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Substrate specificity of novel GH16 endo-β-(1→3)-galactanases acting on linear and branched β-(1→3)-
galactooligosaccharides

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Short running title: novel GH16 endo-β-(1→3)-galactanases
Abstract

Arabinogalactan proteins are proteoglycans located in the plant cell wall. Most arabinogalactan proteins are composed of carbohydrate moieties of β-(1→3)-galactan main chains with β-(1→6)-galactan side chains terminated by other glycans. In this study, three novel endo-β-(1→3)-galactanases were identified and the substrate specificity was further studied using well-defined galactan oligomers. Linear and branched β-(1→3)-linked galactans, which resemble the carbohydrate core of the arabinogalactan protein, were used for the characterization of endo-β-(1→3)-galactanases. The identified enzymes required at least three consecutive galactose residues for activity. Non-substituted regions were preferred, but substituents in the -2 and +2 and in some cases also -1 and +1 subsites were tolerated to some extent, depending on the branching pattern, however at a significantly lower rate/frequency.

Keywords

β-(1→3)-galactanases; gum arabic; β-(1→3)-galactan; type II arabinogalactan; glycosyl hydrolase (GH) family 16

1. Introduction

Arabinogalactan proteins (AGPs) belong to the family of hydroxyproline-rich glycoproteins (HRGPs) and they are characterized as highly glycosylated proteoglycans (Pereira et al., 2015). They are considered as one of the most complex macromolecule families found in plants. Their complexity is revealed from the remarkable diversity of the glycans attached to the protein backbone, compromising 90–95% of the total mass (Ellis et al., 2010). Determining the precise structure of the carbohydrate moiety of the AGPs is of major importance to better understand their biosynthesis and their biological and possible industrial
applications (Ling et al., 2012). AGPs are known to be involved in various processes in plant growth and
development such as cell division (Serpe and Nothnagel, 1994), cell development (Van Hengel and Roberts,
2002), signaling and cell death (Gao and Showalter, 1999).

AGPs consist of a core protein rich in hydroxyproline and large arabinogalactan (AG) domains that
commonly comprise a backbone of β-(1→3)-linked D-galactose with branch points of β-(1→6)-linked D-
galactose of one, two or three residues in length usually terminating in arabinofuranose (Araf),
rhamnopyranose (Rhap) and galactopyranose (Galp) residues (Ellis et al., 2010). Some AGs are additionally
decorated by short Araf oligosaccharide chains and others are rich in uronic acid residues (GalpA, GlcpA)
(Showalter, 2001).

AGPs can be used as food and pharmaceutical products due to their general adhesive, emulsifying and
water-retaining properties (Showalter, 2001). They are important components of various plant gums or
exudates and bestow remarkable properties on these plant products. For instance, gum arabic is a dried
exudate from Acacia Senegal and represents one of the most commercially significant gums. It is widely
used in the pharmaceutical and food industries due to its low viscosity and toxicity (Showalter, 2001). Gum
arabic is a polydisperse molecule, which has morphological similarities with the AGP complex and AG
fractions (Nie et al., 2013). By investigating its structure with 2D NMR spectroscopy, it was proposed that
gum arabic is a branched complex polysaccharide, which constitutes of a backbone of β-1,3-galactan with
extensive branching at the O-2, O-4 or O-6 position. The branches consist of β-galactosyl, β-glucuronosyl, α-
arabinosyl, and α-rhamnosyl residues (Mahendran et al., 2008).

Enzymes hydrolyzing β-1,3-galactans include exo-β-(1→3)-galactanases and endo-β-(1→3)-galactanases.
Exo-β-(1→3)-galactanases from glycosyl hydrolase family 43 (GH43) hydrolyze the β-(1→3)-galactan
backbone of e.g. partially debranched gum arabic or larchwood arabinogalactan in an exo fashion requiring
at least two consecutive galactose residues for activity. Remarkably, they are able to bypass branch points
liberating also intact β-(1→6)-linked galactooligosaccharide side chains (Ling et al., 2012; Sakamoto and
Ishimaru, 2013; Tsumuraya et al., 1990). Endo-β-(1→3)-galactanase activity was observed already around 1970 (Hashimoto, 1971; Hashimoto et al., 1969), but has been much less studied and was first rigorously characterized by Kotake and co-workers in 2011 (Kotake et al., 2011). The authors identified the first endo-β-(1→3)-galactanase (FvEn3GAL) from the winter mushroom Flammulina velutipes which was subsequently cloned, purified and characterized. Two homologous endo-β-(1→3)-galactanases (45-46% sequence homology to FvEn3GAL) from Aspergillus flavus (Af3G) and from Neurospora crassa (NcEn3GAL) were recently expressed in Pichia pastoris, purified and characterized (Yoshimi et al., 2017). All three enzymes specifically hydrolyse β-(1→3)-galactan and β-(1→3)-galactooligosaccharides with degree of polymerization of at least three (Kotake et al., 2011; Yoshimi et al., 2017). However, the enzymes only have trace activity towards native gum arabic, probably because branching at the 6-position prevents them from accessing the β-(1→3)-galactan main chains. These enzymes are classified as EC 3.2.1.181 and belong to GH16 (Sakamoto and Ishimaru, 2013), a family that also includes endo-β-(1→3(4))-glucanases. Apart from FvEn3GAL, NcEn3GAL and Af3G, no other endo-β-(1→3)-galactanases have been described in the literature. Endo-β-(1→3)-galactanases could be relevant industrially due to their potential to process AGP, gum arabic or larchwood arabinogalactan, possibly in combination with auxiliary, debranching enzymes such as endo-β-(1→6)-galactanases, α-L-arabinofuranosidases and α-L-rhamnosidases (Sakamoto and Ishimaru, 2013; Yoshimi et al., 2017).

