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1 **Substrate specificity of novel GH16 endo- β -(1 $\rightarrow$ 3)-galactanases acting on linear and branched β -(1 $\rightarrow$ 3)-**
2 **galactooligosaccharides**

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11 Short running title: novel GH16 endo- β -(1 $\rightarrow$ 3)-galactanases

12

13

14 Abstract

15 Arabinogalactan proteins are proteoglycans located in the plant cell wall. Most arabinogalactan proteins
16 are composed of carbohydrate moieties of β -(1 $\rightarrow$ 3)-galactan main chains with β -(1 $\rightarrow$ 6)-galactan side chains
17 terminated by other glycans. In this study, three novel endo- β -(1 $\rightarrow$ 3)-galactanases were identified and the
18 substrate specificity was further studied using well-defined galactan oligomers. Linear and branched β -
19 (1 $\rightarrow$ 3)-linked galactans, which resemble the carbohydrate core of the arabinogalactan protein, were used
20 for the characterization of endo- β -(1 $\rightarrow$ 3)-galactanases. The identified enzymes required at least three
21 consecutive galactose residues for activity. Non-substituted regions were preferred, but substituents in the
22 -2 and +2 and in some cases also -1 and +1 subsites were tolerated to some extent, depending on the
23 branching pattern, however at a significantly lower rate/frequency.

24 Keywords

25 β -(1 $\rightarrow$ 3)-galactanases; gum arabic; β -(1 $\rightarrow$ 3)-galactan; type II arabinogalactan; glycosyl hydrolase (GH)
26 family 16

27

28 1. Introduction

29 Arabinogalactan proteins (AGPs) belong to the family of hydroxyproline-rich glycoproteins (HRGPs) and
30 they are characterized as highly glycosylated proteoglycans (Pereira et al., 2015). They are considered as one
31 of the most complex macromolecule families found in plants. Their complexity is revealed from the
32 remarkable diversity of the glycans attached to the protein backbone, comprising 90–95% of the total
33 mass (Ellis et al., 2010). Determining the precise structure of the carbohydrate moiety of the AGPs is of
34 major importance to better understand their biosynthesis and their biological and possible industrial

35 applications (Ling et al., 2012). AGPs are known to be involved in various processes in plant growth and
36 development such as cell division (Serpe and Nothnagel, 1994), cell development (Van Hengel and Roberts,
37 2002), signaling and cell death (Gao and Showalter, 1999).

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

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

54 Enzymes hydrolyzing β -1,3-galactans include exo- β -(1 $\rightarrow$ 3)-galactanases and endo- β -(1 $\rightarrow$ 3)-galactanases.
55 Exo- β -(1 $\rightarrow$ 3)-galactanases from glycosyl hydrolase family 43 (GH43) hydrolyze the β -(1 $\rightarrow$ 3)-galactan
56 backbone of e.g. partially debranched gum arabic or larchwood arabinogalactan in an exo fashion requiring
57 at least two consecutive galactose residues for activity. Remarkably, they are able to bypass branch points
58 liberating also intact β -(1 $\rightarrow$ 6)-linked galactooligosaccharide side chains (Ling et al., 2012; Sakamoto and

59 Ishimaru, 2013; Tsumuraya et al., 1990). Endo- β -(1 $\rightarrow$ 3)-galactanase activity was observed already around
60 1970 (Hashimoto, 1971; Hashimoto et al., 1969), but has been much less studied and was first rigorously
61 characterized by Kotake and co-workers in 2011 (Kotake et al., 2011). The authors identified the first endo-
62 β -(1 $\rightarrow$ 3)-galactanase (FvEn3GAL) from the winter mushroom *Flammulina velutipes* which was subsequently
63 cloned, purified and characterized. Two homologous endo- β -(1 $\rightarrow$ 3)-galactanases (45-46% sequence
64 homology to FvEn3GAL) from *Aspergillus flavus* (Af3G) and from *Neurospora crassa* (NcEn3GAL) were
65 recently expressed in *Pichia pastoris*, purified and characterized (Yoshimi et al., 2017). All three enzymes
66 specifically hydrolyse β -(1 $\rightarrow$ 3)-galactan and β -(1 $\rightarrow$ 3)-galactooligosaccharides with degree of polymerization
67 of at least three (Kotake et al., 2011; Yoshimi et al., 2017). However, the enzymes only have trace activity
68 towards native gum arabic, probably because branching at the 6-position prevents them from accessing the
69 β -(1 $\rightarrow$ 3)-galactan main chains. These enzymes are classified as EC 3.2.1.181 and belong to GH16 (Sakamoto
70 and Ishimaru, 2013), a family that also includes endo- β -(1 $\rightarrow$ 3(4))-glucanases. Apart from FvEn3GAL,
71 NcEn3GAL and Af3G, no other endo- β -(1 $\rightarrow$ 3)-galactanases have been described in the literature. Endo- β -
72 (1 $\rightarrow$ 3)-galactanases could be relevant industrially due to their potential to process AGP, gum arabic or
73 larchwood arabinogalactan, possibly in combination with auxiliary, debranching enzymes such as endo- β -
74 (1 $\rightarrow$ 6)-galactanases, α -L-arabinofuranosidases and α -L-rhamnosidases (Sakamoto and Ishimaru, 2013;
75 Yoshimi et al., 2017).

