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original scientific paper

Bioactivity of Cod and Chicken Protein Hydrolysates before and after *in vitro* Gastrointestinal Digestion

Running title: Bioactivity of Cod and Chicken Protein Hydrolysates

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Summary

Bioactivity of cod (*Gadus morhua*) and chicken (*Gallus domesticus*) protein hydrolysates before and after *in vitro* gastrointestinal digestion (GI) was investigated using yeast *Saccharomyces cerevisiae* as a model organism. Both hydrolysates were exposed to *in vitro* GI digestion prior to cellular exposure to simulate digestion conditions in the human body and therefore investigate the role of modulations in the GI tract

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on the cell response. The effect of non-digested and digested hydrolysates was investigated on intracellular oxidation, cellular metabolic energy and proteome. No difference in antioxidant activity was obtained between cod and chicken hydrolysates, while higher antioxidant activity was provided by digested hydrolysates with values of intracellular oxidation for cod $70.2 \% \pm 0.8 \%$ and chicken $74.5 \% \pm 1.4 \%$ compared to non-digested ones, where values for cod and chicken were $95.5 \% \pm 1.2 \%$ and $90.5 \% \pm 0.7 \%$, respectively. For cellular metabolic energy, neither species nor digestion had any effect. At proteome level digested hydrolysates gave again significantly stronger responses than non-digested counterparts; cod peptides here also gave somewhat stronger response compared to chicken. The knowledge of the action of food protein hydrolysates and their digests within live cells, also at proteome level, is important for further validation of their activity in higher eukaryotes to develop new products, in these cases with chicken and cod muscle derived peptides as functional ingredients.

Key words: protein hydrolysates, cod, chicken, *in vitro* gastrointestinal digestion, yeast, proteomics

Introduction

Protein hydrolysates containing bioactive peptides show potential use as functional food ingredients for health promotion and disease risk reduction. They occur naturally *e.g.* in dairy and muscle food sources, and are also released during gastrointestinal (GI) digestion or food processing (1,2). Both chicken and fish derived peptides have earlier been shown for their antioxidative, antihypertensive, antitrombotic, anticoagulant and immunomodulatory effects (for review see Ryan *et al.* (3)), therefore they offer high potential as a source of bioactive ingredients.

In spite of recent findings suggesting bioactivity of fish, chicken and other muscle derived peptides, detailed studies at the cellular and specifically at molecular level, which enable better insight into the action in the cell, are still quite sparse. There are to the best of our knowledge in fact no published studies linking muscle-derived peptides to the proteomic response in cells. This study beside proteome investigated the effects of cod and chicken protein hydrolysates on antioxidant activity and cellular metabolic energy using yeast *S. cerevisiae* in the stationary phase as a model organism, where yeast cells resemble cells of multicellular organisms in important aspects (most energy comes from mitochondrial

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respiration, G₀ phase, oxidative damages accumulate over time) (4). Furthermore, both hydrolysates were exposed to *in vitro* GI digestion prior to cellular exposure to simulate the digestion conditions in the human body and therefore investigate the role of enzymatic breakdown and other modulations in the GI tract on the responses given by the peptides. Namely, for both cod and chicken, such simulated digestion studies with treatment of cells with digests have not been reported earlier.

Materials and Methods

Chemicals and reagents

Immobilized pH gradient (IPG) buffer and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were from GE Healthcare (Little Chalfont, UK). Phosphate-buffered saline (PBS) was from Oxoid (Altrincham, UK). Sodium dodecyl sulphate (SDS), glycerol, thiourea, urea, 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), dithiothreitol (DTT), iodoacetamide (IAA) and bromophenol blue were from Sigma (St. Louis, MO, USA).

Preparation of cod and chicken protein isolates, hydrolysates and determination of their amino acid composition

Fresh cod (*Gadus morhua*) fillets were purchased from Noatun, a grocery store in Reykjavik, Iceland and fresh chicken breasts (*Gallus domesticus*) from the producer Ferskir kjuklingar (Reykjavik) were purchased at Hagkaup, a grocery store in Reykjavik.

Protein isolates of cod as well as chicken were prepared by alkali-aided pH-shift processing according to Kristinsson *et al.* (5) with some modifications described by Jonsdottir *et al.* (6). Briefly, cod fillet/chicken breast was homogenized in water and the pH of the homogenate was adjusted to 11. Insoluble material was removed, while the soluble proteins were precipitated by adjusting pH of the filtrate to 5.5. Cod and chicken protein hydrolysates (CPH, CHPH, respectively) were produced by Protamex (Novozymes, Bagsvaerk, Denmark) in the concentration (E:S 1/50). Hydrolysis was performed at 45 °C, pH=8.1 for 6 hours. Then enzyme was inactivated at 95 °C for 10 min. After putting sample on ice, centrifugation was

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carried out (30 min, 10000xg) to collect the soluble fraction and then pH was adjusted to 7.2. The sample was freeze-dried and kept at -80 °C until further analysis.

