring cereal fungi in the field and at storage, respectively, were also mapped using 2-DE. The surface-associated protein complement of barley kernels was profiled using 2-DE (pH 3−10). Characterization of the fungal community of different barley cultivars by culture-based methods coupled with protein profiles and xylanase activities measurement accordingly enabled grouping of barley cultivars. The mapping of the secretomes of *F. poae* and *A. niger* enabled identification of extracellular proteins in response to two carbon sources, i.e. barley flour and wheat arabinoxylan.

Chapter 5 - describes production, expression and purification of the recombinant xylanase inhibitor protein III (rXIP-III). Biochemical characterization was carried out to assess inhibition activity against commercially available microbial xylanases, washing liquids containing mixture of xylanases/enzymes found on barley surface, and supernatants of the isolated and cultivated cereal fungi. The preliminary dataset shows that the rXIP-III is active and exhibits varying degree of inhibitory action on the tested samples.

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CHAPTER 2

Establishing the protein profile of liquid feed over the steeping period and analysis of application of protease inhibitors and NADPH-dependent thioredoxin reductase/thioredoxin (NTR/Trx) system.

2. ABSTRACT

There has been an increase in application of liquid feed, comprising of barley mixed with water and an incubation period prior to delivery to animals, instead of dry feedstuffs. It is suggested that the steeping period softens the grains and initiate mobilization of seed storage reserves, thereby maximizing nutrient absorption by the animals. The present study set out to profile the water-extractable and water-unextractable protein fractions of liquid feed over the 48 h of steeping period using gel-based proteomics approach. Moreover, we also assessed the protein changes of liquid feed upon application of protease inhibitors to prevent and/or minimize protein degradation taking place during the steeping period. Comparative proteomics analysis of both the water-extractable and water-unextractable protein fractions revealed identification of several full-length proteins in feed containing protease inhibitors, which were decreased in abundance in absence of inhibitors. Thus suggesting that application of protease inhibitors to some extent inhibited seed protein degradation.

Monogastric animals are unable to efficiently utilize grain nutrients including nitrogen from the proteins (estimated that approximately half of the nitrogen is used when fed a cereal-based diet). Thus in the past years several strategies have been devised and applied with the aim of enhancing nutrient availability. Thioredoxins (Trxs) play an important role in germination of cereals by regulating the redox state of proteins. It has been suggested that Trx reductively increase protein solubility and thereby increase their susceptibility to proteolysis. Trxs have been reported to target various proteins and change the activity of several enzymes. In this study, comparative proteomic analysis was carried out to track the protein changes upon application of the NADPH-dependent thioredoxin reductase/Trx system in liquid feed. Prominent changes were found in the water-extractable fraction with identification of a wide range of proteins, including low molecular weight disulphide proteins (e.g. chloroform/methanol soluble inhibitor proteins), enzyme inhibitors and stress-related proteins. Identification of these proteins, which are known Trx targets, support the view that Trx reductively increase solubility of a wide collection of seed proteins. Additionally, spots that increased in abundance in the water-unextractable protein fraction of liquid feed containing NTR/Trx were identified as seed storage proteins

and enzyme inhibitors. Two protein spots were identified as the outer membrane protein X (2 cysteine residues), not previously recognized as Trx target. The results suggest that application of the NTR/Trx system to liquid feed significantly alters the protein profiles and increase the solubility of wide range of seed proteins.

2.1. INTRODUCTION

Cereals crops are major source of feed for livestock. In the feeding industry, there has been an increase in the use of liquid feeding systems instead of dry feedstuffs. The system is based on feed mixed with water and an incubation period until delivery to the livestock. It is suggested that the steeping period softens the grains and mobilizes nutrient components making them available for digestion. A review by Jensen and Mikkelsen (1998) over different feeding studies revealed that pigs fed liquid diet generally had an improved weight gain and feed conversion ratio. Numerous advancements have been implemented to improve the nutritional value of feed with application of microbial enzymes, essential amino acids (e.g. lysine and methionine) and minerals (e.g. phosphate).

Cereal crops are highly valuable for the nutrition of livestock due to the high percentage of carbohydrates, storage proteins, starch, fatty acids and vitamins. Barley seeds contain relatively low amount of protein compared to other crops e.g. legumes. An average of 10-12% of the dry weight barley seed constitute of protein of which 80% represent storage proteins and the remainder 20% metabolically active proteins (Shewry and Halford, 2002). According to the Osborne classification, seed proteins have been classified into four groups based on their solubility, i.e. (1) the water-soluble albumins, (2) the salt-soluble globulins, but water-insoluble, (3) the dilute acid- and alkali-soluble glutelins, and (4) alcohol-soluble prolamins also named hordeins, respectively (Bewley and Black, 1994). The prolamins have been further sub-classified into sulfur-rich and sulfur-poor hordeins on the basis of their amino acid sequence and composition. In barley seeds, the major storage proteins in the endosperm are the hordeins, while in other cereals like wheat it is the glutelins and globulins. The hordeins are deficient in essential amino acids, e.g. lysine, therefor barley seeds are often mixed with other sources of amino acids for animal feed (Shewry, 2007; Shewry et al., 1979).

The hordiens are synthesized and accumulated during seed development and broken down during germination (Brandt et al., 1976; Rahman et al., 1982; Shewry et al., 1979). Seed germination commences upon imbibition and terminates with elongation and protrusion of the radicle through the seed coat. Germination involves activation

and *de novo* synthesis of hydrolytic enzymes (e.g. amylases, glucans, limit dextrinases and proteases) in the aleurone layers that can degrade cell walls and starch, and mobilize reserve storage proteins and carbohydrates accumulated in the endosperm to sustain the growing embryo. The protein reserves are mobilized by proteases released from the aleurone layer as well as by the pre-formed proteases already present in the endosperm (Bewley and Black, 1994). Proteases play a vital role in plant physiology by controlling the synthesis, turnover and function of proteins (Turk, 1999). Proteases have been classified on the basis of the functional groups present at the active site and on the catalytic mechanism into four groups, i.e. serine, cysteine, aspartic, and metallo-proteases (Hartely, 1960). Proteolytic activities have been reported to peak during the germination. The cysteine proteases have been shown to be responsible for 90% of the total proteolytic activities in mobilization of storage proteins in germinating barley seeds. The cysteine proteases are found as pro-enzymes and are activated upon reduction of the cysteine in the active site and cleavage of the pro-domain (Davy et al., 1998). A study by Zhang and Jones (1995) reported identification of forty-two peptidases in germinating barley seeds of which 27 were cysteine peptidases. It was suggested that most of these proteases were involved in mobilization of storage proteins, while some were constitutively present (house-keeping enzymes) also in ungerminated seeds. The cysteine-class proteases also play a predominant role in protein solubilisation in malting barley (Zhang and Jones, 1996). A study by Weiss et al. (1995) showed that the albumins and globulins undergo partial degradation, while the hordeins are relatively rapidly degraded during the malting process. It has been suggested that the cysteine endo-peptidase and carboxylase exo-peptidase act in concert to degrade the storage proteins (Bewley and Black, 1994). More studies are needed to examine and clarify the role of metallo- and aspartic proteases in protein solubilisation.

Proteases are indispensible to the plant, however they can be potentially detrimental when overexpressed (e.g. cause premature sprouting) thus their activities need to be closely and correctly regulated. A means of regulation involves modulation of their activities are by proteinaceous protease inhibitors, which are widely distributed in various tissues of the grain. In general, proteases inhibitors are presumed to be a tool of defense against proteolytic enzymes of the invading microorganisms and insects, as

well as in the animal gut. Beside the regulatory role in proteolysis (Haynes and Feeney, 1967; Warchalewski and Skupin, 1973), they have been hypothesized to confer an inhibitory effect toward autolysis during dormancy (Mansfeld et al., 1959). Despite being highly abundant, the exact physiological function of protease inhibitors still remains to be clarified. Protease inhibitors have been implicated to confer resistance against fungal disease (e.g. *Fusarium* spp.) by inhibiting secreted fungal proteases (Martinez et al., 2003; Pekkarinen et al., 2003). A study by Carrillo et al. (2011) reported a protective role against invading microorganisms and insect pests. Barley serpins (serine protease inhibitors) have been reported to function as defense-related proteins, and on the other hand they act as storage proteins, due to the fact that the serpins are synthesized and deposited during seed development (Finnie et al., 2002). Albeit the synthesis of serpins is not up-regulated upon insect/microbe invasion, they are still considered as pathogen-related proteins (PR-6). Some protease inhibitors i.e. BASI (barley α-amylase/substilisin inhibitor) have been shown to target two different proteins i.e. proteases and/or α-amylases, suggesting possible regulatory function of the inhibitors with endogenous barley proteins (Mosolov et al., 2001).

Thioredoxins (Trxs) are small ubiquitous disulfide reductases involved in many cellular processes by facilitating reduction of other proteins by cysteine disulfide exchange. Thioredoxins have been found in organisms from plants, mammals to bacteria. For most organisms, Trxs are reduced enzymatically by NADPH-dependent thioredoxin reductases (NTR) using NADPH (Williams, 1976). In plants, this tripartite system is highly complex with presence of a diverse selection of Trx isozymes and their temporal- and organelle-specific expression. Additionally, some plants Trxs are reduced by ferredoxin thioredoxin reductase (FTR) and ferredoxin or glutathionine thioredoxin reductase (GTR) (Buchanan, 2001; de la Torre et al., 1979; Gelhaye et al., 2003;). Plant Trxs are involved in photosynthesis, seed development and germination. During seed germination, the cytosolic Trx h facilitates mobilization of reserves via reduction of intramolecular disulfide binds in storage proteins of endosperm, and accordingly increasing their solubility making them more susceptible to proteolysis. Thioredoxins also change the activity of various proteases and amylolytic enzymes (e.g. α-amylase) either directly by reduction or indirectly by inactivation of specific inhibitor proteins (Buchanan, 2001; Besse et al., 1996; Kobrehel et al., 2002; Lozano

et al., 1996; Montrichard et al., 2003; Wong et al., 2004). Reductively inactivating inhibitors of proteases and amylases or activating specific proteases (i.e. thiocalsin) all aim toward breakdown of protein and starch reserves during germination (Besse et al., 1996; Kobrehel et al., 2002). In barley seeds, two forms of each Trx h and NTR have been identified with overlapping spatio-temporal appearance (Maeda et al., 2003; Shahpiri et al., 2008; 2009). Application of proteomics approaches has enabled identification of a wide range of Trx target proteins (Hägglund et al., 2008; Maeda et al., 2003; Wong et al., 2004). A study by Wong et al. (2002) on germinating grains from transgenic barley with overexpression of Trx in the endosperm reported an increase in protein solubility as an effect of thioredoxin. Another study also described the role of Trx in modification of the protein solubility using methanol-soluble and insoluble protein fractions of wheat endosperm (Wong et al., 2004). Facilitating mobilization of the protein and starch reserves or increasing protein solubility is of great interest for feed.

In general, there is a lack of knowledge on susceptibility of the dietary proteins as well as the supplemental enzymes to the proteolytic activities taking place in feed over the steeping period and once in the gastrointestinal tract of animals. In view of this, the aim of the present study was to profile the proteome of the liquid feed over steeping period of 48 h using 2-DE (pH range 4-7), and a comparative study of liquid feed with application of protease inhibitors. Protease inhibitors were applied to liquid feed with the purpose of inhibiting and/or inactivating the proteolytic activities arising during the steeping period and by the microbial inhabitants of the grains. The obtained proteomes of liquid feed over steeping period (onset of germination) were used to track protein mobilization and degradation, and to gain an understanding of the protein abundance dynamics associated with implication of protease inhibitors. Similarly, a gel based proteomics approach was applied to study the alterations in protein solubility of barley by the thioredoxin system (NTR/Trx). The proteome profile of both the water-extractable and water-unextractable protein fractions were analyzed and compared with the control proteomes, and protein spots differing in relative intensity were selected for identification.

2.2. MATERIALS AND METHODS

2.2.1. FEED PREPARATION, PROTEIN EXTRACTION AND 1D-GEL ELECTROPHORESIS

Barley-based diets were made from mature barley seeds (cv. Keops). Four biological replicates of each treatment were prepared for profiling the protein pattern during steeping period and to investigate the effect of protease inhibitors and the thioredoxin system. The experiment was divided into eight parts (two time points x four treatment). Each part comprised of barley mixed with either water, protease inhibitors in DMSO (dimethyl sulfoxide), DMSO (control for the effect of DMSO) or NTR/Trx h. Each combination of sample treatment and incubation time was studied in quadruplicate. Intact barley kernels were ground to flour (roller mill 2 mm), where 100 mg of flour was transferred to 2 mL eppendorf tubes and mixed with 275 µL water and subsequently supplemented with either 17 µL water, protease inhibitors in 5% (v/v) DMSO, DMSO and NTR/Trx h (0.03 mg NTR, 0.06 mg Trx and 0.06 NADPH). Two glass beads were added to aid homogenization and the samples were incubated for 72 h at 20°C under continuous agitation. Samples were withdrawn after 2 and 48 h (based on preliminary experiments). Constant incubation temperature of 20°C was chosen, as it has been found that optimal fermentation of liquid feed can be achieved at that temperature (Jensen and Mikkelsen, 1998). Samples were centrifuged at 20,000 x g for 30 min at 4°C and the supernatants were transferred to new tubes. Both the pellets and supernatants were stored at -80°C until use. For preparation of re-extracts, the pellets were dried under vacuum and ground to fine powder using a ceramic mortar and pestle. The powder was resuspended in 1 mL extraction buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1% (v/v) immobilized pH gradient (IPG) ampholytes 4−7, 200 mM Destreak reagent (hydroxyethyl disulfide), 2% (v/v) glycerol, 5% (v/v) isopropanol) containing protease inhibitor cocktail “complete” (Roche), and incubated for 1 h at 4°C under continuous agitation followed by centrifugation at 20,000 x g for 30 min at 4°C to pellet debris. The supernatants were collected and protein concentrations were quantified by the method of Popov (Popov et al., 1975) using bovine serum albumin (BSA) as standard.

2.2.2. 1D-GEL ELECTROPHORESIS OVER WATER-EXTRACTABLE AND WATER-UNEXTRACTABLE PROTEIN FRACTIONS (SUPERNATANT AND PELLET)

For SDS-PAGE analysis ca. 10 µL of the water-extractable and water-unextractable protein fractions were mixed 1:4 ratio with 4 x LDS sample buffer (2% (w/v) LDS (lithium dodecyl sulphate), 106 mM Tris-HCl, 141 mM Tris-base, 10% (w/v) glycerol, 0.51 mM EDTA, 0.22 mM SERVA blue G-250, 0.175 mM phenol red, pH 8.5), incubated at 95°C for 5 min and separated on 4-12% Bis-Tris NuPAGE gels (Novex system, Invitrogen) using NuPAGE MES running buffer (50 mM Tris Base, 50 mM MES (2-(N-morpholino) ethane sulfonic acid), 1 mM EDTA, 3.5 mM SDS, pH 7.3). Subsequently, the gels were stained with Coomassie Brilliant Blue solution (10% (w/v) ammonium sulphate, 10% (v/v) phosphoric acid, 20% (v/v) ethanol, 0.12% (w/v) colloidal Coomassie Brilliant Blue (CBB) G-250) (Candiano et al., 2004). A broad range protein ladder (Mark 12, unstained standard, Invitrogen) was used for molecular mass determination.

2.2.3. ISOELECTRIC FOCUSING AND TWO-DIMENSIONAL GEL ELECTROPHORESIS (2-DE)

For two-dimensional gel electrophoresis (2-DE) of water-extractable and water-unextractable protein fractions, a volume corresponding to 250 µg of protein was precipitated by addition of 4 volumes of acetone at incubated at -20°C overnight. Precipitated proteins were dissolved in 350 µL of rehydration buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IPG ampholytes (pH 4–7), 200 mM Destreak reagent (GE Healthcare) and a trace of bromophenol blue. Isoelectric focusing (IEF) was performed on 18 cm IPG strips with a linear gradient of pH 4-7 using an Ettan IPGphore (GE Healthcare) for a total of 50,000 kVh (4 h at 150 V, 5 h at 300 V, 1 h at 500 V, 1 h at 1,000 V, gradient to 8,000 V, hold at 8,000 V until a total of at least 50 kVh was reached). The IPG strips were sequentially equilibrated for 2 x 15 min in equilibration buffer (6 M urea, 2 % (w/v) SDS, 30 % (v/v) glycerol, 50 mM Tris-HCl pH 8.8, a trace of bromophenol blue) supplemented with 10 mg/mL dithiothreitol (DTT) and 25 mg/mL iodoacetamide (IAA) in the first and second

equilibration step, respectively. Second dimension, SDS-PAGE (12−14%, 18 cm × 24 cm, GE Healthcare), was performed on a Multiphor II (GE Healthcare) according to the manufacturer's recommendations. Gels were stained with either colloidal Coomassie Brilliant Blue G-250 (Candiano et al., 2004) or silver nitrate (Heukeshoven and Dernick, 1985).

2.2.4. IMAGE ANALYSIS

The gel images (grey scale, 16 Bit) were analyzed by the image analysis software Progenesis SameSpots v4.0 (Nonlinear Dynamics Ltd, Newcastle upon Tyne, UK). The gel images were warped, matched and aligned to a selected reference gel by automated calculation of ten to fifteen manually assigned alignment landmark vectors. Four images representing four biological replicates for each of the eight conditions were grouped to obtain the average volume of each spot. The spot volumes were normalized across different gel images automatically by the software. A 1.5-fold threshold (spot volume ratio change, ANOVA $p \leq 0.05$, power > 0.8 and a false discovery rate of $q \leq 0.05$) was chosen as criterion in the identification of differentially abundant protein candidates.

Power analysis was applied to calculate the minimum sample size required to accept the outcome of a statistical test with a particular level of confidence (Karp et al., 2005). The experimental set up had enough statistical power with the four replicate gels. False discovery rate (5%) estimates the number of false positives within statistically significant changes in the experiment (Karp et al., 2007). Principal component analysis (PCA) and cluster analysis (dendrogram) was applied to compare and analyze the protein patterns among gels and the expression profiles of protein spots fulfilling the above criteria.

2.2.5. IN-GEL ENZYMATIC DIGESTIONS AND MASS SPECTROMTRY

Spots on the gels were excised manually and subjected to in-gel tryptic digestion with minor modifications as described below (Hellman et al., 1995). Briefly, the gel pieces

were washed with 100 µL 40% ethanol (10 min), shrunk in 50 µL 100% ACN and soaked in 2 µL 12.5 ng/µL trypsin (Promega, porcine sequencing grade) in 25 mM (NH_4HCO_3) on ice for 45 min. Gel pieces were rehydrated by addition of 10 µL 25 mM NH_4HCO_3 followed by incubation at 37°C overnight. Tryptic peptides (1 µL) were applied to the AnchorChip^{TM} target plate (Bruker-Daltonics, Bremen, Germany), covered by 1 µL matrix solution (0.5 µg/µL α-cyano-4-hydroxycinnamic acid (CHCA) in 90% ACN, 0.1% TFA) and washed in 0.02% TFA. The tryptic peptides were analyzed by Ultraflex II MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) using Flex Control v3.0 (Bruker-Daltonics, Bremen, Germany) and processed by Flex Analysis v3.0 (Bruker-Daltonics, Bremen, Germany). Peptide mass maps were acquired in positive ion reflector mode with 500 laser shots per spectrum. MS/MS data were acquired with an average of 1000–2000 laser shots for each spectrum. Spectra were externally calibrated using a tryptic digest of β-lactoglobulin (5 pmol/µL). Internal calibration was performed using trypsin autolysis products. Filtering of peaks was also carried out for known keratin. Mass spectrometry (MS) and tandem MS (MS/MS) spectra were analyzed using Biotools v3.1 (Bruker-Daltonics) and searched against the NCBInr (National Center for Biotechnology Information) and HvGI (The Institute for Genome Research - TIGR) barley gene index Release 12.0 (http://compbio.dfci.harvard.edu/tgi) databases using the MASCOT 2.0 software (http://www.matrixscience.com). The following search parameters were applied: Taxonomy: Viridiplantae (Green plants); monoisotopic peptide mass accuracy of 80 ppm, fragment mass accuracy to $\pm$ 0.7 Da; a maximum of one missed cleavage; carbamidomethylation of cysteine and partial oxidation of methionine, respectively. No restrictions with respect to protein Mw and pI were made. The signal to noise threshold ratio (S/N) was set to 1:6. Protein identifications by peptide mass fingerprint (PMF) were confirmed with a MASCOT score of 70, $p \leq 0.05$ and a minimum of 4 matched peptides. Single peptide based protein identification by MS/MS was confirmed with a MASCOT score of 40, $p \leq 0.05$.

Protein sequences were assessed for the presence of cysteine residues using CYS_REC release 2 (http://linux1.softberry.com/berry.phtml).

2.3. Results and Discussion

2.3.1. Amount of water-extractable and water-unextractable protein fractions from the liquid feed samples supplemented with protease inhibitoirs

The water-extractable and water-unextractable proteins were extracted from the same amount of flour supplemented with protease inhibitors in DMSO (dimethyl sulfoxide), DMSO or water, respectively, at 2 and 48 h of incubation. The protein quantifications show that the amount of water-extractable proteins remained relatively steady throughout the 48 h steeping period, Table 2. A slight increase was found when comparing water-extractable proteins of samples containing protease inhibitors (4.47/4.45 µg/µL at 2 and 48h) and DMSO (4.60/4.42 µg/µL) with the controls (4.23/4.09 µg/µL), respectively (Table 2, Figure 9 A). This could be attributed to co-extraction of water-insoluble proteins due to application of ca. 5% (v/v) DMSO, which is an organic solvent used to solubilize the protease inhibitors. Apparent differences were observed when comparing the water-unextractable protein fractions, where there was a significant increase in the amount of extracted proteins over time (Figure 9 B). Furthermore, significant differences were also found between the samples, the amount of water-unextractable proteins at both 2 and 48 hours were lower in samples containing protease inhibitor (1.25/1.65 µg/µL) and DMSO (1.18/1.71 µg/µL) compared to that of the controls (1.57/2.26 µg/µL), respectively. Noticeable, the slight increase in protein content in the water-extractable protein fractions containing protease inhibitors or DMSO is concomitant with the slight decrease found in the water-unextractable protein fractions. This could be related to increased solubility of insoluble (hydrophobic) proteins due to presence of DMSO. Collectively, the total amount of proteins increased for all the samples after 48 h, with no significant differences between the samples. Similar changes in the amount of water-extractable and water-unextractable protein fractions were observed in a SDS-PAGE, when loading equal volumes of protein extracts (Figure 9 C-D). The separated protein bands in the 1D-gels revealed distinct protein profiles. Detection of distinct high molecular weight protein bands and disappearance of low molecular bands in both the water-extractable and water-unextractable protein fractions suggest that implications of

protease inhibitors/DMSO show to some extend inhibition of the proteolytic activities taking place during the steeping period.

Table 2: Water extractable and water-unextractable protein concentrations (µg/µL) of liquid feed samples supplemented with protease inhibitors in DMSO or DMSO.

Sample	Water-extractable proteins (µg/µL)	Water-unextractable proteins (µg/µL)	Sum (µg/µL)
2 h H_2O	4.23 ± 0.06	1.57 ± 0.31	5.74
2 h Inhibitor	4.47 ± 0.02	1.25 ± 0.17	5.72
2 h DMSO	4.60 ± 0.09	1.18 ± 0.20	5.78
48 h H_2O	4.09 ± 0.04	2.26 ± 0.21	6.35
48 h Inhibitor	4.45 ± 0.02	1.65 ± 0.19	6.10
48 h DMSO	4.42 ± 0.04	1.71 ± 0.78	6.13

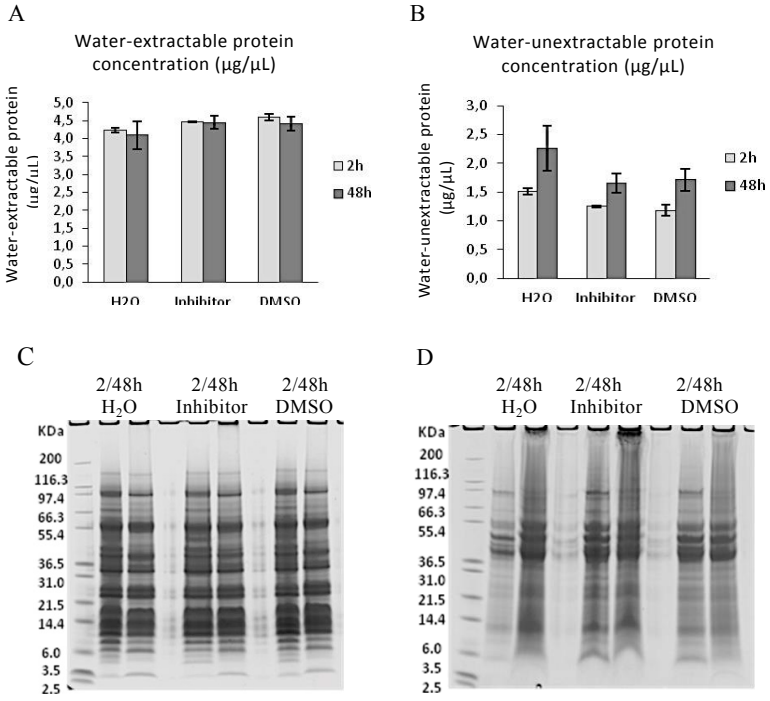

Figure 9: The protein concentration measurements determined by the method of Popov et al. (1975). Water-extractable (A) and water-unextractable (B) proteins from liquid feed samples with or without protease inhibitors and DMSO, at 2 and 48 h of incubation. Each histogram represents the mean ± standard deviations obtained from four independent biological samples. SDS-PAGE loaded with equal volumes (10 µL) of water-extractable (C) and water-unextractable (D) proteins from the different samples after 2 and 48 h. Gels were stained with Coomassie Brilliant Blue G-250. H_2O represent the control samples.

2.3.2. PROTEOME ANALYSIS OF LIQUID FEED SUPPLEMENTED WITH PROTEASE INHIBITORS AND/OR DMSO

To get a more comprehensive picture of the protein profile, two-dimensional gel electrophoresis (2-DE) was carried out for four independent biological replicates of the liquid feed samples with or without different application of protease inhibitors and/or DMSO after 2 and 48 h of incubation. The monitored pH was relatively constant throughout the steeping period (from 6.0 to 5.5). The 2D-gel protein patterns of both the water-extractable and water-unextractable fractions were tracked through the incubation period at 2 and 48 h. The two different fractions were analyzed separately to enrich specific proteins and facilitate identification of protein spots varying in relative abundance elicited by application of protease inhibitors/DMSO to liquid. Separate analysis was also to minimize co-extraction of storage proteins, which would otherwise dominate the 2-DE profile and mask the lower abundance proteins (Görg et al., 1992; Østergaard et al., 2004). Preliminarily protein separations in the pI range 3−10 showed that the majority of proteins migrated in the acidic to neutral pI range (gels not shown). Highly resolved and reproducible gels were obtained in the pI range 4−7. Representative gels of the water-extractable proteomes of liquid feed are shown in Figure 10 and the water-unextractable proteomes in Figure 11. As expected, the protein patterns on 2D-gels of the two fractions were distinctively different. Approximately 300 well-resolved spots were detected on the CBB stained 2D-gels with water-extractable proteins. Fewer spots (ca. 200) were detected in gels with water-unextractable proteins, of which were quite large and intense. Furthermore, high molecular weight proteins and prominent streaking dominated these gels. The re-extracts itself were highly viscous and thus difficult to load the same amount of proteins on the gels. It is not surprising that the 2D-gel profiles for water-extractable protein fraction resemble those of germinating barley seeds reported by others (Bak-Jensen et al., 2007; Bønsager et al., 2007; Østergaard et al., 2002; Perrocheau et al., 2005).

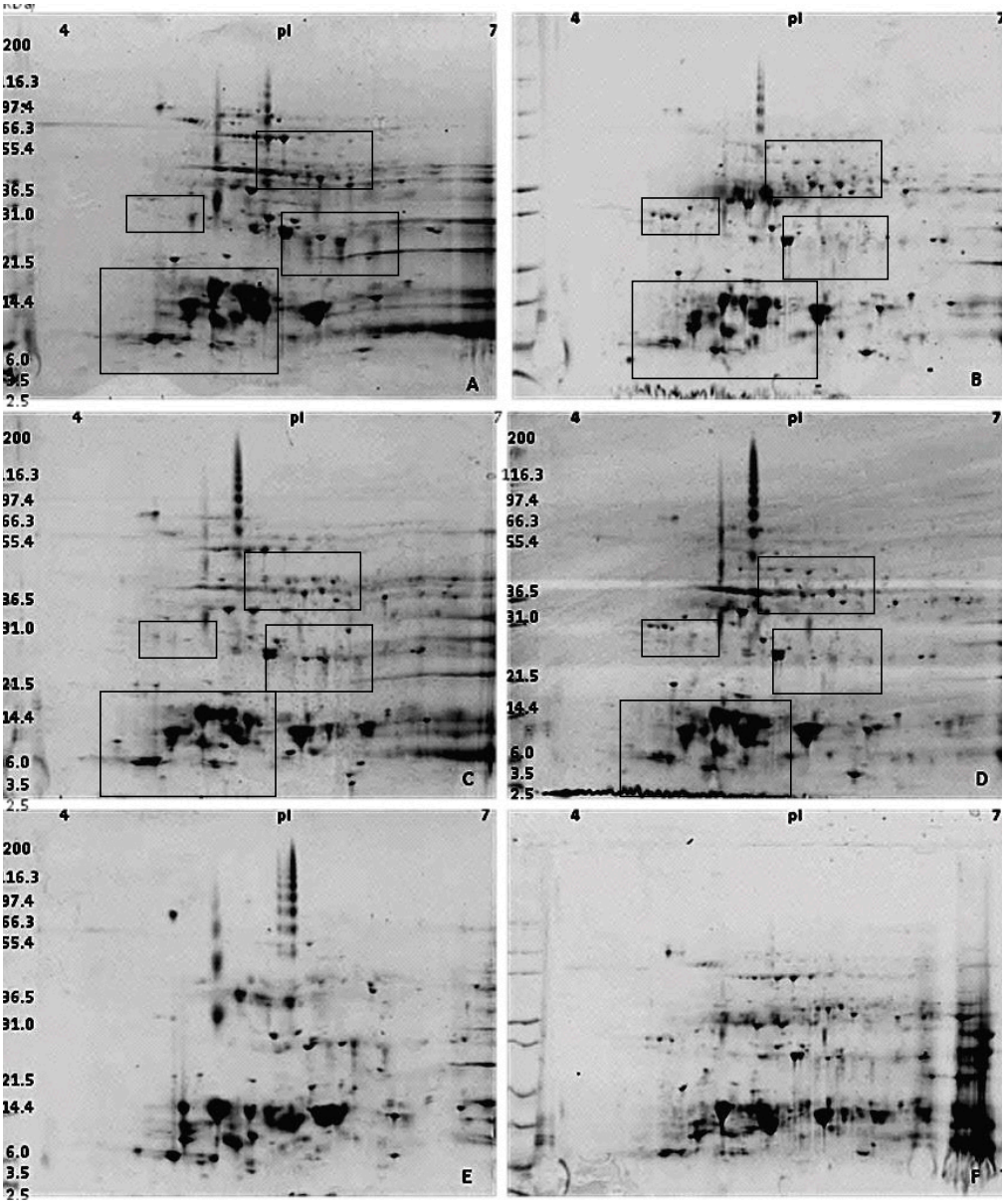

Figure 10: 2-DE of the 250 µg water-extractable proteins (pI 4−7) from liquid feed after 2 and 48 h of incubation. A-B, Control samples (H_2O) at 2 and 48 h; C-D, samples containing protease inhibitors in DMSO at 2 and 48 h; E-F, samples containing DMSO at 2 and 48 h, respectively. Proteins were visualized by colloidal Coomassie Brilliant Blue G-250. The boxes highlight the areas with prominent changes in spot intensities. Molecular markers and pI range are as indicated.

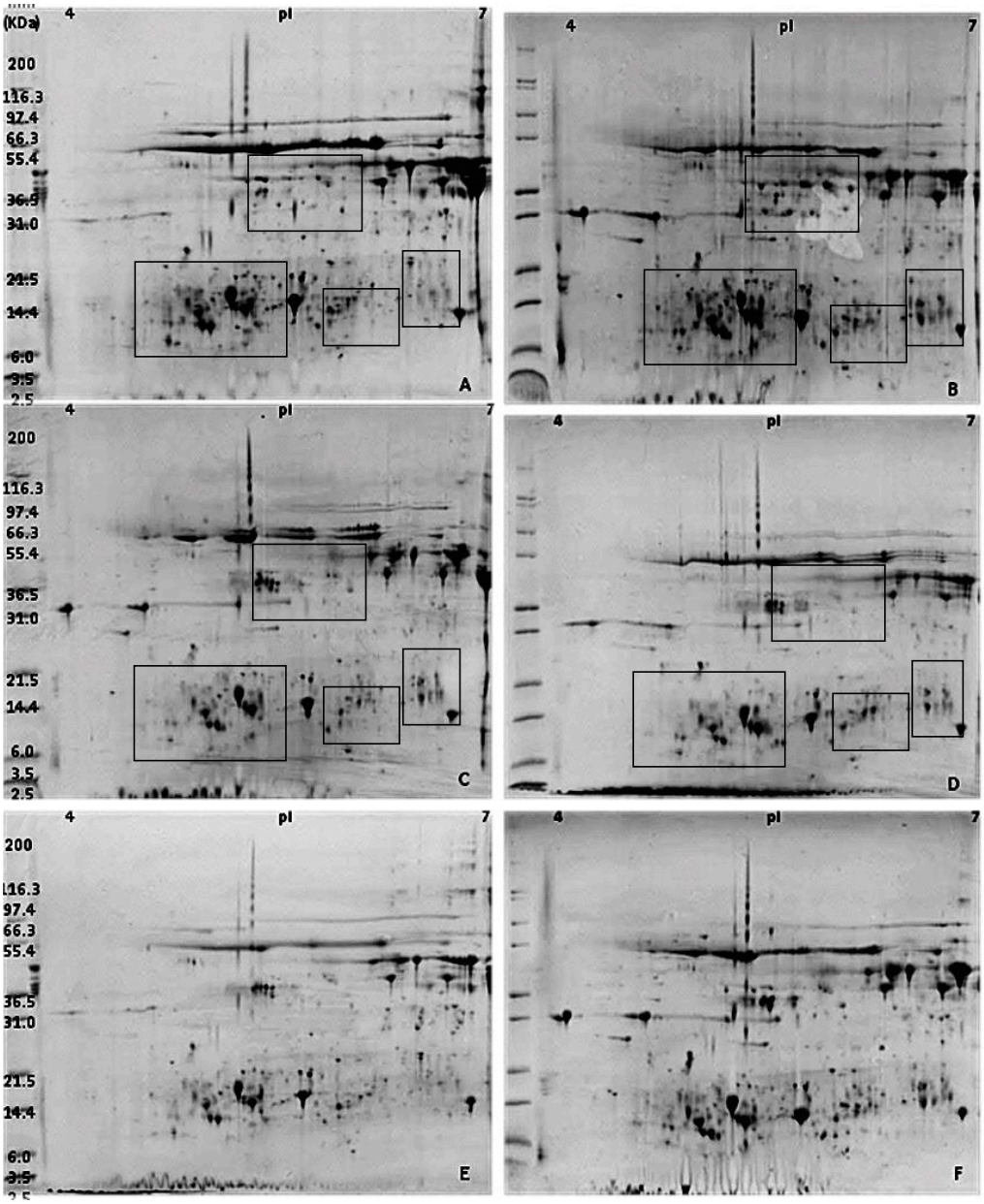

Figure 11: 2-DE of the 250 µg water-unextractable proteins (pI 4−7) from liquid feed over time (2 and 48 h). A-B, Control samples (H_2O) at 2 and 48 h; C-D, samples containing protease inhibitors at 2 and 48 h; E-F, samples containing DMSO at 2 and 48 h, respectively. Proteins were visualized by colloidal Coomassie Brilliant Blue G-250. The boxes highlight the areas with prominent changes in spot intensities. Molecular markers and pI range are as indicated.

The images of the water-extractable and water-unextractable proteins were analyzed separately using Progenesis SameSpot v4.0 to determine differentially expressed spots based on the specified criteria, i.e. ANOVA ($p < 0.05$) and false discovery rate ($q < 0.05$), respectively. Principal component analysis (PCA) was computed to assess reproducibility of the gels and clustering of the spots with the protein pattern of the different samples. From the PCA plots it was evident that the protease inhibitors had pronounced effects on the protein profile with clustering of protein spots in a group separate from the others, Figure 12 (water-unextractable protein fraction). Apparent differences were observed between the samples over time (2 and 48 h). Multiple spots disappeared after the 4 h of incubation, while spot intensity of several high molecular weight proteins decreased and additional spots with lower molecular weight proteins appeared over time (highlighted with boxes in the gel-images, Figure 10 A-D and Figure 11 A-D). The 2-DE portraits of both protein fractions supplemented with protease inhibitors were similar to that of DMSO, which were included in the analysis in order to level out the effect of DMSO. However, the main emphasis was to study the alterations in proteome upon application of protease inhibitors. There are no studies as far as we know of the liquid feed system at the proteome level, regardless of the cultivar, presence of protease inhibitors or anything else.

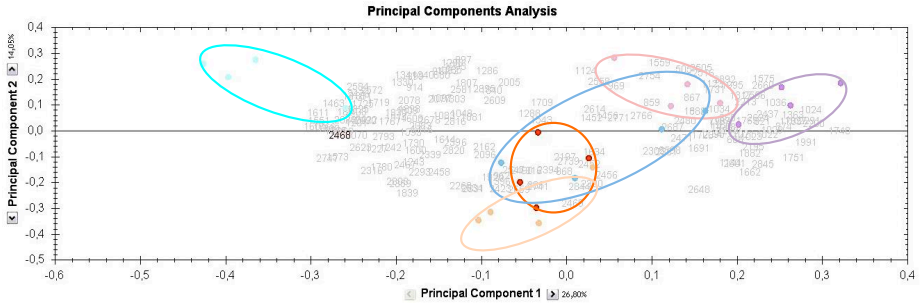

Figure 12: Principal components analysis (PCA) plot over the water-unextractable protein fraction. The enclosed spots represent the gels and the numbers the protein spots on the gels. Pink circles: water at 2 h; Blue: protease inhibitors at 2 h; Purple: DMSO at 2 h; Yellow: water at 48 h; Turquoise: protease inhibitors at 48 h; Orange: DMSO at 48 h. DMSO: dimethyl sulfoxide.

2.3.3. IDENTIFICATION OF PROTEIN SPOTS OF DIFFERENTIAL ABUNDANCE IN THE WATER-EXTRACTABLE PROTEIN FRACTIONS OF LIQUID FEED SUPPLEMENTED WITH PROTEASE INHIBITORS

Comparative proteome analysis was performed separately on the two protein fractions of liquid feed with and without protease inhibitors and DMSO. In the water-extractable protein fractions, a total of fifty-seven protein spots showed differential abundance upon application of protease inhibitors during the 48 h of incubation (Figure 13). The spots were manually excised, tryptic digested and analyzed by MALDI-TOF MS and/or MS/MS. Comparison of the selected spots revealed a wide range of protein abundances (2.5−28.6-fold). Out of the 57 spots, 45 were identified with confidence by MS and/or MS/MS, where 36 increased and 9 decreased in abundance upon application of protease inhibitors. The protein identifications are summarized in Table 3, and their appearances on the images are labeled in Figure 13. The majority of the identified proteins were typically of starchy endosperm and those arising upon imbibition, such as carbohydrate and protein degrading enzymes and others involved in stress-related mechanisms. Several proteins appeared in more then one spot with a shifted molecular mass and pI, suggesting that the proteins were post-translationally modified (PTM), proteolytically degraded or found as similar isoforms. Isoelectric heterogeneity is commonly found in 2-DE, and has been ascribed to PTMs (e.g. glycosylation or phosphorylation) or artifacts arising during processing e.g. carbamylation by urea displayed as string of spots (McCarthy et al. 2003).

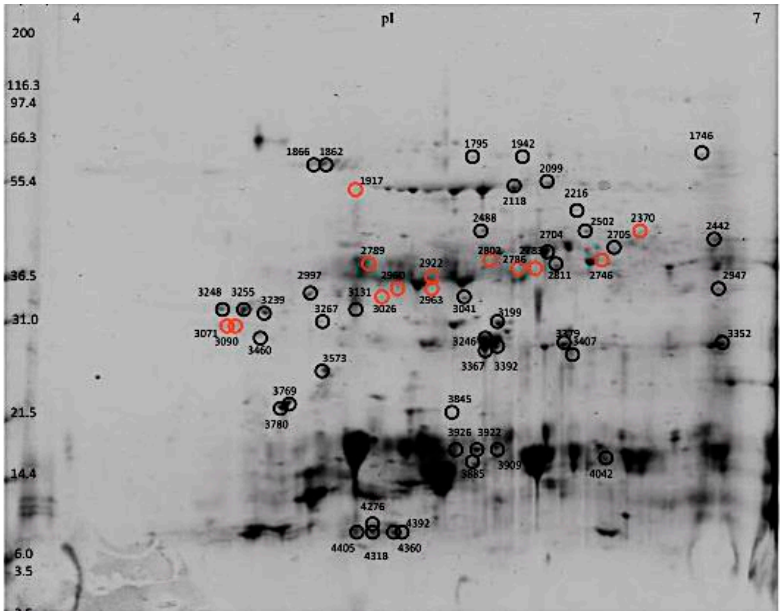

Figure 13: 2-DE of the water-extractable proteins (250 µg, pH 4−7) from liquid feed supplemented with protease inhibitors after 48 h of steeping period. Proteins were visualized by colloidal Coomassie Brilliant Blue G-250 (grey scale, 16 Bit). Numbered circles represent protein spots that changed in abundance (Table 3). The black circles represent an increase in relative spot intensity, while the red circles a decrease in relative spot intensity. Molecular weight markers (kDa) and pI range are as indicated.

Several enzymes involved in the glycolysis were found in spots that increased in abundance, i.e. fructose-bisphosphate aldolase cytoplasmic isozyme (FPA, spot 2442, fold change 7.5), triosephosphate isomerase (TPI, spot 3391, 3367, 3399, fold change 11.1, 8.4, 16.2), phosphoglycerate kinase (PGK, spot 2502, fold change 14.4) and enolase (ENO, spot 2118 and 2099, fold change 10.1 and 4.9), respectively. These enzymes are involved in the breakdown of carbohydrates into pyruvate in multiple steps in the cytoplasm, which then enters the mitochondria to be oxidized to carbon dioxide and water via the tricarboxylic acid (TCA) cycle. Malate dehydrogenase (MDH, spot 2704, fold change 28.6) is a key enzyme in the TCA cycle that catalyzes the oxidation of malate. In addition, NADP-malic enzyme, a member of the oxidoreductase family, was found in spot that increased in abundance (spot 1795, fold

change 6.3). TPIs and ENOs were found in more then one spot with differing pI and/or Mr (Figure 13). Posttranslational modifications common in 2D-gels can change pI and Mr of a protein and form new spots in the gel. These are found as necklaces of spots all containing the same gene product with different pI. Glycolytic enzymes in the mature seeds have been suggested to be in preparation for the rapidly increasing respiratory rate upon imbibition, to provide energy for commencement of germination (Bewley, 1997). Thus it is not surprising to identify these proteins. Moreover, discrepancies in the theoretical and measured molecular weights suggests that several of the identified proteins have been degraded over the steeping period, e.g. spot 2705 (protein z-type serpin) and 2442 (FPA).

One spot identified as β-amylase (spot 1942, fold change 8.7) was found to increase in relative abundance upon application of protease inhibitors. β-amylase belongs to the glycosyl hydrolase family 14 and involved in starch and sucrose metabolism. In mature barley grains, β-amylase occurs both in free and bound form in the starchy endosperm. The bound β-amylase has been proposed to be associated with either the endosperm protein matrix and/or periphery of the embedded starch granules (Sopanen and Lauriére, 1989). In barley, multiple isoforms of β-amylase with different pIs have been identified, all of which are products of proteolysis of the C-terminus of the enzyme. Identification of the full-length β-amylase (spot 1942, fold change 8.7) indicates that application of protease inhibitors to some extend can hamper some of the proteolytic activities during the steeping period. Interestingly, a spot (3573) identified as the granule bound starch synthase also increased in volume (fold change 5.7), illustrating the occurring modulation of the protein matrix and the embedded starch granules of the endosperm. Imbibition is accompanied with mobilization of carbohydrate and protein reserves to support the growth of the new plantlet. Mobilization of protein bodies as well as starch granules could explain the identification of starch granule proteins and proteins bound/associated with the granules, including α-amylase inhibitors (spot 4042) and serpins (spot 2488 and 2705). Moreover, endosperm storage proteins e.g. hordeins (spot 1209) and globulins (spot 3352 and 3885) were identified in the water-extractable protein fraction supplemented with protease inhibitors. Protein spot (2997) predicted as ferritin-like protein increased by 6.5-fold in abundance. Identification of these iron-binding proteins could be

associated with increased availability of e.g. iron in feed.

Another group of the identified proteins constitute of various types of inhibitors suggested to be involved in defense mechanisms against invading microorganisms and insects. The chloroform/methanol (CM) soluble protein, i.e. α-amylase/trypsin inhibitor CMb that selectively inhibits α-amylases and/or proteases from insects, was found in the water-extractable fraction of liquid feed supplemented with protease inhibitors (spot 4042, fold change 4.3). Two different isoforms of the subtilisin-chymotrypsin inhibitors (CI-IB-C, spot 4405 and 4392) were additionally identified, which are known to be involved in plant defense mechanisms (Habib and Fazili, 2007).

A number of identified proteins are involved with maintaining the oxidative and desiccation stress. Two spots were identified as protein disulfide-isomerases (PDIs, spot 1862 and 1866) increased by 9.9- and 5.8-fold in abundance, respectively. The same protein was identified in two adjacent spots, which could be due to overlapping of the spots or PTMs. No *N*-glycosylation sites were found upon further inspection of the protein sequence. PDIs are involved in oxidative protein processing/folding in the endoplasmic reticulum by catalyzing correct disulfide bond pairing. In the control gels, two different PDIs were identified in spot 1917 (69 kDa) and 3026 (32 kDa), respectively, which were absent or low in abundance in the gels of extracts with protease inhibitors. The lower molecular weights of the PDIs suggest protein degradation during the steeping period. In addition, the 27K protein, a hypothetical protein identified to belong to the thiol reductase superfamily, showed a 6.2- and 3.3-fold increase in abundance (spot 3407 and 3379).

Several oxidoreductases were found to increase in abundance, including short-chain dehydrogenase/reductases (SDRs, spot 2942, fold 11.2), which are known to be NAD or NADP-dependent oxidoreductases. Enoyl-(acyl-carrier protein) reductase (spot 2811, fold 7.1), another identified enzyme to be involved in fatty acid biosynthesis. Protein spot 1767 predicted as glutathione S-transferase DHAR1 (also known glutathion-dependent dehydroascorbate reductase) was increased by 2.7-fold. The protein exhibits a dual function, namely glutathione-dependent thiol transferase and dehyrdroascorbate reductase activities. DHAR is involved in the redox homostatis by

scavenging reactive oxygen species (ROS) under oxidative stresses (Dixon et al., 2002). It has also been suggested that glutathione S-transferase plays a role in detoxification of fatty acid peroxidases (Marrs, 1996). Mature seeds have typically acquired desiccation tolerance, thus upon imbibition, the seeds will be under stress. Proteins like late embryogenesis abundant protein (LEA), cold-regulated protein (COR) and glyoxalase (GLX) are found in abundance in the dry seed and play a role in desiccation and oxidative stress (Chen et al., 2002; 2004; Xu et al., 1996). Upon germination, the aforementioned proteins are typically rapidly degraded (Østergaard et al., 2004). The proteins, LEA (spot 3845), COR (spot 3780 and 3759) and GLX (spot 3926), respectively, increased in spot volumes in samples with protease inhibitors. Moreover, desiccation-related proteins (spot 4360, fold 10.3) and seed maturation protein (spot 1766, fold 3.7) were also identified. Appearance and identification of these proteins indicate that application of protease inhibitors to somewhat effectively inhibit proteolysis during the steeping period.

Among the identified stress responsive proteins, several spots exhibiting an increase in volumes were identified as members of the heat shock protein (HSP) family (spot 3460, fold change 3.8). Additional three spots were identified as predicted proteins similar to HSP70 (spot 3239, 3255, 3240). HSP70s are known to exhibit chaperone activity and have been suggested to protect proteins from irreversible aggregation (Whitley et al., 1999). Moreover, two HSP70s found in spot 2370 (52 kDa, pI 9.8) and 3090 (34 kDa, pI 4.9) showed 5.3- and 6.8-fold decrease in abundance. Noticeable the two proteins had a lower experimental molecular mass and slightly shifted pI, suggesting proteolysis and/or PTMs.

2.3.4. IDENTIFICATION OF DIFFERENTIALLY ABUNDANT WATER-UNEXTRACTABLE PROTEINS FROM LIQUID FEED SUPPLEMENTED WITH PROTEASE INHIBITORS AND/OR DMSO

Similarly, the effect of protease inhibitors was evaluated by comparing the proteomes of the water-unextractable fractions of liquid feed with and without protease inhibitors and DMSO, (Figure 11 A−F). A total of thirty-two spots (fold > 1.5, $p < 0.05$) differed

significantly in relative abundance upon application of protease inhibitors. Of these, twenty-one protein spots (5.9−28.2-fold) were identified with confidence by MALDI-TOF MS and/or MS/MS. The protein identifications are summarized in Table 4 and labeled in Figure 14. The majority of the spots that increased in abundance in the water-unextractable fraction were identified as defense/stress-related and storage proteins, such as serpin Z type proteins and hordeins. In addition, a couple of enzymes involved in the glycolysis were also found in spots that increased in abundance in samples supplemented with protease inhibitors, including TPIs (spot 1839 and 2082, fold change 7.9 and 4.5) and ENOs (spot 2070, fold change 2.7).