In the present study, three novel fungal endo-β-(1→3)-galactanases from GH family 16 were identified and their modes of action were investigated by studying their activity on well-defined linear and branched AGP oligosaccharides produced by chemical synthesis (1–4, Figure 1).
2. Material and methods

2.1. Chemicals

All reagents and solvents used were purchased from Sigma-Aldrich (St. Louis, Mo, USA) unless otherwise stated. All solvents were HPLC-grade. Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA). Enzymatic reactions were carried out in Eppendorf safe-lock tubes with a volume of 1.5 mL in an Eppendorf thermomixer. Absorbance at 410 nm was measured using the Infinite M200pro microplate reader (Tecan, Austria). Poly(ethylene glycol) average MW 1500 (PEG 1500) was used as an internal standard.

2.2. Enzymes

Homologues of the *Flammulina velutipes* GH16 endo-β-(1→3)-galactanase (Kotake et al., 2011) were identified in the Swissprot database (Bairoch and Apweiler, 2000) by protein Blast (Altschul et al., 1990). The gene encoding the *Aspergillus terreus* GH16 endo-β-(1→3)-galactanase (AtEn3GAL), including its native signal peptide, was amplified from the genome of *A. terreus* strain S-712 using PCR primers that included 16 bp vector-specific extensions to facilitate cloning (5’ ACACAACTGGGGATCCACCATGTACCTGCTCACACTTCTCGCC 3’ and 5’ CCCTCTAGATCTCGAGGATGGTGGTGTGGTGAA-3’). The PCR amplified fragment was cloned into the Aspergillus expression vector pDau222 behind an inducible Aspergillus natural amylase promoter (Budolfsen et al., 2004) by digestion of the vector with BamHI and XhoI and ligating the fragments with an In-Fusion CF Dry-Down PCR Cloning Kit (Clontech Laboratories Inc., Mountain View, CA, USA). The *Neosartorya fischeri* NRRL181 GH16 endo-β-(1→3)-galactanase (NfEn3GAL) was cloned into pDAu222 by PCR amplification from genomic DNA using PCR primers that included 16 bp vector-specific extensions to facilitate cloning (5’ ACACAACTGGGGATCCACCATGGAATTTTCAACCCTTCTGAGTCTTC 3’ and 5’ CCCTCTAGATCTCGAGCATCAAAACGTATCATTCGGGGTT-3’). The cloned gene sequence was verified by Sanger sequencing. The *Punctularia strigosozonata* HHB-11173 GH16 (PsEn3GAL) was cloned into pDAu222.
by T4 DNA ligation using a synthetic DNA encoding the predicted cDNA sequence flanked by BamHI and XhoI restriction sites.

2.3. Molecular Cloning of AtEn3GAL, NfEn3GAL and PsEn3GAL

The DNA sequence encoding *A. terreus* GH16 endo-β-(1→3)-galactanase (residues 1-245) was amplified from the *A. terreus* S-712 genomic DNA library by PCR using the primers AT-F: 5’ ACACAACTGGGGATCCACCATGTACCTGCTCACACTTCTCGCC 3’ and AT-R: 5’ CCCTCTAGATCTCGAGGATGGTGGTGTGGTGTAATGAA 3’. The amplification reaction was composed of 2.5 µL *A. terreus* S-712 genomic DNA, 10 µL 5x Phusion buffer HF, 1 µL 10 mM dNTP, 2 µL 2.5 µM primer AT-F, 2 µL 2.5 µM primer AT-R, 32 µL H2O, and 0.5 µL 2 u/µL Phusion polymerase (ThermoFisher Scientific, Life Technologies Europe BV, Nærum, Denmark). The amplification reaction was incubated in a PTC-200 DNA Engine Thermal Cycler programmed for one cycle at 94 °C for 2 min; and 35 cycles each at 94 °C for 15 s and 60 °C for 1.5 min. A 1.0 kb PCR reaction product was isolated by 1 % agarose gel electrophoresis using TAE buffer and staining with SYBR Safe DNA gel stain. The DNA band was visualized with the aid of an Eagle Eye Imaging System and a Darkreader Transilluminator. The 1.0 kb DNA band was excised from the gel and purified using a GFX PCR DNA and Gel Band Purification Kit according to the manufacturer’s instructions. The 1.0 kb fragment was cloned into BamHI-XhoI cleaved pDAu222 (Schnorr et al., 2015) using In-Fusion HD Cloning Kit (Clontech, Takara Bio, USA) according to the manufacturer’s instructions. The reaction mixture was transformed into *E. coli* TOP10F competent cells (Invitrogen) according to the manufacturer’s instructions. The transformation mixture was plated onto LB plates supplemented with 50 µg of ampicillin per mL. Plasmid minipreps were prepared from several transformants and sequenced. One plasmid with the correct *A. terreus* GH16 coding sequence was chosen. The DNA sequence (accession number: EMBLEXP:DS027696) encoding *N. fischeri* GH16 endo-β-(1→3)-galactanase (residues 1-245) was amplified from the *Neosartorya fischeri* NRRL 181 genomic DNA library by PCR using the primers NF-F: 5’ ACACAACTGGGGATCCACCATGAAGTTTTCAACCCTTCTGAGTTC 3’ and NF-R: 5’ CCCTCTAGATCTCGAGCATCAAAACGTATCATTCGGGGTT 3’. PCR amplification and cloning of NfEn3GAL was
carried out as described for AtEn3GAL. The cDNA sequence derived from EMBL:XPJ687561 encoding \( P. \)
\textit{strigosazonata} GH16 endo-\(\beta\)-(1\(\rightarrow\)3)-galactanase (residues 1-258) was synthesized at Geneart (ThermoFisher Scientific, Life Technologies Europe BV, Naerum, Denmark). The 0.8 kbp cDNA fragment was cut out of the pUC19 cloning vector with BamHI and XhoI and cloned into pDAu222 using In-Fusion cloning as described above.