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

79

80

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 $\rightarrow$ 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 $\rightarrow$ 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' CCCTCTAGATCTCGAGGATGGTGGTGTGGTGTGGTGAA-3'). The PCR amplified fragment was cloned into the *Aspergillus* expression vector pDau222 behind an inducible *Aspergillus* natural amylase promoter (Budolfson 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 $\rightarrow$ 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' ACACAACTGGGGATCCACCATGAAGTTTTCAACCCTTCTGAGTCTTC 3' and 5' CCCTCTAGATCTCGAGCATCAAACGTATCATTCGGGGTT-3'). The cloned gene sequence was verified by Sanger sequencing. The *Punctularia strigosozonata* HHB-11173 GH16 (PsEn3GAL) was cloned into pDAu222

104 by T4 DNA ligation using a synthetic DNA encoding the predicted cDNA sequence flanked by BamHI and
105 XhoI restriction sites.

106 2.3. Molecular Cloning of AtEn3GAL, NfEn3GAL and PsEn3GAL

107 The DNA sequence encoding *A. terreus* GH16 endo- β -(1 $\rightarrow$ 3)-galactanase (residues 1-245) was amplified
108 from the *A. terreus* S-712 genomic DNA library by PCR using the primers AT-F: 5'
109 ACACAACTGGGGATCCACCATGTACCTGCTCACACTTCTCGCC 3' and AT-R: 5'
110 CCCTCTAGATCTCGAGGATGGTGGTGTGGTGTGGTGAA 3'. The amplification reaction was composed of 2.5 μ L
111 *A. terreus* S-712 genomic DNA, 10 μ L 5x Phusion buffer HF, 1 μ L 10 mM dNTP, 2 μ L 2.5 μ M primer AT-F, 2 μ L
112 2.5 μ M primer AT-R, 32 μ L H₂O, and 0.5 μ L 2 u/ μ L Phusion polymerase (ThermoFisher Scientific, Life
113 Technologies Europe BV, Nærum, Denmark). The amplification reaction was incubated in a PTC-200 DNA
114 Engine Thermal Cycler programmed for one cycle at 94 °C for 2 min; and 35 cycles each at 94 °C for 15 s and
115 60 °C for 1.5 min. A 1.0 kb PCR reaction product was isolated by 1 % agarose gel electrophoresis using TAE
116 buffer and staining with SYBR Safe DNA gel stain. The DNA band was visualized with the aid of an Eagle Eye
117 Imaging System and a Darkreader Transilluminator. The 1.0 kb DNA band was excised from the gel and
118 purified using a GFX PCR DNA and Gel Band Purification Kit according to the manufacturer's instructions.
119 The 1.0 kb fragment was cloned into BamHI-XhoI cleaved pDAu222 (Schnorr et al., 2015) using In-Fusion HD
120 Cloning Kit (Clontech, Takara Bio, USA) according to the manufacturer's instructions. The reaction mixture
121 was transformed into *E. coli* TOP10F competent cells (Invitrogen) according to the manufacturer's
122 instructions. The transformation mixture was plated onto LB plates supplemented with 50 μ g of ampicillin
123 per mL. Plasmid minipreps were prepared from several transformants and sequenced. One plasmid with
124 the correct *A. terreus* GH16 coding sequence was chosen. The DNA sequence (accession number:
125 EMBLEXP:DS027696) encoding *N. fischeri* GH16 endo- β -(1 $\rightarrow$ 3)-galactanase (residues 1-245) was amplified
126 from the *Neosartorya fischeri* NRRL 181 genomic DNA library by PCR using the primers NF-F: 5'
127 ACACAACTGGGGATCCACCATGAAGTTTCAACCCTTCTGAGTCTTC 3' and NF-R: 5'
128 CCCTCTAGATCTCGAGCATCAAAACGTATCATTCGGGGTT 3'. PCR amplification and cloning of NfEn3GAL was