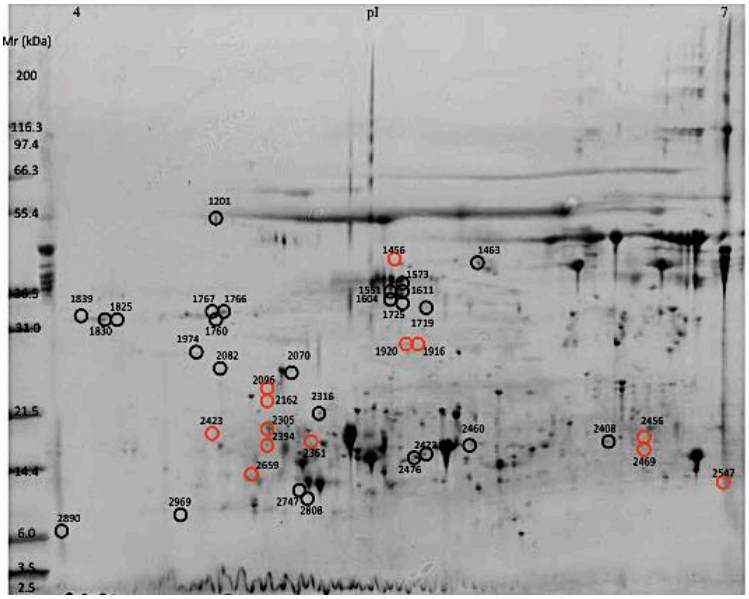

Figure 14: 2-DE of the water-unextractable proteins (250 µg, pH 4−7) from liquid feed supplemented with protease inhibitors after 48 h of steeping period. Proteins were visualized by colloidal Coomassie Brilliant Blue G-250 (grey scale, 16 Bit). Numbered circles represent protein spot that change in relative abundance (Table 4). The black circles represent an increase in relative spot intensity, while the red circles a decrease in relative spot intensity. Molecular markers and pI range are as indicated.

At maturity, the barley grains accumulate large amounts of storage proteins, starch and lipids. Accumulation of storage proteins during grain filling is concomitant with the acquisition of seed vigor. During seed germination, these storage proteins are

degraded to their constituents, i.e. amino acids, to supply the growing seedling (Bewley, 1997). Among proteins showing increased spot volumes in presence of protease inhibitors were identified as, i.e. B hordeins (spot 1780 and 1825), globulin-like (2476), seed maturation protein (1766) and embryo globulin (2316, 2460), respectively. Evidently, the identified storage proteins were in the water-unextractable protein fraction, as they tend to be slightly hydrophobic. Moreover, mobilization of several storage proteins from both the endosperm as well as embryo could be explained by increased stability of the proteins due to inhibition of proteases and reduced susceptibly to proteolysis.

Numerous proteins involved in defense mechanisms were identified, including protease inhibitors. The dimeric form of α-amylase inhibitor (BDAI-1, 2572 and 2584) increased 6.4- and 6.1-fold in spot volume in gels of the water-unextractable protein fraction of samples with protease inhibitors. Serpin proteins Z were identified in multiple spots (1460, 1551, 1573, 1604, 1611, 1719). In the mature grains, serpin protein Z is present in free and thiol-bound forms, which are released during germination. The biological function of these proteins still remains unknown, due to the fact that no target endogenous proteases have been found in plants. Serpins have been proposed to function as storage proteins, due to their high lysine content similar to other storage proteins, and their synthesis and deposition during seed development (Brandt et al., 1990). Serpins mainly target serine proteinases of chymotrypsin and cathepsin family, as well as some cysteine proteases (Roberts and Hejgaard, 2008, Østergaard et al., 2000). The plant serpins act via a suicide mechanism by forming a stable complex with the protease after proteolytic cleavage. In addition, the plant serpins have been shown to provide protection against insect by irreversible inhibition of proteases (Thomas et al., 1995). It is not surprising that there is an increase in endogenous protease inhibitors, as they play important roles against proteolytic enzymes causing digestion of proteins during germination and/or pathogenesis.

2.3.5. Analysis of the proteomes of liquid feed supplemented with the thioredoxin system

To study the protein changes upon application of the thioredoxin system (Trx, NTR and NADPH) to liquid feed, a comparative proteomics approach was applied using 2-DE and MALDI-TOF MS. The 2-DE was carried out using four independent biological replicates of both the water-extractable and water-unextractable protein fractions of the liquid feed with the thioredoxin system after 2 and 48 h of incubation. Similar to before, the two different fractions were analyzed separately to facilitate identification of protein spots varying in relative abundance in presence of NTR/Trx. Representative gels of the water-extractable (2 and 48 h, A–B) and water-unextractable (2 and 48 h, C–D) proteomes of liquid feed containing NTR/Trx resolved in the pH 4−7 are shown in Figure 15. Evidently, the water-extractable proteomes of liquid feed containing NTR/Trx were highly resolved and crowded in both number and intensity of multiple protein spots not observed in the other water-extractable proteome profiles. Thus illustrating that application of NTR/Trx reductively alters the solubility of barley proteins and promotes their mobilization.

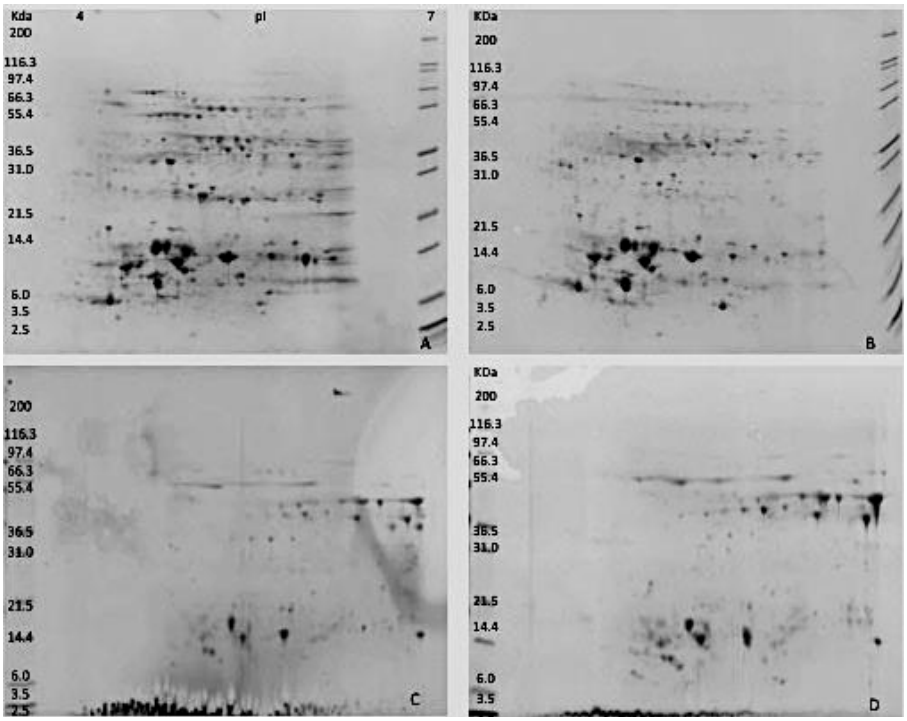

Figure 15: 2-DE of water-extractable and water-unextractable proteins (250 µg, pH 4−7) from liquid feed supplemented with the thioredoxin system over a 48 h steeping period. (A–B) Water-extractable protein fractions at 2 and 48 h. (C–D) Water-unextractable protein fractions at 2 and 48 h. Proteins were visualized by colloidal Coomassie Brilliant Blue G-250 (grey scale, 16 Bit). Molecular markers and pI range are as indicated.

Comparative proteomics analysis of the water-extractable fractions enabled detection of thirty-nine spots increasing in abundance in samples containing NTR/Trx at 48 h when compared to proteome pattern of the control gels (liquid feed at 48 h). These were manually excised, tryptic digested and analyzed by MALDI-TOF MS and/or MS/MS. Out of the thirty-nine spots, thirty-four were identified with high confidence (Figure 16). The majority of the identified proteins were found to be enzyme inhibitors and stress-related enzymes (Table 5). Except a few proteins, all of the identified proteins contained one or more cysteine residues. The bulk group of enzyme inhibitors included CM proteins, such as α-amylase/trypsin inhibitor CMa (spot 1459) and CMd (spot 1266), α-amylase/trypsin inhibitor BDAI-1 (spot 1554 and 1596) and BMAI-1

(spot 1405, 1406, 1408) and trypsin/α-amylase inhibitor pUP38 (spot 1486). Identification of these low molecular weight disulfide CM proteins in the water-extractable protein fractions of liquid feed support the view that application of Trxs alters protein solubility by reducing intra-molecular disulfide bonds of these proteins, thus enhancing their partition in the water-extractable fraction. The CM proteins are known and recognized targets of Trxs and have been previously reported in cereals by others (Hägglund et al., 2008; Wong et al., 2001; 2004). Trxs have been reported to act as a signal early in germination by facilitating mobilization of seed reserves by various means, such as reducing storage proteins and thereby enhancing their solubility and susceptibility to proteolysis, as well as reductively inactivating proteins that inhibit specific amylases and proteases (Kobrehel et al., 1991; 1999; Lozano et al., 1996; Rhazi et al., 2003). Of the protease inhibitors identified included the serpins (spots 705, 714, 747, 1444, 731, 2 Cys) and subtilisin-chymotrypsin inhibitor CI-1B (spot 1691 and 1683, no Cys residues).

In the present study, several stress-related enzymes were identified in the water-extractable protein fraction, including Cu/Zn superoxide dismutase (spot 1468, 3 Cys), glutathione reductase (spot 579, 7 Cys), Glyoxalase (spot 900, 5 Cys) and the plant stress-response enzyme, which is an aldo-keto reductase (spot 1504, 7 Cys). In addition to alteration of protein solubility, Trxs have been reported to reductively activate enzymes participating during germination. In the present study, two spots (505 and 541) were identified as β-amylases (5 Cys). Other Trx target proteins include glucose and ribitol dehydrogenases (spot 918, 5 Cys) and fructose-1,6-bisphosphate aldolase (spot 755 and 798, 7 Cys). Spot 763 was identified as the reversibly glycosylated polypeptide containing up to 9 Cys (3 probable SS bound), whose function is unknown.

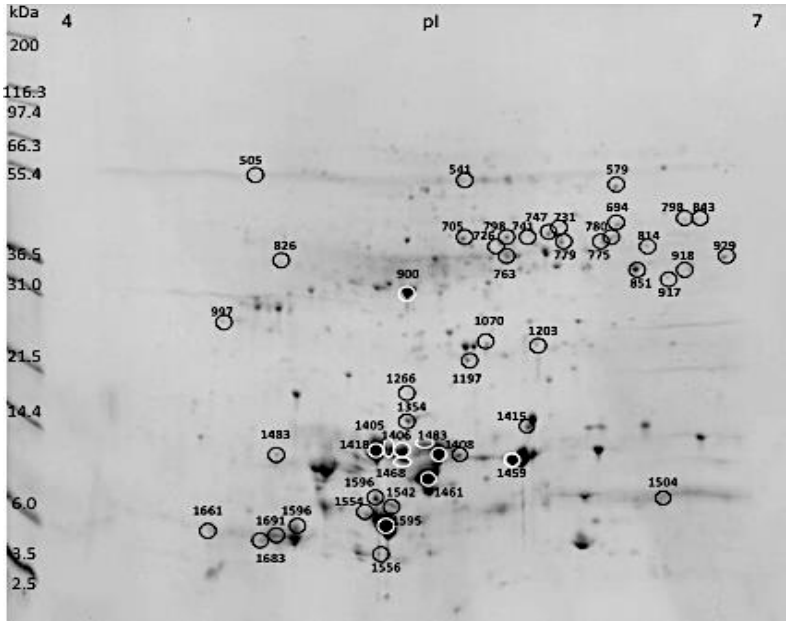

Figure 16: 2-DE of the water-extractable proteins (250 µg, pH 4−7) from liquid feed supplemented with NTR/Trx system after 48 h of steeping period. Proteins were visualized by colloidal Coomassie Brilliant Blue G-250 (grey scale, 16 Bit). Numbered circles represent protein spots that increase in relative intensity (Table 5). Molecular markers and pI range are as indicated.

With increase in both number and intensity of protein spots in gels of the water-extractable protein fraction of liquid feed containing the NTR/Trx system, it is not surprising that the water-unextractable fraction is less crowded (Figure 15). Nevertheless, comparative analysis of the water-unextractable protein fraction containing the NTR/Trx system resulted in detection of twenty-six spots increasing in abundance (Figure 17). Twenty-four were identified with high confidence using MALDI-TOF MS and/or MS/MS (Table 6). Evidently, the majority of the identified proteins were those already identified in the water-extractable fraction, which suggest that not all Trx target proteins were solubilized. It remains to be studied whether the increase in protein solubility is dependent on the extent of reduction (increasing concentration of NTR/Trx system). Similar to the water-extractable protein fraction the enzyme inhibitors dominated the protein identifications, including alpha-amylase/trypsin inhibitor CMd (spot 2082, 2108, 2124, 2212), alpha-amylase/trypsin

inhibitor CMb (spot 2202, 2279, 2234, 2245), alpha-amylase inhibitor BMAI-1 (spot 2163 and 2204) and subtilisin-chymotrypsin inhibitor CI-1A (spot 2562), respectively. Moreover, spot 2273 was identified as the trypsin inhibitor protein BTI-CMe4 not found in the water-extractable fraction.

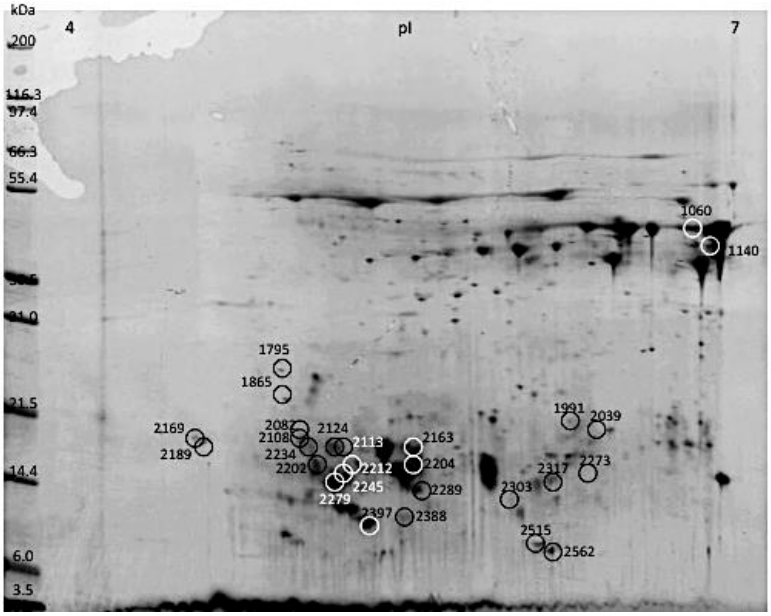

Figure 17: 2-DE of the water-unextractable protein fraction (250 µg, pH 4−7) from liquid feed supplemented with NTR/Trx system over 48 h of steeping period. Proteins were visualized by colloidal Coomassie Brilliant Blue G-250 (grey scale, 16 Bit). Numbered circles represent protein spots that change in relative intensity (Table 6). Molecular markers and pI range are as indicated.

Several storage proteins of the endosperm and embryo showing an increase in spot abundances in presence of Trx included B hordeins (spot 1060 and 1140, 8 Cys), D hordeins (spot 2388, 7 Cys) and embryo globulin (spot 2303, 6 Cys). This is not surprising, since Trx has long been known to enhance protein solubility of several cereal storage proteins (Bewley and Black, 1994; Kobrehel et al., 1992; Marx et al., 2003). Other previously identified Trx targets showing an increase in spot abundance in presence of Trxs included inorganic diphosphatase (spot 1795, 2 Cys), osmotically inducible protein (spot 2113, 2 Cys). In addition, two spots showing an increase in spot abundance in presence of Trxs were identified as the outer membrane protein X

(spot 2169 and 2189). These hydrophobic membrane proteins predicted to contain two Cys residues have not previously been reported as Trx targets. All of the identified proteins in the water-unextractable fraction seemed to contain one or more Cys residues, except for the 17 kDa small heat shock protein (spot 2039) and cold-shock DNA-binding domain protein (spot 2515).

2.4. CONCLUSIONS

Quantitative proteomics analysis enabled profiling the protein pattern of both the water-extractable and water-unextractable fractions of liquid feed over the steeping period of 48 h. This is the first and most comprehensive analysis of the proteomes of the liquid feed. Comparative proteomics analysis of the liquid feed revealed differences in mobilization and/or degradation of proteins upon application of protease inhibitors. The pronounced changes were observed in the water-unextractable protein fractions with identification of a set of proteins that changed more then 1.5-fold in abundance in samples containing protease inhibitors, mainly involved in protein synthesis and storage, as well as in stress-related and defense mechanisms. It is not surprising that we find proteins involved in the primary metabolism and defense mechanisms during the steeping period with the risk of microbial growth. The profiled dynamics of the proteome of the liquid feed over time can be used as a model system for other orthodox seeds (e.g. mung beans) used as feed and to study their response to application of other digestive enzymes over time. The represented dataset for liquid feed can facilitate additional studies as well as complement *in vivo* studies and provide a deeper insight into the various physiological processes at the proteome level.

Application of the NTR/Trx system to liquid feed altered the solubility of many proteins in both the water-extractable and water-unextractable protein fractions. Increased abundance of enzyme inhibitors (low molecular weight disulfide proteins) and stress-related proteins were found in the water-extractable fraction, which are recognized Trx target proteins. The effect of Trx was also apparent in the water-unextractable protein fraction with identification of seed storage proteins as well as enzyme inhibitors. In the present study, two protein spots were identified as the outer membrane protein X (2 Cys residues) not previously recognized as Trx target. It

remains to be assessed whether the identified proteins in the water-unextractable fraction are dependent on extend of reduction, meaning the analysis of the effect of increasing concentrations of Trx.

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Table 3: Identifications of proteins from 2D-gel (pH 4-7) of the water-extractable fraction of liquid feed supplemented with protease inhibitors (≥ 1.5-fold spot volume ratio change and ANOVA ≤ 0.05). Protein identifications were confirmed with a MASCOT score of 70 for peptide mass fingerprint (ANOVA ≤ 0.05) and a minimum of 4 matched peptides or one matched peptide with a MASCOT score of 40 for MS/MS (ANOVA ≤ 0.05). Spot numbers refer to Figure 13. Comb: combined MASCOT score; Mw: molecular weight; Theor: theoretical.

Spot no.[a]	Spot relative intensity[b]	Accession no.	Organism	Protein	Mw theor.	pI theor.	PMF score[c]	Comb. score[c]	Sequence coverage (%)	Unique peptides	MS/MS precursor ions	MS/MS peptides sequences	Ion score[d]
1795	6.3	TC200068	*Oryza sativa*	Cytosolic NADP malic enzyme	73772	8.31		112	19	11			
1746	7.6	TC217857	*Vitis vinifera*	Chromosome chr10 scaffold_81, whole genome shotgun sequence	19425	11.4		74	34	5			
1862	9.9	TC230560	*Hordeum vulgare*	Disulfide-isomerase	64741	5.62		151	25	15			
1866	5.9	TC230560	*Hordeum vulgare*	Disulfide-isomerase	64741	5.62		206	25	16			
1917	-7.9	TC209835	*Hordeum vulgare*	Protein disulfide-isomerase precursor	68582	5.37	158		31	20			
1942	8.7	TC233233	*Hordeum vulgare*	Beta-amylase	68872	5.80		110	31	16			
2118	10.1	gi|326490934	*Hordeum vulgare* subsp. *vulgare*	Predicted protei (enolase)	48601	5.39	91	282	26	9	1790.9117	R.AAVPSGASTGVYEALELR.D	103
											806.4478	K.YNQLLR.I	18
											1577.7797	R.IEEELGDAAVYAGLK.F	49
											978.494	K.FRAPVEPY.-	39
2306	-3.9	TC203837	*Oryza sativa Japonica* Group	Enolase	58053	5.82	96		27	10			
2370	-5.3	TC198009	*Triticum aestivum*	HSP70	51999	9.78	79		20	7			
2442	7.5	TC218799	*Oryza sativa Japonica* Group	Fructose-bisphosphate	54288	8.48		73	18	8			
											838.4336	R.GKFDESR.K	15
											862.5080	K.VLAFEAGR.K	36
2488	7.5	TC204315	*Hordeum vulgare*	Serpin	48709	8.54		93	16	8			
2502	14.4	TC223074	*Triticum aestivum*	Phosphoglycerate kinase	33708	9.15		85	31	8			
2746	-4.5	gi|32651579	*Hordeum vulgare* subsp. *vulgare*	predicted protein	41606	6.49	89		5	2	1124.6101	R.SPAVKPVDGVR.A	41
											900.5494	R.LGTELVLR.K	48
2786	-5.1	gi|297259335	*Macaca mulatta*	Predicted (zinc finger protein 512B-like)	32274	5.79	83		46	9			
2947	11.2	TC203374	*Medicago truncatula*	Short-chain dehydrogenase/reductase SDR	41667	8.6		106	25	12			

3199	9.6	TC216236	*Oryza sativa*	PREDICTED protein (LOC100835371)	31917	6.37		91	28	10			
2704	28.6	TC216967	*Triticum aestivum*	Malate dehydrogenase	29675	6.51		94	45	10			
2705	6.3	TC229089	*Hordeum vulgare*	Protein z-type serpin	50018	6.01		79	18	8			
2802	-4.3	gi|29725933	*Macaca mulatta*	Predicted (zinc finger protein 512B-like)	32274	5.79	75		36	7			
2099	4.9	gi|326490934	*Hordeum vulgare* subsp. *vulgare*	Predicted protein - enolase	48601	5.39	52	192	22	7	1790.9475	R.AAVPSGASTGVYEALELR.D	86
											806.4407	K.YNQLLR.I	16
											978.508	K.FRAPVEPY.-	22
2963	-11.5	TC201243	*Oryza sativa*	Lactoylglutathione lyase	42078	8.46	94		40	13			
2997	6.5	gi|326498119	*Hordeum vulgare* subsp. *vulgare*	Predicted protein (Ferritin_like)	33991	4.90	47	326	26	5	1822.8927	R.DVATQFCYQEVGHLR.A	44
											1711.0024	R.LVAGLLGVESAQDAVIR.A	76
											1958.0791	R.VASYGVGVAEVTAHISELR.N	116
											871.4642	R.FSLAYDR.T	47
3026	-3.9	TC209842	*Zea mays*	Protein disulfide isomerase	32079	5.35	86		5	1	1813.6964	K.AGEEYEAGRDLDDFVK.F	86
3090	-6.8	TC197237	*Triticum aestivum*	HSP70	34273	4.89			3	1	1374.6268	K.NALENYAYNMR.N	54
3239	6.2	gi|326497219	*Hordeum vulgare* subsp. *vulgare*	Predicted protein (heat shock 70 kDa protein)	72202	5.14	48	246	15	11	1017.5408	K.ITITNDKGR.L	50
											1003.518	R.LSKEEIER.M	43
											1153.5109	K.YKSEDEQVR.H	59
											1374.5698	R.NALENYAYNMR.N	45
3246	16.2	gi|2507469	*Hordeum vulgare*	Triosephosphate isomerase, cytosolic	26948	5.39	98	429	37	9	1537.8176	R.RSLLGESSEFVGEK.V	71
											1381.7124	R.SLLGESSEFVGEK.V	65
											1289.6576	K.VIACVGETLEQR.E	37
											1604.9336	K.VATPAQAQEVHANLR.D	87
											1289.6962	K.TNVSPEVAESTR.I	90
3248	4.5	gi|326497219	*Hordeum vulgare* subsp. *vulgare*	Predicted protein (heat shock 70 kDa protein)	72202	5.14	68	432	14	8	2658.2534	K.EQVFSTYSDNQPGVLIQVYEGER.T	184
											1017.5698	K.ITITNDKGR.L	56
											1003.5466	R.LSKEEIER.M	39
											1374.6192	R.NALENYAYNMR.N	59
											1260.6769	K.KIEDSIEDAIK.W	55
3255	7	gi|326497219	*Hordeum vulgare* subsp. *vulgare*	Predicted protein (heat shock 70 kDa protein)	72202	5.14	55	242	14	9	1017.5635	K.ITITNDKGR.L	37
											1003.5453	R.LSKEEIER.M	44
											1153.5523	K.YKSEDEQVR.H	65
											1374.6232	R.NALENYAYNMR.N	46

3352	15.9	TC220483	*Oryza sativa Japonica*	Globulin-like protein	47163	9.31		127	19	9			
3367	8.4	gi|2507469	*Hordeum vulgare*	Triosephosphate isomerase, cytosolic	26948	5.39	108	334	47	11	1381.6645	R.SLLGESSEFVGEK.V	57
											1374.6747	K.VIACVGETLEQR.E	48
											1604.8444	K.VATPAQAQEVHANLR.D	63
											1289.6143	K.TNVSPEVAESTR.I	78
3379	3.3	TC227462	*Hordeum vulgare* subsp. *Vulgare*	27K protein complete	24946	5.54		124	12	3	1212.5626	K.AVTECYKGER.G	41
											1145.5711	R.GHNLSLEYGR.Q	67
3392	11.1	gi|2507469	*Hordeum vulgare*	Triosephosphate isomerase, cytosolic	26948	5.39	76	326	30	7	1537.8616	R.RSLLGESSEFVGEK.V	61
											1381.7508	R.SLLGESSEFVGEK.V K.VATPAQAQEVHANLR.D K.TNVSPEVAESTR.I	65
											1604.9336	K.VATPAQAQEVHANLR.D	58
											1289.6962	K.TNVSPEVAESTR.I	90
3407	6.2	BI957785	*Triticum aestivum*	27K protein	22413	5.80		82	4	1	1145.5726	R.GHNLSLEYGR.Q	82
3460	3.8	gi|476003	*Hordeum vulgare* subsp. *vulgare*	HSP70	67146	5.76		60	3	2	1143.5867	K.FDLSGIPAAPR.G	46
3573	5.7	gi|229610865	*Hordeum vulgare* subsp. *vulgare*	Granule bound starch synthase	22612	5.06	77		47	5			
3759	12.3	gi|10799810	*Hordeum vulgare* subsp. *vulgare*	Cold-regulated protein	17659	4.93	92	317	75	8	1307.6571	R.LVCPPVSAHDGR.L	47
											1543.8134	R.SLALVVQDIDADER.V	119
											1058.5261	R.GPVPDSHGHR.I	59
3780	12.4	gi|10799810	*Hordeum vulgare* subsp. *vulgare*	Cold-regulated protein	17659	4.93	104	327	75	8	1307.6705	R.LVCPPVSAHDGR.L	45
											1543.8257	R.SLALVVQDIDADER.V	127
											1058.5304	R.GPVPDSHGHR.I	58
3845	11.5	TC211434	*Hordeum vulgare*	Late embryogenesis abundant protein B19.3	29594	9.36		82	4	1	1353.6240	K.TLEAQEHLAEGR.S	82
3885	4.1	TC216046	*Brachypodium distachyon*	Globulin-2 precursor	53721	7.14		79	33	3	1029.5896	K.ALAFPQQVR.E	16
3926	2.5	TC206011	*Hordeum vulgare subsp. vulgare*	Predicted protein (Glyoxalase)	16113	5.42	96	401	48	8	1579.7708	K.SVAFYADAFGYNVR.R	117
											2004.8689	R.GPVEICFDYDDVDAAYR.R	89
											1082.5894	K.GAVPVSAPEQK.N	54
											874.4633	R.DVDGITVR.L	52

4042	4.3	TC213888	*Hordeum vulgare*	Alpha-amylase/trypsin inhibitor CMb precursor	16526	5.77	55	21	3	1023.4831	R.EVQMDFVR.I	26
										1039.4922	R.EVQMDFVR.I	40
										943.5628	R.ELADKVIR.A	35
										1711.8026	R.AQTEAVFHDGPQQQR.R	7
4318	13.2	TC216254	*Oryza sativa* subsp. *japonica*	Nucleoporin-like protein, partial (22%)	36396	9.23	70	23	6			
4360	10.9	gi|357163223	*Brachypodium distachyon*	Desiccation-related protein PCC13-62-like	21932	8.92		8	1	1711.0232	R.LVAGLLGVESAQDAVIR.A	95
4392	6.6	gi|124129	*Hordeum vulgare* subsp. *vulgare*	Subtilisin-chymotrypsin inhibitor CI-1C	8253	6.78	82	36	2	1436.6643	K.TSWPEVVGMSAEK.A	61
4405	4	gi|124127	*Hordeum vulgare*	Subtilisin-chymotrypsin inhibitor CI-1B	8958, 0	5.33	161	62	3	2709.2864	R.DKPDAQIEVIPVDAMVPLDFNPNR.I	127

a) Spot numbers refer to Figure 13.
b) -: spots decreasing in intensity upon application of protease inhibitors.
c) Significant MS score above 70 for NCBI and HvGI.
d) Individual ion scores $\geq$ 40 indicate identity or extensive homology.

Table 4: Identifications of proteins of the water-unextractable fraction of liquid feed supplemented with protease inhibitors (≥ 1.5-fold spot volume ratio change and ANOVA ≤ 0.05). Protein identifications were confirmed with a MASCOTascot score of 70 for peptide mass fingerprint (ANOVA ≤ 0.05) and a minimum of 4 matched peptides or one matched peptide with a MASCOT score of 40 for MS/MS (ANOVA ≤ 0.05). Spot numbers refer to Figure 14. Comb: combined MASCOT score; Mw: molecular weight; Theor: theoretical.

Spot no.[a]	Spot relative intensity[b]	Accession no.	Organism	Protein	Mw theor.	pI theor.	PMF score[c]	Comb. score[c]	Sequence coverage (%)	Unique peptides	MS/MS precursor ions	MS/MS peptides sequences	Ion score[d]
1261	2.3	gi|809031	*Hordeum vulgare*	B1 hordein	31240	7.55	45		4	1	1361.7283	K.VFLQQQCSPVR.M	45
1463	2.6	gi|1310677	*Hordeum vulgare*	Protein z-type serpin	43307	5.61	75	166	21	8	925.5209	R.LSIAHQTR.F R.LASAISSNPER.A	27
											1144.5985	K.RLSTEPEFIENHIPK.Q	47
											1809.9634	K.RLSTEPEFIENHIPK.Q	31
1551	5.5	gi|1310677	*Hordeum vulgare*	Protein z-type serpin	43307	5.61	139	602	48	16	925.5339	R.LSIAHQTR.F	52
											1144.6201	R.LASAISSNPER.A	71
											2529.2701	K.ELNALAEQVVQFVLANESS TGGPR.I	210
											1809.988	K.RLSTEPEFIENHIPK.Q	54
											1413.7715	K.ISYQFEASSLLR.A	82
1573	3.6	gi|1310677	*Hordeum vulgare*	Protein z-type serpin	43307	5.61	118	595	43	13	925.5248	R.LSIAHQTR.F	51
											1144.604	R.LASAISSNPER.A	71
											2529.2907	K.ELNALAEQVVQFVLANESS TGGPR.I	226
											1809.97	K.RLSTEPEFIENHIPK.Q	60
											1413.7506	K.ISYQFEASSLLR.A	93
1604	4.6	gi|1310677	*Hordeum vulgare*	Protein z-type serpin	43307	5.61	96	404	26	10	925.5236	R.LSIAHQTR.F	48
											1144.6017	R.LASAISSNPER.A	84
											1809.9621	K.RLSTEPEFIENHIPK.Q	61
											1653.8544	R.LSTEPEFIENHIPK.Q	42
											1413.7423	K.ISYQFEASSLLR.A	84
1611	7	gi|1310677	*Hordeum vulgare*	Protein z-type serpin	43307	5.61	108	411	29	12	925.5251	R.LSIAHQTR.F	52
											1144.5985	R.LASAISSNPER.A	85
											1809.9611	K.RLSTEPEFIENHIPK.Q	65
											1653.8567	R.LSTEPEFIENHIPK.Q	40
											1413.7365	K.ISYQFEASSLLR.A	74
1719	4.3	gi|1310677	*Hordeum vulgare*	Protein z-type serpin	43307	5.61		151	20	7	1809.9765	K.RLSTEPEFIENHIPK.Q	49
											1413.7645	K.ISYQFEASSLLR.A	56
1760	3	TC208227	*Hordeum vulgare* subsp. *vulgare*	B-hordein partial	30146	8.16		80	15	3	1794.8434	K.VFLQQQCSPVAMSQR.I	56

1766	3.7	TC228218	*Hordeum vulgare* subsp. *vulgare*	Late embryogenesis abundant protein	27336	5.24	118	574	69	8	1976.9644	K.YGDVFDVSGDLAAQPVAPR.D	152
											2280.205	R.GQLTGPVADAGVTVTEADLPGRR.V	71
											1300.7017	R.VVTESVAGQVVGR.F	80
											2421.3053	R.FVAPPPVAATEPSGALGQDAVTIGR.A	173
1767	2.7	gi|326496021	*Hordeum vulgare*	Predicted protein (glutathione S-transferase, DHAR1)	23436	5.71		100	23	4	1607.8578	K.ALVDELQALEEHLK.A	62
1825	4.3	BE194281	*Hordeum vulgare* subsp. *vulgare*	B hordein partial	30146	8.16		63	13	2	1794.8726	K.VFLQQQCSPVAMSQR.I K.YAMREAGQSSTLLGWK.N	44
											1813.8501	K.YAMREAGQSSTLLGWK.N	5
1839	7.9	gi|2507469	*Hordeum vulgare* subsp. *vulgare*	Triosephosphate isomerase, cytosolic	26948	5.39	59	338	26	7	1537.8242	R.RSLLGESSEFVGEK.V	20
											1381.721	R.SLLGESSEFVGEK.V	54
											1374.7331	K.VIACVGETLEQR.E	71
											1604.903	K.VATPAQAQEVHANLR.D	54
											1289.6681	K.TNVSPEVAESTR.I	82
2070	2.7	gi|326490934	*Hordeum vulgare* subsp. *vulgare*	Predicted protein (enolase)	48601	5.39	49	217	18	6	1790.9408	R.AAVPSGASTGVYEALELR.D	119
											806.4497	K.YNQLLR.I	22
											978.501	K.FRAPVEPY.-	27
2082	4.5	gi|2507469	*Hordeum vulgare* subsp. *vulgare*	Triosephosphate isomerase, cytosolic	26948	5.39	91	321	26	7	1537.8011	R.RSLLGESSEFVGEK.V R.SLLGESSEFVGEK.V K.VIACVGETLEQR.E	16
											1381.7019	K.VATPAQAQEVHANLR.D K.TNVSPEVAESTR.I	52
											1374.7148	K.VIACVGETLEQR.E	54
											1604.8768	K.VATPAQAQEVHANLR.D	46
											1289.6505	K.TNVSPEVAESTR.I	73
2316	1.8	gi|167004	*Hordeum vulgare* subsp. *vulgare*	Embryo globulin	72551	6.8		339	10	6	2300.9782	R.GSESESEEEEEQQRYETVR.A R.EVQEVFR.A R.AQDQDEGFVAGPEQQSR.E	108
											906.4731	R.EVQEVFR.A	41
											1861.8708	R.AQDQDEGFVAGPEQQSR.E	160
2408	2.2	gi|229464991	*Zea mays*	Heat shock 70 kDa protein	70871	5.22	126	100	6	3	1487.778	R.TTPSYVAFTDTER.L	40
											1680.923	K.NAVVTVPAYFNDSQR.Q	34
											1675.8233	K.ATAGDTHLGGEDFDNR.M	51

2422	3.7	TC163934	*Zea Mays*	Globulin-2 precursor partial (18%)	26610	7.16	84		6	1	1711.8767	R.AQTEAVFHDGPQQQR.R	84
2460	3.7	gi|167004	*Hordeum vulgare* subsp. *vulgare*	Embryo globulin	72551	6.8		113	10	7	1360.7301	R.DTFNLLEQRPK.I	43
											1257.6432	R.SFHALANQDVR.V	51
2476	4.4	TC163934	*Zea Mays*	Globulin-2 precursor partial (18%)	26610	7.16	84		6	1	1711.8767	R.AQTEAVFHDGPQQQR.R	84
2572	6.4	gi|123970	*Hordeum vulgare* subsp. *vulgare*	Alpha-amylase inhibitor BDAI-1	17045	5.36		72	15	4	827.4669	R.VPEDVLR.D	44
2584	6.1	gi|123970	*Hordeum vulgare* subsp. *vulgare*	Alpha-amylase inhibitor BDAI-1	17045	5.36		70	15	4	827.4634	R.VPEDVLR.D	45

a) Spot numbers refer to Figure 14.
b) -: spots decreasing in intensity upon application of protease inhibitors.
c) Significant MS score above 70 for NCBI and HvGI.
d) Individual ion scores $\geq$ 40 indicate identity or extensive homology.

Table 5: Identifications of proteins from 2D-gel (pH 4-7) of the water-extractable fraction of liquid feed supplemented with thioredoxin system (Trx/NTR) ($\geq$ 1.5-fold spot volume ratio change and ANOVA $\leq$ 0.05). Protein identifications were confirmed with a MASCOT score of 70 for peptide mass fingerprint (ANOVA $\leq$ 0.05) and a minimum of 4 matched peptides or one matched peptide with a MASCOT score of 40 for MS/MS (ANOVA $\leq$ 0.05). Spot numbers refer to Figure 16. Comb: combined MASCOT score; Mw: molecular weight; Theor: theoretical.

Spot no.[a]	Spot relative intensity[b]	Accession no.	Organism	Protein	Mw theor.	pI theor.	PMF score[c]	Comb. score[c]	Sequence coverage (%)	Unique peptides	MS/MS precursor ions	MS/MS peptides sequences	Ion score[d]
505	3	gi|29134857	*Hordeum vulgare* subsp. *vulgare*	Endosperm-specific beta-amylase 1	59886	5.58	63	118	24	9	1493.6716	R.DPDIFYTDGHGTR.N	45
											1326.6649	R.YDPTAYNTILR.N	10
541	3.1	gi|38349539	*Hordeum vulgare*	Beta-amylase 1	57883	5.65	89	349	25	12	1493.6948	R.DPDIFYTDGHGTR.N	110
											1701.7376	R.SAVQMYADYMTSFR.E + 2 Oxidation (M)	18
											2013.9994	K.VPSHAAELTAGYYNLHDR.D	35
											1442.7239	R.EGLNVACENALPR.Y	52
											1326.6848	R.YDPTAYNTILR.N	31
											1386.6925	R.DPYVDPMAPLPR.S + Oxidation (M)	39
579	2.1	gi|157362219	*Hordeum vulgare*	Cytosolic glutathione reductase	53445	6.07	82	126	29	11	2901.3093	K.DGEAPVAAADDGGSYDYDLFVI GAGSGGVR.G	54
694	2.3	gi|326497219	*Hordeum vulgare*	Predicted protein	72202	5.14	74	173	21	13	1214.612	R.VEIVANDQGNR.T	4
											1487.7106	R.TTPSYVAFTDTER.L	42
											1691.7437	K.STAGDTHLGGEDFDNR.M	67
											1495.8309	K.TQVHEIVLVGGSTR.I	3
705	4.8	gi|1310677	*Hordeum vulgare* subsp. *vulgare*	Protein z-type serpin	43307	5.61		84	11	5	1809.9441	K.RLSTEPEFIENHIPK.Q	19
											1413.729	K.ISYQFEASSLLR.A	40
726	4.3	TC229089	*Hordeum vulgare* subsp. *vulgare*	Protein z-type serpin	44262	5.42		61	11	4	1413.7276	K.ISYQFEASSLLR.A	43
731	2	gi|1310677	*Hordeum vulgare* subsp. *vulgare*	Protein z-type serpin	43307	5.61	63	217	27	8	925.5235	R.LSIAHQTR.F	41
											1144.598	R.LASAISSNPER.A	44
											1413.7512	K.ISYQFEASSLLR.A	79
741	3.8	gi|1310677	*Hordeum vulgare subsp. vulgare*	Protein z-type serpin	43307	5.61		72	11	5	1413.7465	K.ISYQFEASSLLR.A	43
747	3.5	gi|1310677	*Hordeum vulgare* subsp. *vulgare*	Protein z-type serpin	43307	5.61		57	10	4	1413.709	K.ISYQFEASSLLR.A	39

775	2.6	gi|326511289	*Hordeum vulgare* subsp. *vulgare*	Predicted protein	39243	6.39	99	291	30	13	1662.8328	R.FASINVENVEDNRR.A	57
				Fructose-1,6-bisphosphate aldolase							829.3784	K.YYEAGAR.F	13
											1504.8348	K.KVTPEVIAEYTVR.T	46
											1376.7483	K.VTPEVIAEYTVR.T	84
763	3.8	TC195313	*Hordeum vulgare*	Reversibly glycosylated polypeptide partial	41499	5.82	60	209	25	9	1283.7288	K.DELDIVIPTIR.N	45
											1487.6992	K.VPEGFDYDLYNR.N	94
											839.4292	R.GYPFSLR.E	18
798	2.7	gi|326511289	*Hordeum vulgare* subsp. *vulgare*	Predicted protein	39243	6.39	79	156	30	12	829.3815	K.YYEAGAR.F	19
				Fructose-1,6-bisphosphate aldolase							1376.7507	K.VTPEVIAEYTVR.T	53
814	2,7	gi|326493440	*Hordeum vulgare* subsp. *vulgare*	Predicted protein	36130	8.2		213	15	3	2608.4259	K.GFVGDDQLGEALEGADLVIIPAG VPR.K	187
826	2.4	gi|326511741	*Hordeum vulgare* subsp. *vulgare*	Predicted protein	40517	5.5	55	186	32	8	1372.7074	K.GAGVDIDAGTELVR.R	34
											1155.6315	R.SGLSLTDPLPR.N	17
											1625.8463	K.GLAHITGGGFTDNIPR.V	49
											992.4748	K.IEDAEMLR.T + Oxidation (M)	6
											1164.5928	K.ILEESSPAYR.I	25
843	1.9	TC205675	*Hordeum vulgare*	Os05g0273800 protein	38699	6.07	93	148	44	11	1124.6381	R.SPAVKPVDGVR.A	28
											900.5469	R.LGTELVLR.K	31
											1268.5435	K.SGFTGGMNYYR.A Oxidation (M)	13
851		TC205675	*Hordeum vulgare* subsp. *vulgare*	Os05g0273800 protein	38699	6.07	86	206	41	10	1331.6613	R.HQMPALATAGYR.A Oxidation (M)	46
											1124.6467	R.SPAVKPVDGVR.A	49
											900.5542	R.LGTELVLR.K	31
											1268.5487	K.SGFTGGMNYYR.A Oxidation (M)	17
900	3.2	gi|326493416	*Hordeum vulgare* subsp. *vulgare*	Predicted protein (Glyoxalase_I)	32811	5.34	111	282	48	11	905.4691	R.MLHAVYR.V + Oxidation (M)	23
											2086.9925	K.YDIGAGFGHFAIANEDVYK.L	27
											2397.2186	K.GGSTVIAFAQDPDGYLFELIQR.G	91
											1278.6536	K.ITSFLDPDGWK.V	65

917	2.2	gi|7431022	*Hordeum vulgare*	Glucose and ribitol dehydrogenase	31912	6.54	78	322	34	10	1711.8414	K.GHEDKDAEETLQALR.D	106
											1483.6594	K.ALSGDLGYEENCR.R	54
											1237.6336	R.VVEEVANAHGGR.V	49
											834.4779	K.GAIVAFTR.A	38
											1194.5886	K.QFGSEVPMKR.A + Oxidation (M)	13
918	3.5	gi|7431022	*Hordeum vulgare*	Glucose and ribitol dehydrogenase	31912	6.54	118	349	39	13	1711.831	K.GHEDKDAEETLQALR.D	88
											1145.5797	K.DAEETLQALR.D	57
											1483.6465	K.ALSGDLGYEENCR.R	49
											1237.6314	R.VVEEVANAHGGR.V	58
1266	2.8	gi|585291	*Hordeum vulgare*	Alpha-amylase/trypsin inhibitor CMd	19140	6.07		118	30	3	1967.8761	K.LYCCQELAEIPQQCR.C	48
											1875.0038	R.LLVAPGQCNLATIHNVR.Y	46
1354	2.9	gi|326515048	*Hordeum vulgare* subsp. *vulgare*	Predicted protein	19507	5.69	68	169	40	6	1026.5106	K.AGQAYPTYR.C	43
											926.4307	K.FHHNSER.V	35
											1143.5206	R.EHGYQPPCR.N	35
1405	4.9	gi|2506771	*Hordeum vulgare*	Alpha-amylase inhibitor BMAI-1	16376	5.36		86	23	3	2218.28	K.ELGVALADDKATVAEVFPGCR.T	62
1406	8.6	gi|2506771	*Hordeum vulgare*	Alpha-amylase inhibitor BMAI-1	16376	5.36		248	36	5	1490.7443	K.SQCAGGQVVESIQK.D	80
											2218.1472	K.ELGVALADDKATVAEVFPGCR.T	71
											1206.6135	K.ATVAEVFPGCR.T	57
1468	2	gi|408795920	*Hordeum vulgare* subsp. *vulgare*	Chloroplast Cu/Zn SOD1	19698	5.31	45	159	26	3	2488.2248	K.GTSQVEGVVTLTQDDDGPTTVNVR.I	2
											1471.7417	R.AFVVHELEDDLGK.G	85
											1412.6875	K.GGHELSLSTGNAGGR.L	56
1483	3.3	gi|225103	*Hordeum vulgare* subsp. *vulgare*	Trypsin/amylase inhibitor pUP38	12417	4.94		114	29	2	1967.8872	K.LYCCQELAEIPQQCR.C	65
1504	19.7	gi|255311878	*Arabidopsis thaliana*	Chain A, Crystal structure of the plant stress-response enzyme Akr4c8 (Aldo-keto reductases)	37110	6.97		87	16	4	1768.9549	M.GSSHHHHHHSSGLVPR.G	68
1554	7.2	gi|123970	*Hordeum vulgare*	Alpha-amylase inhibitor BDAI-1	17045	5.36	40	80	28	4	827.4627	R.VPEDVLR.D	59

1542	8.4	gi|186972808	*Hordeum vulgare* subsp. *vulgare*	Chain A, Crystal structure of barley thioredoxin H - isoform 2 in the oxidized state.	13271	5.12	51	183	52	6	1191.6679	R.IMAPVFADLAK.K + Oxidation (M)	58
											935.531	K.FPNAVFLK.V	33
											1257.7542	R.VVGAIKEELTAK.V	42
1408	6.2	gi|2506771	*Hordeum vulgare*	Alpha-amylase inhibitor BMAI-1	16376	5.36		157	36	5	1490.7361	K.SQCAGGQVVESIQK.D	47
											1206.6266	K.ATVAEVFPGCR.T	72
1459	26.8	gi|585289	*Hordeum vulgare*	Alpha-amylase/trypsin inhibitor CMa	16060	5.87		61	6	2	1224.6566	R.RSHPDWSVLK.D	2
											1068.572	R.SHPDWSVLK.D	50
1596	6.9	gi|123970	*Hordeum vulgare*	Alpha-amylase inhibitor BDAI-1	17045	5.36	36	74	28	4	827.4534	R.VPEDVLR.D	50
1691		gi|124127	*Hordeum vulgare*	Subtilisin-chymotrypsin inhibitor CI-1B	8958	5.33		178	62	3	1463.7313	K.YPEPTEGSIGASGAK.R	117
											2709.3815	R.DKPDAQIEVIPVDAMVPLDFNP NR.I + Oxidation (M)	34
1070	3.7	gi|326502266	*Hordeum vulgare* subsp. *vulgare*	Predicted protein	26423	5.51	96	304	40	9	1961.9362	R.QVEAHHFCAHLNEDVR.Q	37
											1419.6914	R.QCLIFDGPDAGAR.L	56
											1529.8444	R.GGVLFMPGVPGVVER.R + Oxidation (M)	55
											1187.5978	K.TVHFWQVDR.G	49
											1213.6261	R.EVDLPAANTGAR.I	45
1683	3	gi|124127	*Hordeum vulgare*	Subtilisin-chymotrypsin inhibitor CI-1B	8958	5.33		235	62	3	1463.7142	K.YPEPTEGSIGASGAK.R	118
											2709.3721	R.DKPDAQIEVIPVDAMVPLDFNP NR.I + Oxidation (M)	90

a) Spot numbers refer to Figure 16.
b) -: spots decreasing in intensity upon application of Trx/NTR.
c) Significant MS score above 70 for NCBI and HvGI.
d) Individual ion scores $\geq$ 40 indicate identity or extensive homology.

Table 6: Identifications of proteins from the 2D-gel (pH 4-7) of the water-unextractable fraction of liquid feed supplemented with thioredoxin system (NTR/Trx) ($\geq$ 1.5-fold spot volume ratio change and ANOVA $\leq$ 0.05). Protein identifications were confirmed with a MASCOT score of 70 for peptide mass fingerprint (ANOVA $\leq$ 0.05) and a minimum of 4 matched peptides or one matched peptide with a MASCOT score of 40 for MS/MS (ANOVA $\leq$ 0.05). Spot numbers refer to Figure 17. Comb: combined MASCOT score; Mw: molecular weight; Theor: Theoretical.