2.4. Expression of Recombinant Protein

The three \textit{endo-\(\beta\)-(1\(\rightarrow\)3)-galactanase} expression constructs were transformed into the \textit{Aspergillus oryzae} strain MT3568 (McBrayer et al., 2011). The \textit{Aspergillus oryzae} strain MT3568 (Biely et al., 2014) was transformed with pDAU222-AtEn3GAL, pDAU222-NfEn3GAL, or pDAU222-PsEn3GAL using standard techniques (Christensen et al., 1988). Transformants were selected on acetamide during regeneration from protoplasts and subsequently re-isolated under selection conditions (Christensen et al., 1988). To assay for recombinant expression, transformants were cultured in 0.75 mL YP + 2% glucose medium and DAP-4C-1 medium (Biely et al., 2014) in 96-well deep well microtiter plates for 4 days at 30 °C, and samples were monitored by SDS-PAGE. Two transformants were selected based on expression in microtiter cultures and cultured in 150 mL of DAP-4C-1 medium in baffled 500 mL flasks shaken at 100 rpm at 30 °C. Samples were taken after 4 days, expression of recombinant \textit{A. terreus} \textit{endo-\(\beta\)-(1\(\rightarrow\)3)-galactanase} protein was monitored by SDS-PAGE, and the highest yielding transformant for each of the three constructs was selected.

For production of recombinant \textit{endo-\(\beta\)-(1\(\rightarrow\)3)-galactanase} enzyme for purification and characterization, Aspergillus transformants were cultured in DAP-4C-1 medium in baffled flasks shaken at 150 rpm at 30 °C. The culture broth was harvested after 4 days and separated from cellular material by passage through a 0.22 µm filter.
2.5. Enzyme purification

The enzymes were purified from the filtered culture broth. The filtered culture broth was supplemented with ammonium sulfate (concentration of 1.5 M for AtEn3GAL and PsEn3GAL; concentration of 2 M for NfEn3GAL) and pH was adjusted (pH 7 for AtEn3GAL and PsEn3GAL; pH 7.5 for NfEn3GAL). The sample was then loaded on a hydrophobic column with a packed bed of butyl Toyo pearl and eluted in a gradient: 0–100 % B in 10 column volumes. Buffer A: 50 mM Mes and ammonium sulphate (concentration in accordance with enzyme samples), buffer B: 50 mM Mes. Buffer A and B had the same pH as the sample. SDS-PAGE under reducing conditions confirmed that the galactanase was not eluted through the column in the flow-through or wash fractions. Based on the SDS-PAGE, fractions containing proteins of the expected MW (Table 1) were pooled, concentrated and buffer-exchanged into 20 mM sodium acetate buffer pH 6.5 by centrifugation in Vivaspin column 3kDa MWCO membrane (3000 x g spin centrifugation).

2.6. Substrates

Gum arabic was purchased from Merck. Larch wood arabinogalactan, pectic lupin type I galactan, galactomannan, mannan, β-glucan (water-soluble β-(1→3/4) polysaccharides) and curdlan (β-(1→3)-D-glucan) were purchased from Sigma Aldrich.

2.7. Heat treated polysaccharides

Heat treated gum arabic and heat treated larchwood arabinogalactan were obtained by heating a 1% substrate solution for 4 h at 100 °C. The reaction was stopped by cooling to room temperature. The pH was adjusted to 7 with 4 M NaOH and the sample was then reduced by treatment with NaBH₄ (2 mg per mg of polysaccharide) for 3 h at room temperature to remove any free reducing sugars contributing to the background level when using the substrate in the reducing end assay) followed by dialysis overnight. Freeze drying afforded a solid product.
2.8. Acid treated polysaccharides

Acid treated gum arabic and acid treated larchwood arabinogalactan were obtained by heating a 1% substrate solution with 0.1 M trifluoroacetic acid (TFA) for 1 h at 90 °C. The reaction was stopped by adjusting the pH to 7 with 4 M NaOH. The sample was then reduced by NaBH₄ (2 mg per mg of polysaccharide) for 3 h at room temperature followed by dialysis overnight. Freeze drying afforded a solid product.