129 carried out as described for AtEn3GAL. The cDNA sequence derived from EMBLEXP:JH687561 encoding *P.*
130 *strigosozonata* GH16 endo- β -(1 $\rightarrow$ 3)-galactanase (residues 1-258) was synthesized at Geneart
131 (ThermoFisher Scientific, Life Technologies Europe BV, Naerum, Denmark). The 0.8 kbp cDNA fragment was
132 cut out of the pUC19 cloning vector with BamHI and XhoI and cloned into pDAu222 using In-Fusion cloning
133 as described above.

134

135 2.4. Expression of Recombinant Protein

136 The three endo- β -(1 $\rightarrow$ 3)-galactanase expression constructs were transformed into the *Aspergillus oryzae*
137 strain MT3568 (McBrayer et al., 2011). The *Aspergillus oryzae* strain MT3568 (Biely et al., 2014) was
138 transformed with pDAU222-AtEn3GAL, pDAU222-NfEn3GAL, or pDAU222-PsEn3GAL using standard
139 techniques (Christensen et al., 1988). Transformants were selected on acetamide during regeneration from
140 protoplasts and subsequently re-isolated under selection conditions (Christensen et al., 1988). To assay for
141 recombinant expression, transformants were cultured in 0.75 mL YP + 2% glucose medium and DAP-4C-1
142 medium (Biely et al., 2014) in 96-well deep well microtiter plates for 4 days at 30 °C, and samples were
143 monitored by SDS-PAGE. Two transformants were selected based on expression in microtiter cultures and
144 cultured in 150 mL of DAP-4C-1 medium in baffled 500 mL flasks shaken at 100 rpm at 30 °C. Samples were
145 taken after 4 days, expression of recombinant *A. terreus* endo- β -(1 $\rightarrow$ 3)-galactanase protein was monitored
146 by SDS-PAGE, and the highest yielding transformant for each of the three constructs was selected.
147 For production of recombinant endo- β -(1 $\rightarrow$ 3)-galactanase enzyme for purification and characterization,
148 *Aspergillus* transformants were cultured in DAP-4C-1 medium in baffled flasks shaken at 150 rpm at 30 °C.
149 The culture broth was harvested after 4 days and separated from cellular material by passage through a
150 0.22 μ m filter.

151

152

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. Larchwood arabinogalactan, pectic lupin type I galactan, galactomannan, mannan, β -glucan (water-soluble β -(1 $\rightarrow$ 3/4) polysaccharides) and curdlan (β -(1 $\rightarrow$ 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 (T_m)

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, T_m (°C), was defined as the top of the denaturation peak (major endothermic peak) in the thermogram (C_p 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 galactooligosaccharides (**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 μ m column (Waters Corporation). The major analytical parameters were 20 °C column temperature, 1.0 mL/min flow rate and 10 μ L 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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 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 $\rightarrow$ 3)-galactanases was

264 carried out in *Aspergillus oryzae* using the endogenous secretion signals as described elsewhere (Biely et
265 al., 2014) and purified to homogeneity using conventional methods (Figure 2).

266 3.2. Physico-chemical properties of endo- β -(1 $\rightarrow$ 3)-galactanases

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

276

277 3.3. Initial experiments on non-, single, double, and triple Smith degraded gum arabic

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

282

283 3.4. Temperature and pH optimum

284 In order to determine the temperature and pH optima for NfEn3GAL, a number of different combinations
285 of temperature and pH were investigated by reducing end assay with triple smith degraded gum arabic. The

286 temperature and pH experiments revealed optimal conditions for NfEn3GAL around 40 °C and pH 5–5.5
287 (Figure 3). For temperatures above 40 °C the activity was rapidly decreasing regardless of the pH of the
288 medium. For pH values below 3 and above 8 the residual activity was less than 10%. Since both AtEn3GAL
289 and PsEn3GAL had higher activity than NfEn3GAL at pH 5 and 40 °C, no further tests to find optimal
290 reaction conditions for AtEn3GAL and PsEn3GAL were conducted.