Spot no.[a]	Spot relative intensity[b]	Accession Number	Organism	Protein name	Mw Theor.	pI Theor.	Comb. score[c]	Sequence coverage (%)	Unique peptides	MS/MS precursor ions	MS/MS peptides sequences	Ion score[d]
1060	1.8	gi|82548223	*Hordeum vulgare*	B Hordein	30593	8.16	157	14	3	1794.8802	K.VFLQQQCSPVAMSQR.I	23
										1666.8255	R.TLPTMCSVNVPLYR.I	51
										997.6100	R.IVPLAIDTR.V	70
1140	3.7	gi|82548223	*Hordeum vulgare*	B Hordein	30593	8.16	122	8	2	1666.8267	R.TLPTMCSVNVPLYR.I	43
										997.6094	R.IVPLAIDTR.V	72
1795	3.2	gi|304396928	*Pantoea* sp. aB	Inorganic diphosphatase	19897	5	321	27	6	1969.0136	K.EYDHIKDVNDLPELLR.A	126
										1183.6317	K.DVNDLPELLR.A	48
										1905.9508	R.AQITHFFEHYKDLEK.G	93
1865	7.3	gi|372274053	*Pantoea* sp. aB	Type VI secretion system, core protein	19703	4.78	110	35	6	1683.8261	R.VQIEYDVEIYGAER.K	56
2039	4.2	gi|1536911	*Hordeum vulgare* subsp. *vulgare*	17 kDa class I small heat shock protein	16832	5.83	257	30	4	1909.9412	R.SIVPAFSGNSETAAFANAR.V	79
										2086.0730	K.EEVKVEVEDGNVLVVSGER.T	79
										1600.8239	K.VEVEDGNVLVVSGER.T	42
										975.5290	R.FRLPEDAK.V	13
2082	1.8	gi|585291	*Hordeum vulgare* subsp. *vulgare*	Alpha-amylase/trypsin inhibitor CMd	19140	6.07	138	28	3	1967.8678	K.LYCCQELAEIPQQCR.C	75
										1876.0108	R.LLVAPGQCNLATIHNVR.Y	44
2108	2	gi|585291	*Hordeum vulgare* subsp. *vulgare*	Alpha-amylase/trypsin inhibitor CMd	19140	6.07	55	19	2	1876.0253	R.LLVAPGQCNLATIHNVR.Y	43
2113	2.4	gi|308185833	*Pantoea vagans* C9-1	OsmC gene product Osmotically inducible protein	15061	5.28	206	38	4	1099.5198	K.GSAHWEGDIK.G	44
										2128.0266	K.GTVSTESGVLSQQPYGFNTR.F	122
2124	2.1	gi|585291	*Hordeum vulgare*	Alpha-amylase/trypsin inhibitor CMd	19140	6.07	84	39	4	1876.0179	R.LLVAPGQCNLATIHNVR.Y	59
2163	4.3	gi|2506771	*Hordeum vulgare*	Alpha-amylase inhibitor BMAI-1	16376	5.36	111	23	3	2218.1234	K.ELGVALADDKATVAEVFPGCR.T	46
										1206.5773	K.ATVAEVFPGCR.T	39
2169	12.9	gi|372276145	*Pantoea* sp. SL1_M5	Outer membrane protein X	18318	4.97	174	30	4	1372.6797	K.GQYYGVTGGPAFR.L	85
										1082.4922	K.FQQNDTSSR.D	63

2202	2	gi|585290	*Hordeum vulgare*	Alpha-amylase/trypsin inhibitor CMb	17199	5.77	106	32	4	1168.4954	R.DYVEQQACR.I	47
										1023.4797	R.EVQMDFVR.I	35
										1039.4914	R.EVQMDFVR.I	33
2204	2.1	gi|2506771	*Hordeum vulgare*	Alpha-amylase inhibitor BMAI-1	16376	5.36	127	23	4	2218.1177	K.ELGVALADDKATVAEVFPGCR.T	53
										1206.5878	K.ATVAEVFPGCR.T	45
2234	2.4	gi|585290	*Hordeum vulgare*	Alpha-amylase/trypsin inhibitor CMb	17199	5.77	121	32	4	1168.5004	R.DYVEQQACR.I	53
										1023.4875	R.EVQMDFVR.I	44
										1039.499	R.EVQMDFVR.I	43
2189	9.8	gi|327393264	*Pantoea ananatis* AJ13355	Outer membrane protein X precursor OmpX	18407	5.36	112	36	4	1386.6867	K.GQYYGITGGPAFR.L	82
2212	6.2	gi|758343	*Hordeum vulgare subsp. vulgare*	CMd preprotein	17894	5.24	147	40	3	1967.8932	K.LYCCQELAEIPQQCR.C	63
										1876.0081	R.LLVAPGQCNLATIHNVR.Y	61
2245	2.6	gi|585290	*Hordeum vulgare*	Alpha-amylase/trypsin inhibitor CMb	17199	5.77	53	11	2	1039.4850	R.EVQMDFVR.I	43
2273	7.8	gi|2707920	*Hordeum vulgare* subsp. *vulgare*	BTI-CMe4 protein	16743	8.05	83	24	2	1614.6671	R.CCDELSAIPAYCR.C	65
2279	1.8	gi|585290	*Hordeum vulgare*	Alpha-amylase/trypsin inhibitor CMb	17199	5.77	105	22	3	1168.4857	R.DYVEQQACR.I	44
										1023.4774	R.EVQMDFVR.I	46
										1039.4899	R.EVQMDFVR.I	41
2303	2.4	gi|167004	*Hordeum vulgare* subsp. *vulgare*	Embryo globulin	72551	6.8	86	6	4	1599.8520	K.LGSPAQELTFGRPAR.E	68
2388	2.2	gi|671537	*Hordeum vulgare* subsp. *vulgare*	D-hordein	51154	7.6	88	7	4	1477.6981	R.ELQESSLEACRR.V	24
										1811.9287	R.DVSPECRPVALSQVVR.Q	48
2397	8.1	gi|186972808	*Hordeum vulgare* subsp. *vulgare*	Chain A, Crystal Structure Of Barley Thioredoxin H Isoform 2 In The Oxidized State	13271	5.12	180	52	6	1191.6533	R.IMAPVFADLAK.K	57
										1257.7459	R.VVGAIKEELTAK.V	63
2515	4.4	TC206849	*Hordeum vulgare* subsp. *vulgare*	Cold-shock DNA-binding domain protein	9271	5.02	58	26	2	1254.5970	K.GFGFITPEDGSK.D	42
2562	6.4	gi|124125	*Hordeum vulgare* subsp. *vulgare*	Subtilisin-chymotrypsin inhibitor CI-1A	8877	5.24	106	62	3	1436.6979	K.TSWPEVVGMSAEK.A	69

a) Spot numbers refer to Figure 17.
b) -: spots decreasing in intensity upon application of Trx/NTR.
c) Significant MS score above 70 for NCBI and HvGI.
d) Individual ion scores $\geq$ 40 indicate identity or extensive homology.

CHAPTER 3

The impact of harvest year and growing site on the variability of microbial, endogenous xylanases and xylanase inhibition activity levels in different barley cultivars and a portrait of the xylanase inhibitors in the barley proteome using 2-DE and immunoblotting

3. ABSTRACT

Xylanases are commonly used as supplements in animal feed and the production of juice and bread. Cereals like barley produce xylanases for remodeling of the cell walls during seed development and germination. In addition, cereals also contain microbial xylanases produced by microbes residing on outer kernel layers of the grains. Accumulating evidence show that microbial xylanases are highly sensitive to inhibition by known proteinaceous xylanase inhibitors (TAXI, XIP and TLXI) present in cereal grains. Rather limited data on the variability of xylanase and xylanase inhibition activities in barley are available. To fill this gap, the variability of xylanases (plant vs. microbial) and xylanase inhibition activities across different barley cultivars were investigated with application of activity assays and gel-based proteomics analysis (2DE and MALDI-TOF MS) coupled with immunoblotting. Considerable inter-cultivar variability in the levels of microbial and endogenous xylanases, as well as at xylanase inhibition activities was found, where the harvest year and cultivar had a significant impact on all three parameters (p-value < 0.05). The occurrence and variability of the different isoforms of the different xylanase inhibitor proteins present in barley cultivars were also detected in immunoblots probed with anti-TAXI, -XIP and -TLXI antibodies. A correlation between microbial xylanase activity and xylanase inhibition activity levels was found for the barley cultivars, 1978 and Power, having high xylanase inhibition activity and low microbial xylanase activity levels. These two cultivars also displayed an additional band in the western blots probed with anti-XIP. To gain an insight into the impact of location on the variability of xylanase and xylanase inhibition activity levels, barley cultivars of two successive harvest years (2010 and 2011) and five different growing sites were analyzed. The growing sites were found to have a significant impact on the variability in endogenous xylanases activities, while cultivar was the main factor to contribute to the variability in the microbial xylanases and inhibition activity levels.

3.1. INTRODUCTION

With the global climate changes there has been an increase in occurrence, duration and severity of abiotic stresses such as drought, salinity, oxidative stress and extreme temperatures. These abiotic stresses represent a serious treat to agriculture as they affect quality properties and characteristics, as well as yield of cereal crops. Grain composition is greatly dependent on the genetic background, climatic and agronomic conditions. Variations in yield are of high importance for the farmers, while functionality is of a broader concern in particular the cereal-processing industry. Considerable efforts have gone into investigation of the impact of variety and growing conditions on the rate and duration of grain filling and on the composition and size of mature grains. The main research area has been on their affect on the content of starch and protein, and their properties (Dupont and Altenbach, 2003). Meanwhile, very little is known about the less abundant constituents, such as cell wall degrading enzymes (e.g. endo-1,4-β-xylanase), enzyme inhibitors (e.g. xylanase inhibitors, non-starch polysaccharides (NSP, e.g. arabinoxylan (AX)) and lipids, respectively, and their effect on the functional properties of the cereal grain (Groseart et al., 2005). AXs are highly abundant non-starch polysaccharides in plant cell walls, composed of a linear β-D-1,4-linked xylopyranosyl backbone substituted with arabinose and/or glucuronic acid residues (Courtin and Delcour, 2002). Studies show that AXs greatly affect the functional properties of cereals e.g. during bread making (Courtin and Delcour, 2002), refrigerated dough (Courtin et al., 2005), gluten-starch separations (Frederix et al., 2004) and animal feeds (Bedford and Schulz, 1998).

In cereal-based industry, microbial xylanases are routinely applied to improve product quality. Endo β-1,4-xylanases (EC 3.2.1.8) hydrolyze, in a random manner, internal β-1,4-glycosidic linkages between xylose units in the AX backbone, and hence drastically impact their molecular mass, solubility and physico-chemical properties (Courtin and Delcour, 2002). Xylanases are produced in high abundance by fungi and bacteria, however they have also been found in plants, marine algae, insects, and protozoa (Dekker and Richard, 1976). Cereals synthesize xylanases for expansion and modulation of the cell wall during cell growth and/or degradation of the cell wall during seed germination (Dekker and Richard, 1976; Simpson et al., 2003). Moreover,

xylanases are also produced by the commensal microorganisms populating the surface of the grains. These grain-associated xylanases occur on the surface and/or outer layers of the grains, while the plant endogenous xylanases can be found inside the kernels (Dornez et al., 2006). It has been reported that the majority of the xylanase activity is contributed by the grain-associated microbial xylanases, while only a minor part is by endogenous xylanases. For wheat, it was estimated that 90-85% of the xylanase activity was due to microbial xylanases, while 10-15% of plant origin (Dornez et al., 2006). However, a large part of the microbial xylanase is significantly inactivated/inhibited by the endogenous xylanase inhibitors.

Cereals have evolved groups of proteinaceous xylanase inhibitors, which are specifically active towards xylanases of microbial origin. Three structurally distinct classes of inhibitors with distinct specificities have been identified, i.e. *Triticum aestivum* xylanase inhibitor (TAXI), xylanase inhibitor protein (XIP), and thaumatin-like xylanase inhibitor (TLXI), respectively (Beaugrand et al., 2006; Gebruers et al., 2004; Juge, 2006). The three inhibitors types have been detected in whole meals of cereals (Beaugrand et al., 2006), and additionally, TAXI- and XIP-type inhibitors have also been purified from several cereals (Gebruers et al., 2002a). XIP-type inhibitors are specifically active against fungal xylanases belonging to GH family 10 and 11 (Payan et al., 2004), whereas TAXI and TLXI-type exclusively inhibit bacterial and fungal xylanases of GH11 (Fierens et al., 2007; Gebruers et al., 2004, Juge, 2006). The three-dimensional structures of TAXI, XIP and recently TLXI-type inhibitors extracted from wheat have been resolved (Payan et al., 2004; Sansen et al., 2004; Vandermarliere et al., 2010). Various roles have been ascribed to these inhibitors including their involvement in seed development and germination, and defence mechanisms (Croes et al., 2008; Gebruers et al., 2004). Due to that fact that xylanase inhibitors are structurally homologous to many defence-related proteins, induced upon pathogen attack and wounding, and show almost exclusive activity towards microbial xylanases (*in vitro*), they are believed to play a role in defence rather any regulatory function in the plant (Flatman et al., 2002; Gebruers et al., 2001; Goesaert et al., 2004). In addition, xylanase inhibitors occur as multi-isoform families, which is typical of defence-related proteins being encoded by related genes. As the presence of multiple genes can ensure the expression of at least one gene member to counteract the

different infection scenarios. Both transciptomics and proteomics studies revealed that xylanase inhibitors occur as polymorphic families with expression of multiple putative TAXI- and XIP-type genes and one TLXI gene (Croes et al., 2008; Igawa et al., 2004; 2005). Considerable variability in xylanase activity and xylanase inhibitor levels has been found between different cereal species and within different varieties, where the variability has been ascribed to genetic, climatic and agricultural factors (Beaugrand et al., 2006; Croes et al., 2009b; Gebruers et al., 2002b; Gebruers et al., 2010). A study by Gebruers et al. (2002b) revealed that the inhibitor levels in different wheat cultivars varied with a factor of 2-3, due to genetic and/or environmental effects. Accumulating research show that xylanase and xylanase inhibitor activity levels significantly impact the variability of functionality of cereals during processing. Due to the fact that the grain-associated xylanases are specifically inactivated by the endogenous xylanase inhibitors, the reported xylanase activity values have been presumably highly underestimated. Rather limited data on the variability of xylanase activity levels in cereals are available, albeit more attention has been received with the coming years (Cleemput et al., 1995; Dornez et al., 2009; Gebruers et al., 2010). In order to quantify the microbial and endogenous xylanases separately, a washing method developed by Dornez et al. (2006) was applied, which enables a physical separation of the microbial, endogenous xylanases as well as the xylanase inhibitors. In this way one can avoid or minimize complex formation between the microbial xylanases and xylanase inhibitors, which typically escape activity measurements, thus representing a more accurate estimation of the activity levels.

In the present study, xylanase (microbial and endogenous) and xylanase inhibition activities in different barley cultivars harvested from two successive years were examined to determine the degree of the variability and the impact of cultivar/genotype and harvest year. A sample set of two different barley cultivars harvested in year 2010 and 2011 in five different locations in Denmark were similarly analyzed for xylanase and xylanase inhibition levels to gain an insight into the impact of growing site/environment and genotype to this variability. A proteomics approach coupled with activity assays and immunoblots were applied on extracts of isolated aleurone layers of barley grains in order to profile and identify the different types and isoforms of xylanase inhibitors.

3.2. MATERIALS AND METHODS

3.2.1. BARLEY GRAINS, GROWING SITES AND HARVEST

Barley grains (Barke, Cabaret, Frederik, Power, Prolog, Scarlett, Simba, and 1978) were grown at the growing sites at Sejet Plant Breeding (Horsens, Denmark) in two successive growing periods (2009 and 2011). Each cultivar was grown in three different plots in the same field in a fully randomized block design and the grains from the different plots were mixed to remove location effects. Meanwhile the two barley cultivars, i.e. Quench and Simba, were grown at five different growing sites in Denmark, namely Abildsgaard, Holeby, Koldkærgaard, Sejet and Tystofte in two successive year 2010 and 2011. It was not possible to get hold of barley grains from Holeby of year 2010, thus these were not included in some of the analysis. All the studied cultivars were spring barley.

3.2.2. APPLICATION OF THE WASHING PROCEDURE

In order to determine the total xylanase activities in barley, a washing procedure developed by Dornez et al. (2006) was implicated with some modifications. The procedure enabled effective separation of surface associated (microbial) and plant endogenous xylanases. Preliminary experiments were performed to test different washing liquids ability to remove microbial xylanases quantitatively from the barley kernels. However, sodium acetate pH 5.0 was considered to efficiently remove most of the surface associated xylanases. The washing liquids were supplemented with sodium azide (0.02% w/v) to inhibit any bacterial growth during the washing period. Different washing periods, i.e. 2, 4, 6, 24 and 48 h, were carried out using sodium acetate (25 mM, pH 5.0) and sodium azide (0.02% w/v). Xylanase activity levels remained relatively constant after 6 h of incubation, but for convenience the grains were typically washed for 15 h or overnight. In addition, two different temperatures (4 and 20°C) during washing were tested, and evidently the lower temperature resulted in a lower xylanase activity. This was probably due to a poor washing efficiency at the low temperature. Commencement of germination is accompanied by production of several

enzymes, including xylanases, thus it could be speculated that the higher xylanase activity could be due to germination of the grains. However, preliminary experiments with incubation of washing liquids with excess of seed extract containing xylanase inhibitors inhibited almost all xylanase activity, thus showing that the xylanases in the washing liquids are almost exclusively of microbial origin and not of plant origin. Moreover, application of seed extract to unwashed whole grain seed extract resulted in no significant reduction in xylanase activity (data not shown). On this basis of these findings, the cause of germination was excluded. Dornez et al. (2006) reported similar findings for wheat, where they tested inhibition of the washing liquids and unwashed seed extract with excess of TAXI- and XIP-type inhibitors.

The initial wash was follow by an additional wash with water for detection of any remaining xylanases on the grains. Activity levels recovered in the secondary wash were rather close to zero and were considered negligible. Endogenous xylanase activity levels were additionally measured in the extract of the washed kernels.

3.2.3. SEED EXTRACT PREPARATION

Unwashed and washed barley kernels were ground on an IKA Labortechnik A10 laboratory mill (Janke & Kunkel, Germany). A weight of 0.5 g flour was suspended in 25 mM sodium acetate (5 mL, pH 5.0) and extraction was performed for 45 min at room temperature under agitation. The suspensions were centrifuged for 20 min at 4,000 x g and the supernatants were filtered through a MN 615 filter paper (Macherey-Nagel, Germany) and kept at -20°C until use.

3.2.4. ENZYME ACTIVITY ASSAY

Xylanase activities were determined with the colorimetric Xylazyme-AX method (Megazyme, Bray, Ireland), which is based on the measurement of the quantity of product released during the enzyme reaction on the substrate i.e. azurine-cross linked wheat arabinoxylan (AZCL-AX). The assay was carried out in 0.5 mL of a reaction volume of either washing liquid or seed extract in 25 mM sodium acetate (pH 5.0). The samples were

pre-incubated at 40°C for 10 min prior to addition of AZCL-AX tablets (30% w/v). After additional 30 min of incubation at 40°C, the reaction was terminated with addition of 5 mL of 2% (w/v) TRIS base solution (pH 9.0) and vigorous mixing. The reaction mixtures were incubated for 10 min at room temperature, filtered and the absorbance values were measured at 590 nm wavelength using UV/visible spectrophotometer (Ultraspec II, Amersham Biosciences) against a blank. The reaction blank was prepared by adding stop solution (2% w/v TRIS, pH 9.0) to the samples prior to addition of substrate. Correction was made for non-enzymatic color release by the AZCL-AX tablets. Control samples were prepared for each reaction sample with addition of specific amount of microbial xylanases (e.g. 5 mU, *Tricoderma longibrachiatum,* pI 9.0, Megazyme). Activity levels were expressed as xylanase units (U). One unit of activity is defined as the amount of enzyme required to release one micromole of D-xylose reducing-sugar-equivalents from wheat arabinoxylan (30% w/v) per minute at 40°C, under defined assay conditions. Xylanase activity levels from the washing liquids were expressed as U per gram originally treated barley seeds. All measurements were performed in quadruplicate.

3.2.5. CALCULATION OF ENZYME ACTIVITY

One unit of xylanase activity is defined as the amount of enzyme needed to release one micromole of xylose reducing sugar equivalents from wheat arabinoxylan per min under defined assay conditions. The level of xylanase activity was calculated as:

$$\text{Enzyme activity } (U/g) = \textit{Added activity} \times \frac{(A_{590}\text{ sample} - A_{590}\text{ blank})}{(A_{590}\text{ control} - A_{590}\text{ blank}) - (A_{590}\text{ sample} - A_{590}\text{ blank})}$$

A_{590} is absorbance reading at 590 nm. Added activity is the amount of xylanase (i.e. 5 mU, *Tricoderma longibrachiatum* pI 9.0, Megazyme) added to the sample at the time of assay. Samples are either the washing liquids or seed extracts. Control is the sample with the added xylanase.

3.2.6. PROTEIN DETERMINATION

The protein content of the washing liquids and seed extracts were estimated by the method of Popov et al. (1975). Bovine serum albumin (BSA) was used as a reference and a concentration range from 0.25 µg/µL to 40 µg/µL was used to plot a standard curve.

3.2.7. XYLANASE INHIBITION ASSAY

Relative inhibition activities of a set of barley cultivars were determined with a variant of Xylazyme AX method as described by Gebruers et al. (2001). It is well known that xylanase inhibitor levels vary considerably across the different barley cultivars. Same amount of protein (containing mixtures of xylanase inhibitors, ca. 16 µg) was applied to washing liquids of barley cultivar Cabaret 2009 (containing mixtures of xylanases), in order to compare the xylanase inhibition efficiency by the different barley cultivars against the microbial xylanases. The washing liquid (Cabaret, 2009) was used as the enzyme source. The assay was performed in 0.5 mL of a reaction mixture volume containing an equal amount of washing liquid (450 µL) mixed with diluted seed extract (inhibited, ca. 22 µg protein) or buffer (uninhibited reference). The seed extracts were appropriately diluted with sodium acetate buffer (25 mM, pH 5.0) to ensure a linear response between the amount of inhibitors and the measured residual activity. The reaction mixtures were pre-incubated for 30 min at room temperature for formation of enzyme-inhibitor complex, followed by addition of the AZCL-AX tablet and further incubation at 40°C for 30 min. The reaction was stopped by addition of 2% w/v Tris solution (pH 9.0) and immediate vortex mixing. After 10 min of incubation, the solutions were filtered and the absorbance values (590 nm) were measured against a blank, prepared by incubating the seed extract with buffer (without enzyme source). The controls allowed correction for non-enzymatic color release, whereas the blanks also corrected for xylanase activity present in the seed extract. All the measurements were performed in duplicate. The inhibition efficiency was expressed as a percentage reduction in xylanase activity and calculated as follows:

$$\text{Inhibition (\%)} = \frac{(A_{590}\text{ reaction wihout XIs} - A_{590}\text{ blank}) - (A_{590}\text{ reaction with XIs} - A_{590}\text{ blank})}{(A_{590}\text{ reaction without XIs} - A_{590}\text{ blank})} \times 100$$

A_{590} is absorbance reading at 590 nm. The control comprises of enzyme and buffer, the sample comprises of enzyme and inhibitors and the blank constitute of seed extract and buffer. The inhibition efficiency towards xylanases corresponds to the amount of inhibitor protein needed to inhibit/reduce the xylanase activity with 50% in the described assay.

3.2.8. PLANT MATERIAL, ISOLATION OF ALEURONE LAYERS AND PROTEINS EXTRACTION

The aleurone layers were prepared from barley cultivar Simba harvested in 2009 provided from Sejet Plant Breeding (Horsens Denmark). The embryo and the distal (1-2 mm) of the grains were removed using a scalpel. The embryoless half-seeds were soaked for 30 min in 50% (v/v) sulfuric acid to dissolve the seed coat and rinsed five times with water. Subsequently, the seeds were washed once with 70% (v/v) ethanol and five times with sterile water. The washed seeds were soaked for four days at 4°C in sterile water containing 50 µg/mL ampicillin and 5 µg/mL nystatin. The starchy endosperm was scraped away from the aleurone layers, keeping the layers as intact as possible. The isolated tissues were frozen in liquid nitrogen and kept at -80°C until use. The aleurone layers (100 mg fresh weight) were freeze-dried and ground using a pre-cooled ceramic mortar. The powder was suspended in 0.5 mL ice-cold buffer (50 mM Tris HCl pH 7.5, 1 mM $CaCl_2$) containing complete protease inhibitor cocktail (Roche), two glass beads were added, and incubated for 30 min at 4°C under agitation. The mixtures were centrifuged (14,000 x g, 15 min, 4°C) and the supernatant collected. Protein concentration of the supernatants was quantified by the method of Popov et al. (1975) prior to storage at -80°C until use.

3.2.9. 1D-GEL ELECTROPHORESIS AND WESTERN BLOTTING

Protein extracts (16 µg) were separated on 4−12% Bis-Tris NuPAGE gels (Novex system, Invitrogen) with NuPAGE MES running buffer (Invitrogen). Samples were prepared by mixing 1 part of 4 x LDS sample buffer (2% w/v LDS (lithium dodecyl sulfate), 106 mM Tris-HCl, 141 mM Tris-base, 10% (w/v) glycerol, 0.51 mM EDTA, 0.22 mM SERVA blue G250, 0.175 mM phenol red, pH 8.5) to 4 parts of protein extract and incubated for 5 min at 95°C. The samples were loaded on to the gel and electrophoresis was carried out with a current of 2 mA per lane. The protein bands were fixed (2% (v/v) phosphoric acid, 70% (v/v) ethanol) for 30 min, stained with colloidal Coomassie Blue (10% (w/v) ammonium sulphate, 10% (v/v) phosphoric acid, 20% (v/v) ethanol, 0.12% (w/v) colloidal Coomassie Brilliant Blue G-250) overnight, and destained by repeated washing with water. A replica gel was prepared, and the separated proteins were transferred to a nitrocellulose membrane (0.20 µm pore size, Hybond N, GE Healthcare) using the Novex system (Invitrogen) according to the manufacturer's instructions. The transfer was carried out for 1 h at 30 V and 160 mA. The free protein binding sites on the blots were blocked for 1 h with tris-buffered saline 20 (150 mM NaCl, 25 mM Tris HCl, pH 7.5, referred to as TBS) with 0.5% (v/v) tween. The membrane was probed with the primary antibodies, i.e. rabbit polyclonal antibodies raised against XIP, TAXI and TLXI from wheat diluted 1:2000 in TBS (0.1% v/v tween) overnight at 4°C (generous gift of Christophe M. Courtin, Laboratory of Food Chemistry and Biochemistry, Catholic University of Leuven, Belgium). The membrane was washed (3 x 10 min) in TBS (0.1% tween), blocked as before, and probed with secondary goat anti-rabbit antibodies conjugated with horseradish peroxidase (1:20000 dilution, DAKO) for 1 h. The membrane was washed again (3 x 10 min) in TBS (0.1% v/v tween) and developed using enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare). Replica gels were prepared for spot picking and peptide sequencing.

3.2.10. 2D-GEL ELECTROPHORESIS AND WESTERN BLOTTING

For 2D-gel electrophoresis, the protein extracts from aleurone layers (ca. 50 µg) were desalted on a NAP-5 column (GE Healthcare) and concentrated with 10% (v/v) TCA/acetone precipitation. This was carried out by adding four volumes of 10% (v/v) TCA in acetone to the extract and incubated overnight at -20°C. The pellet was collected by centrifugation (14,000 x g, 15 min, 4°C) and washed twice with 80% (v/v) acetone and air-dried. The precipitate was dissolved in 100 µL rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS (3-(3-cholamidopropyl) dimethylammonio)-1-propane- sulfonate), 200 mM destreak reagent (bis(2-hydroxyethyl) disulfide), 5% (v/v) glycerol, 10% (v/v) 2-propanol, and 1% (v/v) pharmalyte pH range 6−11 (GE Healthcare, Uppsala, Sweden) and a trace of bromophenol blue). Immobiline pH gradient (IPG) strips (pH 6−11, 11 cm, GE Healthcare) were passively rehydrated (200 µL rehydration buffer) overnight (Immobiline DryStrip Reswelling Tray) at room temperature. Separation by isoelectric focusing (IEF) was carried out on an IPGphor (Ettan™, GE Healthcare). Protein extract (50 µg) in 100 µL rehydration buffer were applied using cup loading (Cup Loading Manifold, GE Healthcare) according to manufacturer's instructions. IEF was performed at 50 V for 5 h, 100 V for 6 h, 1000 V for 1 h, gradient to 8000 V for 3 h, and kept at 8000 V to a total of 20 kVh. After IEF, the strips were equilibrated for 2 × 15 min in 5 mL equilibration buffer (6 M urea, 30% glycerol, 50 mM Tris HCl, pH 8.8, 2% SDS, 0.01% bromophenol blue) supplemented with 1% (w/v) dithiothreitol (DTT) and 2.5% (w/v) iodoacetamide (IAA) in the first and second step, respectively. Prior to second dimension, the strips were cut to give 7 cm in the pH range 7−10. SDS-PAGE was carried out on 4-12% Bis-Tris Zoom gels (NuPAGE, Invitrogen) using NuPAGE MES running buffer (Invitrogen) according to the instructions. A broad range pre-stained protein ladder (SeeBlue, Invitrogen) was used for molecular mass determination. Immunoblotting was performed exactly as described above. Replica gels were prepared for spot picking and peptide sequencing.

3.2.11. IN-GEL ENZYMATIC DIGESTION AND MASS SPECTROMETRY

Spots on the 2D-gels were excised manually and subjected to in-gel tryptic digestion with certain modifications (Hellman et al., 1995). Briefly, the gel pieces were washed with 100 µL 40% ethanol (10 min), shrunk in 50 µL 100% (v/v) acetonitrile (ACN) and soaked in 2 µL 12.5 ng/µL trypsin (Promega, porcine sequencing grade) in 25 mM NH_4HCO_3 on ice for 45 min. The gel pieces were rehydrated by addition of 10 µL 25 mM NH_4HCO_3 followed by incubation at 37°C overnight. Tryptic peptides (1 µL) were loaded on to the AnchorChip™ target plate (Bruker-Daltonics, Bremen, Germany), covered by 1 µL matrix solution (0.5 µg/µL α-cyano-4-hydroxycinnamic acid (CHCA) in 90% v/v ACN, 0.1% v/v TFA) and washed in 0.02% (v/v) TFA. Tryptic peptides were analyzed by Ultraflex II MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) using Flex Control v3.0 (Bruker-Daltonics, Bremen, Germany) and processed by Flex Analysis v3.0 (Bruker-Daltonics, Bremen, Germany). Peptide mass maps were acquired in positive ion reflector mode with 500 laser shots per spectrum. MS/MS data were acquired with an average of 1000-2000 laser shots for each spectrum. Spectra were externally calibrated using a tryptic digest of β-lactoglobulin (5 pmol/µL). Internal calibration was performed by using trypsin autolysis products and keratin contaminants. MS and MS/MS spectra were analyzed using Biotools v3.1 (Bruker-Daltonics) and searched against the NCBInr (National Center for Biotechnology Information) and DFCI (Dana Farber Cancer Institute) barley gene index Release 12.0 (http://compbio.dfci.harvard.edu/tgi) databases using the MASCOT 2.0 software (http://www.matrixscience.com). The following search parameters were applied: Taxonomy: Viridiplantae (Green plants); monoisotopic peptide mass accuracy of 80 ppm, fragment mass accuracy to $\pm$ 0.7 Da; a maximum of one missed cleavage; carbamidomethylation of cysteine and partial oxidation of methionine, respectively. No restrictions with respect to protein Mr and pI were made. The signal to noise threshold ratio (S/N) was set to 1:6. Protein identifications by PMF were confirmed with a MASCOT score of 70, $p \leq 0.05$ and a minimum of 4 matched peptides. A single peptide based protein identifications by MS/MS analysis was confirmed with a MASCOT score of 30, $p \leq 0.05$.

3.2.12. STATISTICS

The data were amalysed using STATGRAPHOCS v4.0. A two-way ANOVA (analysis of variance) was performed to detect significant differences among the cultivars and harvest year. Where harvest year and cultivars were treated as fixed factors. A Fisher's least significant difference (LSD) procedure with a 5% significance level was used to evaluate the significant differences (p-value < 0.05). Another analysis was performed to quantify the contribution of harvest year and genotype/cultivar to the total variance in microbial, endogenous xylanase and inhibition levels. Variance component analysis was performed with harvest and genotype as random factors.

3.3. RESULTS AND DISCUSSION

3.3.1. CHARACTERIZATION OF THE WEATHER CONDITIONS DURING GROWING SEASON

Weather conditions and statistics for year 2009−2011 were obtained from the Danish Meteorological Institute (DMI) website and listed in Table 7. The weather conditions during seed development, maturation and harvest were rather different in the different growing periods (2009−2011), which represent a good opportunity for estimation of the impact of the climatological conditions on the different parameters of the grains. The harvest year 2009 was characterized by a warm spring and summer (average 652 and 700 h of sun, 8.2 and 16.2°C) with intermediate amounts of precipitation (119 and 217 mm). In contrast, the year 2011 had an extremely wet spring and summer (172 and 321 mm), especially July and August, with reduced hours of sun (301 and 573 h). The average temperature during spring 2011 was 10.2°C, which is higher compared to the previous two years, i.e. 8.2 and 6.4°C in 2009 and 2010, respectively. However it should be kept in mind that the local conditions at the different growing sites may have been different. It has been reported that high amounts of rain typically result in increased humidity in the grains, occurrence of pre-harvest sprouting and potential disease pressure (Ringlund, 1993). Elevated humidity of the grains typically favors growth of fungi.

Table 7: The averaged climatological differences between the growing traits/sowing periods of 2009 and 2010. Albeit, local conditions at the growing sites may have been different. Data collected from the Danish Meteorological Institute (DMI) website.

	Spring	Summer	March	April	May	June	July	August
2009								
Average temperature (°C)	8.3	16.2	4.0	9.4	11.5	13.9	17.2	17.4
Rainfall (mm)	119	217	53	10	56	64	86	68
Hours of sun (h)	652	700	106	272	274	280	220	200
2010								
Average temperature (°C)	6.4	16.3	2.8	7.0	9.4	13.9	18.7	16.2
Rain Fall (mm)	123	245	33	26	64	52	69	124
Hours of sun (h)	515	645	127	198	189	248	247	151
2011								
Average temperature (°C)	10.2	15.9	3.1	9.9	11.4	15.1	16.4	16.1
Rain Fall (mm)	172	321	31	16	54	75	113	132
Hours of sun (h)	301	573	143	153	239	252	171	150

3.3.2. VARIABILITY IN XYLANASE (MICROBIAL AND ENDOGENOUS) AND XYLANASE INHIBITION ACTIVITIES IN DIFFERENT BARLEY CULTIVARS

3.3.2.1. MICROBIAL XYLANASES

The commensal microbial community populating the surface of barley grains is known to produce a range of enzymes, including xylanases. Several studies have reported xylanase activity levels in cereals, however, these data are inadequate and underestimated, due to the lack of differentiation between the microbial and endogenous xylanases as well as the presence of xylanase inhibitors, which specifically inhibit the microbial xylanases. Therefore, in the present study, different barley cultivars grown at Sejet Plant Breeding (Horsens, Denmark) harvested in year 2009 and 2011 were subjected to the washing procedure as described by Dornez et al. (2006) to physically separate between the microbial and endogenous xylanases, as well as xylanase inhibitors and screen for xylanase and xylanase inhibition activity levels. An overview of the determined endogenous, microbial and total xylanase activities (which is the sum of the microbial and endogenous xylanases) of the different barley cultivars from harvest year 2009 and 2011 is provided in Table 8. Weather conditions for year 2009−2011 are listed in Table 7.

As can be seen in Figure 18, there is a wide variation in microbial xylanase activity levels between the different barley cultivars (2009 and 2011). In general, slightly higher microbial xylanase activity levels were measured for harvest year 2009 (0.013−0.168 U/g) compared to 2011 (0.019−0.058 U/g). For year 2009, the highest activities were detected for barley cultivars, i.e. Simba, Frederik and Cabaret, where the lowest were found for Barke and 1978, respectively. Meanwhile for 2011, Barke and 1978 had the highest, whereas Scarlett and Simba had the lowest activity levels. Cultivar, harvest year and their interaction were found to have a significant effect on microbial xylanase activities at the 95% confidence level (p-value < 0.05). From the component variance analysis, cultivars were estimated to contribute with 98.8% of the total variance in microbial xylanase activities (Table 9), which is rather surprising, as it is not obvious from Figure 18. The high contribution indicates that barley cultivars show different response. The observed effect of the cultivars can be explained by the differences in disease resistance and/or susceptibility of the different barley cultivars (Edwards, 2004; Hardwick et al., 2001). Moreover, this also suggests that the choice of barley cultivar is of importance when e.g. high microbial xylanase is of preference.

Table 8: Variation in microbial, endogenous and total xylanase activities of different barley cultivars between the year 2009 and 2011.

Barley cultivar	Endogenous xylanase activity (U/g)		Microbial xylanase activity (U/g)		Total xylanase activity (U/g)	
	2009	2011	2009	2011	2009	2011
Barke	1.09E-03 ± 8E-05	1.03E-03 ± 3E-05	0.013 ± 1E-03	0.057 ± 7E-04	0.014	0.058
1978	1.01E-03 ± 2E-05	1.37E-03 ± 6E-05	0.023 ± 2E-03	0.053 ± 8E-04	0.024	0.055
Scarlett	8.42E-04 ± 4E-05	1.06E-03 ± 5E-05	0.038 ± 2E-03	0.015 ± 6E-04	0.039	0.016
Power	9.90E-04 ± 1E-05	6.9E-04 ± 2E-04	0.064 ± 2E-03	0.036 ± 6E-04	0.065	0.036
Prolog	9.33E-04 ± 4E-06	8.3E-04 ± 5E-05	0.099 ± 9E-03	0.031 ± 6E-04	0.100	0.031
Cabaret	9.52E-04 ± 7E-05	9.6E-04 ± 5E-05	0.127 ± 2E-03	0.058 ± 6E-04	0.128	0.059
Frederik	9.36E-04 ± 9E-06	7.3E-04 ± 4E-05	0.171 ± 3E-03	0.031 ± 6E-04	0.172	0.032
Simba	1.03E-03 ± 4E-05	9.1E-04 ± 2E-04	0.168 ± 7E-03	0.019 ± 6E-04	0.169	0.020

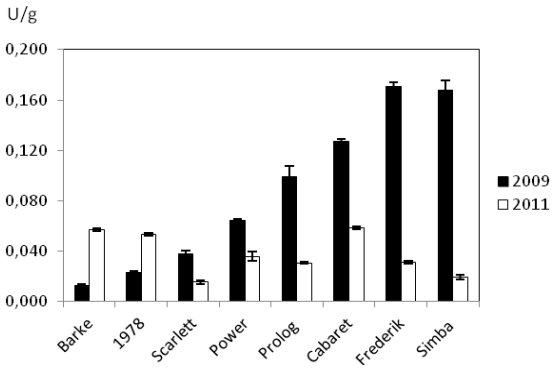

Figure 18: Microbial xylanase activities (U/g) in the washing liquids of different barley cultivars from harvest year 2009 and 2011. The bars represent the standard deviations.

The differences in the surface-associated xylanase activities of the same barley cultivars grown in the same locations indicate the significant impact of harvest year and/or climatological factors. Weather conditions largely impacts the amount and type of commensal microbes populating the surface of the grains, where wet conditions seem to favor microbial growth. Furthermore, with wet weather conditions follow increased risk of pre-harvest sprouting of the grains. The endogenous xylanase activities can be expected to be higher upon sprouting of kernels (Corder and Henry, 1989; Dornez et al., 2006; Elliott et al., 2003).

The effect of the environment on xylanase activities has been extensively studied in wheat. A study by Dornez et al. (2008) reported that the total xylanase activity levels varied up to a factor 20 in the same wheat cultivar grown during different seasons. A study by Salmenkallio-Marttila and Hovinen (2005) reported high xylanase activity levels in rye due to a cold and rainy summer. This was also the case in different wheat cultivars grown during a wet spring and summer (Gebruers et al., 2010). On the contrary, the present study show that a relatively warm summer with intermediate levels of precipitation result in higher levels of microbial xylanase activities in barley than a very rainy summer. Upon harvest the grains were sound with no observation of spouting. The different weather conditions significantly influence the composition of the commensal microflora on grain surface and the dominance of specific types of

microbes over others. A study by Váňová et al. (2004) found that dry and water deficient growing seasons (2001 and 2002) increased the infection incidence by *Fusarium* spp. and resulted in higher mycotoxin content (e.g. deoxynivalenol, DON), in barley cultivars. In addition, the climate and humidity of the crop will also affect the type of the microorganisms available. Physiological differences between wheat and barley e.g. AX content in the outer kernel layers/bran (46% vs. 6.5%, barley and wheat) is also believed to present different requirements for colonization of specific types of fungi (Höije et al., 2005; Shiiba et al., 1993).

3.3.2.2. ENDOGENOUS XYLANASES

The endogenous xylanase activity levels of the different barley cultivars (2009 and 2011) are shown in Figure 19. In comparison to the microbial xylanases, the endogenous xylanase activities were significantly lower (0.69E-03−1.4E-03 U/g). Over 90% of the total xylanase activity was found to originate of the microorganisms populating the surface of the grain, while less than 10% of plant origin. The detected distribution is even more pronounced than that reported for wheat. In wheat, 85% of the xylanase activity was accounted for by microbial and 15% by plant xylanases (Dornez et al., 2006). Moreover, it was reported that when pre-harvest sprouting occurred, the contribution of endogenous xylanase activity exceeded to 40% of the total xylanase activity. The quantified endogenous xylanase activity levels in the mature barley grains were found to vary with a factor of 1.3−2.0 (2009−2011) between the different cultivars. Harvest year was found to have a significant effect on the endogenous xylanase activities at the 95% confidence level. Moreover, from the component variance analysis, it was found that the harvest year and cultivar contributed with 37.6% and 53.0% to the total variance in endogenous xylanase activity levels, Table 9. Dornez et al. (2008) observed a wide variability in endogenous xylanase activity levels in wheat varieties, mainly determined by the interaction between the genotype and harvest year (57%), suggesting that not all wheat varieties showed the same response in the different harvest years. They also reported that genotype and harvest year contributed each for approx. 20% of the total variance in endogenous xylanase activity. Previous studies show the severe effects of weather

conditions on e.g. the composition of the developing grains, germination variability, size, microbial growth, etc. (Etchevers et al., 1977; Flannigan, 2003). Thus it is not surprising that harvest year has an effect on both the microbial and endogenous xylanase activities.

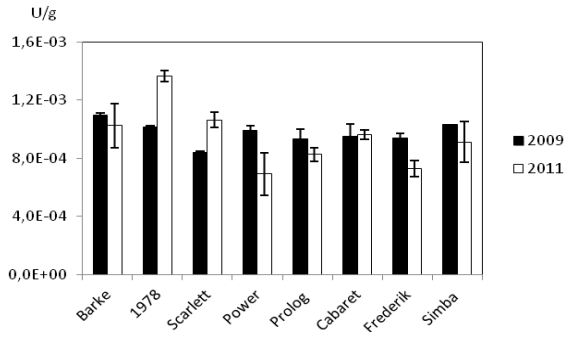

Figure 19: Endogenous xylanase activities in the extracts of milled flour of different barley cultivars from harvest year 2009 and 2011. The bars represent the standard deviations.

Table 9: Component variance analysis for estimation of the contribution by the factors: harvest year and cultivar performed using the statistical program Statgraphics.

	Variance component analysis		
	Dependent variables		
Factors	Endogenous xylanase activity (U/g)	Microbial xylanase activity (U/g)	Xylanase inhibition levels (%)
Harvest year	37.58	0.00	1.92
Cultivar	53.03	98.76	97.3
Error	9.39	1.24	0.79

3.3.2.3. INHIBITION ACTIVITIES

The range of variation of xylanase inhibition activities in the different barley cultivars was determined and the relative inhibition levels (%) are shown in Figure 20. Inhibition assay was performed with the seed extracts of different barley cultivars against the washing liquid of barley cultivar Cabaret (harvest year 2011). Considerable variation in inhibition activity levels was detected between the different cultivars.

Highest inhibition levels were found for the cultivars Power, 1978 and Simba, while lower levels were found for Frederik and Barke. In generally, the range of inhibition levels was slightly higher for harvest year 2009 then 2011, with the exception for Cabaret, while the difference was negligible for Barke and Frederik. Harvest year, cultivar and their interaction were found to have a significant effect on the inhibition levels with the 95% confidence level (p-value < 0.05). It was estimated that the variability of inhibition activity levels is mostly contributed by cultivar (97.3%) and a minor fraction by harvest year (1.92%), Table 9. In wheat, harvest year was found to account for 16% of the variability in TAXI and 3% in XIP levels, while the actual genotype had a much larger role in determining variability in TAXI (77%) and XIP (69%) levels (Bonnin et al., 2005; Dornez et al., 2008; Gebruers et al., 2010). In barley, TAXI and XIP are reported as the most predominant xylanase inhibitor proteins with TLXI being a lesser factor. Wheat cultivars have been shown to have a varied amount of xylanase inhibitor proteins, ranging from 17−200 ppm of TAXI, 156−560 ppm of XIP, and 51−150 ppm for TLXI, respectively (Bonnin et al., 2005; Croes et al., 2009; Dornez et al., 2006; Dornez et al., 2008). Thus, in general, the level of XIP is approx. three times higher compared to TAXI levels across different wheat cultivars.

Harvest year 2009 (Denmark) was dominated by a very wet summer favoring microbial growth, thus it was expected to find higher inhibitor levels due to induction of xylanase inhibitor genes by pathogens and/or insect invasion. Igawa et al. (2004, 2005) reported induction of both TAXI (*taxi-III* and *taxi*-IV) and XIP (*xip-I*) genes by plant disease and wounding in the roots and shoots of wheat. Some the obtained results seem to be in agreement with the reported trends, while others show no change or the opposite, thus making it difficult to draw any evident correlations. Nevertheless, the barley cultivars Power and 1978 were found to have high inhibition levels and low microbial xylanase activity levels. Prolog and Simba had high inhibition as well as microbial xylanases activity levels. Low inhibition levels were found for the cultivars Barke and Frederik, meanwhile these were shown to have low and high microbial xylanase activity levels. The obtained results indicate that the inhibition and microbial xylanase activity levels to some extend are correlated for the barley cultivars Power and 1978. The large discrepancies between the inhibition and microbial xylanase

activity levels can be explained by the fact that the amount of inhibitors may be dependent on the presence of specific type of pathogens or secreted proteins of the invading pathogens (Bellincampi et al., 2004). Moreover, it cannot be excluded that some of the inhibitor types and/or isoforms might be constitutively expressed and have a role other then for plant protection. Overall, these findings suggest that xylanase and their inhibitors are significantly affected by environment/weather conditions and are not very stable components.

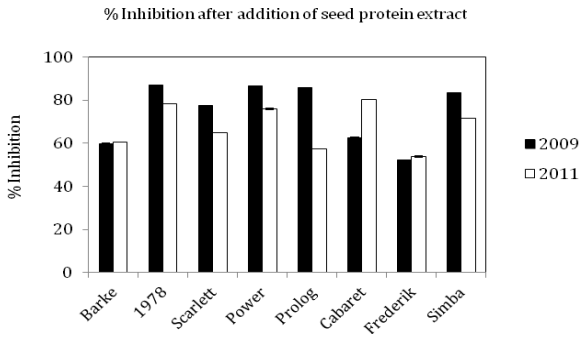

Figure 20: Percentage inhibition of microbial xylanase activities in the washing liquids of the barley cultivar Cabaret (harvest year 2009) by same amount of protein (ca. 16 µg) of the different cultivars from harvest year 2009 and 2011. The microbial xylanases activities in the washing liquids of the cultivar Cabaret were reproducible. The bars represent the standard deviations (which were very small thus masked by the thickness of the lines).

3.3.3. ASSESSMENT OF THE IMPACT OF CULTIVAR AND GROWING SITE/LOCATION ON THE MICROBIAL, ENDOGENOUS XYLANASE AND XYLANASE INHIBITION ACTIVITIES IN DIFFERENT BARLEY CULTIVARS

To gain an insight into the impact of environment (growing site) and genotype (cultivar) on the variability in the microbial, endogenous xylanase and inhibition activity levels in barley, two different barley cultivars, i.e. Quench and Simba, were grown at five different growing sites (i.e. Holeby, Tystofte, Sejet, Abildsgaard and Koldkærgaard) in Denmark of harvest year 2010 and 2011. It was not possible to get hold of barley grains from the growing site Holeby of harvest year 2010, thus these

will be missing in the tables and not included in the data analysis. The microbial, endogenous and total xylanase activities are summarized in Table 10 and Figure 21-23. The microbial xylanase activities varied in the range of 0.079−1.52 U/g and 0.012−0.53 U/g for harvest year 2010 and 2011, respectively, Figure 21. Cultivar, location and their interaction were found to have a significant effect on the variability of microbial xylanase activities with the 95% confidence level (p-value < 0.05). The growing site was found to be the main factor contributing with 98.32% of the total variability in microbial xylanase activity levels. High microbial xylanase activity levels were observed for Quench (1.52 U/g) and Simba (0.34 U/g) harvested in Koldkærgaard in 2011. Whereas the same cultivars of same growing site were lower for harvest year 2010 (Quench 0.53 and Simba 0.17 U/g), thus indicating the significant impact of harvest year and/or environmental conditions. A very wet but mild spring and wet summer characterized the harvest year 2011 in Denmark, which are weather conditions favoring microbial growth.

Table 10: Variation in microbial, endogenous and total xylanase activities of the two different barley cultivars (Quench and Simba) harvested in five different locations in Denmark (Holeby, Tystofte, Sejet, Abildgaard and Koldkærgaard) in harvest year 2010 and 2011.

	Xylanase activity levels					
	Quench			Simba		
Location	Endogenous (U/g)	Microbial (U/g)	Total (U/g)	Endogenous (U/g)	Microbial (U/g)	Total (U/g)
2010						
Holeby	-	-	-	-	-	-
Tystofte	0.73E-3 ± 2E-05	0.0792 ± 0.001	0.080	0.80E-3 ± 2E-05	0.0828 ± 0.001	0.084
Sejet	0.72E-3 ± 4E-05	0.1186 ± 0.002	0.119	0.77E-3 ± 7E-06	0.0828 ± 0.001	0.084
Abildgaard	1.14E-3 ± 2E-05	0.2217 ± 0.002	0.223	0.99E-3 ± 9E-05	0.1445 ± 0.008	0.145
Koldkærgaard	0.97E-3 ± 0E00	1.5160 ± 0.002	1.517	0.97E-3 ± 6E-05	0.3432 ± 0.013	0.344
2011						
Holeby	1.32E-3 ± 8E-06	0.0471 ± 0.001	0.048	1.16E-3 ± 1E-04	0.0123 ± 0.001	0.128
Tystofte	1.24E-3 ± 4E-05	0.0396 ± 0.001	0.041	1.16E-3 ± 1E-04	0.0414 ± 0.003	0.043
Sejet	0.80E-3 ± 3E-05	0.0593 ± 0.002	0.060	1.02E-3 ± 1E-04	0.0414 ± 0.001	0.042
Abildgaard	0.70E-3 ± 8E-05	0.1109 ± 0.004	0.112	1.10E-3 ± 7E-05	0.0723 ± 0.006	0.073
Koldkærgaard	1.92E-3 ± 3E-05	0.5258 ± 0.021	0.528	1.16E-3 ± 1E-04	0.1716 ± 0.001	0.173

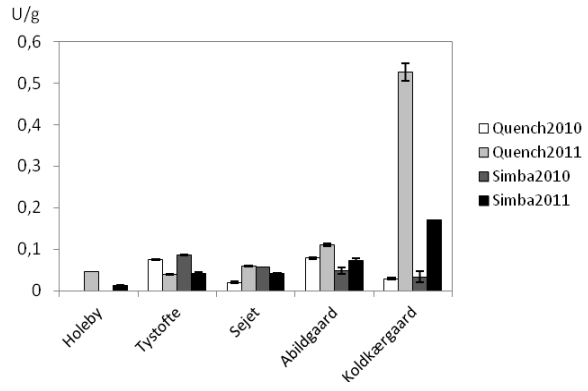

Figure 21: Microbial xylanase activities in the washing liquids of barley cultivars, Simba and Quench, harvested in five different locations in Denmark (Holeby, Tystofte, Sejet, Abildgaard and Koldkærgaard) in year 2010 and 2011. The bars represent the standard deviations.