The amino acid composition of both protein hydrolysates was determined by an external, accredited lab (Eurofins) using standardized methodology; ISO 13903:2005 (7). In short, samples were hydrolyzed in aqueous hydrochloric acid (Sigma Aldrich, St. Louis, MO, USA) or oxidized with hydrogen peroxide (Sigma Aldrich, St. Louis, MO, USA) and formic acid (Sigma Aldrich, St. Louis, MO, USA) at cold temperature. In both cases, amino acids were then separated in an amino acid analyzer Pinnacle PCX derivatization instrument (Pickering Laboratories, Inc., CA, USA) attached to an ultimate 3000 HPLC (Thermo Fisher Scientific, MA, USA) and detection was carried out at 440 and 570 nm following post column derivatization with ninhydrin reagent (Sigma Aldrich, St. Louis, MO, USA). The results are expressed as g amino acids/100 g dry mass.

In vitro gastrointestinal (GI) digestion of cod and chicken protein hydrolysates

CPH and CHPH were digested according to the two-step static *in vitro* GI digestion method described by Tibäck *et al.* (8). In order to avoid the risk that bile salts in the digests would affect the yeast cells with which the digests were to be incubated, samples were digested with only 25 % of the bile extract that was described in the paper by Tibäck *et al.* (8). Blank digestions consisted of digestive juice (electrolyte, digestive enzymes and bile acids) instead of hydrolysate, and were treated exactly as the hydrolysate samples. Final digests were kept at -80 °C until exposure studies in yeast cells.

Yeast strain, cultivation and treatment

The yeast *S. cerevisiae* from Culture Collection of Industrial Microorganisms (Biotechnical Faculty, Slovenia) was used. The yeast were cultivated in YEPD broth (Sigma Aldrich, St. Louis, MO, USA) at 28 °C and 220 rpm until the stationary phase, where the cells were suspended in PBS buffer at a concentration of 10^8 cells/mL (Cigut *et al.* (9)). Yeast cells were exposed to CPH and CHPH (before and

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after digestion) in concentration of 1.0 mg/mL (in yeast suspension) and corresponding controls (control before digestion- water; control after digestion: digestive juice).

After 2-h incubation the effect of CPH/CHPH (before and after digestion) was studied at the cellular level by measuring cellular metabolic energy, intracellular oxidation and at proteome level by analyzing mitochondrial proteins.

Determination of intracellular oxidation in yeast cells

Intracellular oxidation was determined by the method of Jakubowski and Bartosz (10) with some modifications described by Cigut *et al.* (9). Briefly, yeast cells from 2 mL cell suspensions were centrifuged (14000xg, 5 min) and washed three times with 50 mM potassium phosphate buffer (pH=7.8). The dye 2',7'-dichlorodihydrofluorescein diacetate was added to yeast cell suspension prepared in potassium phosphate buffer to reach final concentration of 10 μ M and cells were incubated 20 min at 28 °C and 220 rpm. Then the fluorescence (excitation and emission wavelengths were 488 nm and 520 nm, respectively) was measured using microplate reader Safire II (Tecan, Männedorf, Switzerland). Results are expressed as fluorescence of 80-min kinetic mode measurement relative to corresponding control (yeast cells treated with water in the case of non-digested samples or digestive juice in the case of digested samples).

Determination of cellular metabolic energy

Cellular metabolic energy, related to ATP level, was determined by BacTiter-Glo™ Microbial Cell Viability Assay (Promega, Madison, USA) according to the manufacturer instructions. Briefly, 100 μ L of BacTiter-Glo™ reagent were added to 100 μ L of cell suspension with a concentration of 10^7 cells/mL and after 5-min incubation luminescence was measured using the microplate reader Safire II (Tecan, Männedorf, Switzerland). Results are expressed as luminescence relative to corresponding control (yeast cells treated with water in the case of non-digested samples or digestive juice in the case of digested samples).

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Analysis of mitochondrial proteome

Yeast cells from 20 mL of cell suspension were centrifuged at 4000xg for 3 min and washed once with PBS. Cytosol/Mitochondria Fractionation kit (Calbiochem, Merck, Darmstadt, Germany) was used to extract mitochondrial proteins according to manufacturer instructions with minor modifications. Briefly, zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA) were used to break the yeast cells in 1250 μ L 1x cytosol extraction buffer mix by vortexing five times for 1 min with 1-min intervals on ice. After centrifugation at 800xg for 20 min, 4 °C the supernatant was transferred to a clean microcentrifuge tube and centrifuged at 10000xg for 30 min, 4 °C to obtain cytosolic fraction (supernatant). The pellet was washed once with PBS and then resuspended in 50 μ L mitochondria extraction buffer mix to obtain the mitochondrial fraction, which was used to analyze mitochondrial proteins.

