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Note

Activity of three β-1,4-galactanases on small chromogenic substrates

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ABSTRACT

β-1,4-Galactanases belong to glycoside hydrolase family GH 53 and degrade galactan and arabinogalactan side chains of the complex pectin network in plant cell walls. Two fungal β-1,4-galactanases from *Aspergillus aculeatus, Meripileus giganteus* and one bacterial enzyme from *Bacillus licheniformis* have been kinetically characterized using the chromogenic substrate analog 4-nitrophenyl β-1,4-D-thiogalactobioside synthesized by the thioglycoligase approach. Values of k_{cat}/K_m for this substrate with *A. aculeatus* β-1,4-galactanase at pH 4.4 and for *M. giganteus* β-1,4-galactanase at pH 5.5 are 333 M^{-1} s^{-1} and 62 M^{-1} s^{-1}, respectively. By contrast the *B. licheniformis* β-1,4-galactanase did not hydrolyze 4-nitrophenyl β-1,4-D-thiogalactobioside. The different kinetic behavior observed between the two fungal and the bacterial β-1,4-galactanases can be ascribed to an especially long loop 8 observed only in the structure of *B. licheniformis* β-1,4-galactanase. This loop contains substrate binding subsites −3 and −4, which presumably cause *B. licheniformis* β-1,4-galactanase to bind 4-nitrophenyl -1,4-β-D-thiogalactobioside non-productively. In addition to their cleavage of 4-nitrophenyl -1,4-β-D-thiogalactobioside, the two fungal enzymes also cleaved the commercially available 2-nitrophenyl-1,4-β-D-galactopyranoside, but kinetic parameters could not be determined because of transglycosylation at substrate concentrations above 4 mM.

A plant cell is surrounded by an essential cell wall that has diverse functions in the plant's life. At a physical level it protects the plant against pathogens and herbivores and provides rigid support. At a cellular level it takes part in the cell to cell communication, intracellular signaling and transport processes. The cell wall consists of a primary wall and in many cells also of a secondary wall. The primary cell wall consists, among other components, of the polysaccharide pectin, which is especially abundant in non-woody plants.[1] Pectins are composed of regions of homogalacturonan and regions of rhamnogalacturonans I and II. Rhamnogalacturonan I is a polysaccharide consisting of α-1,5-arabinan, β-1,4-galactan and arabinogalactan type I attached to C4 of the Rha residues in a backbone made up of repeating units of 1,2-α-L-rhamnopyranosyl-1,4-α-D-galacturonic acid.[2] The β-1,4 *O*-glycosidic bond in the side chains of β-1,4-galactan and arabinogalactan type I are hydrolyzed by *endo*-β-1,4-galactanase. Some, but not all, *endo*-β-1,4-galactanases can additionally hydrolyze small galactooligosaccharides (e.g., trisaccharides) formed during the initial polysaccharide degradation,[3] as well as the polysaccharide itself.

β-1,4-Galactanases are glycoside hydrolases that belong to family GH 53 in clan GH A,[4,5] the largest of the glycoside hydrolase clans, according to the CAZY classification.[6–8] All the families in glycoside hydrolase clan GH A share a $(\beta/\alpha)_8$ barrel fold[4,5] and the same retaining catalytic mechanism, a double displacement mechanism involving two carboxylic acid residues, one functioning as a catalytic nucleophile and one as a general acid/base.