As expected, the endogenous xylanase activity levels were much lower in comparison to the microbial xylanase activity levels, and were found to vary in the range 0.72E-03−1.14E-03 U/g and 1.0E-03−1.92E-03 U/g for harvest year 2010 and 2011, respectively (Figure 22). No significant differences in endogenous activity levels were found between the different cultivars. Growing site was found to have a significant impact on the variability of endogenous xylanase activity levels with the 95% confidence level (p-value < 0.05). It was estimated that 98.18% of the total variability in endogenous xylanase activity levels was contributed by the growing site. Highest endogenous xylanase activity levels were found for the cultivars harvested in Koldkærgaard in 2011, which were also characterized by a high microbial xylanase activity. Similar to the microbial xylanases, the endogenous xylanases seem to be highly determined by the interaction between location and harvest year (p-value < 0.05).

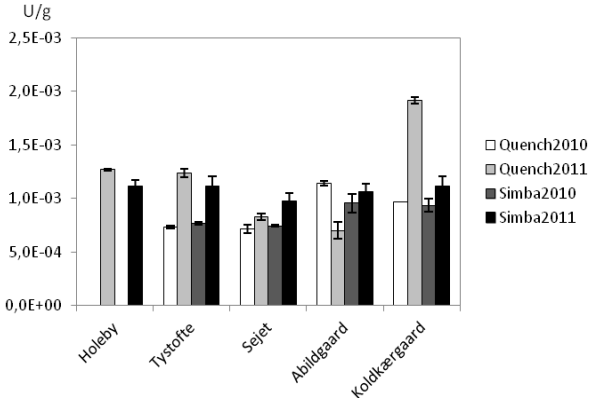

Figure 22: Endogenous xylanase activities of barley cultivars, i.e. Simba and Quench, harvested in five different locations in Denmark (Holeby, Tystofte, Sejet, Abildgaard and Koldkærgaard) in year 2010 and 2011. The bars represent the standard deviations.

Considerable inter-cultivar variation was found for xylanase inhibition activity levels (Figure 23). Cultivar/genotype was found to have a significant impact on the variability of xylanase inhibition levels with 95% of confidence. Low inhibition activity levels in the cultivars harvested in Koldkærgaard in 2011 were also characterized as having high microbial xylanase activities. Cultivar/genotype was the main factor contributing the most variance in xylanase inhibition levels (72.9%). Inhibition levels of the cultivars were highly correlated between the different harvest years, where the range of inhibition levels was higher for year 2010. Previous studies by Dornez et al. (2008) ascribed the variability in TAXI (77%) and XIP (69%) inhibitor levels to the genetic factor. However, Gebruers et al. (2010) observed that the variation in xylanase inhibition activities was attributed to the environment and the interaction between environment and genotype, while only 31-47% of the total variation was explained by genotype. The differences between the two studies were due to the differing weather conditions as well as a higher diversity of environment. The study by Dornez et al. (2008) only differed in harvest year, while the study by Gebruers et al. (2010) differed both in harvest year and growing location.

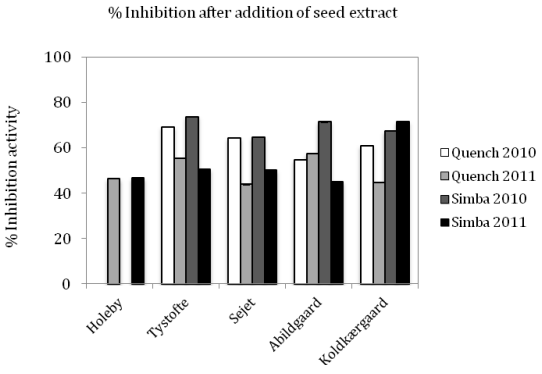

Figure 23: Relative inhibition levels exerted by different barley cultivars, i.e. Simba and Quench, harvested in five different locations in Denmark (Holeby, Tystofte, Sejet, Abildgaard and Koldkærgaard) in year 2010 and 2011. The bars represent the standard deviations (these were rather small thus masked by the thickness of the lines).

3.3.4. DISTRIBUTION OF THE DIFFERENT XYLANASE INHIBITOR TYPES IN BARLEY

The determined variability in xylanase inhibition levels between the different cultivars was also confirmed/observed in the 1D-immunoblots probed with anti-XIP, -TAXI and -TLXI polyclonal antibodies (generous gift of Christophe M. Courtin), Figure 24. Equal amounts of protein extracts from different barley cultivars (ca. 16 µg) were applied in each lane of the 1D-gel and blotted onto a nitrocellulose membrane. Polyclonal antibodies against wheat XIP, TAXI and TLXI were used, which have been shown to cross-react with their homologous inhibitors in cereals (Beaugrand et al., 2006). For each inhibitor type, bands with varying intensities were detected among the different cultivars (Figure 24). The detected bands for each type of inhibitor were built up of several smaller bands with differing molecular masses, representing different iso-forms. For TAXI, both the unprocessed (40 kDa) and processed form (30 and 10 kDa) was detected with varying degree among the different cultivars. For XIP, a band with a molecular mass of ca. 30 kDa was detected with varying intensities among the different cultivars. Noticeably, two bands were detected for XIP for the cultivars 1978 (lane 2) and Power (lane 4, very faint), which were characterized by having high inhibition activity levels (Figure 23). Low intensity bands were detected for Frederik, which was also characterized as having low inhibition activity levels (Figure 23). The

varying band intensities and the presence of additional bands for XIP across the different barley cultivars seem to be highly correlated with the determined inhibition activity levels. Despite the fact that seed protein extracts containing a mixture of the xylanase inhibitor types were used for inhibition activity levels, coupling of 1D-immunoblots suggest that the XIP-type inhibitors might be the most effective against microbial xylanases found on surface of barley grains. As mentioned above, in wheat XIP levels are three times higher compared to TAXI levels (Bonnin et al., 2005 and Croes et al., 2009). TLXI band intensity levels were similar among the different cultivars. The immunoblots suggest that the determined inter-cultivar variability is due to differences in the level and presence of specific members of the xylanase inhibitor families.

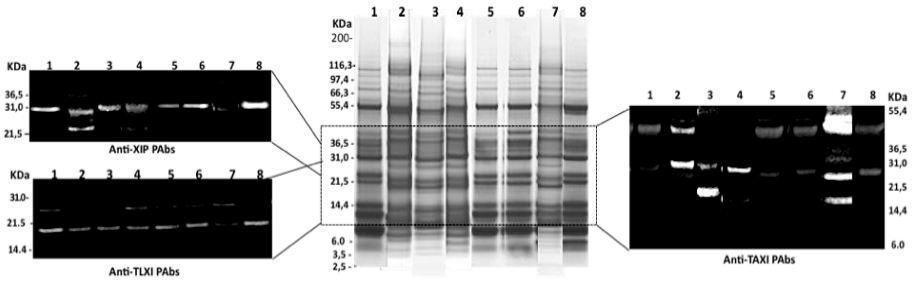

Figure 24: Coomassie-stained SDS-PAGE over seed protein extracts of different barley cultivars (ca. 16 µg) and western blots showing differences in protein band pattern and immunoreactive bands of XIP-, TAXI- and TLXI-type xylanase inhibitors across different cultivars: Lane (1) Barke, (2) 1978, (3) Scarlett, (4) Power, (5) Prolog, (6) Cabaret, (7) Frederik, and (8) Simba. Sizes of molecular weight marker proteins are also indicated.

The obtained protein profiles and 1D-immunoblots of the different xylanase inhibitors in the whole grain extracts reflect the presence and variability of the different iso-forms of the inhibitors among the different cultivars. A single band in a 1D-gel can confine multiple proteins of the same molecular mass. In order determine the 2D protein profile and reveal the presence of the different isoforms of the different inhibitor types, a proteomics approach consisting of 2D-gel electrophoresis, mass spectrometry coupled with immunoblotting was applied. 2D-immunoblots were

performed using aleurone tissue extracts to reduce the large number of non-inhibitor proteins present in whole seed extracts, while concentrating the amount of the inhibitors. Studies with wheat have reported high concentrations of these inhibitors predominantly in the outer kernel layers and the aleurone layer (Croes et al., 2009b; Gebruers et al., 2002b; Jerkovic et al., 2010). Several spots appeared on 2D-blots resolved in the pI range of 7−10 suggesting the appearance of multiple isoforms for XIP, TAXI and TLXI (Figure 25). The visible protein spots on the Coomassie stained 2D-gel were manually excised, tryptic digested and identified by MALDI-TOF MS and/or MS/MS. Despite detection of multiple immune-reactive spots on the 2D-blots, the majority of the spots were identified as non-xylanase inhibitor proteins. Nevertheless, XIP-type inhibitors were found in several spots (53, 58, 65 and 69) in the pI range 7.5−8.5. TLP7−8 were identified in multiple spots (61−62, 63−64, 67, 69, 73−74, 77 and 109) clustering in the pI range 9−10. One spot was identified as TAXI, but was found to have a non-significant PMF score and low sequence coverage (data not shown). Lack of identification of additional xylanase inhibitors is possibly due to low abundance of the protein spots on the gel and not visible to the naked eye. Moreover, the applied antibodies were raised against wheat xylanase inhibitors and therefore might result in non-specific targeting of proteins. Especially for TLXI a lot of non-specific protein spots were detected which are probably proteins sharing high homology to TLPs. In addition, the XIP and TLXI antibodies were known to cross-react, meaning that XIP antibodies might give a weak signal for TLXI and TLXI antibodies for XIP on the immunoblots (email correspondence Kurt Gebruers, KU Leuven, Belgium). Nevertheless, it was possible to identify and locate some of the xylanase inhibitor proteins found in barley. Further work, however, is required to identify the remaining protein spots by e.g. application of a pre-fractionation or concentration step, or deglycosylation of peptides prior to MS analysis. Furthermore, subsequent analysis is needed to determine whether the different spots contain different isoforms or if they have been subject to posttranslational modifications.

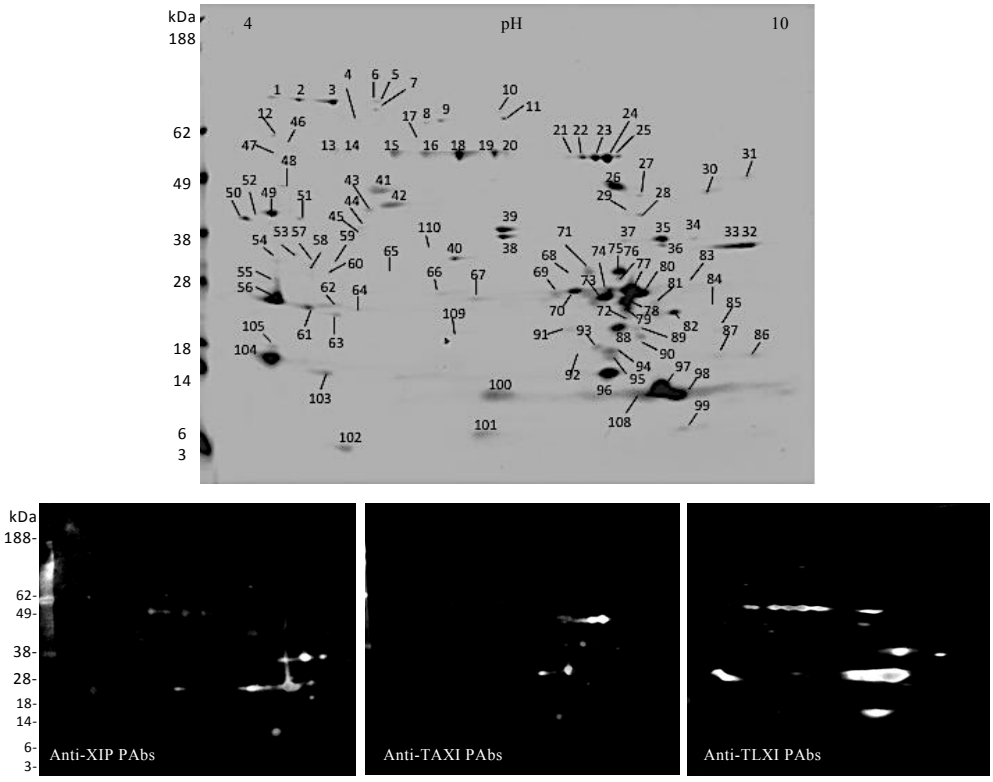

Figure 25: Coomassie-stained 2D-gel of proteins extracted from barley aleurone layers (ca. 50 µg proteins, cultivar Simba harvested in 2009) resolved in the pI range 7-10. Corresponding 2D-immunoblots probed with anti-XIP, -TAXI and -TLXI polyclonal antibodies. The numbered spots represent identified xylanase inhibitor proteins, Table 11. Sizes of the molecular weight marker proteins and pH range are as indicated. PAbs: Polyclonal antibodies.

For barley, this is the first study to report the variability in xylanase inhibition levels across different cultivars. Several comprehensive studies have been performed but mainly on wheat. A comparative proteomics analysis of six different wheat cultivars enabled differentiation between TAXI, XIP and TLXI inhibitor isoforms and quantification of their variation among the different cultivars. The study led to identification of a total 48 xylanase inhibitor protein forms, including 18 TAXI, 27 XIP, and 3 TLXI-type xylanase inhibitors (Croes et al., 2009a). This high

heterogeneity was ascribed to both the presence of multiple *taxi* and *xip* genes and to post-translational modifications, e.g. glycosylation (Croes et al., 2009a). A transcriptomic study by Igawa et al. (2004 and 2005) reported that the expression of some *taxi* and *xip* gene family members were induced in spikelets and leaves infected with fungal pathogens, i.e. *Fusarium graminearum* and *Erysiphe graminis,* respectively, while others were unresponsive (Igawa et al. 2004; 2005). This suggests that the different forms of these inhibitors may be induced by different pathogens and/or other stress conditions, and that they may be tissue specific. However, it is still largely unknown of how and when the different xylanase inhibitor isoforms are regulated at various stages of seed growth and development.

Table 11: Protein identifications of spots resolved in the Coomassie-stained 2D-gel over protein extracts of barley aleurone layers (50 µg protein of the barley cultivar Simba harvested in 2009). The spot numbers correspond to those in Figure 25. Comb.: Combined MASCOT score, Theor.: Theoretical.

Spot no.[a]	Accession no.	Organism	Protein name	MW theor.	pI theor.	PMF score	PMF E-value	Comb. score	E-value	Sequence coverage %	Unique peptides	MS/MS precursor ions	MS/MS peptides sequences	Ion score	Expect
53	TC159027	*Oryza sativa*	Class III chitinases (GH18 Narponin)	42820	7.51			32		2	1	1240,7117	EYIGAQFTGVR	32	0.27
58	gi|326497267	*Hordeum vulgare subsp. vulgare*	Predicted protein (GH18 Hevamine XipI class III)	33357	8.17	51	7.50E+00	166	2.60E-11	16	5	1083,6456 857,5227 1631,6976	R.TPVQLTATPR.C R.ALATGLVGR.I R.FYDDADCAANWQR.Q	37 8 72	1.80E-01 2.50E+02 5.10E-05
65	gi|31615809	*Hordeum vulgare*	Chain A, Crystal structure of xylanase Inhibitor protein (Xip-I)	30494	8.27	83	0.0043			10	2				
61	gi|14164983	*Hordeum vulgare*	Thaumatin-like protein TLP8	25213	7.83			149	1,40E-09	24	4	1269,7128 1769,8208	K.VITPACPNELR.A R.AAGGCNNACTVFKEDR.Y	31 71	1,00E+00 9,30E-05
62	gi|14164981	*Hordeum vulgare*	Thaumatin-like protein TLP7	24541	7.36	75	0.021			51	9				
63	gi|14164983	*Hordeum vulgare*	Thaumatin-like protein TLP8	25213	7.83			149	1,40E-09	30	5	1269,7191 1769,8551	K.VITPACPNELR.A R.AAGGCNNACTVFKEDR.Y	34 84	0.5 5.7e-06
64	gi|14164983	*Hordeum vulgare*	Thaumatin-like protein TLP8	25213	7,83			46	2,60E+01	9	2	1269,6968	K.VITPACPNELR.A	31	1.1
67	gi|326487890	*Hordeum vulgare subsp. vulgare*	Predicted protein (Xylanase inhibitor-I like)	41728	8.42			78	1,80E-02	8	3	1459,6579 1409,6655	K.QGSPAHYVSGTSIR.V R.DVYRPVVDAFTK.A	21 24	9,00E+00 5,00E+00
69	gi|47824814	*Hordeum vulgare*	Xylanase inhibitor	41815	8.58			286	2,70E-23	33	10	1459,7819 873,559 1583,8778 1427,7756	K.QGSPAHYVSGTSIR.V R.LPYVLLR.R R.RDVYRPFVDAFAK.A R.DVYRPFVDAFAK.A	73 40 37 55	7,20E-05 1,00E-01 2,30E-01 4,10E-03
73	TC201229	*Hordeum vulgare*	Thaumatin-like protein TLP7	36262	8.55			150		5	2	1057,4470 1135,4434	R.TGCTFDGSGR.G K.FGGDTYCCR.G	87 62	6.7e-007 0.00013

74	TC201229	*Hordeum vulgare*	Thaumatin-like protein TLP7	36262	8.55	109		5	2	1057,4743 1135,4703	R.TGCTFDGSGR.G K.FGGDTYCCR.G	59 50	0.0006 0.0038
77	TC201229	*Hordeum vulgare*	Thaumatin-like protein TLP7	36262	8.55	150		5	2	1057,4470 1135,4434	R.TGCTFDGSGR.G K.FGGDTYCCR.G	87 62	6.7e-007 0.00013
109	gi|14164983	*Hordeum vulgare*	Thaumatin-like protein TLP8	25213	7.83	118	1.70E-06	19	4	1251,4958 1269,5664	R.CQTGDCGGKLR.C K.VITPACPNELR.A	40 44	4.00E-02 3.60E-02

3.4. CONCLUSIONS

The effect of xylanase and xylanase inhibitors has been largely unexplored in barley. In applications where microbial xylanases are applied to improve the product quality, a variation in xylanase inhibitor levels can cause vast anomalies, and potentially hinder the delivery of a constant product quality by lowering xylanase activity. In the present study, considerable inter-cultivar variability in the level of both microbial and endogenous xylanase activities, as well as at xylanase inhibitor levels. Harvest year/weather conditions and cultivar were found to have a significant impact on the variability of microbial and endogenous xylanases, as well as xylanase inhibition activity levels (p-value < 0.05). The activity assays coupled with the immunoblots indicate a possible correlation between the microbial xylanases and the proteinaceous xylanase inhibitors. Two of the screened barley cultivars, namely 1978 and Power, were found to encompass high xylanase inhibition levels and low microbial xylanases. These cultivars also displayed an additional band in 1D-immunoblots probed with anti-XIP antibodies. These findings suggest that out of the different inhibitors XIP is the predominant form of causing most of the measured inhibition activity.

Analysis of the microbial, endogenous xylanase and xylanase inhibition activities of two different barley cultivars grown in five different locations harvested in year 2010 and 2011 suggest that the extent of variation was mainly contributed by the cultivar and growing site. Cultivar was found to be the main determinant of the variability in the microbial xylanases and inhibition activity levels, while growing site was the main contributor to the variability in endogenous xylanase activities. Application of a proteomics approach enabled profiling the protein pattern and detection of the variability of xylanase inhibitors among different barley cultivars. Moreover, 2D-immunoblotting enabled identification of some of the different xylanase inhibitors (XIP-I and XIP-III and TLP7-8) present in the barley aleurone layer extracts.

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CHAPTER 4

Exploring the plant-microbe interface - Profiling and characterizing the indigenous fungal community and the surface-associated proteins of barley grains

In collaboration with Prof. Jens C. Frisvad, Center for Microbial Biotechnology, Department of Systems Biology, Technical University of Denmark.

4. ABSTRACT

Cereal grains are colonized by a diverse collection of microorganisms including bacteria and fungi. The multifaceted interface of cereal grains and the colonizing and/or infecting microbes involve the interaction between an arsenal of surface-associated proteins and secreted proteins. A metaproteomics study was carried out to explore the composition of the populating fungal community and the surface-associated proteins of barley grains. 2D gel electrophoresis (pH 3−10) coupled with mass spectrometry was applied to profile and map the surface-associated proteome (surfome) of two barley cultivars (Barke and Cabaret, harvest year 2009). This comparative analysis revealed minor but distinct changes in the protein profiles of the two cultivars. The majority of the identified proteins was of plant origin and ascribed to play a role in defense and/or oxidative stress mechanisms. The fungal community of a set of barley cultivars was profiled and the isolated fungi were screened for xylanase activity and secreted 1D protein profiles. Furthermore, the 2D-secretomes of the cereal fungi *Aspergillus niger* (storage) and *Fusarium poae* (field) grown on both barley flour and wheat arabinoxylan (WAX) were mapped. The fungal composition was found to have a significant impact on the production of microbial xylanases, where the cereal storage fungi, e.g. Aspergilli and Penicillia, were found to be prominent producers of xylanases compared to the field fungi. The 1D protein profiles of the isolated cereal fungi were shown to be species-specific, as well as dependent on the media and substrate. The secretomic analysis of *A. niger* grown with barley flour and WAX resulted in identification of a collection of xylanolytic enzymes, as well as others not previously reported in *A. niger*, e.g. melibiase and α-glucuronidase, which are involved in carbohydrate catabolism. In addition, initial steps in mapping and describing the secretome of *F. poae* enabled identification of a set of cell wall degrading enzymes.

4.1. INTRODUCTION

Plants and the populating microbial community are all part of the ecosystem. Over 200 species of microorganisms populate the surface of barley grains, not just fungi but also bacteria, yeast and actinomycetes (Briggs, 1978). The different microorganisms present significantly differ with stage of development, maturation and ripening of the grains and post-harvest storage conditions. The initial colonizers of cereal grains after ear emergence are bacteria, then replaced by yeasts and eventually by fungi after anthesis of the grains (Hill and Lacey, 1983; Magan and Lacey, 1988). Fungi have been reported to have severe effects on the quality of the grains, e.g. causing discoloration, degradation of nutrient constituents, reduced seed germination, production of volatile metabolites and mycotoxins (Lacey, 1980; Lacey and Crook, 1988; Christensen, 1991). Fungi colonizing the grains can be arbitrarily differentiated into two groups, i.e. the field and the storage fungi, based on their ecological requirements, e.g. habits and time of invasion. The field fungi have been classified as those colonizing the developing and mature grains while still on the plant. The major genera comprise *Alternaria* spp., *Cladosporium* spp. and *Fusarium* spp., which typically require high moisture content compared to storage fungi (Christensen, 1991; Lacey, 1988). The storage fungi become more abundant on and within the stored grains, where the moisture level has decreased, these are primarily *Aspergillus* spp. and *Penicillium* spp. The moisture levels prevail for the growth of the different genera of species. Some of the fungi are known as pathogenic, e.g. *Fusarium,* a devastating pathogen of cereals that severely damages the grain and plant. More importantly, however, is the contamination of mycotoxins e.g. aflatoxin and ochratoxin, which poses a critical hazard to food safety and human health, as well as animals (Jayas et al., 1995).

A characteristic of the invading fungi is the secretion of an arsenal of degradative enzymes, which play a role in nutrient acquisition, host colonization and ecological interactions (Carapito et al., 2009). Several extracellular fungal enzymes including xylanases, polygalacturonases, pectate lyases and lipases have been identified and shown to be required for virulence (Bennett and Klich, 2003; Oeser et al., 2002). The availability of complete fungal genome sequences and advances in genomics,

transcriptomics and proteomics approaches have significantly contributed to a better understanding of plant-fungus interactions, fungal pathogenicity and defense mechanisms in plants. Gel-based proteomics have enabled detailed analysis of several fungal secretomes. *Aspergillus oryzae* (traditionally used in production of fermented foods such as sake and soya sauce) grown on wheat bran, was found to specifically produce a complex mixture of cell wall degrading enzymes, e.g. β-glucosidases, α-mannosidases, xylanases, cellulases and peptidases. These were identified with application of MALDI-TOF mass spectrometry (Bouws et al., 2008; Oda et al., 2006). In a study by Lu et al. (2011), the secretome of *A. niger* grown with xylose or maltose as nutrient source was characterized with an identification of 200 proteins and reported to be strongly influenced by the culture conditions and the available nutrient source. The secretory response of *A. niger* to maltose and xylose, and the induction of specific enzymes were confirmed by Ferreira de Oliveira et al. (2011) with identification of e.g. glucoamylase on maltose and β-xylosidase on xylose, as well as microsomal proteins.

An extensive analysis of extra-cellular proteins secreted by *F. graminearum* cultures on a variety of media, including isolated plant cell walls resulted in identification of 229 proteins, such as xylanases, cellulases, proteinases and lipases. It was also reported that the secreted enzymes by the fungus were highly regulated to the respective substrate (Paper et al., 2007; Phalip et al., 2005). A study by Yang et al. (2012) complemented the already established map of the secretome of *F. graminearum* with identification of 24 additional proteins by means of 2-DE and mass spectrometry. One of the key challenges is the further analysis of the expression, regulation and function of the set of proteins encoded by the fungal genome. Moreover, most of the studies of the fungal secretomes have been carried out with complex nutrient media, which is not a representation of the fungus *in planta* situation.

In addition, the plants response to fungus invasion has also been studied. Proteomics analysis focusing on the plant-fungus association has revealed identification of several proteins involved in diverse biological processes, including stress and defense responses, signal transduction, electron transport, photosynthesis and antioxidation (Kim et al., 2003; 2004ab; 2008; Lee et al., 2006; Zhou et al., 2006). It has been reported that wheat cultivars vary in their resistance to biotic and abiotic stresses,

possibly due to differences in defense protein levels (Demeke and Morris, 2002; Yarullina et al., 2005). A study by Jerkovic et al. (2010) of wheat bran tissues revealed identification of several oxidative stress and defense related proteins and inhibitors (e.g. oxalate oxidase, peroxidase and xylanase inhibitors), as well as proteins that improve tissue strength. However, no reports have been found regarding the composition of the naturally occurring surface-associated proteins on seeds. To gain a better understanding of the relationship between the plant and the commensal microbial community, a proteomics approach was applied to describe and map the surface-associated protein complement of two different barley cultivars. The complex composition of the commensal fungal community governing the outer kernel layers of the grains was analyzed and identified at species level with application of culture-based microbiological techniques. In order to understand the effects of the secreted fungal proteins, as well as adaptation of the fungal metabolism to varying carbon sources, the secretome of two cereal fungi grown (field and storage) on wheat arabinoxylan and barley flour were analyzed and the extra-cellular proteins were identified and characterized.

4.2. MATERIALS AND METHODS

4.2.1. SAMPLE PREPARATION AND SDS-PAGE (WASHING LIQUIDS CONTAINING SURFACE-ASSOCIATED PROTEINS)

Surface-associated proteins of barley grains were removed by washing with 25 mM sodium acetate buffer pH 5.0 containing 0.02% (w/v) sodium azide. A detailed description of the washing procedure is described in Chapter 3. The washing liquids were desalted on a NAP-5 column (GE Healthcare) with 10 mM sodium acetate pH 5.0. The protein concentration was determined following the method of Popov et al. (1985) using BSA as a standard. Appropriate amount of protein was precipitated by 4 volumes of ice-cold 100% acetone. SDS-PAGE was performed with a vertical slab mini gel unit (NuPAGE Novex system, Invitrogen) using 4−12% BisTris NuPAGE gels according to the method of Laemmli (1970). Briefly, the protein samples were prepared by mixing one part of 4 x LDS sample buffer (2% (w/v) LDS, 106 mM Tris-

HCl, 141 mM Tris-base, 10% (w/v) glycerol, 0.51 mM EDTA, 0.22 mM SERVA blue G250, 0.175 mM phenol red, pH 8.5) to four parts of protein sample and incubated at 95°C for 5 min. Samples were loaded onto the gel and electrophoresis was performed using NuPAGE MES running buffer (50 mM Tris Base, 50 mM MES (2-(N-morpholino) ethane sulfonic acid), 1 mM EDTA, 3.5 mM SDS, pH 7.3) with a current of 2 mA per lane and constant 200 V for 35 min. The protein bands were fixed with a solution of 2% (v/v) phosphoric acid and 30% (v/v) ethanol for 30 min, stained with Coomassie (0.12% (w/v) colloidal Coomassie brilliant blue (CBB) G-250, 10% (w/v) ammonium sulphate, 10% (v/v) phosphoric acid, 20% (v/v) ethanol) overnight and destained by repeated washing with water (Candiano et al., 2004). A broad range protein ladder (Mark 12, unstained standard, Invitrogen) was used for molecular mass determination.

4.2.2. 2D-GEL ELECTROPHORESIS (SURFACE-ASSOCIATED PROTEINS OF BARLEY GRAINS)

After desalting, an appropriate amount of protein (50 µg) was precipitated by 4 volumes of 100% (v/v) acetone overnight at -20°C and dissolved in 125 µL rehydration buffer (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 200 mM destreak reagent (bis (2-hydroxyethyl) disulfide), 0.5% (v/v) pharmalytes pH range 3−10 (GE Healthcare), trace of bromophenol blue). The protein samples were applied to pH 3−10 IPG strips, 7 cm (GE Healthcare). Separation by isoelectric focusing (IEF) was performed on Ettan™ IPGphor (GE Healthcare). After rehydration for 12 h at 50 mA/strip at 20°C, IEF was performed to a total of 20 kVh (1 h at 150 V, 1 h at 300 V, 1 h at 1000 V, gradient to 8000 V, hold at 8000 V until a total of 20 kVh was reached. Subsequently, the strips were equilibrated for 2 × 15 min in 5 mL equilibration buffer (6 M urea, 30% (v/v) glycerol, 50 mM Tris HCl, pH 8.8, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue) supplemented with 1% (w/v) DTT and 2.5% (w/v) iodoacetamide in the first and second equilibration step, respectively. The strips and molecular weight markers (Mark 12, Invitrogen) were placed on 4−12% Bis-Tris Zoom gels (NuPAGE, Novex system, Invitrogen). Second dimension, SDS-PAGE, was performed in the XCell SureLock mini-cell system (NuPAGE Novex System, Invitrogen) using

NuPAGE MES running buffer (Invitrogen). Gels were fixed in solution of 50% (v/v) ethanol and 7% (v/v) acetic acid for 1 h, rinsed with water 3 times for 10 min, and stained by Sypro Ruby (Invitrogen) overnight. Subsequently, the gels were washed with a solution of 10% (v/v) ethanol and 7% (v/v) acetic acid for 30 min and rinsed with water. Images of the Sypro Ruby-stained gels were captured at excitation/emission wavelengths 532/580 nm, 100 microns (resolution), on a Typhoon scanner (9410 Variable, Mode Imager, GE Healthcare).

4.2.3. IN-GEL ENZYMATIC DIGESTIONS AND MASS SPECTROMETRY

The resolved spots were excised manually from the Sypro Ruby stained gels after post-staining with Coomassie and subjected to in-gel tryptic digestion with certain modifications (Hellman et al., 1995). Briefly, the gel pieces were washed with 100 µL 40% (v/v) ethanol (10 min), shrunk in 50 µL 100% (v/v) acetonitrile (ACN) and soaked in 2 µL 12.5 ng/µL trypsin (Promega, porcine sequencing grade) in 25 mM (w/v) NH_4HCO_3 on ice for 45 min. The gel pieces were rehydrated by addition of 10 µL 25 mM (w/v) NH_4HCO_3 followed by incubation at 37°C overnight. Tryptic peptides (1 µL) were loaded on to the AnchorChip™ target plate (Bruker-Daltonics, Bremen, Germany), covered by 1 µL matrix solution (0.5 µg/µL CHCA in 90% (v/v) ACN, 0.1% (v/v) TFA) and washed in 0.02% (v/v) TFA. Tryptic peptides were analyzed by Ultraflex II MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) using Flex Control v3.0 (Bruker-Daltonics, Bremen, Germany) and processed by Flex Analysis v3.0 (Bruker-Daltonics, Bremen, Germany). Peptide mass maps were acquired in positive ion reflector mode with 500 laser shots per spectrum. MS/MS data were acquired with an average of 1000−2000 laser shots for each spectrum. Spectra were externally calibrated using a tryptic digest of β-lactoglobulin (5 pmol/µL). Filtering of spectra was performed for known trypsin autolysis products and keratin peaks. MS and MS/MS spectra were analyzed using Biotools v3.1 (Bruker-Daltonics). MASCOT 2.0 software (http://www.matrixscience.com) was used for database searches in the NCBInr (National Center for Biotechnology Information), HvGI (The Institute for Genome Research - TIGR) barley gene index Release 12.0 (http://compbio.dfci.harvard.edu/tgi) and Broad Institute for *Fusarium graminearum*

gene index (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum). The following search parameters were applied: Monoisotopic peptide mass accuracy of 80 ppm, fragment mass accuracy to ± 0.7 Da; a maximum of one missed cleavage; carbamidomethylation of cysteine and partial oxidation of methionine, respectively. No restrictions with respect to protein Mw and pI were made. The signal to noise threshold ratio (S/N) was set to 1:6. Protein identifications by PMF were confirmed with a MASCOT score of 70, $p < 0.05$ and a minimum of 4 matched peptides. A single peptide based protein identifications by MS/MS analysis was confirmed with a MASCOT score of 40, $p < 0.05$. Searches in the Broad Institute for *Fusarium graminearum* gene index, a MASCOT score above 62 was considered as a positive identification. All the identified proteins were assessed for signal peptides using SignalP v4.1 (http://www.cbs.dtu.dk/services/SignalP).

4.2.4. AGAROSE PLATE ASSAY FOR ENDO-1,4-XYLANASE ACTIVITY

For screening xylanase activity, plate zymograms were performed by casting dyed substrate-containing agarose plates (0.1% (w/v) Remazol Brilliant Blue (RBB)-dyed wheat arabinoxylan (Megazyme), 1% (w/v) agarose, and 0.2 M sodium citrate-HCl pH 4.8). Wells of 2 mm were punched into the plates, the wells were filled with 5 µL of sample, i.e. washing liquids of different cultivars or supernatants of different fungi, respectively, and incubated overnight at room temperature. Xylanase activities appeared as clearing zones around the wells.

4.2.5. DETECTION OF XYLANASE ACTIVITY BY ZYMOGRAM ANALYSIS

In situ xylanase activity was detected on a 10% (w/v) acrylamide gel using method described by Monrag et al. (1990) with certain modifications. The 10% (w/v) acrylamide resolving gel was supplemented with 0.1% (w/v) wheat arabinoxylan (Megazyme) prior to polymerization. The 10% (w/v) acrylamide resolving gel, 4.5% (w/v) for stacking gel, running buffer and the sample buffer contained SDS (anionic detergent) for movement of samples under charged conditions. The samples for loading onto the gels were prepared and run as described in section (4.2.1). After the

electrophoresis, SDS was removed by washing the gel once with 50 mM sodium phosphate buffer (pH 7.2) supplemented with 25% (v/v) isopropanol at room temperature for 1 h, subsequently with 50 mM sodium phosphate buffer (pH 7.2) for additional 1 h. Renaturation of the proteins were performed by incubation of the gel overnight in 50 mM sodium phosphate buffer (pH 7.2) supplemented with 5 mM β-mercaptoethanol and 1 mM EDTA at 4°C. The gel was then incubated at 50°C for 4 h prior to staining with 0.1% (w/v) Congo Red for 1 h at room temperature and washed with 1 M NaCl for 30 min until excess dye was removed. Zymogram was prepared by soaking the gel in 0.5% (v/v) acetic acid solution, turning the background dark blue. Appearance of clear zones within the gel matrix indicated the presence of xylanolytic activity.

4.2.6. NATIVE PAGE WITH WHEAT ARABINOXYLAN

Native (non-denaturing) gel electrophoresis was performed using 10% acrylamide gels (with 4.5% stacking gel) following the method of Schägger and von Jagow (1991) with certain modifications. The non-denaturing gels (10% (w/v) acrylamide for resolving gel and 4% (w/v) acrylamide for stacking gel) were co-polymerized with 0.1% (w/v) wheat arabinoxylan. Protein samples were prepared in 1x NativePAGE sample buffer (50 mM BisTris buffer pH 7.2, 6 M HCl, 50 mM NaCl, 10% (w/v) glycerol and 0.001% (w/v) Ponceau S) supplemented with 0.5% (w/v) Coomassie Blue G-250 sample additive just prior to loading onto the gel. The dye binds to proteins and confers a net negative charge while maintaining the proteins in their native state without any protein denaturation. Electrophoresis was performed under non-denaturing condition using NativePAGE running buffer (Invitrogen) at a constant 150 V for 2 h until the dye front approached the lowere gel margin. After the electrophoresis, the protein bands were fixed with solution containing 10% (v/v) acetic acid and 40% ethanol for 5 min, then stained for 30 min with Coomassie Blue (0.02% (w/v), 30% (v/v) ethanol, 10% (v/v) acetic acid), and destained by repeated washing with water. NativeMark (unstained, Invitrogen) was used as protein ladder (IgM hexamer, 1236 kDa; IgM pentamer, 1048 kDa; Apoferritin band 1, 720 kDa;

Apoferritin band 2, 480 kDa; β-phycoerythrin, 242 kDa; Lactate dehydrogenase, 146 kDa; Bovine serum albumin, 66 kDa and Soybean trypsin inhibitor, 20 kDa).

4.2.7. NATIVE PAGE OVERLAID WITH SUBSTRATE GEL (ZYMOGRAM ANALYSES)

Native (non-denaturing) polyacrylamide gel electrophoresis was performed using NativePAGE gel system following the method of Schägger and Von Jagow (1991). Pre-cast NativePAGE Novex 4−16% Bis-Tris gels (8 x 8 cm gel, Invitrogen) were used. The protein samples for loading onto the gel were prepared in 1x NativePAGE sample buffer (50 mM BisTris buffer pH 7.2, 6 M HCl, 50 mM NaCl, 10% (w/v) glycerol and 0.001% (w/v) Ponceau S). For running the samples under non-denaturing conditions, the sample buffer did not contain SDS (the cationic detergent for movement of samples under charged conditions) and the sample mixtures were not subjected to heat denaturation. The sample mixture was supplemented with Coomassie G-250 sample additive (0.5%) just prior to loading onto the gel. The G-250 binds to proteins and confers a net negative charge while maintaining the proteins in their native state without any protein denaturation. Electrophoresis was carried out using NativePAGE running buffers (Invitrogen), i.e. cathode buffer (50 mM BisTris, 50 mM Tricine, 0.002 % (w/v) G-250, pH 6.8) and anode buffer (50 mM BisTris, 50 mM Tricine, pH 6.8), respectively, with a current of 2 mA per lane. Zymogram analyses were performed by following the method of Biely et al. (1985) using Remazol brilliant blue (RBB)-dyed wheat arabinoxylan (Megazyme) as substrate. After electrophoresis, the native protein gel was layered over a substrate gel (0.5% (w/v) RBB wheat arabinoxylan, 1.5% (w/v) agarose in 0.2 M sodium citrate-HCl, pH 4.8) and incubated at 50°C for 4 h. Subsequently, the gels were separated and the substrate gel was immersed in a solution of 96% ethanol and 0.2 M sodium citrate-HCl, pH 4.8 (2:1) for 2 h. The presence of endo-1,4-xylanase was detected by the formation of transparent zones in the bluish gel (enzyme degraded substrate zones).

4.2.8. FUNGAL ISOLATION OF BARLEY GRAINS

A collection of different barley cultivars grown in different locations in Denmark in harvest years 2009 and 2010 were used to profile and describe the composition of the populating mycobiota. This was done in collaboration with Prof. Jens C. Frisvad (Center for Microbial Biotechnlogy, Department of Systems Biology, Technical University of Denmark). Fungi were isolated from the surface of barley grains of different cultivars by direct plating on different culture media with different substrates, e.g. PDA (Potato Dextrose Agar, Samson et al., 2004), MEA (Malt Extract Agar, Pitt and Hocking, 1997) and MEA (Oxoid).

For determination of the species present, the isolated fungi were cultivated on different media, i.e. DG18 (Dichloran Glycerol (18%) agar; Hocking and Pitt, 1980), DRYES (Dichloran Rose Bengal Yeast Extract Sucrose agar; Frisvad, 1983), V8 (Vegetable juice water; Simmons, 1992) and CZID (Czapek Dox Iprodione Dichloran agar; Abildgren et al., 1987). DG18 and DRYES were incubated at 25°C in the dark. V8 and CZID plates were incubated in alternating light and dark cycles at 20−23°C (Andersen and Thrane, 2006). A broad range of media was used for fungal identification at subspecies level. For black fungi, such as *Alternaria* species, the following media were used, i.e. V8, DRYES and PCA (Potato Carrot Agar; Simmons, 1992). For *Fusarium* species, PDA, YES (Yeast Extract Sucrose agar; Samson et al., 2004), and SNA (Synthetischer Nährstoffarmer Agar; Nirenberg, 1976) were used. For *Penicillium* species, MEA, YES, CYA (Czapek Yeast Extract Agar; Pitt and Hocking, 1997), and CREA (Creatine Sucrose Agar; Samson et al., 2004) were used, while for *Eurotium* species, CYA, CYA20S (CYA with 20% (w/v) sucrose; Pitt and Hocking, 1997), CZ (Czapek Dox agar; Samson et al., 2004), DG18 and YES, were used. These cultures were incubated for 7 days at different temperatures and alternating dark and light cycles. Fungi were identified based on typical colony form under stereomicroscope (lower magnification and perception of depth) and conidia morphology with light microscope (higher magnification). The obtained results are expressed as percentage of kernels contaminated with fungi.

4.2.9. SOLID MEDIUM

The fungi were cultivated in medium containing 0.67% (w/v) yeast nitrogen base, 2% (w/v) agar and 1% (w/v) WAX as carbon source (Farkas et al., 1985). The fungi were grown at 25°C for 7 days.

4.2.10. LIQUID MEDIUM

Fungi from densely covered agar plates were used to inoculate 8 mL liquid medium composed of 0.67% (w/v) yeast nitrogen base, 0.2% (w/v) asparagine and 0.5% (w/v) KH_2PO_4 supplemented with either 1% (w/v) wheat arabinoxylan (WAX) or finely ground barley flour as carbon source into 50 mL falcon tubes (Biely et al., 1980). The fungi grew on the surface of the medium, and the proteins were secreted into the medium to break down nutrients. Negative controls composed of medium and WAX or barley flour were also prepared. The samples were similarly treated and incubated for 7 days at 25°C. Subsequently, the supernatants were collected by centrifugation at 3200 g for 30 min at 4°C.

4.2.11. MEDIUM FOR DETECTION OF FUNGAL XYLANASE ACTIVITY (DYED-WAX AGAROSE PLATES)

For a quick screening for xylanase activity, plate zymograms were performed by casting dyed substrate-containing agarose (0.1% (w/v) Remazol Brilliant Blue (RBB)-dyed WAX (Megazyme), 1% (w/v) agarose, and 0.2 M sodium citrate-HCl pH 4.8). Wells of 2 mm were punched into the solidified medium and loaded with sample, i.e. supernatants of the different fungi (5 µL), respectively, and incubated overnight at room temperature. Xylanase activity appeared as pale clearing zones around the wells, where the enzymes have degraded the dyed substrate.

4.2.12. ANALYSIS OF THE SECRETED PROTEINS BY SDS-PAGE AND 2-DE

The protein content of the supernatants was quantified following the method of Popov et al., (1985) using BSA as standard. Appropriate amount of protein (10 µg) was precipitated by 4 volumes of ice-cold 100% (v/v) acetone at -20°C overnight. Similarly, the samples were prepared for SDS-PAGE following the method of Laemmli (1970) using 4−12% Bis-Tris NuPAGE Novex Zoom gels (Invitrogen), a vertical slab mini gel unit (NuPAGE Novex system, Invitrogen). Prior to 2-DE, the supernatants were desalted on a NAP-5 column (GE Healthcare) and an appropriate amount of protein (50 µg) was precipitated with ice-cold 100% (v/v) acetone overnight at 20°C. Protein pellets were dissolved in 200 µL rehydration buffer, as described above. The protein samples were applied to 11 cm pH 3−10 IPG strips and IEF was run on Ettan™ IPGphor (GE Healthcare). After rehydration for 12 h at 50 mA/strip at 20°C, IEF was performed at 1 h at 150 V, 1 h at 300 V, 1 h at 1000 V, gradient to 8000 V, hold at 8000 V until a total of 30 kVh was reached. Subsequently, the strips were trimmed to a range covering pH 4−8.5 and applied to the second dimension, as described above. NuPAGE Novex 4−12% Bis-Tris Zoom gels (Invitrogen) were used according the manufacturer´s instructions. Gels were stained with Coomassie Brilliant Blue dye (G-250), and the resolved protein spots were excised, tryptic digested and identified by MALDI-TOF MS and MS/MS, as described above. All the identified proteins were assessed for signal peptides using SignalP v4.1 (http://www.cbs.dtu.dk/services/SignalP).

4.3. RESULTS AND DISCUSSION

4.3.1 MAPPING THE SURFACE-ASSOCIATED PROTEINS NATURALLY FOUND ON TWO BARLEY CULTIVARS

The surface-associated proteomes (surfome) of two different barley cultivars were mapped to characterize and identify the proteins constitutively present on outer kernel layers. It is a prerequisite to have established a reference proteome prior to any studies in describing the dynamics of the proteome in response to e.g. fungal attack, biotic or abiotic stress conditions. Briefly, barley grains of different cultivars were subjected to

the washing procedure developed by Dornez et al. (2006) to physically separate the microbial surface-associated and the plant endogenous xylanases, as well as the proteinaceous xylanase inhibitor proteins (Chapter 3). The resulting fractions were determined for protein contents and xylanase activity levels. The prepared washing liquids were concentrated and run on a 1D SDS-PAGE to obtain the general overview over protein patterns between the different cultivars (data not shown). From initial screening of the protein patterns and microbial xylanase activity levels (data not shown), the barley cultivars Barke and Cabaret, representing low and high xylanase activities, were selected for further thorough investigation. Representative 1D-gels of washing liquids of Barke and Cabaret are shown in Figure 26 (A). Several bands of both high and low molecular sizes appeared in both lanes, but with varying intensities. Zymograms were prepared by running samples in 1D SDS-PAGE incorporated with 0.1% WAX as substrate, followed by staining with Congo Red dye. Detection of transparent/clearing zones against a red background corresponds to xylanolytic activity (Figure 26 B). The zymogram analysis revealed detection of xylanases of high molecular weight bands and suggest the possibility that some of the contained xylanases in the washing liquids migrate in a bound state, meaning that they first hydrolyze the already bound substrate and then migrate to another portion of the incorporated substrate. Moreover, the washing liquids containing xylanases of the barley cultivar Cabaret exhibited higher activity having more intense bands compared to samples of Barke. Another technique using native gels overlaid with substrate gel containing Remazol Brilliant Blue dyed WAX (Figure 26 C) also confirmed the presence of xylanases and that washing liquids of Cabaret displayed higher activity levels. In addition, the gels show that the contained xylanases were able to migrate from the protein gel in order to get to the substrate. In general, several cell wall degrading enzymes have been reported to comprise one or more carbohydrate binding modules (CBMs) used to bind to the plant cell wall polysaccharide (Abou Hachem et al., 2000). Also in this case, it can be suggested that the detected xylanases use their CBMs to bind to the incorporated substrate in the gel once in close proximity.

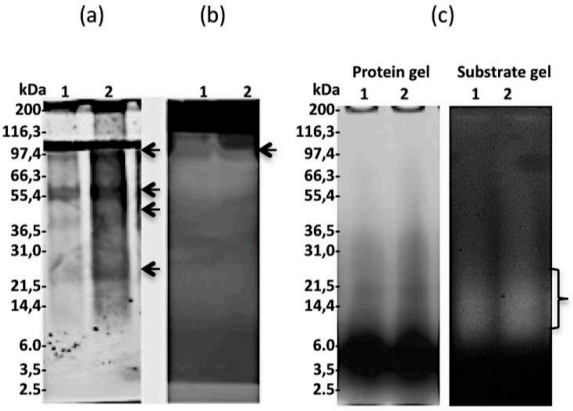

Figure 26: (A) 1D SDS-PAGE of the washing liquids of barley cultivars Barke and Cabaret. Each lane was loaded with equal amount of protein approximately 10 µg, where lane (1) Barke 2009 and (2) Cabaret 2009. (B) Zymogram developed with Congo Red dye, where samples were run in a SDS-PAGE incorporated with 0.1% (w/w) WAX. (C) 4−16% Native PAGE gel and substrate gel (0.5% Remazol Brilliant Blue-dyed WAX and 1.5% agarose). Molecular markers are as indicated.