2.9. Smith degraded gum arabic

Smith degraded gum arabic (SD GA) was obtained according to the Smith degradation procedure (Tsumuraya and Hashimoto, 1984). The oxidation of gum arabic (up to 5 g) with 50 mM aq. sodium metaperiodate (up to 500 mL) was carried out in the dark at 4 °C for 48 h to 96 h. The reaction was terminated by addition of ethylene glycol (up to 4 mL) and stirred for 2 h at room temperature. After dialysis overnight (3.5 kDa MWCO, distilled water), reduction of the oxidized product was performed with NaBH₄ (2 mg per mg of polysaccharide) for 3 h. The reaction was terminated by adjusting the pH to 7 with 10 % (v/v) aq. AcOH followed by dialysis overnight. The sample was hydrolyzed with aq. TFA (0.5 M) for 24 h at room temperature. After evaporating most of the acid and adjusting the pH to 7 with 1 M aq. NaOH, the substrate was precipitated with three volumes of ethanol and recovered by centrifugation and freeze dried. The procedure was repeated 3 times; for each cycle a substrate was obtained: single, double and triple Smith degraded gum arabic (SD I GA, SD II GA and SD III GA). NMR analysis of gum arabic and the three treated samples clearly showed debranching, with SD III GA mainly consisting of signals from the galactan backbone (see supplementary figure 1).

2.10. Defined galactan substrates

The synthesis of the defined linear and branched β-(1→3)-galactans was carried out as described in Andersen et al. (Andersen et al., 2017).
2.11. DSC analyses (Tm)

The thermostability of beta-(1→3)-galactanases was determined by Differential Scanning Calorimetry (DSC) using a MicroCal VP-Capillary Differential Scanning Calorimeter. The thermal denaturation temperature, Tm (°C), was defined as the top of the denaturation peak (major endothermic peak) in the thermogram (Cp vs. T) obtained after heating the enzyme solution (approximately 0.5 mg/mL) in buffer (50mM acetate, pH 5.0) at a constant programmed heating rate of 90 K/hr. Sample- and reference-solutions (approximately 0.2 mL) were loaded into the calorimeter (reference: buffer without enzyme) from storage conditions at 10 °C and thermally pre-equilibrated for 20 min at 20 °C prior to DSC scan from 20 °C to 120 °C. The denaturation temperature was determined at an accuracy of approximately +/- 1 °C.

2.12. Hydrolysis of polysaccharides

The activity of the three enzymes was tested on a range of different polysaccharides. Substrate concentrations were 1.7–5 g/L, enzyme dosage 0.007–0.03 g/L in 6.7 mM acetate buffer pH 5. The reactions were carried out at 40 °C for 45 minutes and terminated at 95 °C for 10 minutes. The activity was determined by reducing end assay, and the activities were adjusted for differences in substrate concentration and enzyme dosage and normalized.

2.13. Temperature and pH optimum

The pH and temperature optimum was determined for NfEn3GAL on the degradation of triple Smith degraded gum arabic. The temperature range was 20–80 °C and pH 2–9. The enzymatic reactions contained 5 g/L substrate, 0.04 g/L enzyme, and 10–30 mM buffer (pH 2: 30 mM KCl/HCl, pH 3–5.5: 10 mM NaOAc, pH 6–9: 25 mM HEPES) and was performed at the desired temperature for 45 minutes and terminated by freezing on dry ice.
2.14. Hydrolysis of defined substrates

The reaction conditions to determine the hydrolysis of the defined linear and branched galacto-oligosaccharides (1–4) were 1 g/L substrate, 0.01 g/L enzyme, 10 mM acetate buffer pH 5 and incubation at 40 °C at 700 rpm in a thermomixer. Sampling was performed at different time points and stored on dry ice. Prior to HPLC analysis samples were thawed and mixed with PEG 1500 solution (0.2 g/L in acetonitrile).

2.15. Reducing end assay

The enzymatic activity on triple Smith degraded gum arabic was determined by reducing end assay, modified from Lever (Lever, 1977) for optimal reaction conditions in a 96 well format. A fresh color reagent was prepared from 106 µL 1 M bismuth solution, 10.6 mL 5% (w/v) 4-hydroxybenzoic acid hydrazide and 89.3 mL 0.536 M NaOH. Enzymatic reactions were terminated by storing the samples on dry ice, and 20 µL of proper diluted reaction was added to 280 µL color reagent and incubated for 10 minutes at 70 °C. After cooling to room temperature, the absorbance was measured at 410 nm against a blank. A standard curve containing galactose was used for quantification.