Enzyme kinetic investigations have been carried out for *endo*-β-1,4-galactanases from several species[9–12] mostly on heterogenous and ill-defined polysaccharide substrates. Here, the activities of *Aspergillus aculeatus* (AAGAL; GenBank ID: AAA32692.1), *Meripileus giganteus* (MGGAL; GenBank: AAQ77827.1) and *Bacillus licheniformis* (BLGAL; GenBank ID: AAO31370.1) *endo*-β-1,4-galactanases towards the well defined thiodisaccharide derivative 4-nitrophenyl 1,4-β-D-thiogalactobioside (4-GTB) as well as the commercially available 2-nitrophenyl β-D-galactopyranoside (2-NPG) were investigated. Small, well defined substrates, as opposed to heterogenous polymers, allow monitoring of hydrolysis of specific

Abbreviations: AAGAL, β-1,4-galactanase from *Aspergillus aculeatus*; BLGAL, β-1,4-galactanase from *Bacillus licheniformis*; MGGAL, β-1,4-galactanase from *Meripileus giganteus*; GH, glycoside hydrolase; 4-GTB, 4-nitrophenyl-1,4-β-D-thiogalactobioside; 2-NPG, 2-nitrophenyl-1,4-β-D-galactopyranoside; 2-NPGB, 2-nitrophenyl-1,4-β-D-galactobioside; DNP, 2,4-dinitrophenol; PNP, 4-nitrophenol.

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glycosidic linkages, and thus a more direct interpretation of activity measurements in terms of molecular mechanisms. The use of a thio-linked disaccharide glycoside ensures that internal degradation of the disaccharide does not occur, simplifying analysis. The results are discussed in the light of the known biochemical characteristics and 3D-structures of AAGAL[13,14] (PDB IDs: 1FHL, 1FOB) and BLGAL[3,15] (PDB IDs: 1R8L, 1UR0, 1UR4, 2CCR, 2GFT, 2J74).

2. Results and discussion

2.1. Production of thiogalactobioside

The thiodisaccharide 4-nitrophenyl -1,4-β-D-thiogalactobioside was produced by the thioglycoligase approach (Scheme 1), which requires a catalytic acid/base mutant of a retaining glycoside hydrolase, an activated sugar donor and a thiosugar acceptor. We used *Agrobacterium* sp. β-glucosidase E170G,[16,17] 2,4-dinitrophenyl β-D-galactopyranoside and 4-nitrophenyl 4-deoxy-4-thio-β-D-galactopyranoside, respectively.

2.2. Activity against polymeric substrates

The specific activities of the enzymes were determined using the dyed substrate azo-galactan by a colorimetric assay involving measurement of the release of dye by monitoring at 540 nm. The specific activities determined for the whole assay duration (20 min) were 0.47 ± 0.02 AU/μg enzyme for MGGAL in 4 mM sodium citrate pH 5.5, 0.12 ± 0.01 AU/μg for AAGAL in 4 mM sodium citrate pH 4.4 and 0.041 ± 0.001 AU/μg for BLGAL in 25 mM MES pH 6.5 and 0.5 mM $CaCl_2$. All measurements were carried out at room temperature.

2.3. Action of β-galactanases on 2-nitrophenyl β-D-galactopyranoside

Initial spectrophotometric monitoring of the reaction of the two fungal β-1,4-galactanases AAGAL and MGGAL showed very low hydrolytic activity on 4 mM 2-nitrophenyl β-D-galactopyranoside (2-NPG) over long reaction times (20 min). AAGAL had under these conditions a specific activity of 0.06 μmol min^{-1} mg^{-1} while MGGAL a specific activity of 0.03 μmol min^{-1} mg^{-1}. However, incubation with higher concentrations of 2-NPG resulted in non-linear progress curves exemplified in Figure 1 (12 mM and 16 mM 2-NPG with AAGAL and MGGAL, respectively). Such behavior is most likely due to the occurrence of an initial transglycosylation to form a disaccharide, 2-nitrophenyl 1,4-β-D-galactobioside (2-NPGB), which serves as a much better substrate for the enzyme and is rapidly cleaved to galactobiose without significant accumulation (Scheme 2). Consistent with this, TLC analysis (Fig. 2) showed that the two fungal galactanases AAGAL and MGGAL