In order to assess the 2D protein distribution, 2D-gel electrophoresis was performed in the pH range 3−10 (7 cm). Subsequent the second dimension, the gels were stained with SYPRO Ruby and thenceforth post-stained with Coomassie Brilliant Blue G-250 for spot picking, tryptic digestion and peptide sequencing. Representative 2D-gels of the washing liquids of Cabaret and Barke are shown in Figure 27. The overall protein patterns of the two barley cultivars were highly similar with minor but distinct spot differences. All the visible protein spots (47 spots of Cabaret and 30 of Barke) on the Coomassie stained 2D-gels were selected for identification. Fifty-seven proteins (30 for Cabaret and 27 for Barke) were identified with confidence by MALDI-TOF MS and/or MS/MS (Table 12). Numerous proteins appeared in more than one spot and in total 16 unique proteins were identified (Table 12), demonstrating a rather simple surface-associated proteome. Some of the spots were too faint to be adequately identified by mass spectrometry analysis. The majority of the identified proteins were found in high abundance and of plant origin. Lack of identifying fungal proteins in the washing liquids are due to low abundance, and consequently masked and obscured by high abundance proteins. One of the major limitations of 2D-gel based proteomics is

the poor detection of low-abundant proteins. The proteins present in more than one spot showing pI and Mw heterogeneity were possibly post-translationally modified (PTMs) or sequence-related isoforms.

Comparison of the surfomes shows the simplicity as well as the similarities between the two barley cultivars. The following proteins were identified, alginate lyases, α-amylase inhibitor/endochitinases, amylase/trypsin inhibitors, 1,3-β-glucanases, glyoxalases, endo-chitinases (different classes), disease resistance proteins, hypothetical protein (UDP-glucose:sterol transferase), metallo-β-lactamase, NADPH-dependent thioredoxin reductase isoform 2 (NTR 2), OprF outer membrane protein and related peptidoglycan-associated (lipo)proteins, pathogenesis-related proteins (PR-1a and PR-4), phytochrome A, polyubiquitins and thaumatin like proteins (TLPs). The majority of the identified proteins was of plant origin and ascribed to have a defensive role and provide resistance to fungal and bacterial colonization. The hypothetical protein similar to UDP-glucose: sterol transferase was designated to *F. graminearum.*

A large number of protein spots (thirty-five) were identified as chitinases (spots 13, 15−17, 20, 22, 25−26, 28−29, 41−43, 47, 66−69, 72, 75−76, 80−82, 86−88), which hydrolyzes chitin, the major cell wall component of fungi as well as in bacteria (Li and Roseman, 2004). Chitinases represent a subgroup of pathogenesis-related (PR) proteins that are produced as part of systemic acquired resistance (Legrand et al., 1987). Plant chitinases have been ascribed to play a role in defense or to enhance competitiveness in plants that lack chitin. Several studies report the anti-fungal properties to chitinases (Gomez et al., 2002). A study by Carranza et al. (2009) reported evidence of an extracellular chitinase in *Chronobacter turicensis* displaying a fungal-inhibiting activity. Others have shown that overexpression of chitinases alone or in synergy with other anti-fungal proteins, such as β-1,3-glucanases and ribosome inactivating proteins, leads to enhanced protection against pathogenic attack. The anti-fungal properties have been attributed to inhibition of the growth of chitin-containing fungi by causing lysis of hyphal tips (Leah et al., 1991; Mauch et al., 1988). Several plant chitinases also exhibit lysozyme activity (EC 3.2.1.17), hydrolyzing the β-1,4-linkages between *N*-acetylglucosamine and *N*-acetylmuramic acid residues in the peptidoglycan of bacteria (Collinge et al., 1993). Plant chitinases have been grouped into six different classes on the basis of sequence similarity to the tobacco chitinases

(Meins et al., 1994). Chitinases of classes I and II have been shown to differ in the presence of a conserved N-terminal cysteine-rich lectin domain. The following classes/types of chitinases were identified, i.e. α-amylase inhibitor/endochitinase (14.6 kDa, pI 6.07), chitinase II (27.5 kDa, pI 9.12), 26 kDa endochitinase (28.5 kDa, pI 8.83), chitinase - GH 19 (34.6 kDa, pI 7.41) and chitinase (36.3 kDa, pI 6.3). Other identified proteins involved in defense mechanisms include, the disease resistance-like protein (spots 2 and 64), pathogenesis related proteins PR-1 (spot 36) and PR-4 (spot 1), thaumatin like protein 4 (TLP4, designated as PR-5, spot 73) and α-amylase/trypsin CMb (spot 30), respectively. Moreover, the metallo-β-lactamase-like protein was identified (spot 58), indicating the plants response to arsenic containing compounds typically found in pesticides, herbicides and insecticides. Polyubiquitins (spot 33) participate in the ubiquitin/proteasome pathway of protein degradation, which has been implicated in plant response to internal and external stimuli, e.g. abiotic stress and pathogen attack (Dong et al., 2006). Identification of these proteins suggests a general role in pathological stress related situations, as well as adaptation to biotic conditions.

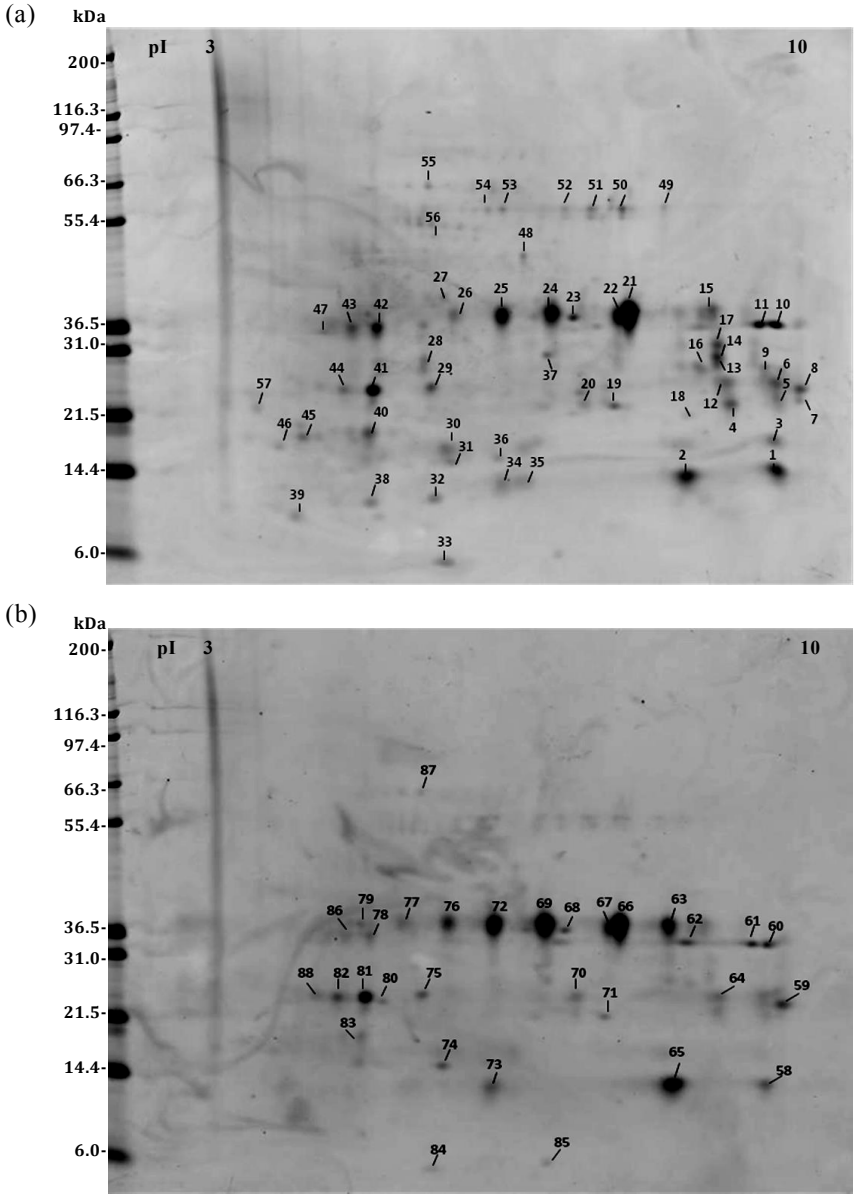

Figure 27: Two-dimensional electrophoresis SYPRO Ruby-stained gels loaded with 50 µg protein of washing liquids of barley cultivar Cabaret (a) and Barke (b) harvest at Sejet at 2009 (greyscale, 16 Bit). After post-staining of the gel with Coomassie Brilliant Blue G-250, the numbered spots were excised and subjected to MALDI-TOF MS and MS/MS. Protein identification can be seen in Table 12. Molecular markers and pI range are as indicated.

Another large group of identified proteins was involved in modulation of plant cell walls, including 1,3-β-glucanases (spots 10−11, 37, 60−62 and 70), which hydrolyze glycosidic linkages in β-glucans. Arabinoxylans and glucans are major constituents of plant cell walls. In barley grains, the highest concentrations of β-1,3;1,4-glucans can be found in endosperm cell walls comprising 70% (Fincher and Stone, 2004; Noots et al., 2003), followed by the aleurone layers of 26% (Fincher, 2009). While arabinoxylans are concentrated in the aleurone layers comprising 70% and endosperm of 20% (Fincher, 2009; Fincher and Stone, 2004; Noots et al., 2003). In addition, β-glucans occur in the cell walls of certain fungi and yeasts. Plant alginate lyases (spot 8−59), also known as mannuronate or guluronate lyases, catalyze the degradation of alginate, a complex gelling polysaccharide (consists of domains of β-1,4-D-mannuronate and α-1,3-L-guluronate) found in abundance as a part of cell wall (Østgaard, 1993; Percival and McDowell, 1967).

One spot (no. 44) was identified as the bacterial outer membrane protein and related peptidoglycan-associated (lipo)protein (OprF), which is a general porin shown to play a role maintaining the cell structure/integrity and growth of bacteria at low osmolarity (Rodriguez-Herva et al., 1996). Spot 27 was identified as fungal hypothetical protein predicted as UDP-glucose:sterol transferase, which is involved in catalyzing the biosynthesis of various steryl glycosides (SGs). These lipids are abundant constituents of the cell membranes of plants, but have also been detected in fungi, bacteria and some animals (Esders et al., 1972; Mayberry and Smith, 1983; Warnecke et al., 1997; 1999). Identification of fungal UDP-glucose:sterol transferases suggests a possible role as effector proteins. Moreover, alterations of the steryl lipid composition of the plasma membrane in plants have been suggested to be a response to the changing environment, in order to maintain homo-viscosity/fluidity of cell membranes (Hazel and Williams, 1990).

Plants have developed numerous adaptation and defense mechanisms to overcome environmental stresses. Enzymatic systems have been developed to provide protection against reactive oxygen species and potentially toxic effects of xenobiotics (Knight and Knight, 2001; Shinozaki and Yamaguchi-Shinnozaki, 2000; Zhu, 2001). Plants under various stresses have been shown to increase the rate of glycolysis leading into an imbalance in the metabolic pathways and consequently an increased production of

unstable triose metabolites (e.g. triose phosphates) and formation of methylglyoxal, a potent cytotoxic compound (Hossain et al., 2011; Thornalley, 1990; Yadav et al., 2005). Plants confer tolerance by detoxifying these compounds with the glyoxalase pathway, which involve two thiol-dependent enzymes, namely glyoxalase I-II (Creighton and Hamilton, 2001; Vander, 1993). Three spots (45, 74 and 84) were confidently identified as glyoxalase. In plants, the NADPH-dependent thioredoxin reductase (NTR) and thioredoxin (Trx) system has been proposed to play a vital role in seed development and germination, defense against invading pathogens, and oxidation stress by thiol redox control. NTR belongs to a superfamily of flavoprotein disulfide oxidoreductases (Reichhelt et al., 2005), and transfer electrons from NADPH to the active site disulfide bridge of oxidized Trx h, which than (reduced form of Trx h) acts as an electron donor to many other target proteins. In barley seeds, two forms of Trx h and NTR have been identified in the embryo and aleurone layer, and these have been characterized (Maeda et al., 2003; Shahpiri et al., 2009). In the present study, NTR 2 was identified in the washing liquids of Cabaret (spot 23), which is surprising as these are cytoplasmic and/or mitochondrial proteins. There is a possibility that some of the grains were abraded during the washing procedure. The grains were incubated for 15 h in the washing liquids, which initiates seed germination. However, in a study by Shahpiri et al. (2008), it was reported that gibberellic acid (GA) down-regulated the expression of NTR 2. Nevertheless the NTR/Trx h system regulate multiple mechanisms, and identification of these proteins show a glimpse of the various strategies of the plant in response to various environmental conditions (e.g. production of reactive oxygen species e.g. H_2O_2).

In general, plants use phytochrome, a photoreceptor, to detect light and regulate the time of flowering based on the day-length perception (photoperiodic) (Johnson et al., 1994; Reed et al., 1994). In this study we identified phytochrome A (spot 65), which regulates the synthesis of chlorophyll and response of seed germination (Childs et al., 1997; Reed et al., 1993; Weller et al., 1997).

Table 12: Identification of proteins spots resolved in the 2D-gel of the prepared washing liquids of barley cultivar Barke and Cabaret by MALDI-TOF MS and MS/MS. Protein identifications were confirmed with a MASCOT score of 70 for peptide mass fingerprint (ANOVA $\leq$ 0.05) and a minimum of 4 matched peptides or one matched peptide with a MASCOT score of 40 for MS/MS (ANOVA $\leq$ 0.05). The spot numbers correspond to the gel image shown in Figure 27. Abbreviation: Comb: combined; meas: measured; PMF: peptide mass fingerprint; theor: theoretical.

Spot no.	Accession no.	Organism	Protein name	Mw theor.	pI theor.	PMF score	PMF E-value	Comb. score	E-value	Sequence coverage %	Unique peptides	MS/MS Precursor ions	MS/MS Sequence	Ion score
Barley cv. Cabaret														
1	gi|6048569	*Triticum aestivum*	PR-4	13442	7	92				10	1	12,997,122	VTNPATGAQITAR	92
2	gi|13310437	*Brassica oleracea*	Disease resistance -like protein	12846	8.94			73	0.033	54	7			
8	gi|326499359	*Hordeum vulgare* subsp. *vulgare*	Predicted protein - Alginate lyase2	24190	9,63			61	7.80E-01	15	2	18,158,633	R.YYNQKPVEDNIYNR.W	53
10	gi|119003	*Hordeum vulgare* subsp. *vulgare*	Resistance endo-1,3-beta-glucosidase GII - GH17	35229	9,01			240	1.10E-18	54	12	14,247,106	R.IYFADGQALSALR.N	85
												16,557,912	R.TYNQGLINHVGGGTPK.K	66
11	gi|167010	*Hordeum vulgare*	(1,3)-beta-glucanase	35243	9,01			121	8.60E-07	17	3	21,380,483	R.DNPGSISLNYATFQPGTT VR.D	96
12	gi|266324	*Coix lacryma-job*	Alpha-amylase inhibitor/ endochitinase	14644	6.07			91	6.2E-04	12	2	1743.9298	R.GPIQISHNYNYGPAGR.A	68
13	gi|326488069	*Hordeum vulgare* subsp. *vulgare*	Predicted protein	34590	7,41			120	1.10E-06	27	5	17,438,744	R.GPIQISHNYNYGPAGR.A	70
14	gi|266324	*Coix lacryma-jobi*	Alpha-amylase inhibitor/ endochitinase	14644	6.07			95	2.2E-04	12	20	1743.9143	R.GPIQISHNYNYGPAGR.A	72
15	gi|116316	*Hordeum vulgare*	26 kDa endochitinase	28537	8,83			93	5.30E-04	13	2	17,439,715	R.GPIQLSHNYNYGPAGR.A	81
16	gi|116316	*Hordeum vulgare*	26 kDa endochitinase	28537	8,83			88	1.70E-03	13	2	17,439,529	R.GPIQLSHNYNYGPAGR.A	74
17	gi|326488069	*Hordeum vulgare* subsp. *vulgare*	Chitinase,GH19	34590	7,41			72	7.30E-02	13	3	17,439,065	R.GPIQLSHNYNYGPAGR.A	50
20	gi|116316	*Hordeum vulgare*	26 kDa endochitinase	28537	8,83			89	1.40E-03	15	3	17,438,817	R.GPIQLSHNYNYGPAGR.A	69
21	gi|266324	*Coix lacryma-jobi*	Alpha-amylase inhibitor/ endochitinase	14644	6.07			106	1.80E-05	12	2	1743.8717	R.GPIQISHNYNYGPAGR.A	84
22	gi|116316	*Hordeum vulgare*	26 kDa endochitinase	28537	8,83			114	4.30E-06	15	3	17,439,378	R.GPIQLSHNYNYGPAGR.A	92
23	gi|159895412	*Hordeum vulgare* subsp. *vulgare*	NADPH-dependent thioredoxin reductase isoform 2	35057	5,73			79	1.30E-02	14	4	1,235,734	K.YGSQVYIIHR.R	56
24	gi|266324	*Coix lacryma-jobi*	Alpha-amylase inhibitor/ endochitinase	14644	6.07			89	8.0E-04	12	2	1743.9038	R.GPIQISHNYNYGPAGR.A	67
25	gi|116316	*Hordeum vulgare* subsp. *vulgare*	26 kDa endochitinase	28537	8,83			67	2.30E-01	13	2	17,438,549	R.GPIQLSHNYNYGPAGR.A	56

26	gi|563489	*Hordeum vulgare*	Chitinase	26898	6.08	80	7.8E-03			40	8			
27	FGSG_12643	*Fusarium graminearum*	Hypothetical protein similar to UDP-glucose,sterol transferase	89370	0.043			55	0.043	11	6			
28	gi|507961	*Hordeum vulgare*	Chitinase	36316	6,3			85	3.50E-03	6	1	23,011,589	R.AIGVDLLNNPDLVATDATVSFR.T	76
29	gi|9501334	*Hordeum vulgare* subsp. *vulgare*	Chitinase II	27509	9,12			122	6.80E-07	20	3	16,898,372	R.GPIQLTGQSNYDLAGR.A	43
												19,339,204	K.DLVSNPDLVSTDAVVSFR.T	46
30	gi|585290	*Hordeum vulgare* subsp. *vulgare*	Amylase/trypsin inhibitor CMb	17199	5.77	89	9.6E-04			40	5			
33	gi|33327284	*Phaseolus vulgaris*	Polyubiquitin 1	6547	6.51	93	3.3E-04			63	5			
36	gi|401831	*Hordeum vulgare* subsp. *vulgare*	PR-1a pathogenesis related protein	17771	8.19					7	1	14,126,798	LQAYAQSYANQR	72
37	gi|809429	*Hordeum vulgare* subsp. *vulgare*	Chain A, The three-dimensional structures of two plant beta-glucan endohydrolases with distinct substrate specificities	32390	8,83			81	7.80E-03	13	2	21,380,043	R.DNPGSISLNYATFQPGTTVR.D	66
41	gi|9501334	*Hordeum vulgare* subsp. *vulgare*	Chitinase II	27509	9,12			167	2.20E-11	20	3	16,899,241	R.GPIQLTGQSNYDLAGR.A	66
												19,340,028	K.DLVSNPDLVSTDAVVSFR.T	73
42	gi|507961	*Hordeum vulgare*	Chitinase	36316	6,3			140	1.10E-08	9	2	13,177,553	R.DLFEQFLLHR.D	47
												23,013,016	R.AIGVDLLNNPDLVATDATVSFR.T	85
43	gi|507961	*Hordeum vulgare*	Chitinase	36316	6,3			56	2.60E+00	15	3	23,011,645	R.AIGVDLLNNPDLVATDATVSFR.T	45
44	gi|332650971	*Pseudomonas abietaniphila*	OprF, partial	33660	4,69			112	1.20E-04	17	3	22,880,376	K.NDGNLFGGSVGYFLTDDVELR.L	53
												13,096,229	K.LYFTDNFYAR.A	32
45	gi|168061933	*Physcomitrella patens* subsp. *patens*	Glyoxalase	13136	7.71			77	0.013	55	5			
47	gi|507961	*Hordeum vulgare*	Chitinase	36316	6.3					3	1	13,177,232	DLFEQFLLHR	56

Barley cv. Barke

58	TC169446	*Oryza sativa*	Metallo-beta-lactamase-like	37674	10.17	71	0.041			24	8			
59	gi|326499359	*Hordeum vulgare* subsp. *vulgare*	Predicted protein - Alginate_lyase2	24190	9,63			83	9.70E-02	15	2	18,159,852	R.YYNQKPVEDNIYNR.W	70
60	gi|809429	*Hordeum vulgare*	Chain A, The three-dimensional structures of two plant beta-glucan endohydrolases with distinct substrate specificities	32390	8,83			207	2.20E-15	41	9	14,247,626 21,380,418	R.IYFADGQALSALR.N R.DNPGSISLNYATFQPGTTVR.D	56 81
61	gi|809429	*Hordeum vulgare*	Chain A, The three-dimensional structures of two plant beta-glucan endohydrolases with distinct substrate specificities	32390	8,83			119	1.40E-06	25	5	21,381,302	R.DNPGSISLNYATFQPGTTVR.D	96
62	gi|809429	*Hordeum vulgare*	Chain A, The three-dimensional structures of two plant beta-glucan endohydrolases with distinct substrate specificities	32390	8,83			289	1.40E-23	36	7	14,247,314 17,118,055 21,380,374	R.IYFADGQALSALR.N R.FDEVANSFPPSAGVFK.N R.DNPGSISLNYATFQPGTTVR.D R.DNPGSISLNYATFQPGTTVR.D	94 51 76
63	gi|116316	*Hordeum vulgare*	26 kDa endochitinase	28537	8,83			112	6.80E-06	16	3	17,438,792	R.GPIQLSHNYNYGPAGR.A	83
64	gi|108740063	*Arabidopsis thaliana*	Disease resistance protein	21325	6.34	78	0.01			51	6			
65	gi|75674190	*Thottea tomentosa*	Phytochrome A	26673	5.75			77	0.014	39	6			
66	gi|18972	*Hordeum vulgare* subsp. *vulgare*	Chitinase GH19	19551	8,55			102	6.80E-05	22	2	17,438,666	R.GPIQLSHNYNYGPAGR.A	86
67	gi|326488069	*Hordeum vulgare* subsp. *vulgare*	Chitinase GH19	34590	7,41			109	1.40E-05	13	3	17,438,351	R.GPIQISHNYNYGPAGR.A	93
68	gi|18972	*Hordeum vulgare* subsp. *vulgare*	Chitinase GH19	19551	8,55			47	2.10E+01	8	1	17,437,997	R.GPIQLSHNYNYGPAGR.A	41
69	gi|326488069	*Hordeum vulgare* subsp. *vulgare*	Chitinase GH19	34590	7,41			101	8.60E-05	13	3	17,438,984	R.GPIQISHNYNYGPAGR.A	82
70	gi|809429	*Hordeum vulgare*	Chain A, The Three-Dimensional Structures Of Two Plant Beta-Glucan Endohydrolases	32390	8.83	105	2.20E-05			37	7			
71	gi|9501334	*Hordeum vulgare* subsp. *vulgare*	Chitinase II	27509	9.12	73	0.035			36	7	17,438,943	R.GPIQISHNYNYGPAGR.A	35
72	gi|326488069	*Hordeum vulgare* subsp. *vulgare*	Chitinase GH19	34590	7,41			99	1.40E-04	16	4	17,439,482	R.GPIQISHNYNYGPAGR.A	80
73	gi|56090131	*Hordeum vulgare* subsp. *vulgare*	Thaumatin-like protein TLP4	18388	5.69	83	0.0037			45				
74	gi|326520285	*Hordeum vulgare* subsp. *vulgare*	Glyoxalase	15398	6,74			86	3.00E-03	10	1	15,797,563	K.SVAFYADAFGYNVR.R	76

75	gi|9501334	*Hordeum vulgare*	Chitinase II	27509	9.12	72	0.031	347		20	9	19,099,851	GPIQLTGQSNYDLAGR	105
												16,899,299	ELAAFFGQTSHETTGGTR	111
												19,340,724	DLVSNPDLVSTDAVVSFR	131
76	gi|326522492	*Hordeum vulgare* subsp. *vulgare*	Chitinase GH19	34446	8,42			64	4.30E-01	8	2	17,439,412	R.GPIQLSHNYNYGPAGR.A	40
78	gi|507961	*Hordeum vulgare*	Chitinase	36316	6,3			82	6.70E-03	9	2	23,012,255	R.AIGVDLLNNPDLVATDA TVSFR.T	74
79	gi|326488069	*Hordeum vulgare* subsp. *vulgare*	Chitinase GH19	34590	7,41			99	1.30E-04	13	3	17,438,523	R.GPIQISHNYNYGPAGR.A	77
80	gi|9501334	*Hordeum vulgare* subsp. *vulgare*	Chitinase II	27509	9,12			158	1.70E-10	24	4	19,098,919	R.ELAAFFGQTSHETTGGTR .G	35
												16,898,386	R.GPIQLTGQSNYDLAGR.A	58
												19,339,747	K.DLVSNPDLVSTDAVVSFR .T	33
81	gi|563489	*Hordeum vulgare* subsp. *vulgare*	Chitinase	26898	6,08			437	2.20E-38	37	7	19,100,248	R.ELAAFFGQTSHETTGGTR .G	116
												10,115,538	K.ATSPPYYGR.G	24
												16,899,618	R.GPIQLTGQSNYDLAGR.A	125
												19,341,237	K.DLVSNPDLVSTDAVVSFR .T	110
82	gi|563489	*Hordeum vulgare* subsp. *vulgare*	Chitinase	26898	6,08			334	4.30E-28	35	5	19,098,989	R.ELAAFFGQTSHETTGGTR .G R.GPIQLTGQSNYDLAGR.A	75
												16,898,545	ELAAFFGQTSHETTGGTR	119
												19,339,879	K.DLVSNPDLVSTDAVVSFR .T	97
84	gi|326520285	*Hordeum vulgare* subsp. *vulgare*	Glyoxalase	15398	6,74			62	6.70E-01	10	1	15,797,585	K.SVAFYADAFGYNVR.R	54
86	gi|507961	*Hordeum vulgare*	Chitinase	36316	6,3			117	2.20E-06	9	2	13,176,778	R.DLFEQFLLHR.D	40
												23,012,158	R.AIGVDLLNNPDLVATDA TVSFR.T	67
88	gi|9501334	*Hordeum vulgare* subsp. *vulgare*	Chitinase II	27509	9,12			302	6.80E-25	26	5	19,098,879	R.ELAAFFGQTSHETTGGTR .G	61
												16,898,315	R.GPIQLTGQSNYDLAGR.A	89
												19,339,738	K.DLVSNPDLVSTDAVVSFR .T	87

4.3.2 THE COMPOSITION OF THE MYCOBIOTA ON BARLEY GRAINS/FUNGAL SPECIES ASSOCIATED TO DIFFERENT BARLEY CULTIVARS

To date, limited research has been performed to provide an integrated picture of protein expression, xylanolytic activities and mycobiota on barley grains. In the fields, cereal grains are exposed to multiple environmental conditions and a wide variety of microorganisms from soil, water, fertilizer, insects, and animal droppings, etc. The surface of the grains can be heavily contaminated by hundreds of microbial species (Hill and Lacay, 1983). In particular, contamination by certain types of fungi of cereal grains is of serious concern, since several species are known to be toxigenic. To assess whether the proteome of the two barley cultivars (Cabaret vs. Barke) differing in microbial xylanase activities can be correlated to the populating microbial community, an array of different barley cultivars were cultivated to profile and characterize the composition of the mycobiota. The study also enabled assessment of the dynamics of the mycobiota on the grains of different harvest years (2009 and 2011) and locations (Sejet and Koldkærgaard). A list of the isolated and identified fungal species on barley grains is shown in Table 13.

Based on the occurrence and composition of the mycobiota, the different cultivars were clustered into five different groups. First group comprising the barley cultivars Frederik and Simba harvested at Sejet in 2009 was dominated by *Alternaria infectoria* and *Gonatobotrys simplex.* In addition, these two cultivars were determined to have high microbial xylanase activity levels compared to the others (0.168−0.170 U/g, Table 13). In the second group, the prevailing species identified were *F. culmorum* and *Cochiobolus sativus* (*Drechslera*), while low percentage levels of *Alt. infectoria* and *Epicoccum nigrum* (Quench and Simba harvested at KKG in 2011 and Quench 2011, Cabaret 2009, 2011 harvested at Sejet). Cultivars in this group had a broad interval of microbial xylanase activity levels from high to intermediate levels (0.172−0.0593 U/g, Table 13). In the third group, high levels of *Chaetomium globosum* and *Epicoccum nigrum*, and low levels of *F. culmorum* and *Penicillium scabrosum* and *Penicillium spathulatum* were identified on Simba (Sejet, 2011) with low microbial xylanase activity levels (0.0414 U/g). Group 4 comprised the cultivars Barke, Frederik and Cabaret (Sejet, 2011), which were dominated by *Eurotium repens* (*Aspergillus*), *Alt. infectoria* and *F. culmorum,* respectively. These cultivars were

characterized to have low microbial xylanase activities (0.031−0.058 U/g). In this group, storage fungi were present in much higher percentage levels compared to the previous groups, even though the identified fungi were isolated from freshly harvested barley (2011). Lastly, *Phoma* sp. and *F. tricinctum* were isolated from Cabaret (Sejet, 2009), which exhibited high microbial xylanase activity levels (0.127 U/g).

Table 13: Examination of fungi isolated from six different barley cultivars (Barke, Cabaret, Frederik, Himalaya, Quench, Simba) by direct plating of 20−34 kernels on different media. All cultivations were performed in collaboration with Professor Jens Frisvad (CMB, Systems Biology, DTU).

Group	Cultivar	Location	Harvest Year	Fungi	Microbial xylanase activity (U/g)
1	Frederik	Sejet	2009	*40% Alternaria infectoria* *5% Fusarium poae* *2% Ulocladium atrum* *2% Trichoderma hamatum* *5% Aspergillus echinulatis* *2% Erotium repens* *20% Gonatobotrys simplex* *5% Harzia vernicosa*	0.168
	Simba	Sejet	2009	*40% Alternaira infectoria* *10% Epicoccum nigrum* *20% Gonatobotrys simplex* *2% Nigrospora* spp./*Arthrinium*	0.171
2	Quench	KKG	2011	*40% Fusarium culmorum* *5% Alternaria infectoria* *20% Cochliobolus sativus (Drechslera)* *5% Epicoccum nigrum* *2% Gonatobotrys simplex* *5% Cladosporium cladsporioides*	0.526
	Simba	KKG	2011	*40% Fusarium culmorum* *2% Penicillum spp.* *1% Eurotium repeas* *2% Epicoccum nigrum* *30% Nigrospora spp.* *20% Cochiobolus sativus (Drechslera)* *5% Alternaria infectoria* *2% Harzia vernicosa*	0.172
	Quench	Sejet	2011	*30% Fusarium culmorum* *30% Alternaria infectoria* *2% Cochiobolus sativus (Drechslera)* *1% Epiccocum nigrum* *5% Penicillium venucorum*	0.06
	Cabaret	Sejet	2010	40% *Alternaria infectoria* 20% *Fusarium culmorum* 7% *Chalastospora gossypii* 7% *Drechslera* sp. 7% *Alternaria infectoria*	0.0593

Continued next page

Group	Cultivar	Location	Harvest Year	Fungi	Microbial xylanase activity (U/g)
3	Simba	Sejet	2011	*20% Epicoccum nigrum* *15% Penicillium scrabosum* *4% Penicillium spathulatum* *4% Fusarium culmorum* *15% Cladosporium cladsporioides (complex)* *5% Alternaria infectoria* *20% Claetomium globusum 15% Drechslera*	0.0414
4	Barke	Sejet	2011	*40% Penicillium spathulatum* *15% Fusarium culmorum* *4% Eurotium repens* *4% Wallemia* *2% Cochiobolus Sativus (Drechslera)* *2% Gonatobotrys simplex* *2% Alternaria infectoria* *2% Nigrospora*	0.057
	Frederik	Sejet	2011	*40% Alternaria infectoria* *10% Aspergillus echinulatus* *2% Gonatobotry simplex* *4% Trichoderma hamatum* *4% Penicillium spathulatum* *30% Erotium repens* *7% Fusarium culmorum* *2% Nigrospora*	0.031
	Cabaret	Sejet	2011	*30% Nigrospora* *2% Cochiobolus sativus (Drechslera)* *10% Alternaria infectoria* *5% Epicoccum nigrum* *7% Fusarium culmorum* *12% Eurotium repens* 5% *Acremoniella verrucosa* 5% *P. brevicompactum* 5% *P. verrucosum*	0.058
5	Cabaret	Sejet	2009	50% *Phoma* sp. 25% *Fusarium tricinctum*	0.127
Others	Cabaret	Sejet	2010	17% *Fusarium culmorum* 7% *Chalastospora gossypii* 7% *Drechslera* sp. 7% *Alternaria infectoria*	0.058
	Himalaya	US	2003	28% *Chalastospora gossypii* (Earlier name: *Alternaria malorum*) 5% *Rhizopus nigricans* 5% *Eurotium repens* 5% *Penicillium freii* 14% *Epicoccum nigrum* 15% *Ulocladium atrum* *5% Penicillium cyclopium*	

In general, the examined grains were colonized by the fungi genera *Alternaria, Dreschlera, Fusarium*, *Nigrospora*, and *Eurotium,* respectively. The universal presence of *Alt. infectoria* on barley grains has also been reported elsewhere (Christensen, 1991; Frisvad and Samson, 1991). Identification of high numbers of

field fungi on stored grains (2 years) indicates that the determined fungal profile is a snapshot of when the grains were in the field and that the fungal spores survived the storage period. Nevertheless the occurrence of specific fungi species in barley grains is strongly dependent on environmental factors. Harvest year 2009, which was characterized to have a warm spring and summer, seems to possess high numbers of fungal genera *Alternaria, Gonatobotrys, Fusarium* and *Phoma*. Harvest year 2011 had an extremely wet spring and summer and dominated by *Fusarium, Alternaria* and *Eurotium.* It should be kept in mind that the characterized fungal species were on Danish barley grains. The profile may be completely different on grains from geographic areas. In addition, the barley cultivar Himalaya harvested in the US in 2003 was also analyzed and exhibited a quite distinct profile compared to the others with high percentage levels of *Chalastospora gossypii* (earlier name *Alt. malorum*), *Ulocladium atrum* and *Epicoccum nigrum.* Thus, the geographical and environmental impact on the level and occurrence of fungal species cannot be neglected.

A study by Andersen and Thrane (2006) reported that the dominant fungi found in Danish barley grains (cultivar Ferment, harvest year 2002) were *Alt. infectoria* species-group*, F. avenaceum, P. aurantiogriseum, P. cyclopium, P. polonicum*. In a two-year study by Hill and Lacey (1983), barley cultivars harvested in UK were reported to be invaded by large numbers of *Alt. alternate, Cladosporium cladosporioides, Aureobasidium pullulans, Epiccocum nigrum*, and several *Penicillium* species, i.e. *P. verrucosum, P. aurantiogriseum*, *P. hordei*, *P. piceum* (correct name *Talaromyces piceus*)*, P. roqueforti*, respectively. In another study, freshly harvested barley seeds in Egypt were found to be dominated by the *Aspergillus* and *Penicillium* species. Other fungi isolated included *Rhizopus, Alternaria*, *Fusarium* and *Drechslera* (Abdel-Kader et al., 1979). Most of the fungi are regarded as non-toxigenic such as *Cladosporium*, *Eurotium* and *Epicoccum*. Both *Alternaria* and *Cladosporium,* have been reported to cause various degree of grey discoloration, which in part can be due to pigments in their mycelium or from melamins produced by plant cells. However, some fungi not only cause staining to the grains, but also produce compounds that are toxic (called mycotoxins) and cause disease to humans and animals (Christensen, 1991; Watson, 1984).

Seeds harboring *Fusarium* in amounts high enough can be harmful, as some species are capable of producing an array of metabolites, i.e. antibiotic Y, aurofusarin, beauvericins, culmorin enniatins, fusarin C, fusarochrome, moniliformin, trichothecenes, zearalenone, respectively (Andersen and Thrane, 2006; Christensen, 1991, Frisvad and Samson, 1991). Other commonly known toxigenic species includes *Alternaria* (except *Alt. infectoria* species-group), *Aspergillus* and *Penicillium* (except *P. solitum)*. *Alternaria* species are known to produce a wide range of toxins, i.e. altenuene, alternariols, altertoxins and tenuazonic acid. Studies have shown that *Aspergillus* species primarily produce, e.g. aflatoxins, aspergillic acid, cyclopiazonic acid, malformins, naphtho-γ-pyrones, terphenyllin and xanthoascin. *Penicillium* species are known to produce, i.e. citrinin, isofumigaclavin, nephrotoxic glycopeptides, ochratoxin A, patulin, penicillic acid, penitrem A, roquefortine C, viridic acid, verrucosidin, viomellein, vioxanthin and xanthomegnin, respectively. Although the biological roles of these mycotoxins and secreted metabolites are not clear in all the cases, they are still considered to be virulence factors in pathogenicity (Bennett and Klich, 2003; Boddu et al., 2003; Maier, 2006). Moreover, mycotoxins produced either in the field or storage should be taken into consideration as they typically persist through storage and downstream processes (Frisvad and Samson, 1991).

4.3.3 XYLANOLYTIC ACTIVITIES AND SECRETED PROTEIN PROFILES OF THE DIFFERENT FUNGI GROWN ON WHEAT ARABINOXYLANS (WAX) AND BARLEY FLOUR

Fungi colonizing the grains must be able to germinate and become established rapidly, as well as to produce necessary extra-cellular enzymes to exploit the nutrients source. Their activity is strongly influenced by abiotic factors, e.g. temperature, water activity and pH. The fungal community actively interacts with the inhabiting barley grains by producing metabolites, plant stimulating hormones (which enhance grain germination) and a collection of amylopectic, proteolytic and cell-wall degrading enzymes, such as β-glucanases and xylanases (Angelino and Bol, 1990; Flannigan 2003; Tuomi et al., 1995). However, fungi have also been shown to interfere with

grain respiration, reduce grain variability and produce mycotoxins (Frisvad and Samson, 1991). Therefore, characterization of the fungal community, their dynamics and activities is of high importance.

To obtain an insight into the plant-microbe relationship, produced fungal proteins and their enzymatic activities, an array of isolated cereal fungal species were grown on two different substrates, i.e. WAX and barley flour, and the harvested culture supernatants were analyzed for secreted proteins and xylanase activities. WAX was used as substrate to induce the production of xylanases by the specific fungal species, while barley flour served as the natural substrate of the different fungi. A list of the grown fungi and determined specific xylanase activities is shown in Table 14. The screening analysis showed that the majority of the fungi exhibited higher xylanase activity levels when grown on WAX compared to barley flour as carbon source with a few exceptions (Table 14). This is not surprising as the amount of arabinose (36%) and xylose (51%) is much higher in WAX (Megazyme) compared to barley flour, which is a complex mixture of nutrients, such as non-starch polysaccharides (i.e. β-glucans), proteins, and lipids. Arabinoxylans constitutes about 7.1−8.0% of barley grains (Han, 2000; Henry, 1988). Moreover, the storage fungi, i.e. *Penicillium*, *Eurotium* and *Aspergillus*, produced significantly higher levels of xylanase in comparison to the field fungi (fungal nr. 1−18, Table 14). In particular, *P. brevicompactum* (fungal no. 19−21), *P. verrucosum*, (fungal no. 22), *P. chrysogenum* (fungal no. 24) and *A. niger* (fungal no. 23) exhibited high activity levels. In nature, the invading fungi have both the seed and plant to live off, while storage fungi only have the seeds. The storage fungi seem to be more specific in nutrient acquisition, which could also explain the fact that they are more competent at producing higher amounts of xylanases. Quite large deviations were found between the biological replicates (different batches), which were probably due to the actual amount of fungi being inoculated into medium.

Plate zymograms prepared with 0.1% dyed substrate (Remazol Brilliant Blue WAX) were used to screen for xylanolytic activity of all the culture supernatants (Figure 28). Xylanases produced by the cultivated fungi were determined by the clarity and size of the zones surrounding the wells, which itself is dependent on variables, such as the ability and rate of diffusion of the xylanases into the medium. Representative plate

zymograms of the culture supernatants can be seen in Figure 28. Prominent clearing zones were seen for culture supernatants of fungal species grown on WAX compared to barley flour. Noticeably, the storage fungi, i.e. *Penicillium* and *Aspergillus* spp., gave rise to large and obscure zones, while the field fungi (e.g. *Fusarium, Alternaria, Acremoniella, Epicoccum* and *Drechslera* spp.) displayed small and clear zones. The differential clearing zones seem to be in accordance with the determined xylanase activity levels. Additionally, the different clearing zones suggest the production of different types of xylanases.

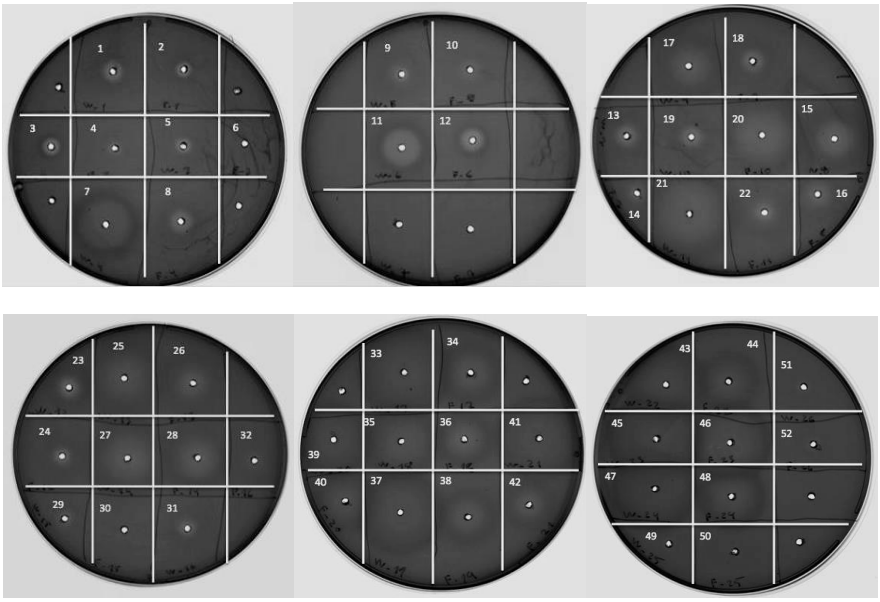

Figure 28: Plate zymograms of the different culture supernatants, where each well is loaded with ca. 5 µL of sample. Well no. (1) W1, (2) F1, (3) W2, (4) F2, (5) W3, (6) F3, (7) W4, (8) F4, (9) W5, (10) F5, (11) W6, (12) F6, (13) W7, (14) F7, (15) W8, (16) F8, (17) W9, (18) F9, (19) W10, (20) F10, (21) W11, (22) F11, (23) W12, (24) F12, (25) W13, (26) F13, (27) W14, (28) F14, (29) W15, (30) F15, (31) W16, (32) F16, (33) W17, (34) F17, (35) W18, (36) F18 (37) W19, (38) F19, (39) W20, (40) F20, (41) W21, (42) F21, (43) W22, (44) F22, (45) W23, (46) F23, (47) W24, (48) F24, (49) W25, (50) F25, (51) W26, (52) F26 (the culture supernatants of fungal no. 27-31 are missing). The numbers refer to fungi listed in Table 14. Abbreviations: W: wheat arabinoxylan and F: flour (barley).

Table 14: Isolated fungi grown with both WAX and barley flour as substrate and their xylanase activity levels (U/g). WAX: wheat arabinoxylan (Screening performed by B.Sc. student Julie Sørensen and Maria Bach). # refers to the batch number.

				Specific xylanase activity (U/g)			
				#1		#2	
Fungal number	**Name**	**Year**	**Cultivar**	**WAX**	**Flour**	**WAX**	**Flour**
1	*F. avenaceum*	2010	Quench	0.80	0.04	6.08	0.11
2	*F. avenaceum*	2011	Quench	0.25	0.01	1.57	0.02
3	*F. avenaceum*	2010	Quench	0.01	0.00	0.55	0.00
4	*F. culmorum*	2011	Quench	9.71	0.04	*-75.57	0.56
5	*F. graminearum*	2010	Simba	3.13	0.03	1.98	0.08
6	*Acremoniella verrucosa*	2011	Carbaret	3.95	0.05	282.45	0.74
7	*Epicoccum nigrum*	2009	Scarlett	0.01	0.00	1.66	*-0.62
8	*F. poae*	2009	Frederik	6.65	0.01	0.01	0.08
9	*F. equiseti*	2010	Quench	2.43	0.04	0.03	0.06
10	*Drechslera* spp.	2011	Scarlett	0.04	3.95	*-0.84	0.01
11	*Alternaria infectoria*	2011	Simba	0.40	2.21	0.03	0.07
12	*Epicoccum nigrum*	2010	Simba	0.20	1.46	0.38	*-0.86
13	*Alternaria tenuissima*	2009	Scarlett	5.04	2.17	0.03	0.02
14	*Alternaria infectoria*	2009	Frederik	0.88	5.86	0.02	001
15	*Cladosporium* spp.	2009	Scarlett	41.22	0.43	*-1.11	*-0.29
16	*Drechslera* spp.	2010	Quench	0.18	2.40	*-1.14	0.18
17	*Cladosporium* spp.	2010	Quench	*-725.42	2.55	0.01	1.02
18	*Cladosporium* spp.	2009	Frederik	*-4114.23	0.26	*-26.10	7.76
19	*P. brevicompactum*	2011	Simba	319.98	0.53		
20	*P. brevicompactum*	2010	Quench	574.32	5.92		
21	*P. brevicompactum*	2011	Cabaret	*-334.57	1.69		
22	*P. verrucosum*	2011	Carbaret	1097.53	22.26	0.95	0.01
23	*A. niger*	2010	Simba	104.86	*-13.19	0.92	0.15
24	*P. chrysogenum*	2011	Scarlett	65.60	11.40	2.91	0.15
25	*Eurotium repens*	2010	Simba	0.13	71.29	1.82	1.03
26	*Eurotium repens*	2011	Scarlett	0.01	0.01	366.99	5.79
27	*Phoma* sp.		Cabaret	0.01	0.70	0.01	0.95
28	*F. tricinctum*		Cabaret	0.18	0.96	0.15	0.92
29	*F. tricinctum*		Cabaret	0.40	5.60	0.15	2.91
30	*P. cyclopium*		Himalaya	1.52	7.77	1.03	1.82
31	*P. freii*		Himalaya	3.25	290.72	5.79	366.99
	Negative control			0.01	0.00		

* Negative values due to background noise.

4.3.4 PROFILING THE SECRETOMES OF *A. NIGER* AND *F. POAE* AND IDENTIFICATION OF PROTEINS INVOLVED IN THE INTERACTION WITH WAX AND BARLEY FLOUR

The proteins of the culture supernatants of the different fungi were analyzed by 1D SDS-PAGE. This allowed a rapid screening of the proteins profiles of the different fungal species grown on both WAX and barley flour. In Figure 29 representative 1D-gel band patterns of the cultivated fungi are shown to be simple and species specific

reflecting secretion of different proteins. All the fungal species grown with WAX resulted in very faints bands on Coomassie Blue staining, despite application of same amount of protein in all the lanes (20 µg). All the fungi grown supernatants with barley flour as carbon source displayed a prominent band of molecular size of 10 kDa, which is likely a barley protein originating from the medium. It is noticeable that some of the samples did not migrate into the gel due to high viscosity, in particular for fungi grown on WAX, thus some of the lanes were loaded insufficiently. Twenty bands (Figure 29, lane 44−53) were excised, tryptic digested and subjected to MALDI-TOF-TOF MS. Analysis resulted in eight confident identifications, of which three (3, 8 and 14) were identified as endo-1,4-β-xylanases (Table 15). The identified endo-1,4-β-xylanases were found to be homologues, e.g. the derived peptides from protein band 3 (*P. freii*) matched the sequence of xylanases from *P. citrinum*. This situation is due to a limited number of fungal gene and/or protein sequences have been annotated and deposited. In general, a threshold of 70% sequence identity is accepted above which cross-species identification is possible (Lester and Hubbard, 2002; Wilkins and Williams, 1997). Based on xylanase activity levels and 1D-gel patterns two fungi were selected for mapping and analysis of their secretomes.

Xylanases can be classified roughly into two groups, namely low molecular weight (< 30 kDa) having basic pI and high molecular weight (> 30 kDa) having acidic pI (Pastor et al., 2007; Wong et al., 1988). Xylanases of fungal origin are typically effective in the acidic pH range 4−6, while xylanases from bacteria have a broader pH optimum 5−9 (Beg et al., 2001). Xylanases are found in five glycoside hydrolase families: 5, 7, 10, 11, and 43 according to the CAZy classification based on protein sequence and structure similarity (Cantarel et al., 2009).

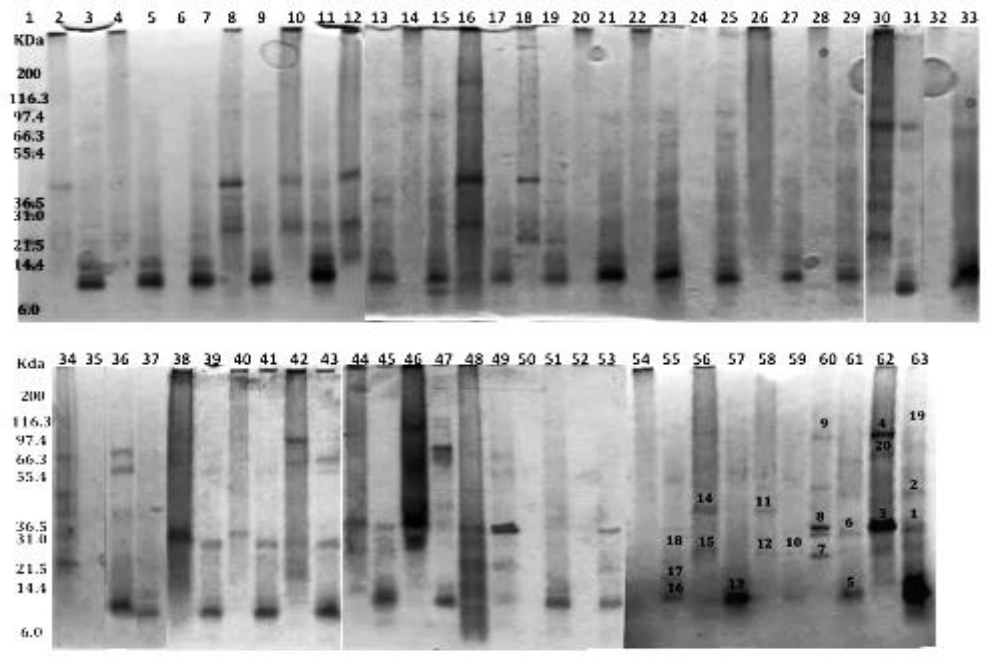

Figure 29: SDS-PAGE of different culture medium, where each well is loaded with ca. 20 µg of protein. The gels were Coomassie Blue stained G-250. Lane (1) Mark12, (2) W1, (3) F1, (4) W2, (5) F2, (6) W3, (7) F3, (8) W4, (9) F4, (10) W5, (11) F5, (12) W6, (13) F6, (14) W7, (15) F7, (16) W8, (17) F8, (18) W9, (19) F9, (20) W10, (21) F10, (22) W11, (23) F11, (24) W12, (25) F12, (26) W13, (27) F13, (28) W14, (29) F14, (30) W15, (31) F15, (32) W16, (33) F16, (34) W17, (35) F17, (36) W18, (37) F18 (38) W19, (39) F19, (40) W20, (41) F20, (42) W21, (43) F21, (44) W22, (45) F22, (46) W23, (47) F23, (48) W24, (49) F24, (50) W25, (51) F25, (52) W26, (53) F26, (54) W27, (55) F27, (56) W28, (57) F28, (58) W29, (59) F29, (60) W30, (61) F30, (62), W31, and (63) F31. The lane numbers (1−63) refer to the isolated fungi listed in Table 14. The band numbers on the gel (lanes 54−63) were excised, tryptic digested and identified by MALDI-TOF MS (Table 15). Molecular marker is as indicated. Abbreviations: W: wheat arabinoxylan and F: flour (barley). The gels were run by B.Sc. students Julie Sørensen and Maria Bach.