2.16. HPLC-MS

Identification of the degradation products was performed on a Waters Acquity UPLC coupled with a photodiode array (PDA) and a single quadrupole (SQD) MS detector (Waters Corporation), equipped with an XBridge Amide, 2.1x150 mm and particle size 1.7 μm column (Water Corporation). Column temperature was 20 °C, flow rate 0.4 mL/min and injection volume 2 µL. Two solvents of elution buffers were used: solvent C, ultra-pure water with 0.1% formic acid; solvent D, acetonitrile with 0.1% formic acid. Gradient elution was employed, from 70% to 10% acetonitrile over 18 min followed by 2 min wash (90% acetonitrile). Chromatographic system control, data acquisition, and chromatographic analysis were done with MassLynx V4.1 software.
2.17. HPLC-ELSD

Separation and quantification of the reaction products were carried out at a Waters e2695 Separations Module HPLC system coupled with an Agilent 1260 Infinity ELSD, equipped with an XBridge Amide, 4.6x150 mm and particle size 3.5 μm column (Waters Corporation). The major analytical parameters were 20 °C column temperature, 1.0 mL/min flow rate and 10 μL injection volume. Solvents, gradients and software were as described for the UPLC-MS analysis.

3. Results

3.1. Identification and purification of three fungal GH16 endo-β-(1→3)-galactanases

The uncharacterized gene CH476601 (SWISSPROT:Q0CJK4) from A. terreus NIH2624 genome of the Broad Institute Aspergillus database was identified by protein BLAST (Altschul et al., 1990) using SWISSPROT:F7J1C8 endo-β-(1→3)-galactanase from Flammulina velutipes (Kotake et al., 2011) belonging to GH16 glycosyl hydrolases as defined by CAZY (Cantarel et al., 2009; Henrissat and Davies, 1997). A refined model of the encoded A. terreus GH16 endo-β-(1→3)-galactanase was used for comparison with GH16 gene models from other fungi. This model was also used as basis for cloning and expression of the A. terreus GH16 endo-β-(1→3)-galactanase from A. terreus S-712. The S-712 strain was originally isolated from soil of a Japanese paddy field. The obtained gene sequence encoded a full-length GH16 of 245 amino acids with 100 % identity to the refined GH16 model of A. terreus NIH2624. Similarly, the putative glycosyl hydrolase SWISSPROT:A1DDC8 from Neosartorya fischeri NRRL181, originally isolated from canned apples in 1923, and the uncharacterized protein SWISSPROT:R7RZP9 from Punctularia strigosozonata HHB-11173, a white rot isolated from Populus tremuloides in USA in 1981, were identified by BLAST as putative GH16 endo-β-(1→3)-galactanases. The Neosartorya fischeri NRRL181 GH16 was cloned by PCR amplification from genomic DNA. The Punctularia strigosozonata HHB-11173 GH16 was cloned using a synthetic DNA encoding the predicted cDNA sequence. Heterologous expression of the GH16 endo-β-(1→3)-galactanases was
carried out in *Aspergillus oryzae* using the endogenous secretion signals as described elsewhere (Biely et al., 2014) and purified to homogeneity using conventional methods (Figure 2).

### 3.2. Physico-chemical properties of endo-β-(1→3)-galactanases

The physico-chemical properties of the purified endo-β-(1→3)-galactanases (two from ascomycete and one from basidiomycete fungi) are listed in Table 1. All enzymes consist of a single catalytic domain (GH16) without any carbohydrate binding domains (CBM). The observed molecular weight of all three enzymes was found to be 3–8 kDa higher than the theoretical molecular weight. This is expected to be due to glycosylation as is commonly observed when proteins are expressed in fungi (Deshpande et al., 2008). This is supported by the presence of predicted glycosylation sites in all three protein sequences (NetNGlyc 1.0 Server, [www.cbs.dtu.dk/services](http://www.cbs.dtu.dk/services), data not shown). Predicted pI ranged from 4–7 and measured thermostability ($T_m$) of all enzymes were well above 50 °C and even around 70 °C for AtEn3GAL and PsEn3GAL.

### 3.3. Initial experiments on non-, single, double, and triple Smith degraded gum arabic

The triple Smith degraded gum arabic was a superior substrate for all three enzymes. Some activity was seen on the heat treated, single- and double Smith degraded gum arabic, especially PsEn3GAL for the latter, but almost no activity was observed on the other substrates tested, including native gum arabic (Table 2).

### 3.4. Temperature and pH optimum

In order to determine the temperature and pH optima for NfEn3GAL, a number of different combinations of temperature and pH were investigated by reducing end assay with triple smith degraded gum arabic. The
temperature and pH experiments revealed optimal conditions for NfEn3GAL around 40 °C and pH 5–5.5 (Figure 3). For temperatures above 40 °C the activity was rapidly decreasing regardless of the pH of the medium. For pH values below 3 and above 8 the residual activity was less than 10%. Since both AtEn3GAL and PsEn3GAL had higher activity than NfEn3GAL at pH 5 and 40 °C, no further tests to find optimal reaction conditions for AtEn3GAL and PsEn3GAL were conducted.