After extraction of proteins 2-D electrophoresis was performed according to Görg (11) with minor modifications described by Cigut *et al.* (9). In brief, the samples were mixed with rehydration solution (7 M urea, 2 M thiourea, 2 % (by mass per volume) CHAPS, 2 % (by volume) immobilised pH gradient (IPG) buffer (pH=4-7), 18 mM dithiothreitol and a trace of bromophenol blue) and put to 13-cm pH=4-7 IPG strips (GE Healthcare, Little Chalfont, UK). Isoelectric focusing was performed using Multiphore II system (GE Healthcare, Little Chalfont, UK) and then the IPG strips were equilibrated in equilibration buffer (75 mM Tris-HCl, pH 8.8, 6 M urea, 30 % (by volume) glycerol, 2 % (by mass per volume) SDS and a trace of bromophenol blue), containing 1 % (by mass per volume) dithiothreitol (15 min), following addition of 4.8 % (by mass per volume) iodoacetamide (15 min). SDS polyacrylamide gel electrophoresis was performed with 12 % running gels using a vertical SE 600 discontinuous electrophoretic system (Hoefer Scientific Instruments, Holliston, MA, USA).

After staining the gels with SYPRO Ruby (Carlsbad, CA, USA), they were documented using a CAM-GX-CHEMI HR system (Syngene, Cambridge, UK).

2-D Dymension software, version 2.02 (Syngene, Cambridge, UK) was used for the gel image analysis where the spots were quantified based on their normalized volumes and compared between different samples to give differentially expressed proteins (9), which were then identified using an Ultraflex II mass spectrometer (Bruker-Daltonik GmbH, Bremen, Germany). Identifications were based on MS/MS spectra using MASCOT to search the NCBI database *S. cerevisiae* as described by Larsson *et al.* (12). Briefly,

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trypsin was used as enzyme, carboxyamidomethyl of cysteine and oxidation of methionine were used as fixed and partial modification, respectively. Fragment ion mass tolerance was 0.5 Da and a precursor mass accuracy was 50 ppm.

Statistical analysis

The experiments (intracellular oxidation, cellular metabolic energy) were performed in triplicates. Data are presented as mean relative values \pm SD. Duncan's multiple range test was used ($p \leq 0.05$) to determine the significant differences among the mean relative values. For proteome analysis two 2-D gels for each sample were done at the same conditions. Expression changes (fold changes) were considered as significant when the intensity of the corresponding spots differed by more than 1.5-fold in a normalized volume compared to the corresponding control (yeast cells treated with – water in the case of non-digested samples or digestive juice in the case of digested samples) and statistically significant (Student's *t*-test) at $p \leq 0.05$. Comparison of fold changes for particular proteins between non-digested and digested samples (cod and chicken) was done using Student's *t*-test ($p \leq 0.05$).

Results and Discussion

Antioxidative activity in the cells and cellular metabolic energy

Many studies have earlier demonstrated that protein hydrolysates from fish proteins are known for their antioxidative activity (6, 13–17). For chicken protein derived peptides there are less reports regarding antioxidative activity (18,19), and only one study has compared fish with avian muscle hydrolysates (20), however, this study is without an *in vitro* digestion step.

In most of the mentioned studies, antioxidative activity has been determined *in vitro* by measuring DPPH radical scavenging capacity, reducing power assay, metal-chelating activity assay or by the ability of hydrolysates to inhibit or delay lipid peroxidation in emulsions or muscle minces, which can give valuable information, when to use peptides as food stabilizing agents. Regarding antioxidative activity at a cellular level it is not necessary that compounds showing antioxidative activity *in vitro* or in foods will possess the same activity in the cells (21). If not injected, there will always be a GI digestion step bridging over from a food situation to an *in vivo* situation, and in this step peptides will be modified *e.g.* via further