Table 15: Glycoside hydrolase identifications of separated bands by 1D SDS-PAGE of culture supernatants of fungi grown on WAX and barley flour as carbon source. Spot numbers correspond to the bands shown in shown in Figure 29. Abbreviation: Comb: combined; meas: measured; PMF: peptide mass fingerprint; theor: theoretical.

Spot no.	Accession no.	Organism	Protein name	Mw theor.	MW meas.	pI theor.	PMF comb. score	E-value	Sequence coverage %	Unique peptides	MS/MS Precursor ions	MS/MS Peptides sequences	Ion score	Expect
2	gi|255931857	*Penicillium chrysogenum* Wisconsin 54-1255	Alpha-amylases GH13	51032	68700	4.87	179	1.9e-12	14	5	1112.5729	R.NIYFALTDR.I	53	0.0089
											1830.8534	R.DLYSINENYGTADDLK.S	61	0.0012
											2312.0883	R.GIPIVYYGTEQGYAGGNDPANR.E	36	0.33
3	gi|169159203	*Penicillium citrinum*	Endo-1,4-beta-xylanase GH11	35338	38000	7.74	134	4.9e-08	24	7	934.5074	R.VIGEDFVR.I	53	0.014
											807.4367	R.IAFETAR.A	40	0.24
4	gi|70996610	*Aspergillus fumigatus* Af293	Beta-xylosidase XylA GH3	86731	121600	4.76	250	1.2e-19	13	9	1612.8372	R.YGLDVYAPNINAFR.S	89	2.3e-06
											1778.9684	R.VLYPGKYELALNNER.S	87	3.00E-06
											892.4683	K.WLVGFDR.L	40	0.29
6	gi|255930951	*Penicillium chrysogenum* Wisconsin 54-1255	Endo-arabinase GH43	36188	33600	5.35	118	1.80E-06	15	3	1795.958	K.VGADGVTPIGDAVQILDR.D	62	0.00086
											1676.7563	K.TGLISPGGGNVCGCGDR.M	28	2.3
8	gi|3915310	*Aspergillus aculeatus*	Endo-1,4-beta-xylanase GH10	35423	38000	8.33	156	3.10E-10	15	4	807.434	R.IAFETAR.A	43	0.12
											1775.9111	K.LYINDYNLDSASYPK.L	90	1.60E-06
9	gi|344228869	*Candida tenuis* ATCC 10573	Hypothetical protein 116822	76355	113600	4.9	76	0.031	16	8				
11	gi|310699603	*Fusarium oxysporum*	Alpha-D-galactopyranosidase GH31	45222	46600	8.01	94	0.00055	14	5	984.4848	K.FGLYGDGGAK.T	59	0.0021
14	gi|374253734	*Fusarium oxysporum*	Endo-1,4-beta-xylanase A GH10	36403	48600	8.57	95	0.00038	13	4	935.541	R.LVKSYGLR.I	40	0.19
											1155.6408	K.QYFGTALTVR.N	26	6.4
15	gi|302913666	*Nectria haematococca* mpVI 77-13-4	Hypothetical protein GH11	24103	32600	9.13	65	0.35	17	3	1989.9925	K.KGEVTVDGSVYDIYTSTR.T	46	0.035
20	gi|119481903	*Neosartorya fischeri* NRRL 181	Alpha-L-arabinofuranosidase A GH43	70046	75100	5.21	100	0.00014	3	2	1561.7308	R.FPGGNNLEGDTIDGR.W	93	9.00E-07

A proteome changes continuously and a large amount of information on the functional responses of the organism can be obtained by characterizing the proteome under different physiological conditions. Based on the microbial xylanase activities and the 1D protein patterns, the field and the storage fungi, *F. poae* and *A. niger*, respectively, were selected for a further elaborate analysis. To access the secretomes of *A. niger* and *F. poae*, 2-DE was performed to map and identify the secreted proteins in the culture medium containing WAX and/or barley flour. Representative 2D-gels (pH 4−8.5) of the secretomes of *A. niger* grown on WAX and/or barley flour are shown in Figure 30 (A-B) and of *F. poae* in Figure 30 (C-D). The resolved protein patterns of the *A. niger* growing either on WAX or barley flour revealed striking similarities. Approximately 105 protein spots were resolved on both representative 2D-gels of *A. niger* grown on WAX and barley flour while 54 spots were displayed for *F. poae*. All visible spots for both *A. niger* and *F. poae* were excised for identification by MALDI-TOF MS and MS/MS, which resulted in 82 and 31 confident protein identifications (Table 16). According to the predicted biological function, the identified proteins were assigned to categories including glycoside hydrolases (GH), proteases (P), oxidoreductases (OR), esterases (E), nucleases (N), lyases (L), housekeeping enzymes (HK), hypothetical proteins (HP) or proteins with unknown function.

Functional assignment of the secreted proteins indicates that *A. niger* grown on either WAX or barley flour as substrate produce an arsenal of proteins targeted towards plant cell wall degradation or carbohydrate catabolism. The majority of the identified proteins were of xylanolytic enzymes, namely 1,4-β-arabinoxylan arabinofuranohydrolase (axhA, GH43, spots 4−5, 33, 35−36, 62), α-L-arabinofuranosidase A and E (GH51, spots 51, 86 and GH43, spot 89), α-glucuronidase A (GH67, spot 101), β-glucuronidase (GH2, spot 105), endo-1,4-β-xylanases F1 (GH10, spots 6, 10, 39, 43), endo-1,4-β-xylanases A (GH11, spots 8, 12, 19, 21, 24−25, 42, 47−48, 51, 63) and B (GH11, spot 46), xyloglucanase (GH16, spot 84), α- and β-xylosidase (GH31, spot 102 and GH3, spot 94). It should be noted that some of the protein identifications were found for homologous *Aspergillus* species. Feruloyl esterase A was identified in spot 34. This enzyme removes ferulic acid from plant cell wall polysaccharides, and is known to act synergistically with xylanolytic enzymes and facilitate access to the backbone of cell wall polymers. Endo-1,4-β-xylanases randomly cleave glycosidic bonds in the xylan backbone generating substituted or unsubstituted xylo-oligosaccharides (XOS), while β-xylosidases cleave the

xylo-oligomeric chain from the non-reducing end, liberating xylose (Biely, 1985; Coughlan and Hazlewood, 1993). It has been reported that these key enzymes are regulated at the transcriptional level by the activator XlnR. The genes encoding these xylanolytic enzymes are induced upon growth on XOS (Courtier et al., 2012; Van Peij et al., 1998).

Another major group is constituted by cellulolytic enzymes, including α-1,3-glucanase (mutanase, GH71, spot 78), β-1,4-glucanase (cellulase, GH12, spots 11−15, 44), β-1,4-glucan cellobiohydrolase B (GH7, spot 86), exoglucanase CBHII (GH7, spot 84), α-glucosidase AglU (GH31, spots 1, 74−76) and β-glucosidase (GH92, spots 95, 98). The polysaccharides, cellulose and hemicellulose xylan, are one of the major structural components of plant cell walls, and both the xylanolytic and cellulolytic enzymes work in concert upon degradation of cellulose to glucose. Starch degrading enzymes, i.e. glycoamylase (GH15, spots 2, 32) and α-amylase (GH13, spot 85), as well as polysaccharide hydrolyzing α-mannosidase (GH92, spot 70), β-mannosidase MndA (GH2, spot 65) and α-galactosidase C and D (melibiase, GH36, spots 72, 99 and GH27 spot 93), were identified in the culture medium of *A. niger* grown on WAX or barley flour. In addition, β-galactosidase LacA (GH35, spot 68), which hydrolyzes lactose into galactose and glucose, was also identified in both media (Figure 30, Table 16). The most abundant proteins identified on the 2D-gels were represented by arabinofuranosidases and xylanases for both cultures. These enzymes were present in multiple spots with varying pI values, which is probably due to PTMs or existence of closely related gene products/isoforms. Spots migrating with similar apparent molecular mass and varying pI have been suggested to arise by phosphorylation (Rosen et al., 2004) or deamidation (Robinson, 2002). Collectively, the cell wall degrading enzymes, also termed pathogenicity/virulence factors, have been predicted to function in the penetration and maceration of plant tissues for nutrient acquisition (Paper et al., 2007).

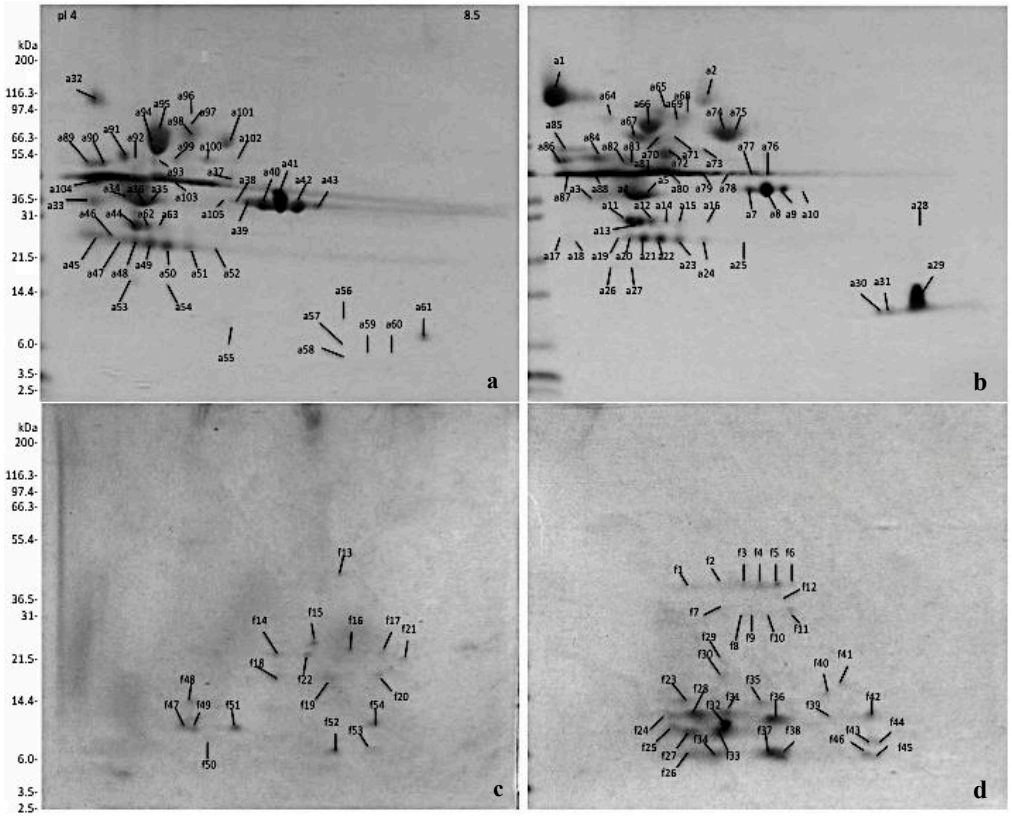

Figure 30: 2D-gel electrophoresis of the secretome (50 µg) of *Aspergillus niger* grown in medium containing wheat arabinoxylan (a) and barley flour (b) and of *Fusarium poea* grown in presence of wheat arabinoxylan (c) and barley flour (d). The numbered spots were selected for in-gel tryptic digestion and analysis by mass spectrometry. Spot numbers a (*A. niger*) and f (*F. poae*) refer to Table 16. The gels were run by B.Sc. students Julie Sørensen and Maria Bach. Molecular markers and pI range are as indicated.

The enzymes, α-xylosidase, β-xylosidase, α-glucuronidase, β-glucuronidase and feruloyl esterase, were only identified from cultures grown on WAX. The higher xylanase activity levels determined for *A. niger* grown on WAX (104.9 U/mg protein) could be correlated with the presence and identification of these key enzymes by 2DE. The xylanase activity levels were much lower for cultures grown on barley flour (0.15 U/mg protein). It was noticeable that for *Aspergillus* grown on barley flour, cellulases were produced in much higher amounts. α-amylase was only detected in cultures grown on

barley flour. It is not surprising as the utilized substrate/carbon source is based on barley grains of which 87% is constituted by starchy endosperm (Finnie and Svensson, 2003). Moreover, peptides derived from the protein spot 87 (barley flour) matched the protein sequence of glucoamylase (GH15), which are known to catalyze the breakdown of α-(1,4)-linked malto-oligosaccharides to glucose.

Other proteins identified in the medium of *A. niger* grown on WAX or barley flour include catalase, peptidase, glutaminase and acyltransferase. Growth on barley flour resulted in confident identification of tripeptidyl-peptidase, carboxypeptidases S1 and CpdS, while peptidase S10 was identified in the culture grown on WAX. The proteolytic activities appearing by cultivating the fungi are probably involved in nutrient acquisition as well as in enhancement of fungal pathogenicity (Monod et al., 2002; Naglik et al., 2003). Moreover, proteases have been reported to be involved in infection processes in fungi, such as *Aspergillus fumigatus* and *Candida albicans* in plant as well as in animal hosts (Krikstaponis et al., 2001; Naglik et al., 2004). Mycelial catalase Cat1 (oxidoreductase, spots 73 and 100) is involved in removing reactive oxygen species and protecting the cells from oxidative damage. Glutaminase (GtaA, spot 90), which catalyze the hydrolysis of glutamine to glutamate and release of ammonia, was also confidently identified for *A. niger* growing on barley flour. Glutaminases have been proposed to belong to the large group of serine β-lactamases and penicillin-binding proteins on the basis of a common protein fold, structural motifs, and catalytic mechanism. β-Lactamases produced by microorganisms confer resistance to antibiotics like penicillin by hydrolysing the β-lactam ring of antibiotics and inactivating the antibacterial properties (Brown et al., 2008; Goodwin et al., 2011). The enzyme acyltransferase (spot 80) is required for cutin synthesis, which is a waxy polymer and one of the main components of the plant cuticle that occur in seed coats. The hydrophobic cuticle protects the seeds from desiccation and functions in defence by forming a physical barrier that resists penetration by microorganisms (Li et al., 2004; Ranathunge et al., 2010). Cholinesterases (spot 81) are potent neurotoxins, and the enzyme hydrolyses the neurotransmitter acetylcholine into choline and acetic acid. Several microbes and fungi have been reported to utilize acetic acid as carbon source (Augustinsson, 1948).

A. niger is a well studied filamentous fungi due to its high secretion capacity of a number of hydrolytic enzymes and metabolites. With the recent annotation of the genome sequence, several studies were performed to analyze and characterize the

proteome of *Aspergilli* under a variety of conditions (Van Peij et al., 1998). In a study by Lu et al. (2010), the intra- and extra-cellular proteins expressed by *A. niger* grown in presence of xylose and maltose were compared and analyzed. It was shown that the intracellular proteomes of *A. niger* were highly similar, while the secretomes were strongly influenced by the utilized substrate. The secretome of the xylose-grown culture was found to contain a variety of plant cell wall degrading enzymes, with xylanase and ferulic acid esterase being the most abundant. In another study by Braaksma et al. (2010), a comparative analysis of the secretomes of *A. niger* grown on complex medium with sorbitol and galacturonic acid and upon depletion of the carbon source resulted in identification of 209 secreted proteins and revealed dynamic changes in the secretome upon variations in growth condition/substrate (Braaksma et al., 2010). A comparison of the present dataset of the secretome of *A. niger* grown on WAX with the xylose-grown cultures reported by Lu et al. (2010) revealed a large overlap in the identified proteins with a few exceptions. This is expected since the backbone of arabinoxylan is a xylan composed of xylose units, but the degree and position of arabinosyl substitutions require specific enzymes for liberation. The enzymes e.g. melibiase (spot 93) and α-glucuronidase (spot 101) identified in our dataset, were not observed in the study by Lu et al. (2010), while e.g. the cell wall protein PhiA essential for phialide and conidium-spore development, was only found in cultures grown with xylose. Evidently, discrepancies are found when comparing to two different datasets, due to differences in the experimental designs, procedures and culture conditions. The applied culture condition were completely different than that in the study of Lu et al. (2010), e.g. complex culture medium (Vogel medium), cultivation volume (0.5 L) and substrate concentration (2%). To our knowledge, this is the first study to profile the secretomes of *A. niger* growing in medium with WAX and barley flour that mimic the natural hosts of cereal fungi.

Table 16: Proteins of the extracellular proteome of *A. niger* and *F. poae* growing on medium with wheat arabinoxylan (WAX) or barley flour as carbon source and identified by MALDI-TOF MS and/or MS/MS. Protein identifications were confirmed with MASCOT score of 70 ($p \leq 0.05$) of peptide mass fingerprint, and a minimum of 4 matched peptides and a MASCOT score of 40 for MS/MS analysis ($p \leq 0.05$). The numbers correspond to the resolved spots in 2D-gels shown in Figure 30. Comb.: combined MASCOT score; Theor.: Theoretical.

Spot no.	Accession no.	Organism	Protein name	Mw theor.	pI theor.	PMF score	E-value	Comb score	E-value	Sequence coverage %	Unique peptides	MS/MS Precursor ions	MS/MS Peptides sequences	Ion score	Expect
***A. niger* on flour**															
a1	gi|358375153	*Aspergillus kawachii* IFO 4308	Extracellular α-glucosidase (AglU) GH31	108867	5.06	82	0.0085	347	2.5e-29	12	12	1504.8621	R.NSHGLEILLRPQK.L	54	0.0048
												1623.7994	K.NIYGQHPFYLDTR.Y	16	37
												2412.0339	R.NFDNDQNRFSYSEGDEFLSK.L	116	1.30E-09
												1697.8361	R.NHNELSTIPQEPYR.W	91	9.8e-07
a2	gi|224027	*Aspergillus niger*	Glucoamylase G1 GH15	65448	4.19	69	0.17	346	3.20E-29	16	8	1633.8136	-.ATLDSWLSNEATVAR.T	89	1.70E-06
												1966.9777	R.SIYTLNDGLSDSEAVAVGR.Y	123	5.50E-10
												863.4936	K.TLVDLFR.N	31	1.6
												1983.9589	K.FNVDETAYTGSWGRPQR.D	56	0.0025
a4	gi|3913152	*Aspergillus tubingensis*	α-L-arabinofuranosidase axhA GH43_62_32_68	36027	4.74			154	5.00E-10	33	5	1929.0079	K.ANSGATWTEDISHGDLVR.N	110	1.00E-08
a5	gi|3913152	*Aspergillus tubingensis*	α-L-arabinofuranosidase axhA GH43_62_32_68	36027	4.74			158	2.00E-10	23	5	1928.8824	K.ANSGATWTEDISHGDLVR.N	123	4.30E-10
a6	gi|317028138	*Aspergillus niger CBS 513.88*	Endo-1,4-β-xylanase F1 GH10	34908	6.17			150	1.30E-09	11	4	807.4319	R.IAFETAR.A	40	0.2
												950.5073	K.VIGEDYVR.I	45	0.063
												961.4576	K.WDATEPSR.G	32	1.3
a8	gi|358375979	*Aspergillus kawachii* IFO 4308	Endo-1,4-β-xylanase A	34234	5.82	104	6.20E-05			58	14				
a10	gi|317028138	*Aspergillus niger* CBS 513.88	Endo-1,4-β-xylanase F1 GH10	34908	6.17			124	5.00E-07	15	5	950.4925	K.VIGEDYVR.I	38	0.31
												807.4445	R.IAFETAR.A	39	0.28
a11	gi|1362263	*Aspergillus niger*	Cellulase GH12	25753	4.63			76	0.029	25	6	1218.5741	K.SYSNSGLTFDK.K	33	0.77
a12	gi|145228427	*Aspergillus niger* CBS 513.88	Endo-1,4-β-xylanase A GH11	24042	5.2			123	6.30E-07	7	1	1805.8813	K.GTVTSDGSVYDIYTATR.T	116	2.90E-09

a13	gi|1362263	*Aspergillus niger*	Cellulase GH12	25753	4.63			208	2.00E-15	25	6	1346.67	K.SYSNSGLTFDKK.L	79	2.50E-05
a14	gi|1362263	*Aspergillus niger*	Cellulase GH12	25753	4.63			112	7.90E-06	13	4	1346.6808	K.SYSNSGLTFDKK.L	79	2.40E-05
a15	gi|1362263	*Aspergillus niger*	Cellulase GH12	25753	4.63			99	0.00015	9	3	1346.5961	K.SYSNSGLTFDKK.L	69	0.00022
a16	gi|19919756	*Aspergillus niger*	Endo-1,4-β-xylanase GH11	24112	5.23			84	0.0045	7	1	1805.8192	K.GTVTSDGSVYDIYTATR.T	72	5.80E-05
a19	gi|145228427	*Aspergillus niger* CBS 513.88	Endo-1,4-β-xylanase A GH11	24042	5.2			102	7.90E-05	7	1	1805.8032	K.GTVTSDGSVYDIYTATR.T	89	1.00E-06
a20	gi|19919756	*Aspergillus niger*	Endo-1,4-β-xylanase A GH11	24112	5.23			142	7.90E-09	7	1	1805.8969	K.GTVTSDGSVYDIYTATR.T	132	7.10E-11
a21	gi|145228427	*Aspergillus niger* CBS 513.88	Endo-1,4-β-xylanase A GH11	24042	5.2			140	1.30E-08	7	1	1805.9005	K.GTVTSDGSVYDIYTATR.T	134	4.50E-11
a22	gi|9858848	*Aspergillus niger*	Xylanase GH11	7893	4.53			132	9.90E-08	23	1	1806.0281	K.GTVTSDGSVYDIYTATR.T	124	8.50E-10
a23	gi|19919756	*Aspergillus niger*	Endo-1,4-β-xylanase GH11	24112	5.23			142	7.90E-09	7	1	1805.8953	K.GTVTSDGSVYDIYTATR.T	135	4.40E-11
a24	gi|145228427	*Aspergillus niger* CBS 513.88	Endo-1,4-β-xylanase A GH11	24042	5.2			140	1.30E-08	7	1	1805.8555	K.GTVTSDGSVYDIYTATR.T	131	9.50E-11
a25	gi|145228427	*Aspergillus niger* CBS 513.88	Endo-1,4-β-xylanase A GH11	24042	5.2			81	0.011	7	1	1805.7848	K.GTVTSDGSVYDIYTATR.T	65	0.00025
a30	gi|358369412	*Aspergillus kawachii* IFO 4308	Hypothetical protein AKAW_04140 Anti-fungal protein	10495	6.26			80	1.70E-02	22	3	2277.9054	R.HHCEYDDHHKTVDCQTPV.-	53	2.40E-03
a31	gi|328864038	*Melampsora larici-populina* (strain 98AG31)	Hypothetical protein MELLADRAFT_58483 Putative uncharacterized protein	1010567	6.41			106	3.20E-05	24	14	1960.9834	K.HLNRLHQQRPETFER.L	0	1.20E+03
a64	gi|317025187	*Aspergillus niger* CBS 513.88	Xyloglucanase	89951	4.32			170	2.10E-10	5	4	1881.8903	R.STDQGDTWEETKLPFK.V	73	1.10E-03
												1764.8993	R.LAVDPNDNSILYFGAR.S	79	3.00E-04
a65	gi|358369379	*Aspergillus kawachii* IFO 4308	β-mannosidase (MndA)	104329	4.84			244	8.22E-18	9	7	941.4663	K.SQIQFYR.R	43	1.00E+00
												1489.6744	K.GSNLIPPDSFWTR.V	52	1.20E-01
												1929.9047	R.AGVIDEPYHGLNDFNLR.W	67	4.00E-03
												1821.9345	R.TVPAEFPSQVHLDLLR.A	64	9.60E-03
a68	gi|358370052	*Aspergillus kawachii* IFO 4308	β-galactosidase (LacA) GH35	109966	5.09	66	3.90E-01	333	1.00E-26	13	12	935.5291	K.SLFINGER.I	28	5.10E+01
												1665.8722	K.LTGNLGGEDYEDKVR.G	113	1.10E-07
												1275.6777	R.GPLNEGGLYAER.Q	96	7.60E-06
												1608.8112	R.QGFHQPEPPSGNWK.S	40	2.30E+00

a69	gi|461623	*Aspergillus niger*	β-galactosidase GH35	109663	5.12			209	2.60E-14	5	5	1665.8345	K.LTGNLGGEDYEDKVR.G	86	6.30E-05
												1275.6511	R.GPLNEGGLYAER.Q	92	1.80E-05
												935.5009	K.SLFINGER.I	10	3.40E+03
a70	gi|134077473	*Aspergillus niger*	α-mannosidase GH92	86733	4.82			202	1.30E-13	16	10	1146.59	R.HEGYLPDCR.M	42	1.90E+00
												2294.1461	K.TLNYIPTDDFDPYGVGLHTR.S	99	2.40E-06
												1063.6188	R.LGGPQTFTSR.L	34	9.50E+00
a71	gi|350633946	*Aspergillus niger* ATCC 1015	Hypothetical protein ASPNIDRAFT_50997	86104.0	4.90			467	4.10E-40	15	8	1752.8499	K.AAVGDAATVNYAQGCER.W	119	2.60E-08
												1521.8079	K.GEFGVLVGSSSVDIR.G	124	9.50E-09
												1334.7562	K.LIHSPEAVELAR.T	53	0.01
												2268.2449	K.IPELVESGQLDIEVVNTAVSR.V	127	3.40E-09
a72	gi|358373696	*Aspergillus kawachii* IFO 4308	α-galactosidase C GH36	81707	4.88	79	2.70E-01	359	2.60E-29	14	10	2318.1578	R.GIHETPSPSTDHQYMLGLYR.V	52	1.10E-01
												952.5105	R.VFDTLTTR.F	59	3.00E-02
												1607.7311	R.FPDVLWEGCASGGGR.F	115	5.60E-08
												1372.7404	K.VNPIVLNGDLYR.L	83	1.2e-05
a73	gi|358375222	*Aspergillus kawachii* IFO 4308	Mycelial catalase Cat1	80261	5.13	138	3.30E-07	490	2.10E-42	26	18	1184.5341	R.FDHERVPER.A	24	6.20E+01
												1287.5769	R.HVDGFGVHTYR.L	39	1.80E+00
												1446.6741	R.GVDFTEDPLLQGR.L	75	7.20E-04
												1635.7219	R.STSPTFQDVWSQPR.L	71	1.40E-03
												2718.4829	R.NAGIDDEREGVVVGQTVGAEFVDSVR.E	143	5.60E-11
a74	gi|358375153	*Aspergillus kawachii* IFO 4308	Extracellular α-glucosidase (AglU) GH31	109267	5.06	64	5.50E-01	394	8.20E-33	12	9	1504.9484	R.NSHGLEILLRPQK.L	59	6.00E-03
												2151.2004	R.TLGGGIDLTFYSGPNPADVTR.Q	151	1.20E-11
												1022.4939	R.NFDNDQNR.F	66	7.60E-03
												1697.9536	R.NHNELSTIPQEPYR.W	89	2.40E-05
a75	gi|358375153	*Aspergillus kawachii* IFO 4308	Extracellular α-glucosidase (AglU) GH31	109267	5.06	82	1.20E-01	528	3.30E-46	13	11	1504.8825	R.NSHGLEILLRPQK.L	60	1.60E-02
												2151.0755	R.TLGGGIDLTFYSGPNPADVTR.Q	152	1.20E-11
												1022.4415	R.NFDNDQNR.F	69	1.70E-03
												858.5363	R.RPFIIGR.S	44	1.30E+00
												1697.8763	R.NHNELSTIPQEPYR.W	89	3.10E-05
												1613.8553	R.GGNILPMQEPALTTR.E	69	2.90E-03

a76	gi|358370756	*Aspergillus kawachii* IFO 4308	α-glucosidase GH31	97683	4.96			203	1.00E-13	8	7	1731.9011	R.NHNELGDIPQEFYR.W	95	6.30E-06
												1016.5469	R.WPTVAESAR.K	57	6.40E-02
												825.4912	R.GGNIIPVR.T	30	1.80E+01
a77	gi|358370756	*Aspergillus kawachii* IFO 4308	α-glucosidase GH31	97683	4.96			189	2.60E-12	10	7	1731.8177	R.NHNELGDIPQEFYR.W	90	2.20E-05
												1016.5153	R.WPTVAESAR.K	54	1.30E-01
												825.4941	R.GGNIIPVR.T	24	5.40E+01
a78	gi|358368862	*Aspergillus kawachii* IFO 4308	α-1,3-glucanase/mutanase GH71	54886	5.57	64	5.90E-01	209	2.60E-14	26	9	1258.7155	R.WFEILNLAPR.F	66	5.60E-03
												1380.8335	K.LGPAGLSALQQGLR.V	91	8.80E-06
a79	gi|350633883	*Aspergillus niger* ATCC 1015	Hypothetical protein ASPNIDRAFT_123586 XynE_like	45989	5.09			87	4.30E-02	4	2	1607.755	K.AEFDSGDHLHLNPR.G	81	1.80E-04
a80	gi|350633910	*Aspergillus niger* ATCC 1015	Hypothetical protein ASPNIDRAFT_54865 Fatty_acyltransferase_like	39983	5.03			176	5.20E-11	10	3	1590.8313	K.TLVTFGDSYTDDTR.I	101	2.00E-06
												1918.0037	R.MWEQVSTVNQVYDYR.T	65	6.00E-03
	gi|145231236	*Aspergillus niger* CBS 513.88	phospholipase C PLC-C	49880	5.11			117	4.10E-05	18	5	1603.9407	K.LTPSQQGFVTEQLR.L	44	0.044
												1675.9573	R.VEYAPSFEHLVQTR.A	49	0.019
a81	gi|358367957	*Aspergillus kawachii* IFO 4308	Cholinesterase	61991	4.75	58	2.40E+00	278	3.30E-21	17	10	1121.5881	R.VPSEGYQWR.R	44	1.30E+00
												2022.8788	R.QCEAWTETSTPAYCYR.F	97	2.50E-06
												1770.9176	K.EGIDYINSVANAYWR.-	88	3.80E-05
a82	gi|317036371	*Aspergillus niger* CBS 513.88	Carboxypeptidase S1	61628	4.51			346	5.20E-28	16	5	1848.0627	R.YPVIDATIIDTTDIAAR.A	142	8.60E-11
												1501.7846	R.DNVEGPYYQFGGR.G	88	4.10E-05
												1916.9382	R.AAGYTPMTVDGVEYGETR.E	95	6.50E-06
												1932.9434	R.AAGYTPMTVDGVEYGETR.E	71	1.70E-03
a83	gi|358365618	*Aspergillus kawachii* IFO 4308	Carboxypeptidase CpdS	56265	4.49			80	2.10E-01	8	3	1343.6235	K.HFVDTFDLHGR.K	69	0.00026
a84	gi|308212489	*Aspergillus niger*	Exoglucanase CBHII GH7	55235	4.02			326	5.20E-26	15	5	1463.5462	K.YGTGYCDSQCPR.D	85	6.90E-06
												1939.7443	R.YSGTCDPDGCDYNPYR.L	86	8.00E-06
												2904.4273	R.GTCSTDSGVPATVEADSPNAYVTYSNIK.F	129	1.50E-09
a85	gi|350631148	*Aspergillus niger* ATCC 1015	α-amylase A GH13	64819	4.08	95	6.30E-03	449	2.60E-38	14	6	1356.7297	R.TQSIYFLLTDR.F	63	0.0011
												1671.7957	K.IYDVNSNFGTADDLK.S	99	2.70E-06
												2558.1776	K.SVASDCSDPTLLGNFIENHDNPR.F	156	3.80E-12

												2084.9447	R.EYTVPECGSGETVVDTWR.-	104	6.10E-07
a86	gi|189484494	*Aspergillus niger*	α-L-arabinofuranosidase E	67706	4.13			129	2.60E-06	3	1	2245.9881	K.YLEVGNEDNLNSGCGTYANR.F	129	1.70E-09
	gi|74698498	*Aspergillus niger*	1,4-β-D-glucan cellobiohydrolase B GH7	57495	4.09			83	1.10E-01	2	1	1939.8668	R.YSGTCDPDGCDYNPYR.L	83	7.30E-06
a87	gi|157829865	*Aspergillus Awamori* Var. X100	Chain A, Refined structure for the complex of acarbose with glucoamylase	50791	4.26	48	2.30E+01	242	1.30E-07	21	7	1633.819	-.ATLDSWLSNEATVAR.T	68	3.50E-03
												1966.9914	R.SIYTLNDGLSDSEAVAVGR.Y	133	1.10E-09
a88	gi|55670667	*Aspergillus kawachii*	Chain A, Crystal structure of arabinofuranosidase	51280	4.21			340	2.10E-27	21	7	2278.125	M.GPCDIYEAGDTPCVAAHSTTR.A	122	1.00E-08
												1405.7398	K.AYGVFMSPGTGYR.N	49	3.10E-01
												2140.2541	K.YVVGSLVSGPSFTSGEVVSLR.V	112	5.70E-08
												896.4827	R.SWSYPTR.Y	19	2.80E+02
***A. niger* on WAX**															
a32	gi|40313280	*Aspergillus awamori*	Glucoamylase, GH15	68347	4.26			330	1.30E-27	12	7	863.5025	K.TLVDLFR.N	34	0.65
												1966.9526	R.SIYTLNDGLSDSEAVAVGR.Y	161	9.20E-14
												1485.7462	R.ALANHKEVVDSFR.S	68	0.00022
												851.4222	K.EVVDSFR.S	35	0.52
a33	gi|358375978	*Aspergillus kawachii* IFO 4308	Arabinoxylan arabinofuranohydrolase axhA GH43_62_32_68	35947	4.63			72	0.078	7	2	1928.923	K.ANSGATWTEDISHGDLVR.N	56	0.0028
a34	gi|259016351	*Aspergillus awamori*	Feruloyl esterase A	30381	4.59			107	2.50E-05	5	5	982.4799	R.LYTFGEPR.S	35	0.42
a35	gi|3913152	*Aspergillus tubingensis*	Arabinoxylan arabinofuranohydrolase axhA GH43_62_32_68	36027	4.74			140	1.30E-08	31	6	1928.9909	K.ANSGATWTEDISHGDLVR.N	93	6.50E-07
a36	gi|3913152	*Aspergillus tubingensis*	Arabinoxylan arabinofuranohydrolase axhA GH43_62_32_68	36027	4.74			129	1.60E-07	17	3	1928.9946	K.ANSGATWTEDISHGDLVR.N	110	1.10E-08
a37	gi|300706143	*Nosema ceranae* BRL01	Hypothetical protein NCER_101765	25574	8.96			65	0.41	49	8				

a38	gi|254571817	*Komagataella pastoris* GS115	Hypothetical protein	75848	8.9			83	0.0066	27	12	950.5856	K.IFTDLVDK.D	0	8.10E+02
a39	gi|317028138	*[Aspergillus niger* CBS 513.88]	Endo-1,4-β-xylanase F GH10	34908	6.17			183	6.30E-13	9	3	1775.9382	K.LYINDYNLDSASYPK.L	87	2.60E-06
												950.5388	K.VIGEDYVR.I	42	0.11
												961.5071	K.WDATEPSR.G	45	0.082
a42	gi|358375979	*Aspergillus kawachii* IFO 4308	Endo-1,4-β-xylanase A GH11	34234	5.82	111	1.20E-05			63	16				
a43	gi|317028138	*Aspergillus niger* CBS 513.88	Endo-1,4-β-xylanase F1 GH10	34908	6.17			218	2.00E-16	9	3	950.5364	K.VIGEDYVR.I	50	0.017
												961.5025	K.WDATEPSR.G	43	0.14
												1775.9269	K.LYINDYNLDSASYPK.L	116	3.20E-09
a44	gi|1362263	*Aspergillus niger*	Cellulase GH12	25753	4.63			193	6.30E-14	25	6	1346.6987	K.SYSNSGLTFDK.K	79	2.40E-05
												1207.5982	K.WTWSGGEGTVK.S	74	7.40E-05
a45	gi|19919756	*Aspergillus niger*	Endo-1,4-β-xylanase GH11	24112	5.23			69	0.16	15	2	1805.8588	K.GTVTSDGSVYDIYTATR.T	57	2.50E-03
a46	gi|380865431	*Aspergillus kawachii* IFO 4308	Endo-1,4-β-xylanase B GH11	24167	5.74			94	0.0005	24	3	1805.9417	K.GTVTSDGSVYDIYTATR.T	69	0.00017
a47	gi|145228427	*Aspergillus niger* CBS 513.88	Endo-1,4-β-xylanase A GH11	24042	5.2			137	2.50E-08	7	1	1805.9705	K.GTVTSDGSVYDIYTATR.T	131	9.00E-11
a48	gi|145228427	*Aspergillus niger* CBS 513.88	Endo-1,4-β-xylanase A GH11	24042	5.2			141	1.00E-08	7	1	1805.9158	K.GTVTSDGSVYDIYTATR.T	136	3.50E-11
a49	gi|13242071	*Aspergillus niger*	Xylanase	11059	8.18			140	1.60E-08	44	2	1805.908	K.GTVTSDGSVYDIYTATR.T	121	1.50E-09
a50	gi|13242071	*Aspergillus niger*	Xylanase	11059	8.18			98	2.80E-04	33	2	1805.9151	K.GTVTSDGSVYDIYTATR.T	77	4.60E-05
a51	gi|145228427	*Aspergillus niger* CBS 513.88	Endo-1,4-β-xylanase A GH11	24042	5.2			131	1.00E-07	7	1	1805.9145	K.GTVTSDGSVYDIYTATR.T	124	4.50E-10
a52	gi|19919756	*Aspergillus niger*	Endo-1,4-β-xylanase, GH11	24112	5.23			145	4.00E-09	7	1	1805.8682	K.GTVTSDGSVYDIYTATR.T	138	2.00E-11
a56	gi|9858848	*Aspergillus niger*	Xylanase GH11	7893	4.53			78	0.021	23	1	1805.8842	K.GTVTSDGSVYDIYTATR.T	64	0.00045
a63	gi|145228427	*Aspergillus niger* CBS 513.88	Endo-1,4-β-xylanase A GH11	24042	5.2			110	1.30E-05	15	2	1805.9155	K.GTVTSDGSVYDIYTATR.T	98	2.20E-07
a89	gi|189484494	*Aspergillus niger*	α-L-arabinofuranosidase E GH43	67706	4.13			213	1.00E-14	7	3	1531.7503	R.FPGGNNLEGATPSDR.W	85	8.40E-05
												2245.9907	K.YLEVGNEDNLNSGCGTYANR.F	118	2.10E-08
a90	gi|189484494	*Aspergillus niger*	α-L-arabinofuranosidase E GH43	67706	4.13			317	4.10E-25	7	4	1081.5956	K.GDYSGTVNLR.L	48	3.30E-01
												1531.8037	R.FPGGNNLEGATPSDR.W	104	1.20E-06
												2246.1176	K.YLEVGNEDNLNSGCGTYANR.F	152	1.20E-11
	gi|145234270	*Aspergillus niger* CBS 513.88	Glutaminase GtaA	75641	4.29			100	2.10E-03	7	4	1773.9147	K.ADWEMFTAAIASTSTR.D	91	1.5e-06

a91	gi|358370298	*Aspergillus kawachii* IFO 4308	Six-hairpin glycosidase	75936	4.37			359	2.60E-29	13	6	2250.121	R.IPLFETDLSDIQDVYYYR.W	128	2.70E-09
												1360.7374	R.QYAQLHYNPAR.G	62	1.50E-02
												2452.1675	R.GGVLDIEEDYYSDTGSPIVGLPR.S	144	5.70E-11
a92	gi|134083763	*Aspergillus niger*	Peptidase_S10	61715	4.51			266	5.20E-20	16	5	1847.9753	R.YPVIDATIIDTTDIAAR.A	98	3.50E-06
												1501.6657	R.DNVEGPYYQFGGR.G	74	6.60E-04
												1916.8753	R.AAGYTPMTVDGVEYGETR.E	66	4.10E-03
a93	gi|358370493	*Aspergillus kawachii* IFO 4308	Melibiase D	68129	4.7			406	5.20E-34	15	6	1543.6866	R.YVTTDCGWTVADR.L	73	7.70E-04
			GH27 or 13									2966.3158	K.YDNCYSDAATNYPNVNYAPSTSPEPR.F	157	2.10E-12
												1154.6215	R.IGNDIIPAWR.T	44	9.70E-01
												1966.895	R.WTEEGYEVWSGPLSNGR.T	108	2.60E-07
a94	gi|4235093	*Aspergillus niger*	β-xylosidase GH3	87558	4.76	101	1.60E-03	534	8.20E-47	27	13	1663.8192	R.SHLICDESATPYDR.A	77	4.50E-04
												2025.1284	R.LGLPAYQVWSEALHGLDR.A	115	5.70E-08
												1642.8822	R.YGLDVYAPNINTFR.H	74	8.30E-04
												1898.8963	K.HYAGYDIENWHNHSR.L	79	2.30E-04
												931.495	K.WLVGWDR.L	42	2.30E+00
												1060.6015	R.VPVEVGSFAR.V	74	1.20E-03
a95	gi|7009581	*Aspergillus niger*	β-glucosidase	93687	4.7	138	3.30E-07	351	1.20E-29	28	23	1392.7253	K.GADIQLGPAAGPLGR.S	92	1.70E-06
												1492.6726	K.HYIAYEQEHFR.Q	79	2.40E-05
												921.4962	R.LYDELIR.V	43	1.50E-01
a96	gi|118582212	*Aspergillus niger*	lactase, partial	108056	5.02	100	2.10E-03	384	8.20E-32	16	14	935.5032	K.SLFINGER.I	39	4.10E+00
												1665.8407	K.LTGNLGGEDYEDKVR.G	110	2.40E-07
												1275.67	R.GPLNEGGLYAER.Q	106	6.60E-07
												1608.7838	R.QGFHQPEPPSGNWK.S	45	7.50E-01
a97	gi|358367698	*Aspergillus kawachii* IFO 4308	Hypothetical protein AKAW_02431	101022	5.01	69	2.80E+00	216	5.20E-15	17	14	1198.6247	R.SVNGPVLQDNR.V	48	3.90E-01
												1244.6182	R.RYWDFTTAGK.L	24	1.00E+02
												1795.9452	R.VLYGENKVEFDLVDR.A	75	6.40E-04
a98	gi|358376345	*Aspergillus kawachii* IFO 4308	β-glucosidase	85815	4.9	111	1.60E-04	292	1.30E-22	19	10	1752.8344	K.AAVGDAATVNYAQGCER.W	94	8.10E-06
												1101.5751	K.VKDLGLWDR.K	37	5.60E-01
												1521.8134	K.GEFGVLVGSSSVDIR.G	95	7.20E-06
99	gi|358373696	*Aspergillus kawachii* IFO 4308	α-galactosidase C	81707	4.88			292	1.30E-22	14	9	848.3897	R.EFPDQGR.G	38	3.60E+00
												952.5154	R.VFDTLTTR.F	48	3.70E-01
												1607.7549	R.FPDVLWEGCASGGGR.F	117	4.10E-08

a100	gi|35837522 2	*Aspergillus kawachii* IFO 4308	Mycelial catalase Cat1	80261	5.13	168	3.30E-10	616	5.20E-55	34	22	1307.7272	R.GPTLLEDFIFR.Q	70	2.30E-03
												1287.6514	R.HVDGFGVHTYR.L	54	1.10E-01
												1367.6912	R.QDLWTSIEYGR.F	79	3.50E-04
												2250.0336	R.FPEWELGVQIMDEEDQLR.F	89	2.10E-05
												1446.7566	R.GVDFTEDPLLQGR.L	74	1.10E-03
												1635.8107	R.STSPTFQDVWSQPR.L	99	3.10E-06
a101	gi|3912991	*Aspergillus tubingensis*	α-glucuronidase A GH67	94188	5.36	105	6.50E-04	398	3.30E-33	24	16	1377.6585	R.YAPVSCDLHCR.Q	52	1.70E-01
												2516.315	R.DINLPELDPDGFWLQSEGDTVR.I	123	7.10E-09
												1339.7588	R.VAYTTNPHAPIR.W	36	5.10E+00
												1779.9078	K.DGTVVEDMAPVEQYAR.L	47	4.70E-01
												1795.9018	K.DGTVVEDMAPVEQYAR.L	43	1.10E+00
												1094.6076	K.YGPIDFQVR.E	44	1.10E+00
a102	gi|35837500 6	*Aspergillus kawachii* IFO 4308	α-xylosidase GH31	82481	5.27	65	6.60E+00	201	1.60E-13	13	8	1227.5123	R.TTGWGGTTAGYR.L	20	9.70E+01
												1978.9131	R.VNDDGSETLLTNEYAPLK.S	83	8.80E-05
												1124.4495	R.NYDSTNPSAR.K	34	2.80E+00
103	gi|35837025 9	*Aspergillus kawachii* IFO 4308	EstA precursor	93754	5.37			139	2.60E-07	7	4	1322.6828	R.LEGGVDEFLGMR.Y	56	7.70E-02
												1721.846	R.YAAAPEWNTWGDGQR.L		1.30E-03
	gi|35837044 2	*Aspergillus kawachii* IFO 4308	Tripeptidyl-peptidase	66337	5.3			76	5.30E-01	5	4	1404.7342	K.VGFASYLEEYAR.Y	64	0.00097
a104	gi|55670667	*Aspergillus kawachii*	Chain A, Crystal structure of arabinofuranosidase	51280	5.21			189	2.60E-12	8	3	1405.7475	K.AYGVFMSPGTGYR.N	32	1.80E+01
												2140.285	K.YVVGSLVSGPSFTSGEVVSLR.V	145	1.90E-11
a105	gi|35836780 5	*Aspergillus kawachii* IFO 4308	β-glucuronidase	59044	5.19			120	2.10E-05	9	3	1779.8246	R.VGGNTQDFALYDPNLR.A	93	9.00E-06
***F. poae* on barley**															
f2	gi|1310677	*Hordeum vulgare* subsp. *vulgare*	Protein z-type serpin	43307	5.61	95	3.80E-04	179.00	1.40E-12	20	8	925.5249	R.LSIAHQTR.F	26	2.60E+00
												1144.5938	R.LASAISSNPER.A	41	1.40E-01
												1809.9352	K.RLSTEPEFIENHIPK.Q	17	5.20E+01
f3	gi|1310677	*Hordeum vulgare* subsp. *vulgare*	Protein z-type serpin	43307	5.61	123	5.60E-07	202	7.00E-15	24	9	925.5292	R.LSIAHQTR.F	38	1.60E-01
												1144.5895	R.LASAISSNPER.A	40	1.70E-01
f4	gi|1310677	*Hordeum vulgare* subsp. *vulgare*	Protein z-type serpin	43307	5.61	144	4.40E-09	421	8.80E-37	30	13	925.5233	R.LSIAHQTR.F	50	1.10E-02
												1144.5985	R.LASAISSNPER.A	61	1.60E-03
												1089.5339	K.TQSVDFQHK.T	47	3.20E-02
												1525.693	K.GAWDQKFDESNTK.C	61	9.40E-04
												1809.9503	K.RLSTEPEFIENHIPK.Q	14	4.90E+01
												1653.8548	R.LSTEPEFIENHIPK.Q	43	6.00E-02