3.5. Mode of action on defined substrates

The substrate specificity and the mode of action were investigated for all three enzymes on defined linear and branched oligosaccharides (see Figure 1). Linear substrates were β-(1→3)-linked galactopentaose (1) and galactoheptaose (2), designated gal₅ and gal₇ respectively. The branched molecules were two nonasaccharides with a linear β-(1→3)-linked galactoheptaose backbone carrying a β-(1→3)-linked galactobiose linked to either the 6’’’-position (3) or the 6’’-position (4), designated Gal₂βGal₇ and Gal₂βGal₉ respectively.

The identity of the degradation products was determined by UPLC-MS. The hydrolysis products were analyzed in a time-course experiment by HPLC-ELSD analysis. The chromatographic peaks in the HPLC-ELSD were identified by comparing their retention times with reference compounds. Due to the lack of Gal₂, Gal₃ and Gal₄ samples, the reference compounds used were D-galactose, D-lactose and 1,4-β-D-cellotetraose. The representative chromatograms of each compound and their mixture are shown in supplementary figure 2. The results indicated that sugars were well separated on the HPLC-HILIC column and that determination of the degradation products was possible.

All three enzymes were able to degrade Gal₅ following the same major degradation pattern (Figure 4A), where the pentamer was degraded into Gal₃ and Gal₂. For both AtEn3GAL and PsEn3GAL, Gal₃ was further degraded into Gal₂ and Gal, while no further degradation of Gal₃ was observed for NfEn3GAL. A higher activity towards Gal₅ compared to Gal₃ was observed, indicating a greater affinity for the longer oligomer.
No further degradation of Gal2 was observed in any of the experiments (Figure 5, supplementary figure 3A, and supplementary figure 4A). For the linear (unbranched) substrates 1 and 2 it is not known if a preference for hydrolysis closer to the reducing versus non-reducing end exists. Degradation patterns are arbitrarily drawn closer to reducing end for simplicity.

For the linear (unbranched) substrates 1 and 2 it is not known if a preference for hydrolysis closer to the reducing versus non-reducing end exists. Degradation patterns are arbitrarily drawn closer to reducing end for simplicity.

The degradation of the heptamer Gal7 (2) followed the same major pattern for all the three enzymes (Figure 4B). Gal7 was degraded to Gal4 and Gal3, but in the case of NfEn3GAL, the degradation products Gal5 and Gal2 were observed as well. For AtEn3GAL and PsEn3GAL, Gal4 was almost immediately degraded further to Gal2, whereas Gal3 was degraded to Gal2 and Gal, but at a much slower rate. (Figure 5B, supplementary figure 3B, supplementary figure 4B).

The degradation pattern of the branched oligomers revealed some variation among the three enzymes. When AtEn3GAL and NfEn3GAL acted on Gal25Gal7 (3), the initial products observed were Gal22Gal4 and Gal3, with further hydrolysis into Gal22Gal2, Gal2 and Gal only for AtEn3GAL (Figure 6A). Given the similarity in sequence among GH16 endo-β-(1→3)-galactanases it is not likely that the difference in reactivity of AtEn3GAL and NfEn3GAL is a sign of overall subfamily specificity in GH16, but rather a result of differences in individual enzyme properties. The absence of further degradation products for NfEn3GAL might be due to the relative low activity compared to AtEn3GAL and PsEn3GAL, even though increased enzyme dosage and prolonged reaction time did not result in further degradation (data not shown). Contrary to this, the main degradation pattern of PsEn3GAL was the formation of Gal22Gal5 and Gal2, with further hydrolysis into Gal22Gal2, Gal2 and Gal (Figure 6B). The degradation pattern shown in Figure 6A was also observed for PsEn3GAL, but to a much lesser extent.

The second branched oligomer Gal23Gal7 (4) also resulted in some variation between the enzymes. All three were able to degrade Gal22Gal7 into Gal22Gal4 and Gal3 (Figure 7A). Both AtEn3GAL and PsEn3GAL further degraded Gal3 into Gal2 and Gal, along with further hydrolysis of Gal22Gal4 to either Gal2, Gal2 or Gal22Gal3.

No degradation of Gal22Gal4 was observed for NfEn3GAL, which again, as for Gal22Gal7, can be due to the
relative low activity of NfEn3GAL. Even though AtEn3GAL hydrolyzed \( \text{Gal}_2^3\text{Gal}_7 \) as described, a second degradation pattern was observed with the formation of \( \text{Gal}_2^3\text{Gal}_3 \) and \( \text{Gal}_4 \), with the latter immediately hydrolyzed to \( \text{Gal}_2 \) (Figure 7B). Likewise, small amounts of \( \text{Gal}_2^3\text{Gal}_5 \) were observed for PsEn3GAL, indicating another pattern with the release of \( \text{Gal}_2 \) from the non-reducing end (not shown in Figure 7).

4. Discussion

The three GH16 endo-\( \beta-(1\rightarrow3) \)-galactanases presented here all displayed low activity towards the different polysaccharide substrates tested, except for triple smith degraded gum arabic. This was expected, since accessible, unsubstituted \( \beta-(1\rightarrow3) \) linked galactan is mainly present in the latter substrate (see also supplementary figure 1). Also, in the case of gum arabic, it was clear that partial or full removal of \( \beta-(1\rightarrow6) \)-linked branching by Smith degradation was a necessity in order for the enzymes to catalyze the hydrolysis of the \( \beta-(1\rightarrow3) \) linked galactan backbone.