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proteolysis. Therefore, in this study, cod and chicken protein hydrolysates before and after *in vitro* GI digestion were determined for antioxidative activity on a cellular level by measuring intracellular oxidation in the yeast *S. cerevisiae* exposed to non-digested and digested samples. Before digestion both hydrolysates showed a slight decrease in intracellular oxidation compared to control - 95.5 % \pm 1.2 % (cod) and 90.5 % \pm 0.7 % (chicken), which was more pronounced after digestion, for both cod and chicken samples (70.2 % \pm 0.8 % and 74.5 % \pm 1.4 %, respectively). As before, even after digestion, no difference between both hydrolysates were observed (Fig. 1). In the study of Centenaro *et al.* (20); fish (*Umbrina canosai*) hydrolysates generally showed higher antioxidative activity in *in vitro* tests (e.g. ABTS and DPPH radical scavenging) and in a meat system than chicken hydrolysates, which was ascribed more sulfur containing amino acids and hydrophobic amino acids in the fish hydrolysate. Here both protein hydrolysates showed similar amino acid profiles (Table 1) except the content of histidine, serine and methionine differed by ≥ 20 % between samples; CPH had 20 % lower content of histidine compared to CHPH, while the content of serine and methionine was higher in CPH than CHPH (20 and 23 % respectively). All these 3 aminoacids are known to have antioxidant properties (22–24). Although the content of 2 aminoacids, methionine and serine was higher in CPH compared to CHPH, their antioxidative activity was comparable, and it was more pronounced after digestion. This indicates that GI digestion contributed by modification or further hydrolysis of both hydrolysates to higher antioxidative effect and/or cells modulated digests in more antioxidative way. Further hydrolysis was confirmed by measuring the degree of hydrolysis occurring during GI digestion of CHPH and CPH, which showed 2.8-fold and 2.6-fold higher content of free amino groups in both hydrolysates, respectively, after digestion compared to in non-digested samples (data not shown). Namely, antioxidative ability of peptides depends on peptide size, its amino acid composition and presence of free amino acids within the hydrolysate (3). Similarly, You *et al.* (13), Zhu *et al.* (25), Nalinanon *et al.* (26) and Teixeira *et al.* (27) simulated the process of human GI digestion to determine the changes in antioxidant activities of different fish species. Results showed that GI digestion in general increased their antioxidative properties measured by different *in vitro* assays. In contrast Borawska *et al.* (28) showed that high degree of hydrolysis of carp muscle tissue led to products with lower free radical scavenging activity.

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Additionally, we measured cellular metabolic energy in the cells exposed to non-digested and digested hydrolysates, where no significant difference between cod and chicken samples or between digested and non-digested samples were observed (Fig. 2) indicating also no cytotoxic effects of hydrolysates on yeast cells before and after digestion.

Proteome changes after exposing yeast cells to non-digested and digested cod and chicken protein hydrolysates

To study the effects of protein hydrolysates, before and after GI digestion, at a proteome level, the protein profile of yeast cells treated with both, non-digested and digested protein hydrolysates was analyzed. In our previous work, yeast has been already shown to be a good model to investigate the effects of bioactive compounds at a proteome level (11,29).

Proteins from yeast cells exposed to either chicken or cod protein hydrolysates before and after *in vitro* GI digestion were analyzed by 2-D electrophoresis followed by mass spectrometry to identify differentially expressed proteins. Mostly down-regulation of proteins according to the corresponding blanks was observed. In the cells exposed to non-digested and digested chicken or cod protein hydrolysates, yeast proteins down-regulated by hydrolysates (fold change >1.50, $p \leq 0.05$) were identified as peptidyl-prolyl cis-trans isomerase (PPIase), elongation factor 1-beta (Efb1), elongation factor 2 (Eft2), translationally-controlled tumor protein homolog (Tma19), peroxiredoxin (Tsa1) and cytochrome c oxidase subunit 6 (Cox6) (Table 2, Fig. 3). Additionally, proteins such as Efb1 and Tma19 were identified in two spots reflecting different isoforms, post-translational modifications or alternative mRNA splice forms, which could be related to their regulation. Additionally, exposure of cells to both non-digested and digested chicken and cod hydrolysate induced two proteins Bhm1 and Bhm2, that were absent in untreated cells (Table 2, Fig. 3).

By comparing fold change before and after digestion; digests have higher effect on protein abundance. In contrast to measuring antioxidant activity, where no difference between cod and chicken hydrolysates were observed, here at proteome level somewhat stronger effect was observed for cod. Since the targets are also proteins related to oxidative stress response, this difference in cellular response to cod and chicken

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digests at proteome level could be connected to higher content of two aminoacids, serine and methionine in cod compared to chicken protein hydrolysates.