f5	gi|1310677	*Hordeum vulgare* subsp. *vulgare*	Protein z-type serpin	43307	5.61	129	1.40E-07	399	1.40E-34	27	12	925.5223	R.LSIAHQTR.F	51	8.30E-03
												1144.5943	R.LASAISSNPER.A	55	5.30E-03
												1089.5338	K.TQSVDFQHK.T	52	1.00E-02
												1525.6909	K.GAWDQKFDESNTK.C	60	1.20E-03
												1809.9524	K.RLSTEPEFIENHIPK.Q	34	4.70E-01
												1653.852	R.LSTEPEFIENHIPK.Q	43	6.70E-02
f11	gi|46120810	*Gibberella zeae* PH-1	Hypothetical protein FG04936.1 aminopeptidase Y	52033	5.93			58	1.8	2	1	1149.6073	R.LASVPSEFSGR.S	54	0.008
f12	gi|358375979	*Aspergillus kawachii* IFO 4308	Endo-1,4-β-xylanase A GH10	34120	5.82			110	1.30E-05	7	3	950.4952	K.VIGEDYVR.I	30	1.9
f23	gi|225102	*Hordeum vulgare* subsp. *vulgare*	Trypsin/amylase inhibitor pUP13	15307	5.35	104	8.20E-04	274	4.40E-22	52	7	962.3753	R.DYGEYCR.V	32	1.70E-01
												1237.692	K.SIPINPLPACR.E	15	5.00E+01
												1205.5581	R.ELSDLPESCR.C	30	1.40E+00
												1918.9921	R.CDALSILVNGVITEDGSR.V	121	9.30E-10
f24	gi|123970	*Hordeum vulgare*	α-amylase inhibitor BDAI-1	17045	5.36	61	1.60E+01	260	2.10E-19	39	6	1655.8797	K.LECVGNRVPEDVLR.D	55	7.20E-02
												827.4604	R.VPEDVLR.D	34	9.10E+00
												1940.816	R.DCCQEVANISNEWCR.C	106	2.60E-07
												1024.4618	R.CGDLGSMLR.S	12	1.10E+03
f25	gi|123970	*Hordeum vulgare*	α-amylase inhibitor BDAI-1	17045	5.36	55	5.90E+01	228	1.70E-17	38	5	1655.8403	K.LECVGNRVPEDVLR.D	56	3.10E-03
												1940.7848	R.DCCQEVANISNEWCR.C	125	1.40E-10
f26	gi|123970	*Hordeum vulgare*	α-amylase inhibitor BDAI-1	17045	5.36	54	7.80E+01	257	4.10E-19	25	5	1655.9277	K.LECVGNRVPEDVLR.D	46	4.20E-01
												827.4596	R.VPEDVLR.D	46	5.40E-01
												1940.8476	R.DCCQEVANISNEWCR.C	107	2.80E-07
												1024.4868	R.CGDLGSMLR.S	17	4.60E+02
f27	gi|123970	*Hordeum vulgare*	α-amylase inhibitor BDAI-1	17045	5.36			254	4.40E-20	25	5	1655.9448	K.LECVGNRVPEDVLR.D	49	1.20E-02
												827.459	R.VPEDVLR.D	45	4.20E-02
												1940.8597	R.DCCQEVANISNEWCR.C	104	3.10E-08
												1024.488	R.CGDLGSMLR.S	17	2.40E+01
f28	gi|123970	*Hordeum vulgare*	α-amylase inhibitor BDAI-1	17045	5.36	76	5.30E-01	268	1.70E-21	53	7	1655.8828	K.LECVGNRVPEDVLR.D	47	2.60E-02
												827.4555	R.VPEDVLR.D	45	4.50E-02
												1940.8164	R.DCCQEVANISNEWCR.C	100	6.00E-08
												1024.4573	R.CGDLGSMLR.S	21	8.00E+00
f29	gi|585290	*Hordeum vulgare*	α-amylase/trypsin inhibitor CMb	17199	5.77			200	1.10E-14	32	4	1168.5361	R.DYVEQQACR.I	50	2.10E-02
												1861.8548	K.QQCCGELANIPQQCR.C	67	2.40E-04
												1039.5089	R.EVQMDFVR.I	52	9.50E-03

f30	gi|225102	*Hordeum vulgare* subsp. *vulgare*	Trypsin/amylase inhibitor pUP13	15307	5.35	101	1.60E-03	239	2.60E-17	52	7	1237.6655	K.SIPINPLPACR.E	14	1.00E+03
												1205.5382	R.ELSDLPESCR.C	24	7.60E+01
												1918.9614	R.CDALSILVNGVITEDGSR.V	127	3.80E-09
f31	gi|123970	*Hordeum vulgare*	α-amylase inhibitor BDAI-1	17045	5.36	87	4.40E-02	358	3.30E-29	55	8	1655.9171	K.LECVGNRVPEDVLR.D	51	1.50E-01
												827.4618	R.VPEDVLR.D	48	4.00E-01
												1940.8606	R.DCCQEVANISNEWCR.C	104	6.50E-07
												2232.3301	K.LLVAGVPALCNVPIPNEAAGTR.G	91	4.80E-06
f32	gi|123970	*Hordeum vulgare*	α-amylase inhibitor BDAI-1	17045	5.36	72	7.20E-02	221	1.60E-15	53	8	1940.7816	R.DCCQEVANISNEWCR.C	115	2.20E-08
												2232.2492	K.LLVAGVPALCNVPIPNEAAGTR.G	51	1.20E-01
f33	gi|123970	*Hordeum vulgare*	α-amylase inhibitor BDAI-1	17045	5.36	70	2.30E+00	226	5.20E-16	38	6	1655.883	K.LECVGNRVPEDVLR.D	54	9.20E-02
												1940.8088	R.DCCQEVANISNEWCR.C	105	3.10E-07
												1024.4722	R.CGDLGSMLR.S	22	1.50E+02
f34	gi|149237516	*Lodderomyces elongisporus* NRRL YB-4239	Ubiquitin	17499				116	5.20E-05	37	5	1523.7918	K.IQDKEGIPPDQQR.L	83	7.9e-06
f36	gi|1405736	*Hordeum vulgare*	Trypsin inhibitor cme precursor	16341	7.49			141	1.60E-07	14	3	1614.7227	R.CCDELSAIPAYCR.C	76	4.00E-04
												845.3613	K.DSPNCPR.E	44	3.60E-01
f37	gi|123970	*Hordeum vulgare*	α-amylase inhibitor BDAI-1	17045	5.36	50	1.90E+02	267	4.10E-20	25	5	1655.8939	K.LECVGNRVPEDVLR.D	54	8.60E-02
												827.4629	R.VPEDVLR.D	45	7.70E-01
												1940.808	R.DCCQEVANISNEWCR.C	105	3.00E-07
												1024.4622	R.CGDLGSMLR.S	30	1.80E+01
f38	gi|123970	*Hordeum vulgare*	α-amylase inhibitor BDAI-1	17045	5.36			201	8.70E-15	25	5	1655.8429	K.LECVGNRVPEDVLR.D	56	3.70E-03
												1940.7872	R.DCCQEVANISNEWCR.C	107	8.50E-09
	gi|326503930	*Hordeum vulgare* subsp. *vulgare*	Predicted protein	18038	9.8	49	1.30E+01	206	2.70E-15	30	5	1523.7771	K.IQDKEGIPPDQQR.L	84	5.50E-06
			Ubiquitin									1039.5055	K.EGIPPDQQR.L	47	3.00E-02
												1067.6047	K.ESTLHLVLR.L	46	3.10E-02
f39	gi|1405736	*Hordeum vulgare*	Trypsin inhibitor cme precursor	16341	7.49			102	1.30E-03	14	3	1614.7148	R.CCDELSAIPAYCR.C	70	1.60E-03
f40	gi|68305063	*Triticum aestivum*	Ubiquitin, partial	7901	5.69			150	1.10E-09	53	4	1523.7437	K.IQDKEGIPPDQQR.L	71	1.30E-04
												1039.4963	K.EGIPPDQQR.L	29	1.70E+00
	gi|1588926	*Triticum aestivum*	Pathogenesis-related protein	14013	6.97			103	5.50E-05	26	2	1299.6642	R.VTNPATGAQITAR.I	82	8.60E-06
f41	gi|1405736	*Hordeum vulgare*	Trypsin inhibitor cme precursor	16341	7.49	44	4.70E+01	97	4.50E-03	23	4	1614.6531	R.CCDELSAIPAYCR.C	55	2.20E-02

f44	gi|326499596	*Hordeum vulgare* subsp. *vulgare*	Predicted protei, Gamma-thionin	9580	8.49			56	5.20E+01	16	1	1604.6091	K.GACFSDTNCASVCR.T	46	0.0062
f45	gi|225465030	*Vitis vinifera*	Ubiquitin-NEDD8-like protein RUB2	17185	5.77			220	1.10E-16	45	6	1523.809	K.IQDKEGIPPDQQR.L	76	3.50E-05
												1039.5259	K.EGIPPDQQR.L	39	1.90E-01
												1067.6282	K.ESTLHLVLR.L	50	8.50E-03
f46	gi|326503930	*Hordeum vulgare* subsp. *vulgare*	Predicted protein	18038	9.8	66	2.70E-01	324	4.40E-27	30	5	1764.0132	K.TITLEVESSDTIDNVK.S	96	2.20E-07
			Ubiquitin									1523.8653	K.IQDKEGIPPDQQR.L	82	6.40E-06
												1039.5669	K.EGIPPDQQR.L	50	1.30E-02
												1067.6746	K.ESTLHLVLR.L	47	7.90E-03
***F. poae* on WAX**															
f48	gi|46115498	*Gibberella zeae* PH-1	Hypothetical protein FG03591.1 GH18_chitinase	45250	5.35			234	8.20E-17	8	2	1704.7548	R.ADGTVYTGDSYADLEK.H	82	9.20E-05
												2170.9157	K.HYTGDSWEEPGTNAYGCVK.Q	137	2.00E-10
f50	gi|46138441	*Gibberella zeae* PH-1	Hypothetical protein FG10735.1	10689	5.11			60	1.40E+00	15	1	1815.0141	K.ALEIVQINHEDHQIR.V	49	2.10E-02
f52	gi|46138969	*Gibberella zeae* PH-1	Hypothetical protein FG10999.1 Endo-1,4-β-xylanase GH11	25886	6.5			125	6.50E-06	12	3	844.4194	K.GWNPGTGR.T	48	4.30E-01
												1464.624	R.MGEGSHYQVDWR.N	46	2.90E-01
												1480.6366	R.MGEGSHYQVDWR.N	60	1.20E-02

Fusarium poae has been reported as one of the most frequent *Fusarium* species isolated from cereal grains in Finland, Japan, Norway and Sweden (Liu et al., 1998; Pettersson et al., 1995; Sugiura et al., 1993; Yli-Mattila et al., 2008). *F. poae* is a pathogenic filamentous fungus and has been reported to produce several mycotoxins, including trichothecenes, such as deoxynivalenol (DON), nivalenol (NIV) and fusarenone-x (FX) (Desjardins, 2006; Desjardin and Proctor, 2007; Rotter et al., 1996) and inhibits mitochondrial function and protein synthesis (Oldham et al., 1980; Rosenstein and Lafarge-Frayssinet, 1983). Little work has been performed on *F. poae,* despite its pathogenicity and hazard imposed to human health. In the present study, we initiated analysis of the extra-cellular proteins of *F. poae* growing on either WAX or barley flour. The protein content of *F. poae* cultures was lower compared to *A. niger*, despite being inoculated with the same amount of spores. The differences could be due to different growth rates of the fungi in the medium and accordingly differences in secretory capacity. Moreover, the protein content is influenced by several factors, including the inoculum size and composition of the fungal mycelium

The secretome profiles of *F. poae* grown on medium with WAX or barley flour as carbon source show considerable differences, indicating that each of the assayed substrate elicits a specific secretion response (Figure 30 c-d). A significant number of spots were quite faint and thus difficult to identify by mass spectrometry due to the low amount of protein. Nonetheless, *F. poae* grown with either WAX or barley flour resulted in 31 confident identifications of secreted proteins (Table 16). Wheat arabinoxylan induced fungal proteins, i.e. endo-1,4-β-xylanase (GH11, spot 52), chitinase (GH18, spot 48) and a hypothetical protein (FG10735.1, spot 50) with unknown function, respectively. The secretome profile of *F. poae* growing with barley flour was dominated by plant proteins, while only three spots were identified as fungal proteins, i.e. endo-1,4-β-xylanases A (GH10, spot 12), ubiquitin (spot 38) and a hypothetical protein (FG04936.1, spot 11) displaying homology to aminopeptidase Y, respectively. Ubiquitin are small regulatory protein, which direct proteins for degradation/recycling and other functions. The protein identifications were found for the homolog *F. graminearum, F. oxysporum* and *Lodderomyces elongisporus* (the latter is a yeast).

Xylanase activity level for *F. poae* grown with WAX was determined to 6.7 U/mg, which is much higher compared to the cultures grown with barley flour of 0.08 U/mg. The high xylanase activity on WAX could be explained by induction of different and/or higher numbers of GHs, even though only a few of them were identified in this study, and the fact that GHs are typically regulated by catabolite repression and/or substrate availability (Magasanik and Neidhard, et al. 1987; Paper et al., 2007). Moreover, the specific xylanase activity was determined per amount of protein, and barley flour itself represents a very important source of protein. It is also worth mentioning that barley flour contain xylanase inhibitors, which have a significant impact on the fungal xylanases. It was noticeable that xylanases of GH11 were detected in the secretome of *F. poae* growing on WAX, while GH10 was detected in secretomes for cultures growing on barley flour. The higher xylanase activity on WAX could be explained by the presence of a higher number of xylanases of GH11. A study by Beaugrand et al. (2004) showed that GH11 xylanases were more efficient than the GH10 in the hydrolysis of wheat bran and displayed two-fold higher affinity for wheat bran and 6-fold turnover rate. Moreover, xylanases of GH11 are known to have a lower catalytic versatility than GH10 and preferentially cleave unsubstituted regions of AX. While GH10 xylanases have broader substrate specificity and cleave within decorated regions of AX.

The majority of the identified proteins appearing when *F. poae* grown on barley flour were of plant origin, indicating high abundance of proteins of the medium that mask the secreted fungal proteins. Several protease inhibitors, such as trypsin inhibitor CMb (spot 30) and CMe (spots 36, 39, 41) and α-amylase/trypsin inhibitor pUP13 (spots 23, 30) were identified, which selectively inhibit proteases and/or α-amylases from bacteria, fungi and insects. A train of spots 2−5 was identified as protein Z−type serpins, which are known serine protease inhibitors. The dimeric form of α-amylase inhibitor (BDAI-1) was identified in multiple spots (24−28, 31−33, 37−38), suggesting post-translational modifications. γ-thionins or defensins (spot 44) characterized to possess anti-fungal and anit-bacterial activities were also identified (Pellegrini and Franco, 2005). Plant ubiquitins were identified (spots 40, 45−46), which have been shown to participate and regulate in a variety of cellular functions including protein degradation. Collectively, the identification of these inhibitors of

microbial proteases and amylases and defense-related proteins suggests the presence of high plant biomass, which masks the fungal proteins that are low abundant.

As can be seen from the 1D SDS-PAGE (Figure 29), the different *Fusarium* species exhibit distinctly different band patterns. The secretome pattern of e.g. *F. graminearum* (lanes 10 and 11) differs from that of *F. poae* (lanes 15 and 16), indicating different proteins to be secreted andsecretory capacity. In a study by Paper et al. (2007), the secretome of *F. graminearum* was mapped with identification of 229 proteins by analysis of cultures growing on 10 different carbon sources. Six proteins were found from all the sources, while the rest dependent on the respective culture condition and substrate. The high number of protein identifications was facilitated due to the fact that the genome of *F. graminearum* was fully sequenced. Another study by Yang et al. (2012) complemented the already generated data by Paper et al. (2007) by identification of additional 24 fungal proteins from *F. graminearum* grown on barley or wheat flour. Moreover, they also reported identification of two plant proteins, i.e. a serpin and a fasciclin-like protein, which appeared in several spots. In our dataset, the plant proteins dominated the secretome of *F. poae* grown on barley flour and only a few fungal proteins involved in cell wall degradation were identified. An important number of proteins could not be identified, probably due to low abundance as well as the lack of a genome sequence. An annotated genome sequence would generally facilitate protein identification in proteomics studies. Nevertheless, even with that availability of the genome, the identity and function of the secreted proteins remains to be elucidated.

4.4. CONCLUSIONS

A proteomic approach applying 2-DE coupled with mass spectrometry was used to profile and map the surface-associated proteome of barley grains (cultivars Barke and Cabaret, harvest year 2009). To our knowledge this is the first report to describe the proteome/surfome constitutively present on and within the outer kernel layers. The comparative proteomics analysis revealed minor but distinct changes in the protein profiles of the two different barley cultivars. The majority of identified proteins was of

plant origin and ascribed to play a role in defense and/or oxidative stress mechanisms. To assess whether the proteome differences could be correlated with the differences in fungal community, the present study set out to analyze and describe the composition of the populating fungal community of selected barley cultivars, as well as to identify their secreted proteins. Cultivation and microscopic evaluation enabled profiling of the fungal community populating the surface of the grains and their composition was found to have a significant impact on the production of microbial xylanases.

The cereal storage fungi, e.g. Aspergilli and Penicillia, were found to be prominent producers of xylanases compared to the field fungi. The 1D protein profiles of the isolated cereal fungi were shown to be species-specific, as well as dependent on the medium and substrate. A comprehensive analysis of the secretomes of *A. niger* and *F. poae* grown on WAX or barley flour using 2-DE showed induced production of specific enzymes involved in cell wall degrading and carbohydrate catabolism. *A. niger* is a well-studied filamentous fungus and secretomes in grown on different substrates, e.g. xylose and maltose, have been characterized. However, the present study provides a map of the secretomes of *A. niger* grown on WAX and barley flour, and identification of proteins not previously reported, e.g. melibiase and α-glucuronidase, that are involved in carbohydrate catabolism. The proteome of *F. poae* has not previously been characterized, thus the present study is the initial step of mapping and describing the extra-cellular proteins of *F. poae* grown on WAX or barley flour. Only a handful of fungal proteins were identified in the secretome of *F. poae,* and there is room for optimization of the experimental procedures and conditions to obtain higher protein identification coverage and establish a comprehensive reference map of *F. poae*. Availability of the genome sequence of *F. poae* would most definitely complement secretomics/proteomics analysis and in general facilitate protein identifications. Moreover, analysis of the intracellular proteins is also of significance as they provide insight into the fungal physiology and enzymes involved in utilization of the carbon source. The fungal secretome analyses are significant since they provide improved understanding of strategies and synergistic effects of fungal proteins and the host/cereal, as well as insight into the underlying mechanisms involved in the secretory capacity and pathogenicity of the studied organisms.

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CHAPTER 5

Secretory expression of functional barley xylanase inhibitor protein (XIP-III) by yeast *Pichia pastoris*

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5. ABSTRACT

Cereals have been found to contain high amounts of xylanase inhibitors that specifically inhibit xylanases of microbial origin. Hitherto, three types of xylanase inhibitors have been identified, namely xylanase inhibitor proteins (XIP), *Triticum aestivum* xylanase inhibitors (TAXI) and thaumatin-like xylanase inhibitors (TLXI), all occurring as multi-isoform families. XIP inhibit fungal xylanases belonging to glycoside hydrolase families 10 and 11, but not the enzymes of bacterial origin. In the current study, barley XIP-III was produced recombinantly by the methylotrophic yeast *Pichia pastoris*. The expressed rXIP-III was purified from the *P. pastoris* culture medium using nickel affinity chromatography. The rXIP-III having a molecular mass of 33.4 kDa was confirmed by SDS-PAGE and immunoblotting probed with anti-penta-His and anti-XIP antibodies. rXIP-III showed inhibition against microbial xylanases of different sources, such as naturally occurring xylanases recovered from the surface of barley grains and secretomes of grain-associated fungi grown on wheat or barley flour. The cross-inhibitory activity assays indicate that rXIP-III is specific towards certain types xylanases present in varying degree in the different samples. More research is needed to unravel the presence and types of xylanases in secretomes of grain-associated fungi. Moreover, xylanase-containing secretomes of *F. tricinctum* were found to be moderately resistant to the rXIP-III, with high residual xylanase activity levels.

5.1. INTRODUCTION

Cereals are exposed to various biotic stress conditions, such as fungal and bacterial invasion. Plant cells are protected from its environment by the cell wall, which functions as a structural barrier e.g. against pathogenic attack. The indigenous community of cereal grains harbors a diverse range of microbes including species of the genera bacteria, yeasts and fungi, which colonize during different grain stages from development to maturation, harvest and storage. The microbial contamination of grains comes from the environment, such as soil, air and dust during harvest and handling (post-harvest storage). The colonizing microbial community includes both symbionts and pathogens actively interacting with the barley grain by various means, such as production of metabolites, plant stimulating hormones as well as a diverse array of enzymes for degradation of plant cell walls (Noots et al., 1999). One type of these cell wall degrading enzymes are endo-β-1,4-xylanases (EC 3.2.1.8), which depolymerize arabinoxylan by catalyzing the hydrolysis of the β-1,4-glycosidic linkages between xylose units in the backbone of arabinoxylan, a predominant constituent of cell walls in plants (Beliën et al., 2006; Collins et al., 2009). The majority of the xylanases are classified into glycoside hydrolase families 10 and 11 (GH10 and GH11) on the basis of similarities in the amino acid sequences of catalytic domains. However, other xylanases have been found in GH5, 7, 8, and 43 (Collins et al., 2009). In addition, plants themselves are known to produce xylanases, which play an important physiological role by modulating or degrading cell walls during fruit ripening, seed development and germination. Plant xylanases identified thus far all belong to GH10 (Collins et al., 2009; Juge, 2006).

Proteinaceous inhibitors of cell wall degrading enzymes including xylanases naturally occur in various cereals, and have been ascribed to play various roles from defence to metabolism regulation. Hitherto, three types of xylanase inhibitors have been identified in cereals, namely xylanase-inhibiting protein (XIP), *Triticum aestivum* xylanase inhibitor (TAXI) and thaumatin-like xylanase inhibitor (TLXI), respectively (Debyser et al., 1997; Gebruers et al., 2004; Juge, 2006; Juge and Svensson, 2006; McLauchlan et al., 1999; Rouau and Surget, 1998). All three types of xylanase inhibitors occur as multi-isoform families and have been detected in various cereals

including wheat, durum wheat, barley, rye, maize and oat (Beaugrand et al., 2006; Elliot et al., 2003; Goesaert et al., 2002). The concentrations of these xylanase inhibitors in cereals are highly dependent on variety (cultivar genetic differences), milling fraction, growing conditions and environment (Bonnin et al., 2005). The XIP type inhibitors specifically inhibit xylanases of fungal and not bacterial origin (GH10 and 11). Whereas both TAXI and TLXI exclusively inhibit xylanases of GH11 independent of whether they are of fungal or bacterial origin (Fierens et al., 2005; 2007).

The TAXI type inhibitor proteins occur as monomeric (40 kDa) and heterodimeric (29+11 kDa) forms as detected in wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), barley (*Hordeum vulgare*) and rye (*Secale cereale*) (Gebruers et al., 2004; Payan et al., 2004). TAXI-type inhibitors are described as basic (pI ≥ 8.0) proteins with minor glycosylation and specific competitive inhibitory activity towards xylanases of GH11 (Gebruers et al., 2004; Goesaert et al., 2003). The TAXI-I displays structural homology with a pepsin-like protease, but is proteolytically non-functional (Fierens et al., 2003; Sansen et al., 2004). TLXI type inhibitors share high sequence identity (60%) with the thaumatin-like proteins (TLPs) and belong to pathogenesis-related proteins of family 5 (PR-5), which are involved in various mechanisms of plant defence. In wheat, TLXI occurs with a molecular mass of approximately 18 kDa, high pI (≥ 9.3) and varying extent of glycosylation. These inhibitors exhibit non-competitive inhibition of xylanases of GH11, while they are inactive towards GH10 xylanases (Fierens et al., 2007; Reiss et al., 2006).

The XIP type proteins are characterized as monomeric with a molecular mass of 29 kDa, high pI (8.7−8.9) and one *N*-glycosylation site. They competitively inhibit type GH10 and GH11 xylanases. It has been revealed that a XIP possess two independent enzyme-binding sites: one for each xylanase of GH10 and GH11 families (Goesaert et al., 2005; Juge et al., 2004; Payan et al., 2004). Based on their amino acid sequence homology, XIP type inhibitors have been related to chitinases of GH18 family, though they exhibit no activity toward chitin. Chitinases belonging to the PR-8 family provides protection to the plant by attacking chitin, a principal component of fungal cell walls (Durand et al., 2005). Furthermore, it has been demonstrated that XIPs can inhibit barley α-amylases (AMY1 and AMY2) belonging to the GH13 family (Sancho

et al., 2003).

All three types of inhibitors predominantly occur in high abundance in the outer layers of cereal grains. A study by Gebruers et al. (2002) revealed 2−3 fold higher inhibition activities in fractions containing wheat shorts and bran than in flour. Croes et al. (2009), however, detected high concentrations of these inhibitors in the wheat aleurone layer. Analysis of the spatial distribution of TAXI and XIP in wheat grains demonstrated high occurrence in the nucellar tissue adjacent to the aleurone tissue (Jerkovic et al., 2010). Various roles have been ascribed to these inhibitors including involvement in development, germination and defence against pathogens. Due to the specific inhibitory activity towards xylanases secreted by pathogens e.g. *Botrytis cinera and Fusarium graminearum*, and high sequence similarities to many defence-related proteins, it is likely that these inhibitors form part of the plant's defence mechanisms, however a direct role remains to be elucidated (Bellincampi et al., 2004; Brutus et al., 2005; Croes et al., 2008; Flatman et al., 2002; Gebruers et al., 2004; 2001; Goesaert et al., 2004). Several genes encoding for XIP-like proteins, subgroups *xip-I-III*, have been identified in wheat and other cereals, such as barley, maize, rye and rice. A study by Igawa et al. (2005) reported induced expression of *xip* in *Erysiphe graminis*-infected leaves as well as in wounded leaves, although the enhanced transcription was ascribed to *xip-I* and not *xip-III*. Transcripts of *xip-I* and *xip-III* were found in immature wheat embryos three weeks after pollination and detected in young shoots after germination, as well as in the roots. However, the *xip-I* was the most abundant isoform detected. Thus far, xylanase inhibition activity has been demonstrated for recombinant XIP-I and II from wheat and RIXI from rice (Durand et al., 2005; Elliott et al., 2002; 2009; Juge et al., 2004).

The present study reports the cloning and production of functional recombinant barley rXIP-III using the methylotrophic yeast *Pichia pastoris* as heterologous host. The secreted rXIP-III was purified from the *P. pastoris* culture supernatant using metal affinity chromatography. The rXIP-III was assessed for inhibition against microbial xylanases of GH11 (*Aspergillus nidulans, Aspergillus niger* and *Tricoderma longibrachiatum*) and xylanases naturally found on barley grains as produced by the populating indigenous microbial community (surface associated). In addition inhibition activities of rXIP-III were tested towards extracellular proteins (secretomes)

of selected grain-associated fungi, i.e. *Fusarium trincinctum*, *Penicillium freii*, and *Phoma* sp., respectively, grown on wheat arabinoxylan or barley flour.

5.2. MATERIALS AND METHODS

5.2.1. CLONING OF XYLANASE INHIBITOR ISOGENES FROM BARLEY (XIP)

Developing (21−35 days after pollination) and germinating (six days on wet filter paper) barley grains (cv. Golden Promise) were used as starting material for mRNA purification. The mRNA was isolated using the Dynabeads kit as described by the manufacturer (Life Technologies). Single-strand cDNA was synthesized from the pool of mRNA using the Superscript III reverse transcriptase and oligod(T)18 oligonucleotide primers according to the manufacturer´s instructions (Life Technologies). The Open Reading Frames (ORF) cDNA of the *xip* type xylanase inhibitors were obtained by PCR using the Phusion DNA polymerase (New England BioLabs) and the primers Fw (5´-GAAGAAAACTCATCGATCTAACAATG-3´) and Rv (5´-GGCAATACTTATTCACTGTAAGGTAAGC-3´), Table 17. Moreover, the aim was also to express the other two *xip* genes with the primers listed in Table 17. The PCR product was purified from the gel and cloned into pCR4-blunt TOPO vector (Life Technologies). The resulting constructs were transformed into One Shot TOP10 *E. coli* competent cells (Life-Technologies) by heat shock. The transformed cells were grown on Luria-Bertani (LB) plates containing 35 µg/mL zeocin for selection at 37°C overnight. The positive clones were sequenced at Eurofins MWG-Operon (Germany) before use as template for the *Pichia* constructs. The mRNA isolation, cDNA synthesis and primer design was performed by Dr. Giuseppe Dionisio (Department of Molecular Biology and Genetics, Faculty of Agricultural Sciences, University of Aarhus).

Table 17: Cloning and construct primers used for barley xylanase inhibitor proteins (XIP). The start codons are indicated in bold.

Cloning primers	Sequences (5´-3´)	PCR product (bp)
AK371069		1021
Hv_XIP-I_fw	TACGTGGAGAAACCAAAA**ATG**GC	
Hv_XIP-I_rv	CAAGTACAAGCCTCACTTTATTCTCGC	
AK360178		934
Hv_XIP-II_fw	GAGTACAGTACGTACTAATCAGAA**ATG**G	
Hv_XIP-II_rv	GAGCTCAAGCCCAGTTCTTGA	
AK35730		1021
Hv_XIP-III_fw	GAAGAAAACTCATCGATCTAACA**ATG**	
Hv_XIP-III_rv	GGCAATACTTATTCACTGTAAGGTAAGC	
Construct primers		
Hv_XIP-III_EcoR_I_fw	GCTGAATTCGGCGCCACTGGTAAGACCG	835
Hv_XIP-III_Afl_II_rv	GATGCTTAAGTGGAGCCCAGTTCTTGATGTAG	

5.2.2. RECOMBINANT PRODUCTION OF BARLEY XIP-III IN *PICHIA PASTORIS*

XIP-III was produced extracellularly in *Pichia pastoris* using the pPICZαA vector (Invitrogen) as fusion protein along with the alpha-mating factor and driven by the alcohol oxidase promoter. Among the three isoforms of barley *xip*, the less homologous to wheat *xip-I* and *xip-II* was chosen as a new isoform to characterize. It also contained a different potential *N*-glycosylation site close to the C-terminus of the protein (which is supposed to interact with GH10 xylanases) rather than close to the N-terminus as in the other two isoforms (Figure 31). Barley XIP-III translated protein (BAJ88515) has a leader peptide of 29 amino acids as predicted by the SignalP v4.0 server (Petersen et al., 2011). For transformation in *Pichia*, the leader peptide was removed and fused with the alpha-factor mating secretion signal peptide of the vector pPICZαA (Invitrogen), a C-terminal His_6-tag was included in the expression constructs.

BAJ88515 is predicted to be glycosylated at the asparagine residue of the motif NYS close to the C-terminus. In order to evaluate whether this residue is glycosylated and if the glycosylation and the hyperglycosylation have an effect on the inhibitory function of barley XIP-III, two recombinant rXIP-III were expressed in two different *Pichia*

strains, KM71H and its derivative strain KM71H och1::G418R, where the och1 gene responsible for the hyperglycosylation has been knocked out. The effect of no glycosylation was tested after the treatment with endoglycosidase, Endo H (This is under investigation by Dr. Giuseppe Dionisio). The *xip-III* construct was obtained by PCR using the cloned AK357301 PCR product in pCR4-Blunt as template. The PCR primers used for the construct are described in Table 17. The PCR was performed using Phusion DNA polymerase (New England BioLabs) and 36 cycles of 95°C as denaturation temperature, 64°C as annealing temperature and 1 min as elongation time. The PCR product was purified and digested with EcoRI and AflII and inserted into the destination vector pPICZαA opened with the same restriction sites and dephosphorylated, coming from a previous engineered vector (JN635739). The insert was ligated into the vector using 10 U of T4 Ligase (Fermentas, Thermo Scientific) for 12 h at 4°C to result in pPICZαA-XIPIII. Bacterial chemical transformation was performed in *E. coli* TOP10 competent cells (Life Technologies). Positive colonies were identified by restriction analysis and sequenced (EurofinsMWG-Operon, Germany).

The *Pichia* transformation was carried out using 5 µg of plasmid miniprep (pPICZαA-XIPIII) linearized with *Dra*I and electroporated (1.5 kV, 200 Ω and 25 µF and 5 ms) into competent *Pichia pastoris* KM71H cells according to Lin-Cereghino et al. (2005). After 3 days growth at 30°C, zeocin resistant colonies on YPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol, 2% (w/v) agar, supplemented with 100 µg/mL zeocin) were picked up and screened for the presence of rHvXIP-III insert by PCR on the genomic DNA using the same construct primers. Positive colonies were selected for expression using the feed-batch method according to Life Technologies fermentation guidelines. Positive colonies were grown in buffered minimal glycerol medium (1% (w/v) yeast nitrogen base, 1% (w/v) casaminoacids, 100 mM phosphate buffer, pH 6.0, 2% (v/v) glycerol, 50 mM $ZnSO_4$, 450 mM $FeSO_4$, 150 mM $MnSO_4$, 200 mM $MgCl_2$, and 200 mM $CaCl_2$) for 24 h at 30°C under continuous shaking at 300 rpm. After 24 h pre-growth the *Pichia* cells were inducted with 1% (v/v) methanol for the first day and the induction continued with a final concentration of 2% methanol every 24 hours for 4 days. After 4 days, the cultures were centrifuged (3000 x g for 15 min) to remove yeast cells and the

supernatants were stored at 4°C. The presence of the rHvXIP-III was detected in the medium by assessing the inhibitory activity against *A. niger* GH11 xylanase (Megazyme, Ireland) and SDS-PAGE (4−12%) followed by western blotting using anti-penta His monoclonal antibodies (Qiagen).

For large scale purification the workflow started with two liters of *P. pastoris* culture liquid (4 days methanol induced) and the recombinant proteins were collected in the supernatant after centrifugation (5000 x g, 20 min) and dialysed against 10 mM sodium acetate pH 4.5. The dialysed supernatant was applied onto a SP-Sepharose column (HiLoad 16/10, 20 ml bed resin, GE Healthcare, Sweden) using an ÄKTA FPLC (GE Healthcare, Sweden) and recombinant proteins were eluted with a linear gradient of NaCl from 0 to 0.5 M. The fractions with xylanase inhibitory activity against *A. niger* GH11 xylanase (Cat. num. E-XYAN4, Megazyme, Ireland) were loaded onto Ni-NTA (nickel nitriloacetic acid) Sepharose (HisPrep FF 16/10, GE Healthcare, Sweden) washed with 20 mM Tris-HCl, 0.5 M NaCl and 10 mM imidazole, pH 7.3. The rXIP-III bound proteins were eluted with 0.5 M imidazole, containing 0.5 M NaCl and 20 mM Tris-HCl pH 7.3. The pooled fractions were dialysed against 10 mM sodium acetate pH 5.0 and concentrated using Vivaspin 20 columns (cut off 5 kDa, GE-Healthcare, Sweden) and stored at 4°C until use. A fraction of the eluate was deglycosylated by Endo H performed under native conditions as described by the supplier (New England Biolabs).

Figure 31: Three translated ORF of the barley *xip* isoform clones. In green is highlighted the leader peptide as predicted by the SignalP v4.0 server (Petersen et al., 2011). Putative glycosylation sites are in light blue (*xip-I*, NRS; *xip-II*, NQS; *xip-III*, NYS). In yellow are highlighted the region that by homology are responsible of the interaction with type GH11 xylanases. In light green are also indicated the homologous regions that should interact with type GH10 xylanases, according to Payan et al. (2004). GenBank accession numbers of cloned XIP protein isoforms: B25-F1 (HvXIP-I): BAK02267; B25-G1 (HvXIP-II): BAJ91387; B25-H4 (HvXIP-III): BAK02218.

5.2.3. Recombinant production of *Aspergillus nidulans* xylanase GH10 and GH11 in *Pichia pastoris* expression vector

Xylanases GH11 (DQ490490) and GH10 (DQ490475) from *A. nidulans* (Baueret al., 2006) were obtained as *Pichia* X-33 expression clones from the Fungal Genetics Stock Center (http://www.fgsc.net/). The expression and the purification of these enzymes by one step Ni-NTA chromatography was carried out as described above and dialyzed and concentrated similarly to rXIP-III.

5.2.4. Protein quantification, SDS-PAGE and western blotting

The protein concentration was determined using the Coomassie Brilliant Blue method of Bradford (1976), where bovine serum albumin (BSA) was used as a standard.

Protein purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE Novex Bis-Tris 4−12% gels (Invitrogen). Reducing agent was added in the sample preparation according to the manufacturer's recommendation (Invitrogen). SDS-PAGE was carried out using 1 X NuPAGE MES running buffer at 200 V, 120 mA for 35 min. Gels were stained with Coomassie Brilliant Blue G-250 (Candiano et al., 2004). Mark12 (Invitrogen) was used as molecular weight marker. In parallel, an identical gel loaded with same amount of proteins was run and the separated proteins were blotted onto a nitrocellulose membrane (0.20 µm pore size, Hybond N, GE Healthcare) using the Novex system (Invitrogen) according to the manufacturer's instructions. The transfer was performed for 1 hour at 30 V and 160 mA. The free protein binding sites on the blots were blocked for 1 h with tris-buffered saline 20 (150 mM NaCl, 25 mM Tris HCl pH 7.5, referred to as TBS) with 0.5% (v/v) tween. The membrane was probed with the primary antibodies, i.e. rabbit polyclonal antibodies raised against XIP (generous gift of Prof. Christophe M. Courtin, Laboratory of Food Chemistry and Biochemistry, Catholic University of Leuven, Belgium) from wheat or mouse monoclonal anti-penta-HIS antibodies (Thermo scientific), diluted 1:1000 in TBS (0.1% v/v tween) for 1h at room temperature. The membrane was washed (3 x 10 min) in TBS (0.1% v/v tween), blocked as before, and probed with secondary goat anti-rabbit or anti-mouse antibodies conjugated with horseradish peroxidase (1:20,000 dilution, DAKO) for 1 h. The membrane was washed again (3 x 10 min) in TBS (0.1% v/v tween) and developed using enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare).

5.2.5. PREPARATION OF WASHING LIQUIDS

In order to estimate the total xylanase activities (microbial and endogenous xylanases) of barley seeds, a washing procedure developed by Dornez et al. (2006) was utilized. The procedure enabled effective separation of surface associated (microbial) and plant endogenous xylanases. The washing liquids were supplemented with sodium azide (0.02% w/v) to inhibit any bacterial growth during the washing period, even though *in situ* production of microbial xylanases and/or microbial growth was considered

unlikely. A washing period of 15 h (overnight) was carried out using 25 mM sodium acetate pH 5.0 and 0.02% (w/v) sodium azide, at room temperature. The initial wash was followed by second wash with water to detect remaining xylanases on the grains. Activity levels recovered in these second washes, however, were rather close to zero and considered negligible. The recovered washing liquids were used for measurement of xylanase activity levels.

5.2.6. PREPARATION OF FUNGAL SECRETED PROTEINS

The isolated fungi were used to inoculate fluid medium composed of 0.67% (w/v) yeast nitrogen base, 0.2% (w/v) asparagine and 0.5% (w/v) KH_2PO_4 supplemented with either 1% (w/v) wheat arabinoxylan (WAX) or finely ground barley flour as carbon source (Biely et al., 1980). The inoculums were incubated for 7 days at 25°C after which the secreted proteins in the supernatant were collected after centrifugation (3200 x g for 30 min, 4°C). Negative controls composed of medium and WAX or barley flour was also prepared.

5.2.7. IN-GEL DIGESTION AND MALDI-TOF MASS SPECTROMTRY

The resolved protein bands were excised manually from the Coomassie Brilliant Blue G-250 stained gels and subjected to in-gel tryptic digestion with certain modifications (Hellman et al., 1995). Briefly, the gel bands were washed with 100 µL 40% (v/v) ethanol (10 min) and shrunk in 100 µL 100% (v/v) acetonitrile (ACN). The gel pieces were incubated in presence of 50 mM dithiotritol (DTT) at 56°C for 45 min, followed by 125 mM iodoacetamide (IAA) for 30 min at room temperature. The gel pieces were washed with 100 µL 50% (v/v) ACN for 10 min, shrunk in 100 µL 100% (v/v) ACN for 10 min and soaked in 3 µL 12.5 ng/µL trypsin (Promega, porcine sequencing grade) in 25 mM (w/v) NH_4HCO_3 on ice for 45 min. The gel pieces were rehydrated by addition of 10 µL 25 mM (w/v) NH_4HCO_3 followed by incubation at 37°C overnight. Tryptic peptides (1 µL) were loaded on to the AnchorChip target plate (Bruker-Daltonics, Bremen, Germany), covered by 1 µL matrix solution (0.5 µg/µL CHCA in 90% (v/v) ACN, 0.1% (v/v) TFA) and washed in 0.02% (v/v) TFA. Tryptic

peptides were analyzed by Ultraflex II MALDI-TOF mass spectrometer (Bruker-Daltonics, Bremen, Germany) using Flex Control v3.0 (Bruker-Daltonics, Bremen, Germany) and processed by Flex Analysis v3.0 (Bruker-Daltonics, Bremen, Germany). Peptide mass maps were acquired in positive ion reflector mode with 500 laser shots per spectrum. MS/MS data were acquired with an average of 1000−2000 laser shots for each spectrum. Spectra were externally calibrated using a tryptic digest of β-lactoglobulin (5 pmol/μL). Filtering of spectra was performed for known trypsin autolysis products and keratin peaks. MS and MS/MS spectra were analyzed using Biotools v3.1 (Bruker-Daltonics). MASCOT 2.0 software (http://www.matrixscience.com) was used for database searches in the NCBInr (National Center for Biotechnology Information) and Broad Institute for *Fusarium graminearum* gene index (http://www.broad.mit.edu/annotation/genome/fusarium_graminearum). The following search parameters were applied: Monoisotopic peptide mass accuracy of 80 ppm, fragment mass accuracy to $\pm$ 0.7 Da; a maximum of one missed cleavage; carbamidomethylation of cysteine and partial oxidation of methionine, respectively. No restrictions with respect to protein Mw and pI were made. The signal to noise threshold ratio (S/N) was set to 1:6. Protein identifications by PMF were confirmed with a MASCOT score of 70, $p \leq 0.05$ and a minimum of 4 matched peptides. A single peptide based protein identifications by MS/MS analysis was confirmed with a MASCOT score of 40, $p \leq 0.05$. Searches in the Broad Institute for *Fusarium graminearum* gene index, a MASCOT score above 62 was considered as a positive identification.

5.2.8. XYLANASE ACTIVITY ASSAYS

Xylanase activity was assayed toward 1% (w/v) wheat arabinoxylan (WAX, low viscosity, Megazyme). The amount of reducing sugar released by the reactions was quantified by the 3,5-dinitrosalicylic acid (DNS) method of Bailey et al. (1992). The substrate was prepared in 50 mM sodium acetate pH 5.0 and heated until clear in a microwave. The enzyme (5 mU xylanase, diluted to a total volume 40 μL) was incubated with the substrate solution (360 μL) at 40°C for 5 min. Each reaction

mixture was mixed with DNS solution (600 µL), heated to boiling point (95°C for 15 min), cooled and the absorbance was measured at 540 nm. Xylose was used as a standard. One unit of xylanase activity was defined as the amount of enzyme liberating 1 µmol of xylose per min under the assay condition. All measurements were performed in triplicate.

5.2.9. XYLANASE INHIBITION ASSAYS

rXIP-III was assayed for inhibition of xylanases from various sources, such as *A. niger* (GH11, Megazyme), *T. longibraciatum* (GH11, Megazyme), *A. nidulans* (recombinant xylanase of GH10 and GH11 by *P. pastoris*, clones obtained from Chris R. Somerville, Stanford University, USA, Bauer et al., 2006), washing liquids containing mixture of xylanolytic enzymes found on barley grain surface of different cultivars (details of preparation is described in Chapter 3), secretomes of grain-associated fungi grown on wheat arabinoxylan or barley flour (details of fungal growth, isolation and sample preparation are described in Chapter 4). Increasing amounts of rXIP-III (5-60 µg) were tested against 5 mU xylanases of *A. niger* or *T. longibraciatum*. Cross-inhibition by rXIP-III (25 µg) was assessed against of xylanases contained in washing liquids (10 µL) of different barley cultivars or xylanases contained in the secretomes of selected cereal fungi (5 mU).

The inhibitory activity was determined with wheat arabinoxylan (WAX, Megazyme) as substrate for xylanase using a modified reducing sugar analysis, DNS method (Bailey et al., 1992). Reaction mixtures (end volume of 50 µL) of suitably diluted enzymes in 50 mM sodium acetate (pH 5.0) and different amounts of purified rXIP-III (5−70 µg) were incubated for 1 h at room temperature. The substrate (1% w/v WAX, 360 µL) was equilibrated at 40°C for 10 min and the reaction were initiated with addition of the xylanase/rXIP-III mixtures (50 µL). After 5 min of incubation at 40°C, the reactions were terminated by addition of 3,5-dinitrosalicylic acid solution (600 µL) and boiled at 95°C for 15 min. After cooling, the reaction mixtures absorbance was measured at 540 nm against a blank composed of buffer and DNS reagent. For each reaction mixture, enzyme blanks were prepared without XIP-III. The experiment was carried out in triplicate and the results were presented as means and standard

deviations of percentage of XIP-III induced inhibition relative to the control (absence of inhibitor).

5.3. RESULTS AND DISCUSSION

5.3.1. CLONING AND EXPRESSION OF XIP-III

Barley XIP-III (BAJ88515) translated protein sequence has a leader peptide of 29 amino acids with a peptidase recognition site between the amino acids Ala29-Ala30 as predicted by the SignalP v4.1 server (Petersen et al., 2011). The calculated molecular mass and pI of the mature plant protein were determined to be 30132.84 Da and 7.35, respectively. The leader peptide was removed and a C-terminal His_6-tag included to the sequence prior cloning and transformation. After removal of the leader peptide, the XIP-III mature protein in *P. pichia*, after Kex2 and Ste13 processing of the N-terminal, is predicted to have a molecular mass of 31294.13 and pI 7.84. The sequence includes a potential glycosylation site at Asn^{288} (*N*-glycosylation site: NYS). The barley XIP-III harbors a glycosylation site close to the C-terminus (which is thought to interact with GH10 xylanases) than at the N-terminal as the other two isoforms, XIP-I and II, (Figure 31) (Payan et al., 2004). The XIP-III sequence includes five cysteine residues, four of which are involved in intra-molecular disulphide bonds (Figure 31).

The coding sequence of *xip-III* from barley was hence subcloned into the pPICZαA vector and transformed in *P. pastoris* strain KM71H. The positively transformed colonies were validated by PCR prior expression. The initial attempt in expression of rXIP resulted in loss of the His_6-tag, as no immunoreactive polypeptides were detected on the immunoblots probed with anti-penta His monoclonal antibodies (blots not shown). It has been reported that during growth of yeast cells, several *P. pastoris* proteases are secreted, which can influence the recombinant protein, e.g. due to removal of the His_6-tag. These proteases typically cleave at the C-terminal or at the linker region between the protein and tag. In order to prevent proteolysis, a home made cocktail of protease inhibitor, 1 mM PMSF (phenylmethylsulfonyl fluoride), 50 µM TLCK (Nα-Tosyl-L-lysine chloromethyl ketone) and 20 µg Pepstatin A (Sigma-Aldrich, Germany), was added to the induction media in order to minimize

proteolysis, which enabled subsequent purification of the recombinant protein with nickel affinity chromatography.

5.3.2. GEL ELECTROPHORESIS AND IMMUNOBLOTTING

After 96 h of induction with methanol, an active and functional rXIP-III was expressed and purified. Assessment of the purity and identity of the purified inhibitor protein was carried out by SDS-PAGE and western blotting. Under reducing conditions, the recombinant purified proteins appeared as a single band with a molecular mass of approximately 35−36 kDa with a minor intensity lower band of about 31 kDa (Figure 32 A). The calculated molecular mass of 31.29 kDa fit with the lower band and corresponds well to the recombinant protein produced with the C-t His_6-tag. The slight difference in molecular mass could be accounted to presence of a sugar residue at the predicted *N*-glycosylation site (Asn^{288}) or additional *O*-glycosylation. The identity of the prominent band as rXIP-III was verified by peptide mapping using MALDI-TOF MS with estimation of a molecular mass of 33,357 Da and 40% protein sequence coverage (MASCOT score 112; E-value: 0.00013). Deglycosylation with Endo H resulted in a decrease in molecular mass of 1.7 compared to the untreated rXIP-III, thus confirming a M8 type ($Man_8GlcNAc_2$) *N*-glycosylation of rXIP-III. The slightly higher molecule mass than expected can also be ascribed possibly to *O*-glycosylation.

The protein band visualized on the SDS-PAGE gel was confirmed as His_6-tagged rXIP-III, on a western blot probed with anti-penta-His antibodies (Figure 32 B). Similarly, an immuno-reactive polypeptide of a molecular mass of 33−34 kDa was detected in a western blot using antibodies raised against native XIP from wheat (Figure 32 C). On both immunoblots, in addition to the prominent band for rXIP-III, also observed on SDS-PAGE gel, a trace protein band of slightly lower molecular mass was visible, suggesting the presence of an unglycosylated form of the secreted rXIP-III.

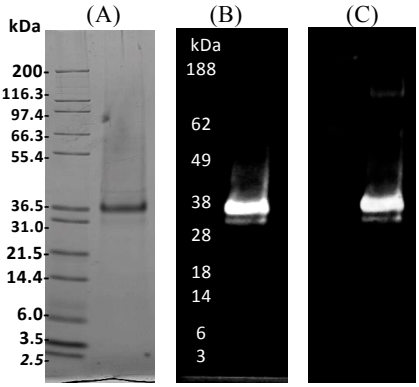


Figure 32: (a) Coomassie Blue stained SDS-PAGE of the purified His_6-tagged XIP-III. The recombinant proteins were purified by affinity chromatography using Ni-NTA resin. Lane 1: Mark12 protein ladder with bands in kDa; Lane 2: 10 µg of the eluted His_6-tagged rXIP-III. (b) Western blot probed with anti-Penta-His monoclonal antibodies. Lane 1: Prosieve colour protein ladder; Lane 2: 10 µg of the eluted His_6-tagged rXIP-III. (c) Western blot probed with anti-XIP polyclonal antibodies. Lane 1: Prosieve color protein ladder; Lane 2: 10 µg of the eluted His_6-tagged rXIP-III. The molecular masses (kDa) of the protein ladder are as indicated.

The native XIPs have been reported to be weakly glycosylated (Elliott et al., 2002), and in general *P. pastoris* is known to be capable of introducing a *N*- and *O*-type glycosylation to foreign proteins, albeit with differences in the glycosylation pattern compared to that of the original organism (Dionisio et al., 2011; Gemmill and Trimble, 1999). The minor degree of glycosylation of the native XIP-I (approximately 2%) has been reported to have a stabilizing effect on the protein (McLauchlan et al., 1999). This was evident in the unglycosylated *E. coli* produced rXIP-I, which was highly unstable and lost its activity soon after purification (Elliott et al., 2002).

5.3.3. INHIBITION SPECIFICITY

In planta, the XIP-type inhibitors exclusively inhibit xylanases of fungal origin (GH10 and 11), but no bacterial or the plant endogenous xylanases. This is also one of the reasons the inhibitors have been ascribed to be involved in the plants defense mechanisms, in particular upon fungal attack. A study by Igawa et al. (2005) demonstrated induced expression of the transcripts of *xip-I* by the pathogen *Erysiphe*

graminis, but not for *F. graminearum*. Thus indicating that the expression of *xip-I* is dependent on the pathogenic fungi.