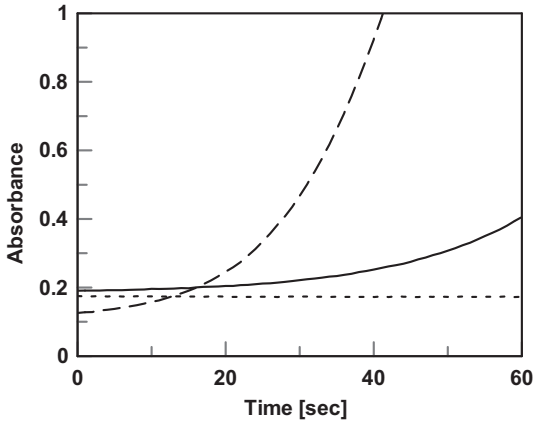
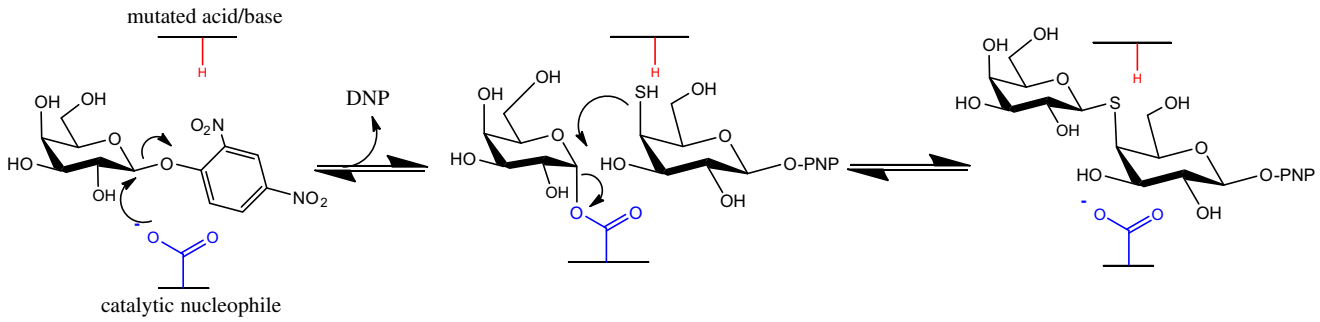


Figure 1. Progress curves for cleavage of 2-NPG by β-1,4-galactanases. Measurements with AAGAL are shown with solid a line, MGGAL with dashed line and BLGAL with a dotted line. The figure was generated using the software package Grafit.[18]

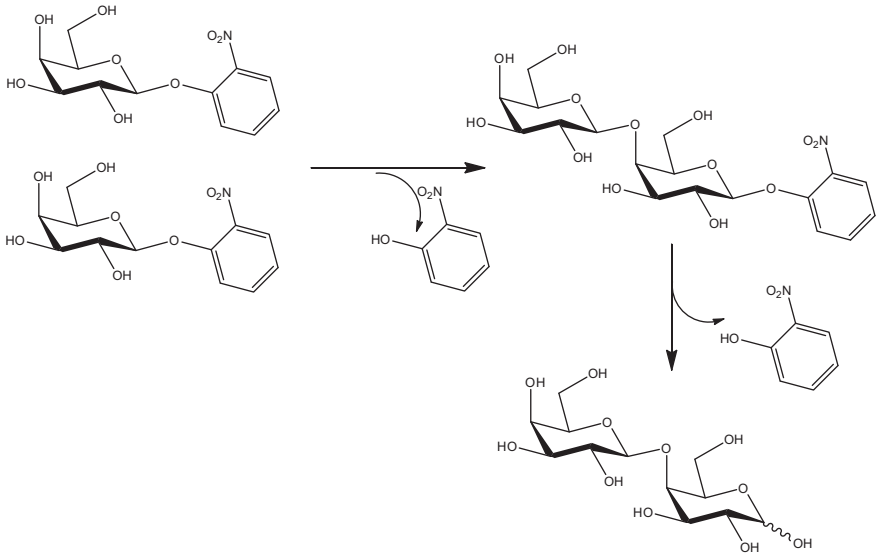
produce galactose and galactobiose when incubated with 2-NPG. The free galactose arises from hydrolysis, occurring in competition with transglycosylation, of the initially formed galactosyl enzyme intermediate. This experiment demonstrates that transglycosylation occurs and explains the accelerating progress curves. No such behavior is seen with BLGAL either in the spectrophotometric or TLC assay (Figs. 1 and 2). Most probably this is because BLGAL does not bind 2-NPG productively, but rather at distal-subsites. This is consistent with earlier investigations,[3] which revealed that the smallest oligosaccharide BLGAL can hydrolyze is β-1,4-galactotetraose.