Function of identified proteins

Elongation factor 1-beta (EF1B) and elongation factor 2 (EF2) belong to proteins related to protein synthesis. Elongation factor 1-beta is a subunit of the EF-1 complex and a highly conserved protein that has a major role in elongation regulation by regenerating a GTP bound EF 1-alfa, necessary for each elongation cycle. Control at the level of EF-1 can modulate the general rate of protein synthesis (30). eEF2 stimulates the GTP-dependent translocation of the nascent protein chain on the ribosome from the A-site to the P-site. There are different factors that control the peptide chain elongation in eukaryotic cell by inhibiting or activating eEF2 (31). The elongation stage of protein synthesis normally consumes a great deal of energy and amino acids (31). It is well established that yeast cells adapt to oxidative stress conditions by changing general gene expression patterns, including transcription and translation of genes related to antioxidants and other stress-protective defences. There are many highly abundant proteins involved in oxidative stress response to enable cells to cope with high ROS level and survive (32). In the cells treated with peptides a decrease in oxidant level was determined meaning that peptides with antioxidative activity might take a part in maintaince of oxidative resistance. Thus, cells might slow down synthesis of oxidative stress response proteins via down-regulation of eEF and thus conserve energy. Additionally, Olarewaju *et al.* (33) reported that eEF1Bgamma (subunit of EF1B) in *S. cerevisiae* gave resistance to oxidative stress. Additional roles for eEF1 complex outside the translation system show a tendency for further studies and thus understanding their biological relevance (34).

On the other side, there are reports suggesting that the machinery of protein synthesis may provide targets for anticancer drugs, since aberrations in protein synthesis (*e.g.* overexpression of translation factors) are commonly encountered in established cancers (35). Thus testing of our protein hydrolysates in the context of elongation factors' down-regulation in different cancer cell lines could be interesting.

Cod as well as chicken protein hydrolysate before and after digestion caused downregulation of Tma19 protein. Tma19 is the yeast orthologue of mammalian translationally controlled tumor protein (TCTP).

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TCTP is an evolutionarily highly conserved protein, it shows about 50 % amino acid sequence identity with its most distantly related orthologs in higher organisms (36). Rinnerthaler *et al.* (36) showed that yeast orthologue of TCTP - Tma19 after a mild oxidative stress and various other stress factors is translocated from cytosol to the outer surface of the mitochondria. A stress-induced up-regulation of TCTP expression has been reported in many organisms at different stress conditions such as oxidative stress (37), exposure to heavy metals (38). Based on data obtained by Rinnerthaler *et al.* (36) the downregulation of Tma19 in our study could mean its transfer from mitochondria back to cytosol, since decreased intracellular oxidation is present in the yeast cells exposed to cod and chicken peptides, especially their digests. Like in our study, TCTP was the target in resveratrol treated MCF-7 breast cancer cells, where also downregulation was observed (39).

Decrease in intracellular oxidation after exposure to cod and chicken peptides is reflected also on protein Tsa1, which plays an important role in oxidative stress response and redox homeostasis and whose expression decreased. Similarly, expression of protein peptidyl-prolyl cis/trans isomerase (Ppi), which is involved in protein re/folding and thus might be indirectly connected to oxidative stress, decreased. Additionally, cytochrome c oxidase subunit 6 was downregulated. This is a subunit of cytochrome c oxidase (Cox) or complex IV of the mitochondrial respiratory chain, which has a fundamental role in energy production of aerobic cells. This multimeric enzyme catalyzes the transfer of electrons from cytochrome c to molecular oxygen. Eukaryotic COX is formed by 11–13 subunits (11 in the yeast *Saccharomyces cerevisiae* and 13 in *Homo sapiens*) of dual genetic origin. The assembly of COX made of subunits is a highly regulated process. The regulation involves the availability of subunits and assembly factors regulated at the transcriptional and translational levels, availability of cofactors, protein import into mitochondria and membrane insertion, as well as coordination of sequential or simultaneous steps of the process (40). Therefore, bioactive peptides could be an additional factor in regulation. There are already some studies indicating that antioxidants such as flavonoids can modulate respiratory chain components and inhibit hydrogen production (41). Since antioxidative activity in the cells treated with digested cod and chicken protein hydrolysates increased (Fig. 1), there are no indications showing inhibition of respiration due to down-regulation of Cox6. Additionally, none of the protein hydrolysates changed cellular metabolic energy in the yeast cells (Fig. 2).

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Bmh1 and Bmh2 proteins whose abundance was observed in only treated yeast cells (Table 2), irrespective to treatment with non-digested or digested hydrolysates from cod or chicken belong to the protein family 14-3-3 proteins. 14-3-3 proteins are highly conserved and have been found in all eukaryotes investigated. They are involved in many different cellular processes, and interact with hundreds of other proteins (42). Therefore it is difficult to exactly explain their abundance in treated yeast cells and further studies are needed.

Conclusion

This study revealed that *in vitro* GI digestion of protein hydrolysates, both from cod and chicken muscle, contributed to higher antioxidative activity in the cell as well as to more pronounced effect at proteome level compared to non-digested samples. No significant differences in antioxidant activity were recorded between the two muscle sources, but at proteome level slightly stronger effect was observed for digests from cod compared to chicken. Proteins targeted by both hydrolysates belong to different cellular processes (*e.g.* oxidative stress response, protein folding, protein synthesis) and knowledge of their identity is important also in the context of quality as well as safety of chicken and cod protein hydrolysates as potential functional ingredients. Since we used yeast in the stationary phase, where yeast cells resemble cells of multicellular organisms, our results present a good basis for further validation of cod and chicken protein hydrolysates activity in higher eukaryotes. This is important for development of new products with chicken and cod proteins derived peptides as functional ingredients.