The observed pH and temperature optima for NfEn3GAL at pH 5 and 40 °C were similar to those reported by Kotake et al. (Kotake et al., 2011) and Yoshimi et al. (Yoshimi et al., 2017), where the pH optima for the endo-\( \beta-(1\rightarrow3) \)-galactanases FvEn3GAL, NcEn3GAL and Af3G were between pH 4 and 5.5 and the temperature optima for their enzymatic reaction were found to be 40–45 °C. In pH values below 3 and over 8 NfEn3GAL was not active.

On the basis of the mode of action of the three enzymes and their specificity towards the studied \( \beta-(1\rightarrow3) \)-galactans, all three can be classified as endo-\( \beta-(1\rightarrow3) \)-galactanases. The endo-acting fashion was verified by the formation of several oligosaccharides during the initial phase of the reactions. The inability to hydrolyze galactobiose is further evidence that all the enzymes are endo-\( \beta-(1\rightarrow3) \)-galactanases. On the contrary, exo-\( \beta-(1\rightarrow3) \)-galactosidases would have released high amounts of galactose in the initial phase of the reaction.

Also, as larger oligomers were not observed during the experiments with any of the defined linear or
branched oligosaccharides, the occurrence of transglycosylation reaction was unlikely, but cannot be ruled out.

The degradation of the branched oligomers followed different primary pathways, but with overlap among the three enzymes suggesting slightly different substrate preferences of the different enzymes. Furthermore, the particular substrate specificity of all three enzymes showed a preference for hydrolysis of the \(\beta-(1\rightarrow3)\)-linkage of \(\beta-(1\rightarrow3)\)-galactooligosaccharides having at least three consecutive galactosyl units which is in accordance with the observations of Kotake et al. (Kotake et al., 2011). Furthermore, the relative reaction rate was higher on Gal\(_4\), Gal\(_5\) and Gal\(_7\) than on Gal\(_3\) which supports the hypothesis of Kotake that longer unbranched \(\beta-(1\rightarrow3)\)-galactooligosaccharides serve as better substrates than Gal\(_3\). None of the enzymes were able to hydrolyze the \(\beta-(1\rightarrow6)\)-linkage, since all the proposed degradation products were observed with a different retention time compared to linear oligosaccharides, hence can be assumed to be branched. The \(\beta-(1\rightarrow6)\) branching of the \(\beta-(1\rightarrow3)\)-galactan backbone did reduce the degree of hydrolysis, but did not completely prevent the enzymes from acting on substrates and degradation products carrying a \(\beta-(1\rightarrow6)\) branch. Furthermore, subtle differences between the substrates were observed: both AtEn3GAL and PsEn3GAL tolerated substitutions in subsites -2, -1, +1, and +2, but AtEn3GAL was not able to hydrolyze Gal\(_2\)Gal\(_3\) (Figure 7A) even though a substitution in the -2 subsite was accepted during hydrolysis of Gal\(_2\)Gal\(_7\) (Figure 6A). Likewise, PsEn3GAL was able to hydrolyze Gal\(_2\)Gal\(_4\) with the substitution in the -1 subsite, but was not able to hydrolyze Gal\(_2\)Gal\(_3\). This might be due to a requirement of at least four consecutive \(\beta-(1\rightarrow3)\)-linked galactosyl units, if one of them is substituted. Although able to tolerate a single substituent at either -2, -1, +1 or +2 subsites, it is currently not known how two closely positioned substituents affect reactivity. In any case, significant substitution of the \(\beta-(1\rightarrow3)\)-galactan backbone clearly impedes enzyme efficiency by blocking access to the backbone as observed with native gum arabic and the single, double and triple Smith degraded gum arabic samples having decreasing degrees of branching (Table 2).
In conclusion, three novel GH16 endo-β-(1→3)-galactanases were purified and characterized with a set of well-defined linear and branched β-(1→3)-galactooligosaccharides providing important knowledge on substrate specificity of this class of enzymes in particular with respect to their ability to accommodate branches in vicinity to the active site; a feature that is highly desirable in connection with industrial processing of gum arabic and other substituted β-(1→3)-galactans. Since the currently known sequence diversity of putative GH16 endo-β-(1→3)-galactanases is phylogenetically classified in one clade with taxonomically contingent distribution, it is not likely that the observed differences in specificity among the tested endo-β-(1→3)-galactanases can justify classification in functional GH16 endo-β-(1→3)-galactanase subfamilies; rather the observed differences likely arise from individual enzyme properties and preferences among the different tested substrates.

5. Conflict of Interest

The authors declare no conflict of interest. NS and RNM are employees of Novozymes A/S.

6. Acknowledgements

We acknowledge financial support from the Danish Council for Independent Research (Grant Case no.: 107279), Danish Council for Strategic Research (GlycAct and SET4Future projects), the Villum Foundation (PLANET project), the Carlsberg Foundation and the Novo Nordisk Foundation (Biotechnology-based Synthesis and Production Research). The 800 MHz NMR data were recorded on the NMR spectrometers of the NMR Center • DTU supported by the Villum Foundation.