291

292 3.5. Mode of action on defined substrates

293 The substrate specificity and the mode of action were investigated for all three enzymes on defined linear
294 and branched oligosaccharides (see Figure 1). Linear substrates were β -(1→3)-linked galactopentaose (**1**)
295 and galactoheptaose (**2**), designated gal₅ and gal₇ respectively. The branched molecules were two
296 nonasaccharides with a linear β -(1→3)-linked galactoheptaose backbone carrying a β -(1→3)-linked
297 galactobiose linked to either the 6'''-position (**3**) or the 6''-position (**4**), designated Gal₂⁵Gal₇ and Gal₂³Gal₇,
298 respectively.

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

306 All three enzymes were able to degrade Gal₅ following the same major degradation pattern (Figure 4A),
307 where the pentamer was degraded into Gal₃ and Gal₂. For both AtEn3GAL and PsEn3GAL, Gal₃ was further
308 degraded into Gal₂ and Gal, while no further degradation of Gal₃ was observed for NfEn3GAL. A higher
309 activity towards Gal₅ compared to Gal₃ was observed, indicating a greater affinity for the longer oligomer.

310 No further degradation of Gal₂ was observed in any of the experiments (Figure 5, supplementary figure 3A,
311 and supplementary figure 4A). For the linear (unbranched) substrates **1** and **2** it is not known if a
312 preference for hydrolysis closer to the reducing versus non-reducing end exists. Degradation patterns are
313 arbitrarily drawn closer to reducing end for simplicity.

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

319 The degradation pattern of the branched oligomers revealed some variation among the three enzymes.
320 When AtEn3GAL and NfEn3GAL acted on Gal₂⁵Gal₇ (**3**), the initial products observed were Gal₂²Gal₄ and
321 Gal₃, with further hydrolysis into Gal₂²Gal₂, Gal₂ and Gal only for AtEn3GAL (Figure 6A). Given the similarity
322 in sequence among GH16 endo-β-(1→3)-galactanases it is not likely that the difference in reactivity of
323 AtEn3GAL and NfEn3GAL is a sign of overall subfamily specificity in GH16, but rather a result of differences
324 in individual enzyme properties. The absence of further degradation products for NfEn3GAL might be due
325 to the relative low activity compared to AtEn3GAL and PsEn3GAL, even though increased enzyme dosage
326 and prolonged reaction time did not result in further degradation (data not shown). Contrary to this, the
327 main degradation pattern of PsEn3GAL was the formation of Gal₂³Gal₅ and Gal₂, with further hydrolysis into
328 Gal₂²Gal₂, Gal₂ and Gal (Figure 6B). The degradation pattern shown in Figure 6A was also observed for
329 PsEn3GAL, but to a much lesser extent.

330 The second branched oligomer Gal₂³Gal₇ (**4**) also resulted in some variation between the enzymes. All three
331 were able to degrade Gal₂³Gal₇ into Gal₂³Gal₄ and Gal₃ (Figure 7A). Both AtEn3GAL and PsEn3GAL further
332 degraded Gal₃ into Gal₂ and Gal, along with further hydrolysis of Gal₂³Gal₄ to either Gal₂¹Gal₂ or Gal₂²Gal₃.
333 No degradation of Gal₂³Gal₄ was observed for NfEn3GAL, which again, as for Gal₂⁵Gal₇, can be due to the

334 relative low activity of NfEn3GAL. Even though AtEn3GAL hydrolyzed Gal₂³Gal₇ as described, a second
335 degradation pattern was observed with the formation of Gal₂³Gal₃ and Gal₄, with the latter immediately
336 hydrolyzed to Gal₂ (Figure 7B). Likewise, small amounts of Gal₂³Gal₅ were observed for PsEn3GAL, indicating
337 another pattern with the release of Gal₂ from the non-reducing end (not shown in Figure 7).

338

339 4. Discussion

340 The three GH16 endo- β -(1 $\rightarrow$ 3)-galactanases presented here all displayed low activity towards the different
341 polysaccharide substrates tested, except for triple smith degraded gum arabic. This was expected, since
342 accessible, unsubstituted β -(1 $\rightarrow$ 3) linked galactan is mainly present in the latter substrate (see also
343 supplementary figure 1). Also, in the case of gum arabic, it was clear that partial or full removal of β -(1 $\rightarrow$ 6)-
344 linked branching by Smith degradation was a necessity in order for the enzymes to catalyze the hydrolysis
345 of the β -(1 $\rightarrow$ 3) linked galactan backbone.