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Fig. 1. Intracellular oxidation of yeast *Saccharomyces cerevisiae* exposed to non-digested and digested cod (CPH-ND, CPH-D, respectively) and chicken protein hydrolysate (CHPH-ND, CHPH-D, respectively). Results are expressed as average values of relative fluorescence \pm SD, $n = 3$. Values followed by the different letter (a-b) are statistically different ($p \leq 0.05$), as measured by Duncan's test

Fig. 2. Cell energy metabolic activity of yeast *Saccharomyces cerevisiae* exposed to non-digested and digested cod (CPH-ND, CPH-D, respectively) and chicken protein hydrolysate (CHPH-ND, CHPH-D, respectively). Results are expressed as average values of relative luminescence \pm SD, $n = 3$. Values followed by the same letter (a) are not statistically different ($p \leq 0.05$), as measured by Duncan's test

Fig. 3. Representative protein profiles of yeast cells exposed to A: control - water; B: CPH-ND; C: CHPH-ND; D: control - digestive juice; E: CPH-D; F: CHPH-D. Differentially expressed proteins are circled. Details of indicated spots are listed in Table 2. For each gel (from left to right is pI: 4-7, respectively and from top to down is Mw: 220-10 kDa, respectively)

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Table 1. Amino acid composition w/(g/100g dry mass, n=1) of cod and chicken protein hydrolysates. The third column shows ratio between CPH and CHPH for each amino acid or groups of amino acids.

Amino acid	CHPH w/(g/100g)*	CPH w/(g/100g)*	CPH/CHPH
Alanine	4.83	5.12	1.06
Arginine	5.19	5.44	1.05
Asparagine	8.45	9.70	1.15
Glutamine	12.90	14.70	1.14
Glycine	3.08	3.30	1.07
Histidine	2.39	1.92	0.80
Isoleucine	4.08	3.89	0.95
Leucine	6.96	7.35	1.06
Lysine	8.07	8.98	1.11
Phenylalanine	3.45	3.25	0.94
Proline	2.71	2.77	1.02
Serine	3.29	3.95	1.20
Threonine	3.89	4.09	1.05
Tyrosine	2.87	3.21	1.12
Valine	4.27	4.40	1.03
Cysteine	0.88	0.88	1.00
Methionine	2.05	2.52	1.23
HAA	32.10	33.39	1.04
PCAA	15.65	16.34	1.04
NCAA	28.53	32.44	1.14
AAA	6.32	6.46	1.02
SAA	2.93	3.40	1.16

CPH - cod protein hydrolysate.

CHPH - chicken protein hydrolysate

HAA=hydrophobic amino acids (alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, proline, methionine and cysteine)

PCAA= positively charged amino acids (arginine, histidine, lysine)

NCAA= negatively charged amino acids (aspartic + asparagine, glutamic + glutamine, threonine, serine)

AAA=aromatic amino acids (phenylalanine, and tyrosine)

SAA=sulfur containing amino acids (methionine, cysteine)

*Analytical variation of the amino acid analyses ranged from 6-11 %.

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Table 2. Fold changes of expression relative to corresponding control (water or digestive juice) of proteins after 2-h exposure of yeast cells to cod and chicken protein hydrolysates. * indicates, where the difference of fold changes for particular proteins between non-digested and digested samples (cod and chicken) is statistically different ($p \leq 0.05$) as measured by Student's t-test ($p \leq 0.05$).

Spot No.	Fold change relative to the corresponding control				Total score (number of matched peptides)	Protein name/ Acc. No. in NCBI
	CHPH-ND	CPH-ND	CHPH-D	CPH-D		
1	-1.81	-1.10	-1.76	-1.78*	158 (6)	PPIase/gi151941057
2	-1.49	-1.63	-1.19*	-1.88*	217 (7)	EF2/gi6320593
3	-1.49	-1.49	-1.86*	-1.75*	173 (10)	EF1B/gi6319315
4	-1.97	-1.43	-2.09	-1.59*	157 (4)	EF1B/gi6319315
5	-2.69	-2.54	-1.51*	-3.09*	143 (7)	Tma19/gi6322794
6	-1.43	-1.48	-1.30	-1.77*	208 (9)	Tma19/gi6322794
7	-2.36	-1.53	-2.25	-1.92*	238 (4)	Tsa1/gi6323613
8	-1.46	-2.09	-1.94*	-1.86*	118 (4)	Cox6/gi6321842
9	De novo	De novo	De novo	De novo	181 (7)	Bmh2/683696
10	De novo	De novo	De novo	De novo	219 (5)	Bmh1/gi671634