7. Literature list

Altschul, S.F., Gish, W., Miller, W., Meyers, E.W., Lipman, D.J., 1990. Basic Local Alignment Search Tool


Table 1. Physico-chemical properties of GH16 endo-\(\beta\)-(1→3)-galactanases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>%ID to FvEn3GAL</th>
<th>%ID to FvEn3GAL</th>
<th>%ID to FvEn3GAL</th>
<th>%ID to FvEn3GAL</th>
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<tbody>
<tr>
<td></td>
<td>(Kotake et al., 2011)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AtEn3GAL</td>
<td>A. terreus(^a)</td>
<td>50</td>
<td>26.7</td>
<td>35</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NfEn3GAL</td>
<td>N. fischerr(^a)</td>
<td>51</td>
<td>26.8</td>
<td>30</td>
<td>7.1</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>PsEn3GAL</td>
<td>P. strigosozonata(^b)</td>
<td>59</td>
<td>27.7</td>
<td>35</td>
<td>4.0</td>
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</tbody>
</table>

\(^a\) Ascomycete; \(^b\) Basidiomycete
Table 2. Relative activity of purified endo-β-(1→3)-galactanases on various polysaccharide substrates at 40 °C, pH 5. The activity was determined by reducing ends and adjusted with respect to substrate concentration and enzyme dosage and normalized.

<table>
<thead>
<tr>
<th>Substrate1</th>
<th>AtEn3GAL</th>
<th>NfEn3GAL</th>
<th>PsEn3GAL</th>
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<tbody>
<tr>
<td>β-(1→3)-galactans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gum arabic (acacia)</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
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<tr>
<td>Heat treated gum arabic</td>
<td>5.8</td>
<td>5.5</td>
<td>10</td>
</tr>
<tr>
<td>Acid treated gum arabic</td>
<td>0.1</td>
<td>1.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Single Smith degraded gum arabic</td>
<td>5.0</td>
<td>5.0</td>
<td>0.2</td>
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<tr>
<td>Double Smith degraded gum arabic</td>
<td>6.6</td>
<td>6.6</td>
<td>49</td>
</tr>
<tr>
<td>Triple Smith degraded gum arabic</td>
<td>100</td>
<td>42</td>
<td>98</td>
</tr>
<tr>
<td>Larchwood arabinogalactan</td>
<td>0</td>
<td>2.8</td>
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</table>

*Other glycans*

<table>
<thead>
<tr>
<th></th>
<th>AtEn3GAL</th>
<th>NfEn3GAL</th>
<th>PsEn3GAL</th>
</tr>
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<tbody>
<tr>
<td>Galactan (lupin)</td>
<td>1.2</td>
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<tr>
<td>Galactomannan (locust bean)</td>
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<td>0.6</td>
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<td>Mannan (ivory nut)</td>
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<td>Glucan (curdlan)</td>
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</table>

1) Substrate concentrations were 3.3 g/L except for triple Smith degraded gum arabic (SD III GA) (1.7 g/L), heat treated gum arabic (1.7 g/L), mannan (2.3 g/L) and gum arabic (5 g/L); Relative activities are indicated in % activity as compared to the activity of AtEn3GAL on triple Smith degraded gum arabic.
Figure legends:

Figure 1. Chemical structure of defined linear and branched substrates together with Consortium for Functional Glycomics symbols (β-(1→3) linkages omitted for clarity). 1: Gal5, 2: Gal7, 3: Gal5Gal7, 4: Gal5Gal7.

Figure 2. SDS-PAGE gel of purified endo-β-(1→3)-galactanases. M: Marker (200, 116.3, 97.4, 66.3, 55.4, 36.5, 31.0, 21.5, 14.4, 6.0, 3.5, 2.5 kDa; 1: AtEn3GAL; 2: NfEn3GAL, 3: PsEn3GAL.

Figure 3. Effect of pH and temperature on the relative activity of NfEn3GAL.

Figure 4. Suggested degradation patterns for linear Gal5 (A) and linear Gal7 (B).

Figure 5. Progression profiles for degradation of A: Gal5, B: Gal7, C: Gal5Gal7, and D: Gal5Gal7 by PsEn3GAL.

Figure 6. Degradation patterns of gal5gal7 by AtEn3GAL (A, red), NfEn3GAL (A, green), and PsEn3GAL (B blue).

Figure 7. Degradation patterns of Gal5Gal7 by AtEn3GAL (blue), NfEn3GAL (green), and PsEn3GAL (red) (A) and alternative degradation pattern of PsEn3GAL (red) (B).
Figure 1.
Figure 2.
<table>
<thead>
<tr>
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Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.

A

\[ \text{Gal}_2^3\text{Gal}_4 \]

\[ \text{Gal}_2^1\text{Gal}_2 \]

\[ \text{Gal}_2^2\text{Gal}_3 \]

B

\[ \text{Gal}_2^3\text{Gal}_3 \]