To assess the specificity of the purified rXIP-III towards microbial xylanases, the inhibitory activity towards a range of xylanases of various sources were determined using the DNS method (Bailey et al., 1992). The expressed recombinant xylanases of *A. nidulans* (GH10 and 11) showed hydrolytic activity on WAX (data not shown), and were used for determining rXIP-III inhibition specificity. rXIP-III was shown to be active towards most of the tested fungal xylanases, including from *A. nidulans, A. niger* and *T. longibrachiatum* (5 mU). Figure 33 shows the measured residual activity of xylanases after incubation with 25 µg of rXIP-III. Xylanases of *T. longibrachiatum* seem to be more sensitive to inhibition by rXIP-III compared to the other two fungi.

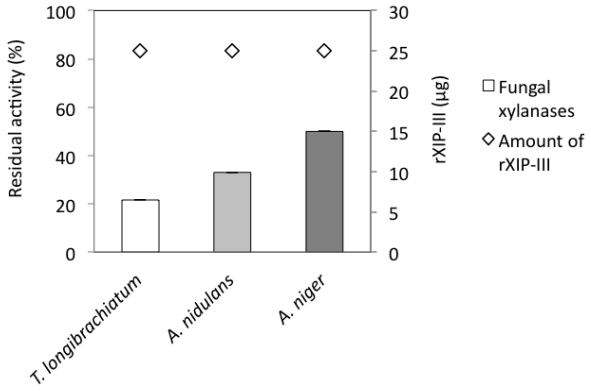


Figure 33: Residual xylanase activity after addition of recombinant rXIP-III (25 µg) to xylanases from *A. niger, A. nidulans* and *T. longibrachiatum* (5 mU). The xylanases activity levels were determined by the DNS method using 1% (w/v) WAX as substrate. *A. niger* and *T. longibrachiatum* xylanases were purchased from Megazyme, while *A. nidulans* was produced recombinant. Error bars represent standard deviations.

The xylanase inhibition as a function of rXIP-III concentration is shown in Figure 34. Increasing amounts of rXIP-III (5−70 µg) were tested against xylanases from *A. niger* and *A. nidulans* of GH11. The lowest amount of rXIP-III (5 µg) resulted in 20% inhibition of *A. niger* xylanases, while approximately 20−25 µg inhibited the enzyme by 50%. At the same amount of inhibitor (20 µg), the *A. nidulans* xylanase seems to be

more sensitive to the inhibitor than the *A. niger* xylanase (which is expressed by Megazyme as fusion protein with an unknown solubilization tag).

The native XIP-I from wheat has been reported to be more effective in its inhibition towards *A. nidulans* GH11 xylanases than *A. niger* GH11. This was also the case with the produced rXIP-III exhibiting similar inhibition pattern towards the same enzymes, as also verified for the wheat XIP-I by surface plasmon resonance studies (Flatman et al., 2002).

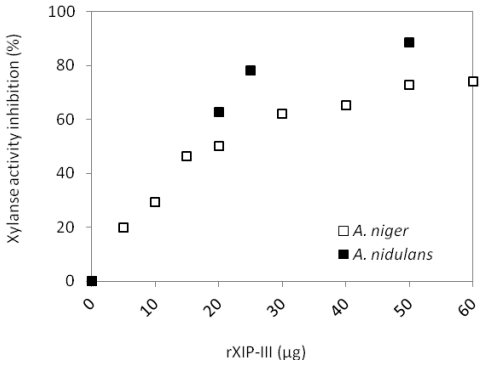


Figure 34: Inhibitory activity of the purified rXIP-III toward xylanases from *A. niger* (Megazyme) and *A. nidulans*. Inhibition of xylanase activity levels was determined after incubation with increasing amounts of rXIP-III by the DNS method using WAX as substrate. All measurements were performed in triplicates (error bars represent standard deviations).

With the aim of obtaining knowledge of the effect of rXIP-III on fungal xylanases, the rXIP-III was tested against xylanases produced by the populating commensal microbial community of barley grains. These samples were prepared by subjecting the barley grains to a washing procedure (Dornez et al., 2008) using 25 mM sodium acetate pH 5.0 (details in Chapter 3). After a washing period of 15 h, the barley grains were removed and the recovered washing liquids of different barley cultivars were used for the xylanase activity and inhibition assessment. Appropriate volumes of washing liquids of different barley cultivars were incubated with rXIP-III (20 µg) followed by determination of the residual xylanase activities (Figure 35).

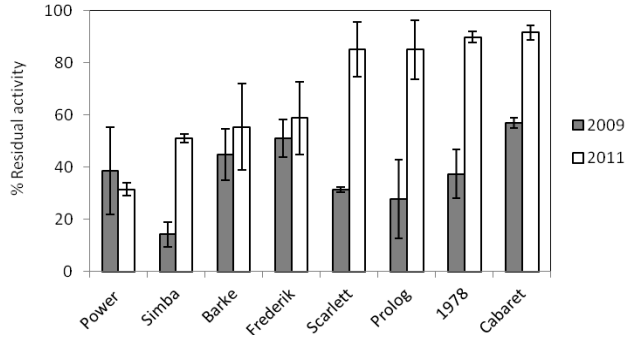


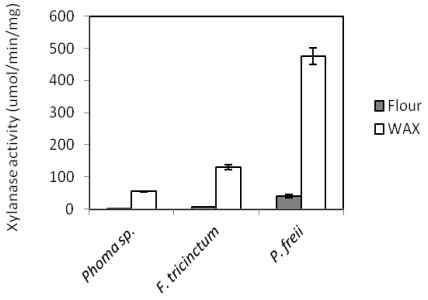
Figure 35: Residual xylanase activity after addition of recombinant rXIP-III (20 µg) towards xylanases present in washing liquids of different barley cultivars. Residual xylanases activity was determined using WAX as substrate. Different barley cultivars of harvest year 2009 and 2011 were subjected to a washing procedure after which the recovered washing liquids were used for assessment xylanase activity and inhibition.

Varying degrees of inhibition was observed upon addition of rXIP-III, in general, xylanases contained in washing liquids of barley harvest year 2009 were more sensitive to inhibition by rXIP-III, compared to year 2011. An amount of 20 µg of rXIP-III resulted in ≥ 50% inhibition of xylanases present in samples of different cultivars of harvest year 2009. Xylanases from the surface of Simba (2009) were strongly inhibited with 10−15% residual xylanase activity. Meanwhile, samples of harvest year 2011, particularly the cultivars Cabaret, Prolog, Scarlett and 1978, were inhibited with only 5−15% by rXIP-III. Xylanases of Barke, Frederik and Power were inhibited to the same extent independent of harvest year. These results suggest that the inhibitory specificity of rXIP-III greatly depends on the composition and types of xylanases/hydrolases present in the washing liquids.

Similarly, the inhibitory activity of rXIP-III was examined on xylanases contained in the secretomes of cereal-associated fungi. Isolated barley grain-associated fungi, both field (*F. tricinctum* and *Phoma* sp.) and storage fungi (*P. freii*), were grown on either WAX or barley flour with the aim of profiling their secretomes and assessing inhibition by rXIP-III. Varying levels of xylanase activity were determined for the cultivated fungi (Figure 36 A). In general, higher xylanase activity levels were

determined for fungi grown on WAX, than that on barley flour, in particular for *P. freii*.

(A)



(B)

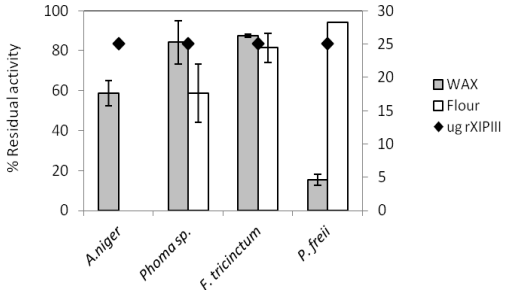


Figure 36: (A) Determination of xylanase activity levels present in the supernatant of secreted proteins from cereal-associated fungi (i.e. *F. trincinctum*, *P. freii* and *Phoma* sp.) grown on WAX or barley flour. Activity levels were quantified by the DNS method of Bailey et al. (1992) using WAX as substrate. (B) The effect of purified rXIP-III (25 µg) on xylanase activity levels of cereal fungi grown on WAX or barley flour. Residual xylanase activity after incubation with the purified inhibitor was determined by the DNS method using WAX as substrate. The analyzed fungi were isolated from barley grains in collaboration with Prof. Jens C. Frisvad (CMB, Systems Biology, DTU).

Incubation of the rXIP-II (25 µg) with the secretomes of the different fungi (ca. 5 mU of xylanase activity) resulted in varying degree of inhibition (Figure 36 B). A residual xylanase activity of 15% was determined for *P. freii* grown on WAX after incubation with rXIP-III, while growth on barley flour had a residual xylanase activity of 95%, thus indicating the presence of different xylanases with varying sensitivity towards

rXIP-III. *F. tricinctum* grown on either substrate exhibited the same level of sensitivity with a residual xylanase activity of 85−90%. The *F. tricinctum* xylanases seem to be relatively insensitive to inhibition by rXIP-III compared to that of *Phoma* sp. with residual activities of 55−70%. The varying degree of inhibition by rXIP-III demonstrates the occurrence of different xylanases in the tested secretomes, which are highly dependent on the fungal species and the utilized substrate for fungal growth.

The cross-inhibitory effect of the rXIP-III toward xylanases contained in the secretomes of the different fungi or the washing liquids of different cultivars should be interpreted as indicative, as these are composed of mixtures of hydrolases and not just xylanases. This was also evident from the meta-proteomics study of barley proteomes and the surface-associated fungi (Chapter 4), where the fungal secretomes were dominated by xylanases and endoglucanases. Moreover, a study by Sancho et al. (2003) reported cross-inhibitory activity by XIP against other hydrolases than xylanases, namely α-amylases (Sancho et al., 2003). However, further analysis is required to elucidate these inhibiting activities.

The xylanases in the secretomes of *F. tricinctum* and *P. freii* (barley flour) represent potential candidates for producing xylanases that are relatively resistant to XIP-III. However more research is required to unravel the presence and types of xylanases in the secretomes of the individual grain-associated fungi. From an application point of view, *Fusarium* species are known to produce mycotoxins, thus there is a requirement to determine the metabolite profile of the fungi of interest prior to any kind of application.

5.4. CONCLUSIONS

In the present study, a functional and stable recombinant XIP-III was produced in a *P. pastoris* system. The identity of the secreted protein with a molecular mass of 33.4 kDa was confirmed by gel electrophoresis and immunoblotting probed with anti-penta-His monoclonal antibodies and anti-XIP polyclonal antibodies. The secreted rXIP-III showed varying inhibition activities towards fungal xylanases of various sources, including samples containing xylanases removed from the surface of barley

grains and secretomes of surface-associated fungi grown on WAX or barley flour. Xylanases in the secretomes of *F. tricinctum* were relatively resistant to the rXIP-III, thus representing a potential source of xylanases insensitive to rXIP-III. With successful expression of rXIP-III protein, *P. pastoris* is proven as a suitable expression system for future large-scale production.

5.5. ACKNOWLEDGEMENTS

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CHAPTER 6

Conclusions

6. CONCLUDING REMARKS

The present study is part of a larger effort with the goal of providing detailed understanding of the cereal grain enzyme activities and providing new insight to their potential use for improvement of feed ("*Exploiting barley first wave enzyme activities for better feed*" embedded in the Food Research Program). The project is multi-disciplinary covering several aspects of barley as feed at protein and molecular level involving *in vitro* studies of protein degradation/digestion of liquid feed and the fate of application of amino acids (Christensen JB, Ph.D. student, Department of Animal Health Welfare and Nutrition, University of Aarhus), proteome analysis of liquid feed, screening for xylanolytic activities, purification and characterization of feed enzyme and protein candidates, i.e. endoprotease B2 (Rosenkilde A, Postdoc, Department of Molecular Biology and Genetics, Faculty of Agricultural Sciences, University of Aarhus) and XIP-III (Sultan A) and a portrait of the cereal grains-associated microbial community. The main focus of the present thesis has been on proteome analysis and characterization of barley combined with a portrait of the commensal cereal-associated fungal community and their secretomes.

The work presented in this thesis contributes with establishment of the proteomes of liquid feed over 48 h of steeping period using gel-based proteomics and comparative proteome analysis to identify the changes in the proteomes of liquid feed with application of protease inhibitors and the thioredoxin system (NTR/Trx). When using cereal-based diet as feed, the main concern has been the inefficient utilization of nitrogen from the protein by the monogastric animals and subsequent secretion of large amounts of nitrogen in the environment. To improve protein absorption, several strategies such as liquid feed with supplements have been applied. Small-scale production of liquid feed supplemented with protease inhibitors or NTR/Trx system was applied to track the changes of both the soluble and insoluble protein fractions. Applications of protease inhibitors were shown to some extent hamper protein degradation. Prominent protein changes were found upon application of the NTR/Trx system with increased solubility of various proteins, including CM-proteins and protease inhibitors. These findings show a glimpse of the diverse range of proteins targeted by Trxs. In addition, the different classes of xylanase inhibitors are also

potential targets of Trxs, e.g. TAXI (14 Cys), which was also found in the water-soluble protein fraction of liquid feed containing Trx, but with low sequence coverage). Application of the NTR/Trx system represents great potential in improvement of the potential of feed either directly or indirectly alteration of protein and enzyme activities.

A battery of barley cultivars of different harvest years and growing sites were screened for xylanase (microbial and endogenous) and xylanase inhibition activity levels using different activity assays combined proteome analysis and immunoblotting. Considerable inter-cultivar variability was found at microbial and endogenous xylanase, as well as xylanase inhibition levels. Harvest year was found to have a significant impact on the variability in all the three parameters. Nonetheless, a correlation was found for the two barley cultivars, namely 1978 and Power, which exhibited high xylanase inhibitory activity and low microbial xylanase activity levels. The same cultivars displayed an additional immune-reactive band in the 1D-immunoblots. Moreover, on the basis of the analysis of two barley cultivars grown at five different locations of two harvest years, growing site was found to be the main contributor to the variability in microbial and endogenous xylanase activities levels. Cultivar/genotype was found to be main determinant for the variability in inhibitory activity levels.

A metaproteomics approach was applied to characterize and map the surfomes of barley grains and to unravel the composition of the commensal fungal community populating the grain surface. A similar protein pattern was observed for the two barley cultivars with identification of proteins involved in plant defense and/or stress-related mechanism. From the comparison of microbial xylanase activity levels with the composition of fungal community, the high activity levels were linked to presence of the storage fungi, i.e. Aspergilli and Penicillia. Furthermore to gain a better understanding of the relation between barley and fungi, the secretomes of two cereal fungi, i.e. *F. poae* and *A. niger* grown on WAX and barley flour, were mapped and various xylanolytic, amylopectic and cell-wall degrading enzymes were identified. Along with secretion of an array protein and enzymes, fungi are known to produce metabolites that greatly influence the invading host and/of competitive outcomes. Some metabolites are toxic to humans and animals, while others can alter the growth

and metabolism of plants. Profiling the expressed metabolites of the isolated cereal-associated fungi and their linkage with the identified secretomes will contribute with new knowledge.

APPENDIX

Review: From protein catalogues towards targeted proteomics approaches in cereal grains

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Review

From protein catalogues towards targeted proteomics approaches in cereal grains

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ABSTRACT

Due to their importance for human nutrition, the protein content of cereal grains has been a subject of intense study for over a century and cereal grains were not surprisingly one of the earliest subjects for 2D-gel-based proteome analysis. Over the last two decades, countless cereal grain proteomes, mostly derived using 2D-gel based technologies, have been described and hundreds of proteins identified. However, very little is still known about post-translational modifications, subcellular proteomes, and protein–protein interactions in cereal grains. Development of techniques for improved extraction, separation and identification of proteins and peptides is facilitating functional proteomics and analysis of sub-proteomes from small amounts of starting material, such as seed tissues. The combination of proteomics with structural and functional analysis is increasingly applied to target subsets of proteins. These "next-generation" proteomics studies will vastly increase our depth of knowledge about the processes controlling cereal grain development, nutritional and processing characteristics.

Contents

1. Introduction

Cereal grains provide a large proportion of the food supply for the growing human population. Their composition has therefore been analysed at many levels, for identification of determinants of nutritional quality, resistance to pathogens and pests, and suitability for industrial processing. Over the past years, proteome analysis has been increasingly applied to the study of cereal grains. The aim of many proteomics studies in cereal grains is to provide

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; DIGE, difference gel electrophoresis; ESI, electrospray ionisation; GH, glycosyl hydrolase; GR, glutathione reductase; HMG, high mobility group; ICAT, isotope-coded affinity tag; LCM, laser capture microdissection; LC, liquid chromatography; MALDI, matrix associated laser desorption ionisation; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NTR, NADPH-dependent thioredoxin reductase; RBP, RNA-binding protein; RT-PCR, reverse transcriptase-polymerase chain reaction; TAXI, *Triticum aestivum* xylanase inhibitor; TLXI, thaumatin-like xylanase inhibitor; XIP, xylanase inhibitor protein.

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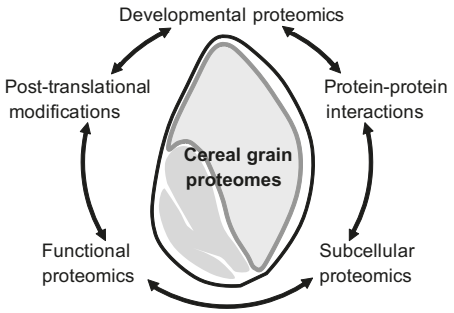

Fig. 1. Proteomics implies a temporal and spatial characterisation of proteins during development, together with post-translational modifications, interactions, structures and functions. Although we are still far from this goal, recent studies are reaching deeper into the cereal grain proteome.

knowledge that will facilitate improvement of crop quality, either in terms of resistance to biotic and abiotic stresses, or in terms of nutritional or processing quality (reviewed by Salekdeh and Komatsu, 2007). In the early days of proteome analysis, 1D and 2D-gel electrophoretic separations of protein extracts were used to characterise and classify barley cultivars (Weiss et al., 1991a,b; Görg et al., 1992a,b). With the advent of advanced protein mass spectrometry, it became possible to identify the proteins observed on the gels, and this, combined with the rapidly increasing amounts of sequence information in databases, led to a quantum leap in plant proteome analysis. A full proteome analysis now implies a temporal and spatial cataloguing of proteins, together with their post-translational modifications, interactions, structures and functions (Fig. 1). Proteome analysis has been applied extensively for analysis of cereal grain proteins, especially in rice, wheat and barley (Agrawal and Rakwal, 2006; Skylas et al., 2005; Finnie and Svensson, 2009) but also maize (Méchin et al., 2004, 2007). Many studies have provided catalogues of proteins (Østergaard et al., 2004; Méchin et al., 2004), an overview of temporal changes in protein abundances during seed development and germination (Østergaard et al., 2002; Bak-Jensen et al., 2004; Finnie et al., 2002, 2006; Méchin et al., 2007), a comparison of the proteomes of different seed tissues (Finnie and Svensson, 2003; Bønsager et al., 2007) or an analysis of changes induced by stresses such as heat (Skylas et al., 2002; Hurkman et al., 2009; Laino et al., 2010) salt (Witzel et al., 2010) or pathogen attack (Zhou et al., 2006; Yang et al., 2010). Cereal grains pose challenges for extraction and analysis of proteins due to the high storage protein and starch content, which may have limited the progress towards more in-depth analyses. In addition, many proteomic studies do not go beyond the mass spectrometric identification of proteins of interest, in which case no functional information about the identified proteins is obtained. It is also apparent when comparing the lists of identified proteins from the different studies, that there is a high degree of overlap. This strongly suggests that new and additional approaches are needed, both to reach deeper into cereal grain proteomes and to obtain functional information about the proteins identified. Fractionation and enrichment techniques decrease sample complexity and enable analysis of lower-abundance proteins. Such techniques can be based on spatial fractionation (e.g. by tissue or organelle isolation) or functional fractionation (exploitation of specific structural or functional properties of a subset of proteins). The workflow for a targeted proteomics study is thus similar to that for a classical proteomics experiment, requiring techniques for tissue isolation, protein extraction, separation and identification, however will be specifically tailored to the subproteome of interest (Fig. 2). In order to achieve this, prior knowledge of the proteins of interest is often required such as solubility characteristics, cellular location or binding specificity. Thus, classical proteome studies provide important data on which to base targeted analyses. Combining proteome analysis with other protein chemical techniques such as immunofluorescence microscopy, activity assays, production of recombinant proteins and protein structural analysis can also be a powerful approach for analysis of selected proteins or groups of proteins. Employing protein/protein interaction studies may provide insight into the interactome of seed proteins and therefore, can supplement other functional proteomics approaches. This review will focus on recent studies that lead in different ways towards a more in-depth view of cereal grain proteomes (Fig. 3).

2. Spatial sub-proteomes of cereal grains

Grains from different cereals share many structural and developmental characteristics, and the grain structure is central to its biological function as well as processing quality (Evers and Millar, 2002). The cereal grain at maturity consists of the embryo and endosperm tissues, where the endosperm can further be subdivided into the starchy endosperm and aleurone layer. The embryo, which results from the fusion of pollen and egg cells, consists of the shoot, mesocotyl, radicle and scutellum. The scutellum provides contact with the starchy endosperm and has an important roles in nutrient uptake and hormone signalling during germination. The starchy endosperm comprises the major part of the seed and contains most of the storage proteins, starch granules and other polysaccharides that will fuel the growing seedling until autotrophy. Starchy endosperm cells undergo cell death during seed maturation and desiccation, and are no longer living tissue in the mature seed. Surrounding the starchy endosperm is the aleurone layer, which in contrast gains desiccation tolerance during seed maturation and thus survives as a living tissue in the mature seed. The major role of the aleurone layer during germination is to synthesise and secrete hydrolytic enzymes for degradation of storage products in the starchy endosperm. This is triggered by uptake of water by the seed and gibberellic acid produced by the embryo. Surrounding the aleurone layer are several outer layers including the nucellar tissue, the testa (or seed coat), the pericarp comprising tube cells and cross cells, and the outer layer consisting of epidermis and hypodermis (Evers and Millar, 2002). The molecular composition of the aleurone layer and outer layers are of interest due to their high content of vitamins and minerals (Hemery et al., 2007).

2.1. Microdissection of seed tissues

Cereal seeds are complex organs in which the different tissues have different developmental programs and different functions during seed development and germination. Analysis of whole seed proteomes, therefore, has limitations since proteins are not evenly distributed throughout the seed and the protein profile is the averaged result of contributions from different tissues. Dissection of barley seeds to separate starchy endosperm, embryo and aleurone layers (Finnie and Svensson, 2003; Bønsager et al., 2007) gave a clear illustration that the proteomes of seed tissues reflects their differing functions. However, the proteomes at this level are still an averaged contribution from different cell types. Manual microdissection of the bran fraction from wheat kernels into outer layers (containing epidermis and hypodermis), intermediate layers (containing cross cells, tube cells, testa and nucellar tissue) and inner layer (aleurone) demonstrated a distinct profile of defence-related proteins in each layer to provide protection for the nutrient-rich embryo and starchy endosperm (Jerkovic et al., 2010). The

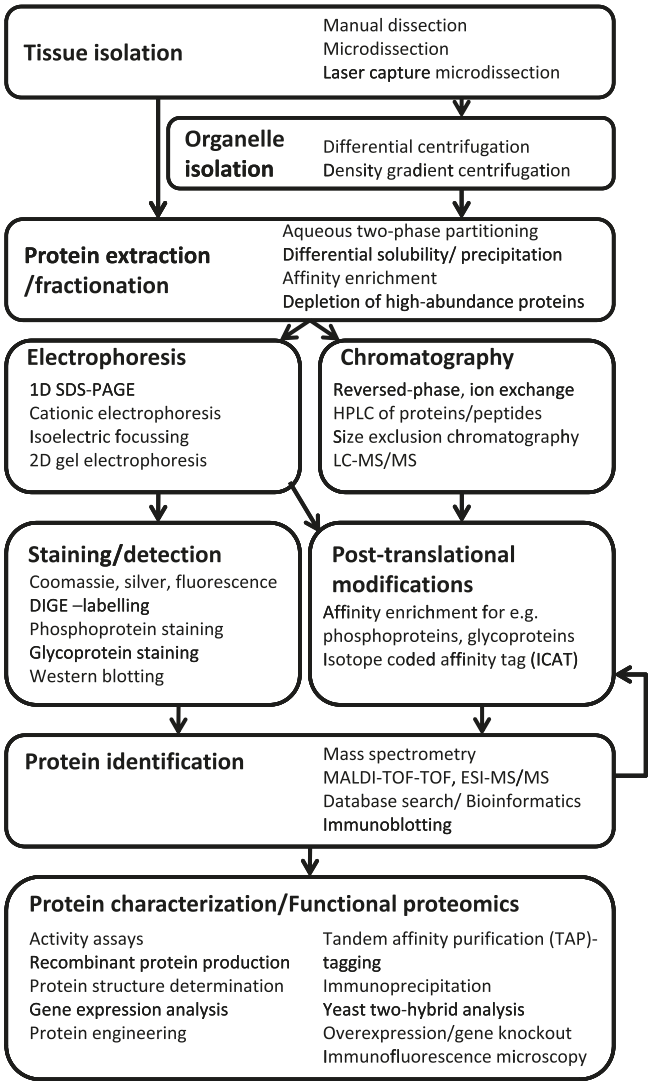

Fig. 2. Experimental proteomics workflow. The classical proteomics workflow is adapted towards a targeted analysis of spatial or functional subproteomes. A selection of techniques are indicated, many of which have been used in the studies discussed in this review.

differential localisation of the pathogenesis-related protein PR-4 in aleurone layer, xylanase inhibitor protein XIP-1 in the intermediate fraction, and oxalate oxidase in the outer layers was validated by fluorescence immunolocalisation (Jerkovic et al., 2010).

Seed tissues contain highly specialised cell-types which are not amenable to manual dissection, and analysis of these specialised proteomes would be highly informative. Laser-capture microdissection (LCM) is a highly promising technique in this respect, since specific areas can be excised and collected from tissue sections. The technique has been more widely used in combination with gene expression analysis, since in this case the small amount of material for analysis can be boosted by PCR amplification. In cereal grains, LCM has been applied to analyse transcripts from embryo, aleurone layer and starchy endosperm potentially involved in zinc trafficking (Tauris et al., 2009) and to analyse transcript profiles from endosperm transfer cells and nucellar projection (Thiel et al., 2008). Recently, the analysis of endosperm transfer cells and nucellar projection was extended to include identification and quantification of proteins (Kaspers et al., 2010) in one of the very few studies so far to apply proteomics to analysis of LCM-dissected plant tissue. Proteins were extracted and digested with trypsin prior to analysis by multiplexed LC–MS. This required material from between 40 and 75 tissue sections per sample, corresponding to a tissue area of 2,500,000–4,500,000 μm^2. Three independent extractions showed highly reproducible chromatograms. The analysis resulted in reliable identification of 137 proteins from the endosperm transfer cells and 44 from the nucellar projection. Thirty-one proteins were identified in both tissues. Absolute

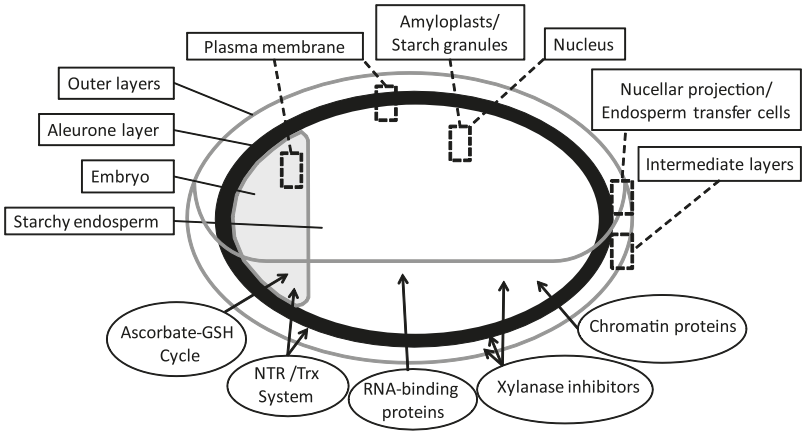

Fig. 3. Current knowledge of cereal grain subproteomes. A highly simplified cereal grain is shown with the major seed tissues represented. Boxes indicate spatial subproteomes and ovals indicate functional subproteomes.

quantification of these proteins in the two tissues was achieved based on the mean intensities of the three most abundant peptides of the protein per injection, relative to 100 fmol of yeast enolase, added as an internal standard. The analysis demonstrated that these tissues differ greatly in their proteome, and demonstrated the huge potential of LCM for proteome analysis of tissues and cells that are beyond the reach of manual isolation.

2.2. Subcellular proteomes – Membrane proteins

Analysis of membrane proteins is complicated by their low abundance and low solubility. Combined with the challenges of extracting proteins from small amounts of seed tissues, this means that there are so far very few published studies of seed membrane proteomes. However, membrane proteins are of great importance during seed development and germination. In particular, the plasma membrane is expected to have an important role in signalling, communication and uptake of nutrients. Plasma membrane-enriched fractions were prepared from seed tissues by aqueous two-phase partitioning. Using a combination of reversed-phase chromatography, SDS–PAGE and LC–MS/MS, integral membrane proteins were identified from plasma-membrane-enriched fractions prepared from barley aleurone layers (Hynek et al., 2007) and germinated embryos (Hynek et al., 2009). Due to the limited amount of material available for analysis, it is likely that those proteins identified were among the most abundant. Despite this, several of them were not homologous to proteins of known function, illustrating that our knowledge of the plant plasma membrane is still very limited. Although a comparative analysis of the plasma membrane proteomes from different seed tissues or cell types remains to be carried out, it is expected that they will vary at least as much as the soluble (mainly cytoplasmic) protein fractions that have been analysed to date.

2.3. Subcellular proteomes – Starch granules/amyloplasts

Starch, composed of amylose (linear α(1–4) D-glucan) and amylopectin (branched α(1–4) and α(1–6) D-glucan), is the major storage product and main component of cereal seeds, and is synthesised by a series of enzymes in the amyloplasts of the starchy endosperm and stored in the form of starch granules. Analysis of the proteome of isolated starch granules requires careful washing procedures to remove weakly- and non-specifically associated proteins. This has so far been achieved in barley (Borén et al., 2004) maize (Grimaud et al., 2008) and wheat (Bancel et al., 2010). Multiple forms of starch synthetic enzymes are observed, which were demonstrated to be in the interior of the granule by proteolytic shaving of the granules, whereas other proteins such as storage proteins and proteins with defence-related functions were located on the granule surface (Borén et al., 2004). In a recent study in which water-washed starch granules were treated with trypsin to release peptides from surface-associated proteins (Wall et al., 2010), several peptides originating from plant pathogens were identified in addition to plant defence-related proteins. Maize mutants affected in starch biosynthesis demonstrated possible protein–protein interactions since lack of certain biosynthetic enzymes had pleiotropic effects on the level of other specific proteins in the starch granules (Grimaud et al., 2008). Evidence for phosphorylation of the granule-bound starch synthase, branching enzyme IIb and starch phosphorylase was also obtained by Pro-Q Diamond-staining (Grimaud et al., 2008; Bancel et al., 2010).

Several studies of the amyloplast proteome have demonstrated that amyloplasts have a metabolic capacity beyond starch synthesis (Andon et al., 2002; Balmer et al., 2006a; DuPont, 2008) and also contain a functional ferredoxin/thioredoxin regulatory system analogous to that found in chloroplasts (Balmer et al., 2006b). However, isolation of amyloplasts from cereal grains is challenging and limited to a small developmental window where sufficient starchy endosperm can be isolated, but prior to the onset of extensive storage protein and starch synthesis (Dupont, 2008), making proteomic studies of amyloplast development and function highly challenging using current techniques.

2.4. Subcellular proteomes – Nuclear proteomes

Detailed knowledge about the nuclear proteome (and changes in protein modifications) during cereal seed maturation is highly desirable to aid in a better understanding of seed development. Towards that goal, both global proteome studies and specific targeted approaches addressing a distinct subset of proteins may provide valuable information. There are promising examples illustrating the power of these experimental approaches for studying plant nuclear and chromosomal proteins (see also Section 3.2). A wide range of nuclear proteins were examined in a global analysis of

chromatin-associated proteins from rice suspension cultured cells (Tan et al., 2007), while many details about post-translational modifications (and the possible combinations of them) were clarified by an in-depth study of *Arabidopsis* histone H3 (Johnson et al., 2004). However, a major problem studying nuclear proteomes in cereal seed tissue is the abundance of storage products (i.e. starch, storage proteins), especially when low-abundant proteins are analysed. Using rice endosperm as model, Peng and colleagues studied the nuclear proteome and developed a method of removing starch grains by cotton filtration and abundant storage proteins by excision from SDS–PAGE gels followed by recovery of the remaining proteins by phenol extraction from the gel. This approach allowed identification of over 450 proteins including those of lower abundance such as transcription factors and histone modifying proteins (Li et al., 2008). Therefore, one can expect that in the coming years proteomic studies will provide important insight into cereal seed development and its regulation.

3. Functional sub-proteomes of cereal grains

3.1. Xylanase inhibitors

The xylanase inhibitors in cereal grains provide a good illustration of the targeted use of proteomics for understanding complex functional groups of proteins. Cereal grains have evolved groups of proteinaceous xylanase inhibitors which have a strong impact on the xylanases regularly used in cereal processing applications such as baking. In cereals, three structurally distinct classes of proteinaceous xylanase inhibitors have been identified: *Triticum aestivum* xylanase inhibitor (TAXI), xylanase inhibitor protein (XIP), and thaumatin-like xylanase inhibitor (TLXI) (Beaugrand et al., 2006; Gebruers et al., 2004; Juge, 2006). TAXI, XIP or TLXI-type inhibitors have been detected in whole meals of cereals by immunoblotting (Beaugrand et al., 2006). In addition, TAXI- and XIP-type xylanase inhibitors have been purified from several cereals including wheat (Gebruers et al., 2002a).

The three distinct types of inhibitors are basic proteins and are active against glycoside hydrolase (GH) family 10 and 11 xylanases with varying specificities. XIP-type inhibitors are specific for fungal xylanases belonging to GH family 10 and 11 (Payan et al., 2003), whereas TAXI and TLXI-type exclusively inhibit bacterial and fungal xylanases (GH11) (Fierens et al., 2007; Gebruers et al., 2004). The three-dimensional structures of TAXI, XIP and recently TLXI-type inhibitors extracted from wheat have been determined by X-ray crystallography analysis and NMR (Payan et al., 2003; Sansen et al., 2004; Vandermarliere et al., 2010). In addition, the structures of TAXI-I and XIP-I in complex with fungal xylanases have also been resolved and key residues involved in the interaction between the inhibitors and target proteins determined (Juge, 2006). Various roles have been ascribed to these inhibitors including involvement in development, germination and defence against pathogens (Croes et al., 2008; Gebruers et al., 2004). Since they show almost exclusive activity towards microbial xylanases (*in vitro*) and are structurally homologous to many defence-related proteins, it is likely that xylanase inhibitors form part of the plant's defence mechanisms (Flatman et al., 2002; Gebruers et al., 2001; Goesaert et al., 2004). In cereals, TAXI- and XIP-type xylanase inhibitors widely occur as multi-isoform families. Transciptomic studies revealed expression of multiple putative TAXI- and XIP-type genes and one TLXI gene in wheat grains (Igawa et al., 2004, 2005). The presence of TAXI and XIP proteins in wheat as polymorphic families was also demonstrated by comprehensive proteomics analyses using 2-DE and subsequent MALDI-TOF tandem MS analysis (Croes et al., 2008). In addition, a 2D-DIGE approach coupled with LC-ESI tandem MS in extracts from six different wheat cultivars enabled differentiation between TAXI, XIP and TLXI inhibitor isoforms and quantified their variation among the different cultivars. This study led to identification of a total 48 xylanase inhibitor protein forms, including 18 TAXI, 27 XIP, and 3 TLXI-type xylanase inhibitors (Croes et al., 2009a). This high heterogeneity was ascribed to both the presence of multiple *taxi* and *xip* genes and to post-translational modifications, e.g. glycosylation (Croes et al., 2009a), and is most easily characterised using 2-DE-based proteomics. Transcriptome analysis suggested that the expression of some *taxi* and *xip* gene family members were induced in spikelets and leaves infected with fungal pathogens, i.e. *Fusarium graminearum* and *Blumeria graminis*, respectively, while others were unresponsive (Igawa et al., 2004, 2005). Thus, the different forms of these inhibitors may be induced by different pathogens or other stress conditions, and are tissue specific. However, little is known about how and when the different xylanase inhibitor isoforms are regulated at various stages of seed growth and development.

Xylanase inhibition activity assays combined with immunoblotting on extracts from wheat kernels demonstrate considerable variation in inhibitor levels among different wheat cultivars (Beaugrand et al., 2006; Croes et al., 2009b). Inhibition activity measurements of 19 different European wheat cultivars revealed that the inhibitor levels in the different cultivars varied with a factor of 2–3, due to genetic and/or environmental effect (Gebruers et al., 2002b).

All three types of inhibitors display increased affinity for arabinoxylans (Fierens et al., 2009). This indicate that the inhibitors are found in or close proximity of the cell wall where they bind to the substrate which they protect from cell wall degrading enzymes. Analysis of wheat milling fractions revealed that the three types of inhibitors are highly abundant in the outer layers of wheat kernels, in particular in the caryopsis. Gebruers et al. (2002b) reported 2–3 folds higher inhibition activities in wheat shorts and bran fractions than in flour. A study by Croes et al. (2009b) detected that the highest concentrations of these inhibitors occur in the aleurone layer. Recently, the spatial distribution of TAXI and XIP in wheat grains was demonstrated to be predominantly found in nucellar tissue adjacent to the aleurone tissue by using proteomics and immunofluorescence microscopy (Jerkovic et al., 2010). Thus, proteomics in combination with classical protein chemistry, immunochemistry and activity measurements is providing a detailed picture of a complex protein family.

3.2. Chromosomal proteins

In the genetic reprogramming during seed development, chromosomal proteins are thought to play important roles by altering the accessibility of genes for regulatory factors in the chromatin context. Different abundant chromosomal proteins influence chromatin structure and dynamics including (i) post-translational modifications of nucleosomal core histones and exchange of core histones with non-canonical variants by histone replacement (Fuchs et al., 2006; March-Díaz and Reyes, 2009), and (ii) the presence and post-translational modification of proteins associated with the internucleosomal linker DNA and nucleosomes such as linker histone H1 and high mobility group (HMG) proteins (Grasser, 2003; Jerzmanowski, 2004). The role of core histone modifications on gene expression was studied in detail in maize endosperm development, revealing that upon gene activation along with DNA demethylation various histone H3 modifications change. Thus, a reduction in repressive modifications such as H3K9me2 and H3K27me2 was paralleled by elevated levels of H3K4me2/3, H3K14ac and H3K9ac that promote gene transcription (Locatelli et al., 2009).

Plants typically have several genes coding for linker histone H1 variants (e.g. six in tobacco, six in maize, three in *Arabidopsis*), and

the encoded proteins have a three-domain structure typical of higher eukaryotes. Linker histones are important determinants of chromatin properties and can confer a more condensed chromatin structure (Jerzmanowski, 2004). In *Arabidopsis* and tobacco, altered levels of linker histone variants could influence certain plant developmental programs underscoring the importance of these general chromatin proteins for plant development (Prymakowska-Bosak et al., 1996, 1999). Analysis of the linker histone levels during maize kernel development demonstrated that their overall levels are relatively constant (Zhao and Grafi, 2000; Kalamajka et al., 2010). However, when linker histones isolated before and after onset of grain filling were analysed by 2D-gel electrophoresis followed by identification of protein spots using MALDI-TOF MS, a massive shift from acidic to more basic protein forms was observed (Kalamajka et al., 2010). This suggests a reduction in the level of post-translational modifications during kernel development. Interestingly, linker histones isolated from kernels after onset of grain filling displayed a lower affinity for double-stranded DNA immobilized on cellulose, when compared with the proteins isolated before onset of grain filling (Kalamajka et al., 2010). Therefore, the affinity for DNA (and presumably chromatin) appears to be regulated by post-translational modification(s). The difference in DNA/chromatin binding may result in a more open chromatin structure during storage synthesis, facilitating the massive transcription of storage-related genes.

In plants, HMG proteins of the HMGA and the HMGB families have been identified. Depending on the species, plants have 1 or 2 *HMGA* genes, while they encode around 6 HMGB proteins. HMGA proteins contain four AT-hook DNA-binding motifs and a plant-specific N-terminal domain with similarity to linker histones, while the HMGB proteins have a distinctive HMG-box DNA-binding domain. HMG proteins as architectural factors are involved in the regulation of various nuclear processes including transcription (Grasser, 2003). Altered levels of both HMGA and HMGB can cause developmental abnormalities in higher eukaryotes indicating that these chromosomal proteins have important cellular roles (Grasser, 2003; Pedersen and Grasser, 2010). HMG proteins (like linker histones) are soluble in dilute acids and can be efficiently extracted from plant tissue for biochemical studies using 2% TCA or 5% PCA, which removes the majority of other proteins and facilitates the analysis of HMG proteins and linker histones (Grasser et al., 1991, 1996). Immunoblot analyses of acid-extracted proteins showed that the expression of maize HMGA is significantly upregulated during kernel development (Zhao and Grafi, 2000). Since *in vitro* transcription assays revealed that HMGA could relieve the inhibitory effect of linker histone H1 on transcription, the authors suggest that HMGA is involved in transcriptional activation during maize endosperm development (Zhao and Grafi, 2000), perhaps in combination with the above-mentioned reduced affinity of linker histones for DNA/chromatin (Kalamajka et al., 2010).

HMGB proteins were biochemically characterised from maize revealing that the different family members are expressed at different levels. HMGB3 is the most abundant HMGB protein in kernels, while HMGB1 is most abundant in leaves, and HMGB4 is clearly least abundant in all tested tissues. Determination of the masses of the HMGB proteins isolated from kernels by MALDI-TOF MS indicated that the HMGB proteins are post-translationally modified (Stemmer et al., 1999). Purified HMGB proteins of maize suspension cultured cells were examined in more detail for modifications. Mass spectrometric analyses of tryptic digests demonstrated that serine residues within the acidic C-terminal domains were phosphorylated. Since the same sites could be phosphorylated by recombinant maize protein kinase CK2 *in vitro*, it is very likely that this protein kinase modifies HMGB proteins *in vivo* (Stemmer et al., 2002). CK2-mediated phosphorylation increased the thermal stability of the HMGB proteins (presumably by facilitating intramolecular interactions) and it reduced the affinity of the phosphorylated proteins for linear DNA substrates (Stemmer et al., 2002). Mass spectrometric analysis of the post-translational modifications of the HMGB proteins from maize kernels revealed (i) that the majority of HMGB1 and HMGB2/3 proteins were phosphorylated and (ii) that compared to suspension cells a larger number of differentially phosphorylated protein species occurred. This also indicated that a protein kinase other than CK2 may be involved in the modification of maize HMGB proteins (Stemmer et al., 2003). Since CK2 could phosphorylate also several *Arabidopsis* HMGB proteins, it appears that CK2-mediated phosphorylation is a general mechanism of regulating plant HMGB proteins (Stemmer et al., 2003). Altered levels of HMG proteins during seed development in combination with their post-translational modifications may be involved in establishing the transcriptional changes required for developmental reprogramming.

3.3. RNA-binding proteins

RNA-binding proteins (RBPs) have important roles, both in the nucleus for chromosome remodelling, rRNA- and mRNA-processing and the cytoplasm where they are involved in RNA transport, localisation, storage and metabolism. However, very little is known about RBPs in plants. Mature dry seeds contain stored mRNAs that are thought to be used as templates for transcription in the early stages of germination since germination does not require *de novo* transcription (Rajjou et al., 2004). In a study of mature rice grains, nucleic acid-binding proteins were isolated by binding to a single stranded DNA-cellulose affinity matrix (Masaki et al., 2008) and nine different RBPs belonging to six different RBP families were identified from 2D-gels. When the grains were imbibed in water for 48 h, a putative RBP-containing spot and a glycine-rich RBP decreased in intensity, while another containing a different RBP remained unchanged. When the seeds were imbibed in abscisic acid to suppress germination, the disappearance of the putative- and glycine-rich RBPs was also suppressed, strongly implying a role for these proteins in controlling germination (Masaki et al., 2008).

A cytoskeletal-bound RBP is suggested to be important for transport and localisation of storage protein mRNAs in developing rice endosperm (Wang et al., 2008). In order to expand the catalogue of plant RBPs, a cytoskeleton-enriched fraction was prepared from developing rice grains and applied to a polyU-Sepharose affinity column (Doroshenk et al., 2009). A number of nucleic acid binding proteins were identified from 2D-gels, including several involved in RNA metabolism and protein translation, metabolism and transport, and many proteins of unknown function, illustrating that much research into plant RBPs is still needed. Proteins identified from various studies are available in the Rice RBP database http://www.bioinformatics2.wsu.edu/RiceRBP.

3.4. Redox-regulation, thioredoxin and the ascorbate–glutathione cycle

Redox regulation is of great importance during seed development and germination. It is suggested that during germination, disulphide bonds in seed proteins become progressively more reduced (Marx et al., 2003) by the cytosolic protein disulphide reductase thioredoxin h (Trxh). The activity of thioredoxin has been exploited to develop proteomics-based methods for identification of Trx-reduced proteins in complex extracts. These techniques are either based on labelling of target proteins via the thiol groups released by Trx treatment (Yano et al., 2001; Maeda et al., 2004), or on affinity to immobilised Trx mutants in which one of the catalytic cysteines is mutated to serine, resulting in stabilisation of

an intermolecular disulphide (Wong et al., 2004). A large number of such targeted proteomics studies in cereals and other plants has resulted in identification of several hundred proteins potentially reduced by Trx (reviewed in Montrichard et al., 2009). A major challenge is to validate these potential Trx targets *in* vivo, and to determine the molecular basis for the interaction of Trx with so many diverse proteins. In barley, a thioredoxin system consisting of two Trxh and two NADPH-dependent thioredoxin reductases (NTR) has been characterised in grain proteomes and at the structural and functional level (reviewed in Shahpiri et al., 2009) including the first three dimensional structure of a complex between Trx and a target protein, the barley α-amylase/subtilisin inhibitor (Maeda et al., 2006), structures of both barley Trxh proteins and one NTR (Maeda et al., 2008; Kirkensgaard et al., 2009). An isotope-coded affinity tag (ICAT)-based method for quantification of peptide reduction by Trx has also been developed and applied to proteins from barley embryo and aleurone layer (Hägglund et al., 2008, 2010). This enables identification of the cysteine residues that are effectively reduced, as well as those not reduced by Trx. Together with the unique structural information available for the barley Trx system, this information has great potential to advance our understanding of interactions and specificity between Trx and its target proteins in cereal seeds.

Ascorbate–glutathione cycle enzymes ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) are among those that have been suggested as targets of Trx reduction (Montrichard et al., 2009). Analysis of proteomes during germination and seedling growth (Bønsager et al., 2007) showed that three ascorbate–glutathione cycle enzymes identified in barley seed proteomes APX, DHAR, and glutathione reductase (GR) displayed differing appearance profiles in barley embryos. Activities of APX, DHAR, GR and the fourth enzyme of the cycle, monodehydroascorbate reductase were measured in the protein extracts (Bønsager et al., 2010). By combining these measurements with 2D-gel electrophoresis and western blotting, apparent protein abundances and activities were compared. A complex array of APX forms arising from two gene products was observed. The results clearly illustrated that enzyme activity cannot necessarily be inferred from appearance of 2D-gel spots. While an increase in protein spot intensity was consistent with increased APX activity, contrasting activity and 2D-gel spot appearance suggested possible post-translational regulation of DHAR activity. Whether this is achieved by the Trx-mediated reduction of the active site cysteine residue that was suggested by ICAT-based redox proteomics (Hägglund et al., 2008) remains to be investigated.

3.5. Post-translational modifications

Analysis of post-translational modifications, including phosphorylation, typically requires strategies for enrichment of modified peptides, due to their low abundance and often non-stoichiometric modification of proteins. Phosphorylation is the most extensively studied post-translational modification in proteomics, due to its ubiquitous regulatory importance. However, to date no studies have been directed towards systematic identification of post-translational regulations in cereal seeds. In order to determine whether phosphorylation events were involved in seed germination in cereals, an LC–MS/MS-based analysis was carried out in extracts from germinating maize embryos (Lu et al., 2008). Protein extracts were digested with trypsin and fractionated by strong cation exchange chromatography prior to analysis by LC–MS/MS. Among the proteins identified were several protein kinases and phosphatases. Quantitative real-time RT-PCR demonstrated that transcripts encoding 10 of the kinases increased during seed germination (Lu et al., 2008). Although enrichment for phosphopeptides was not performed in this study, 36 phosphorylation sites in 33 proteins involved in DNA repair, RNA splicing, transcription and protein synthesis and folding were suggested by examination of MS/MS spectra (Lu et al., 2008).

Methylation and acetylation of histones comprise part of the "histone code" regulating chromatin status and gene expression (see Section 3.2). In an examination of MS/MS spectra obtained from analysis of the rice endosperm nuclear proteome (Li et al., 2008), 59 proteins with putative acetylations and 40 with putative methylations on lysine or arginine residues were identified.

It is clear from these studies that targeted enrichment for cereal grain proteins carrying post-translational modifications would be of great value.

4. Concluding remarks

Proteomics has been used successfully to catalogue the major proteins in cereal grains, and to address changes in grain proteomes during development, germination, and in response to stresses. Such studies, apart from providing fundamental knowledge also provide the basis for improvement of cereal resistance to biotic and abiotic stresses, for industrial applications such as malting and baking, as well as quality of grains and flour for human and animal nutrition. An increasing number of studies are targeting subcellular compartments or functional protein subsets, going deeper into the proteome and beyond protein identification lists (Fig. 3). This requires a wide diversity of approaches for fractionation of grain extracts and enrichment for the subproteome of interest. Proteomics techniques are applied in combination with a variety of other approaches to obtain data on protein activities, structures and functions. Although some highly challenging proteomes such as plasma membranes and organelles are being tackled in cereal grains, there are some areas in which knowledge is scarce. For example, post-translational modifications and protein–protein interactions have not yet been characterised in a systematic manner in cereal grains, although these aspects are certainly of major importance in seed development, germination and determination of grain quality. It is likely that future targeted studies will be highly fruitful.

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