CPH-ND - cod protein hydrolysate before digestion

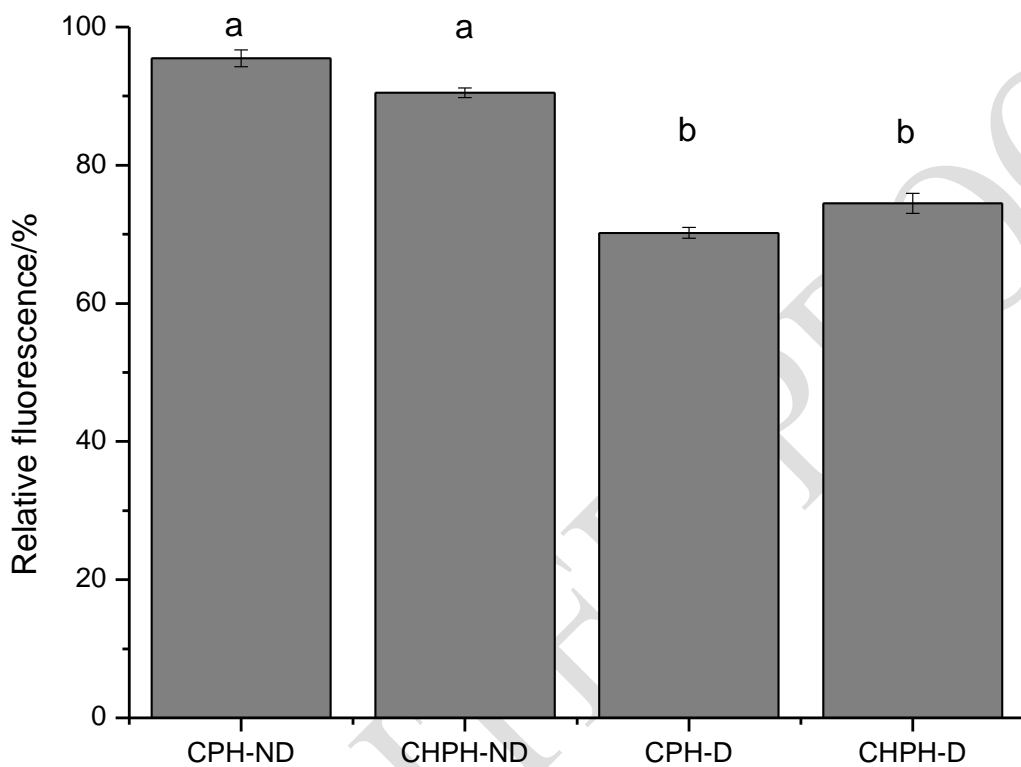
CHPH-ND - chicken protein hydrolysate before digestion

CPH-D - cod protein hydrolysate after digestion

CHPH-D - chicken protein hydrolysate after digestion

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Fig. 1.



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Fig. 2.