346 The observed pH and temperature optima for NfEn3GAL at pH 5 and 40 °C were similar to those reported
347 by Kotake *et al.* (Kotake et al., 2011) and Yoshimi *et al.* (Yoshimi et al., 2017), where the pH optima for the
348 endo- β -(1 $\rightarrow$ 3)-galactanases FvEn3GAL, NcEn3GAL and Af3G were between pH 4 and 5.5 and the
349 temperature optima for their enzymatic reaction were found to be 40–45 °C. In pH values below 3 and over
350 8 NfEn3GAL was not active.

351 On the basis of the mode of action of the three enzymes and their specificity towards the studied β -(1 $\rightarrow$ 3)-
352 galactans, all three can be classified as endo- β -(1 $\rightarrow$ 3)-galactanases. The endo-acting fashion was verified by
353 the formation of several oligosaccharides during the initial phase of the reactions. The inability to hydrolyze
354 galactobiose is further evidence that all the enzymes are endo- β -(1 $\rightarrow$ 3)-galactanases. On the contrary, exo-
355 β -(1 $\rightarrow$ 3)-galactosidases would have released high amounts of galactose in the initial phase of the reaction.
356 Also, as larger oligomers were not observed during the experiments with any of the defined linear or

357 branched oligosaccharides, the occurrence of transglycosylation reaction was unlikely, but cannot be ruled
358 out.

359 The degradation of the branched oligomers followed different primary pathways, but with overlap among
360 the three enzymes suggesting slightly different substrate preferences of the different enzymes.
361 Furthermore, the particular substrate specificity of all three enzymes showed a preference for hydrolysis of
362 the β -(1 $\rightarrow$ 3)-linkage of β -(1 $\rightarrow$ 3)-galactooligosaccharides having at least three consecutive galactosyl units
363 which is in accordance with the observations of Kotake *et al.* (Kotake et al., 2011). Furthermore, the relative
364 reaction rate was higher on Gal₄, Gal₅ and Gal₇ than on Gal₃ which supports the hypothesis of Kotake that
365 longer unbranched β -(1 $\rightarrow$ 3)-galactooligosaccharides serve as better substrates than Gal₃. None of the
366 enzymes were able to hydrolyze the β -(1 $\rightarrow$ 6)-linkage, since all the proposed degradation products were
367 observed with a different retention time compared to linear oligosaccharides, hence can be assumed to be
368 branched. The β -(1 $\rightarrow$ 6) branching of the β -(1 $\rightarrow$ 3)-galactan backbone did reduce the degree of hydrolysis,
369 but did not completely prevent the enzymes from acting on substrates and degradation products carrying a
370 β -(1 $\rightarrow$ 6) branch. Furthermore, subtle differences between the substrates were observed: both AtEn3GAL
371 and PsEn3GAL tolerated substitutions in subsites -2, -1, +1, and +2, but AtEn3GAL was not able to hydrolyze
372 Gal₂³Gal₃ (Figure 7A) even though a substitution in the -2 subsite was accepted during hydrolysis of
373 Gal₂⁵Gal₇ (Figure 6A). Likewise, PsEn3GAL was able to hydrolyze Gal₂³Gal₄ with the substitution in the -1
374 subsite, but was not able to hydrolyze Gal₂³Gal₃. This might be due to a requirement of at least four
375 consecutive β -(1 $\rightarrow$ 3)-linked galactosyl units, if one of them is substituted. Although able to tolerate a single
376 substituent at either -2, -1, +1 or +2 subsites, it is currently not known how two closely positioned
377 substituents affect reactivity. In any case, significant substitution of the β -(1 $\rightarrow$ 3)-galactan backbone clearly
378 impedes enzyme efficiency by blocking access to the backbone as observed with native gum arabic and the
379 single, double and triple Smith degraded gum arabic samples having decreasing degrees of branching (Table
380 2).

381 In conclusion, three novel GH16 endo- β -(1 $\rightarrow$ 3)-galactanases were purified and characterized with a set of
382 well-defined linear and branched β -(1 $\rightarrow$ 3)-galactooligosaccharides providing important knowledge on
383 substrate specificity of this class of enzymes in particular with respect to their ability to accommodate
384 branches in vicinity to the active site; a feature that is highly desirable in connection with industrial
385 processing of gum arabic and other substituted β -(1 $\rightarrow$ 3)-galactans. Since the currently known sequence
386 diversity of putative GH16 endo- β -(1 $\rightarrow$ 3)-galactanases is phylogenetically classified in one clade with
387 taxonomically contingent distribution, it is not likely that the observed differences in specificity among the
388 tested endo- β -(1 $\rightarrow$ 3)-galactanases can justify classification in functional GH16 endo- β -(1 $\rightarrow$ 3)-galactanase
389 subfamilies; rather the observed differences likely arise from individual enzyme properties and preferences
390 among the different tested substrates.