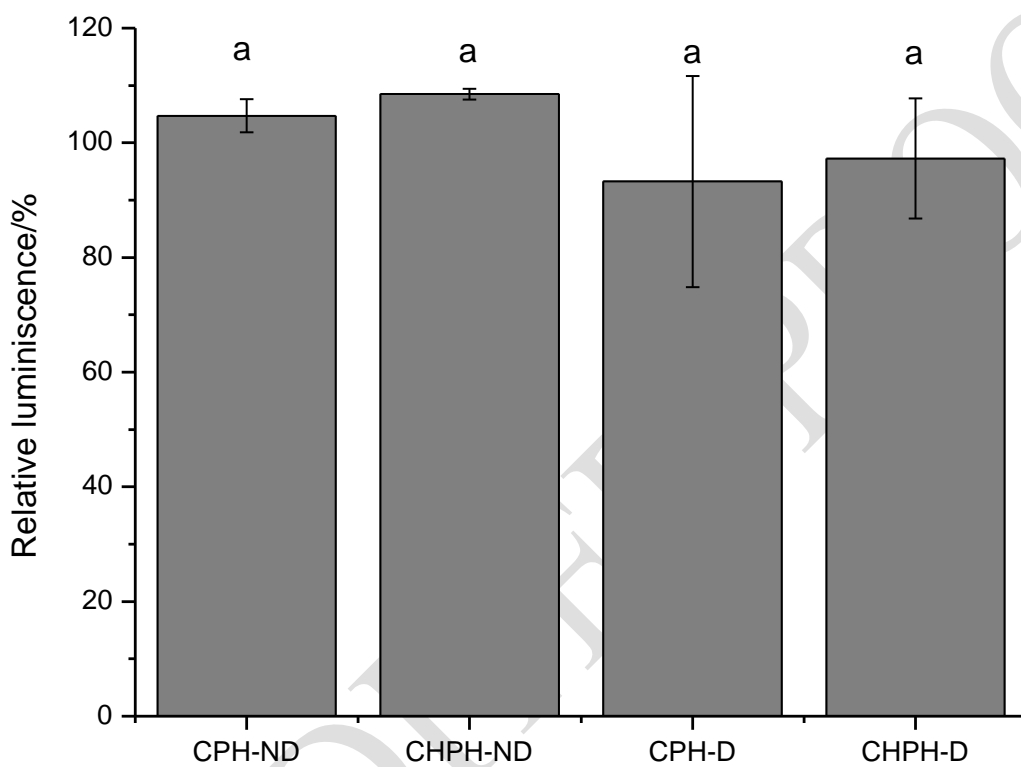
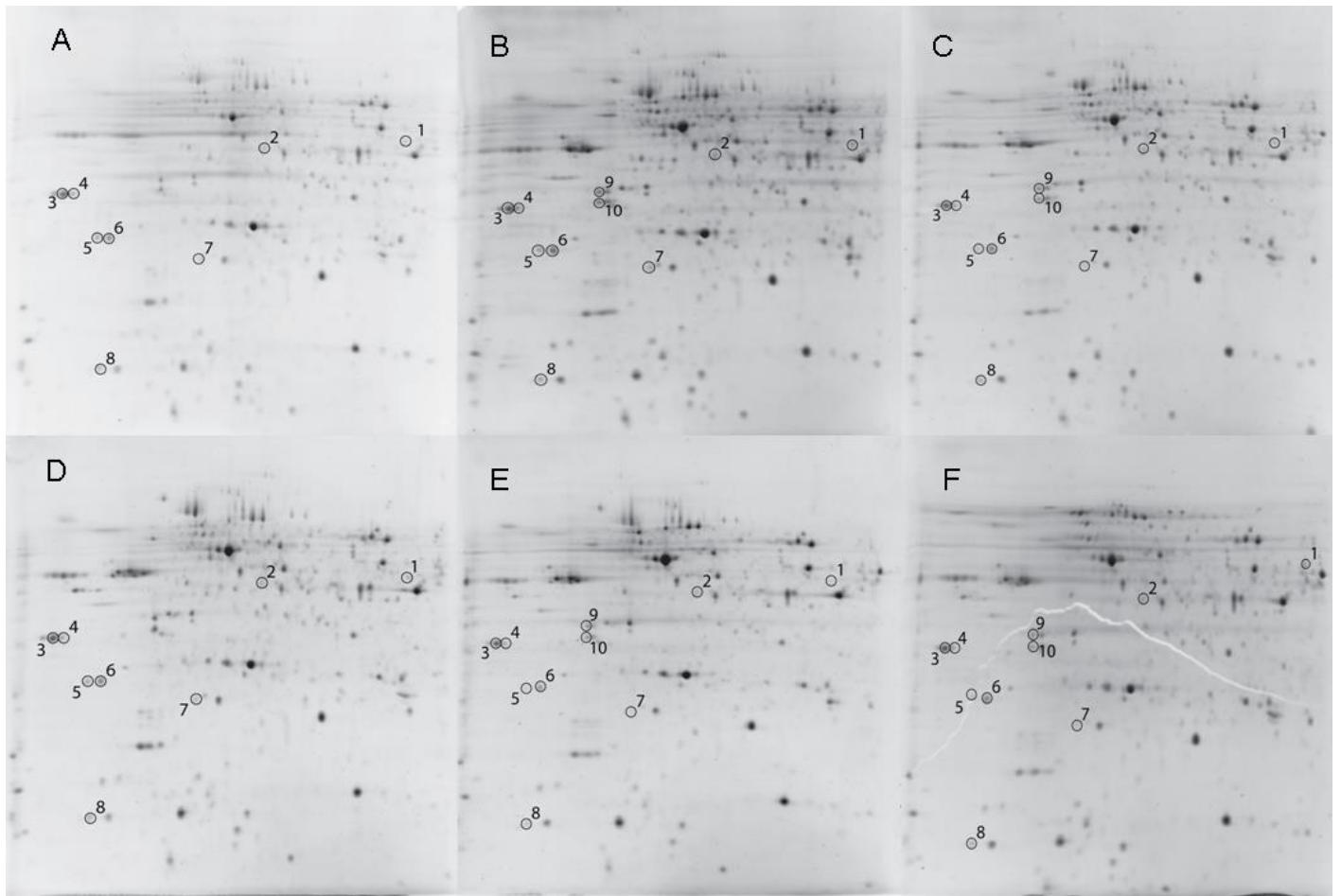


Fig. 3.



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