391

392 5. Conflict of Interest

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

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400

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473

474

475 [Tables](#)

476 Table 1. Physico-chemical properties of GH16 endo-β-(1→3)-galactanases.

Enzyme	Source	%ID to FvEn3GAL (Kotake et al., 2011)	Theoretical	Observed		Tm (°C)
			MW (kDa)	MW (kDa)	Predicted pI	
AtEn3GAL	<i>A. terreus</i> ^A	50	26.7	35	3.9	73
NfEn3GAL	<i>N. fischeri</i> ^A	51	26.8	30	7.1	53
PsEn3GAL	<i>P. strigosozonata</i> ^B	59	27.7	35	4.0	67

477 ^A Ascomycete; ^B Basidiomycete

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480

481 Table 2. Relative activity of purified endo- β -(1 $\rightarrow$ 3)-galactanases on various polysaccharide substrates at 40
482 $^{\circ}$ C, pH 5. The activity was determined by reducing ends and adjusted with respect to substrate
483 concentration and enzyme dosage and normalized.

Substrate ¹	Relative activities (%)		
	AtEn3GAL	NfEn3GAL	PsEn3GAL
<i>β-(1$\rightarrow$3)-galactans</i>			
Gum arabic (acacia)	0.2	0.3	0.2
Heat treated gum arabic	5.8	5.5	10
Acid treated gum arabic	0.1	1.2	0.2
Single Smith degraded gum arabic	5.0	5.0	0.2
Double Smith degraded gum arabic	6.6	6.6	49
Triple Smith degraded gum arabic	100	42	98
Larchwood arabinogalactan	0	2.8	0
<i>Other glycans</i>			
Galactan (lupin)	1.2	1.2	0
Galactomannan (locust bean)	0	0.6	2.6
Mannan (ivory nut)	0.7	0.9	1.4
Glucan (barley)	0	0	0
Glucan (curdian)	0	1.2	2.0

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

487

488

489 Figure legends:

490 Figure 1. Chemical structure of defined linear and branched substrates together with Consortium for
491 Functional Glycomics symbols (β -(1 $\rightarrow$ 3) linkages omitted for clarity). 1: Gal₅, 2: Gal₇, 3: Gal₂⁵Gal₇, 4:
492 Gal₂³Gal₇.

493

494 Figure 2. SDS-PAGE gel of purified endo- β -(1 $\rightarrow$ 3)-galactanases. M: Marker (200, 116.3, 97.4, 66.3, 55.4,
495 36.5, 31.0, 21.5, 14.4, 6.0, 3.5, 2.5 kDa; 1: AtEn3GAL; 2: NfEn3GAL, 3: PsEn3GAL.

496

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

498

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

500

501 Figure 5. Progression profiles for degradation of A: Gal₅, B: Gal₇, C: Gal₂⁵Gal₇, and D: Gal₂³Gal₇ by PsEn3GAL.

502

503 Figure 6. Degradation patterns of gal₂⁵gal₇ by AtEn3GAL (A, red), NfEn3GAL (A, green), and PsEn3GAL (B
504 blue).

505

506 Figure 7. Degradation patterns of Gal₂³Gal₇ by AtEn3GAL (blue), NfEn3GAL (green), and PsEn3GAL (red) (A)
507 and alternative degradation pattern of PsEn3GAL (red) (B).

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512 Figure 1.

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516 Figure 2.

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		pH									
		2	3	4	4.5	5	5.5	6	7	8	9
temperature	20	0	2	49	57	80	42	34	29	5	2
	30	3	8	77	83	85	58	54	21	8	5
	40	7	0	9	54	100	99	76	33	4	0
	50	7	6	8	16	19	18	18	9	7	4
	60	0	0	0	0	0	1	2	0	0	0
	70	0	0	1	1	0	1	3	1	0	0
	80	0	0	5	0	0	0	3	3	0	2

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520 Figure 3.

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524 Figure 4.

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Figure 5.

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533 Figure 6.

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537 Figure 7.