2.4. Enzymatic hydrolysis of 4-nitrophenyl -1,4-β-D-thiogalactobioside (4-GTB)

The two fungal fungal β-1,4-galactanases AAGAL and MGGAL had considerably higher specific activity on 4 mM 4-GTB than on 2-NPG, 1.8 μmol min^{-1} mg^{-1} for AAGAL and 0.44 μmol min^{-1} mg^{-1} for MGGAL. The initial velocities observed for cleavage of a range of concentrations of the substrate 4-GTB by AAGAL (33 μM) and MGGAL (11 μM) are plotted in Figure 3. Normal saturable Michaelis–Menten kinetics are observed for AAGAL, while a linear dependency is seen for MGGAL, in the measurable concentration range of 4-GTB. The maximum measurable substrate concentration was 12.5 mM, due to the high background signal. Fitting of data for AAGAL to the Michaelis–Menten equation yielded K_m and k_{cat} values of 18.6 ± 5.5 mM and 6.2 ± 1.3 s^{-1}, respectively, using the software package Grafit.[18] The k_{cat}/K_m was calculated as 333 M^{-1} s^{-1}. The linear dependence for MGGAL in the v versus [S] plot indicates a very high K_m value (≫12.5 mM) and does not allow determination of individual values of k_{cat} and K_m. However a reliable value for



Scheme 1. Thioglycoligase reaction mechanism.



Scheme 2. Galactanase-catalyzed formation of galactobiose by transglycosylation.

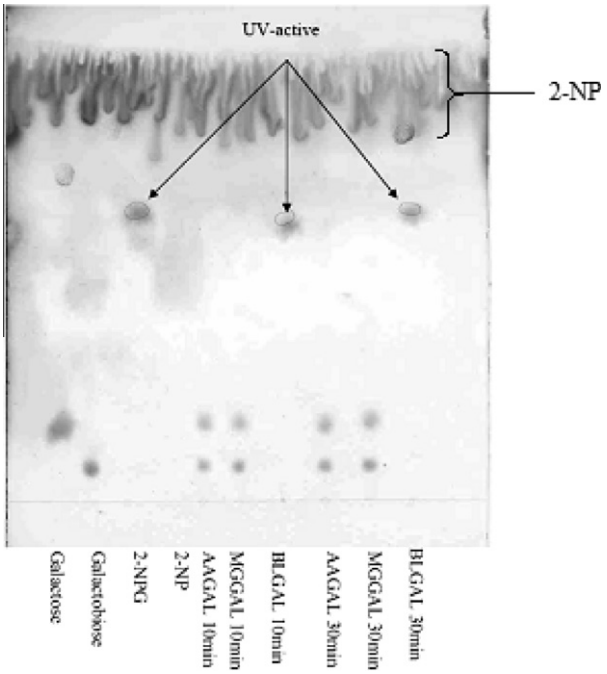


Figure 2. Thin layer chromatographic analysis of the products of reaction of β-1,4-galactanases with 2-NPG. Galactose, galactobiose, 2-NPG and 2-nitrophenol are shown as standards. UV-active compounds are marked with a circle. 2-Nitrophenol is in the broad band at the top of the plate.

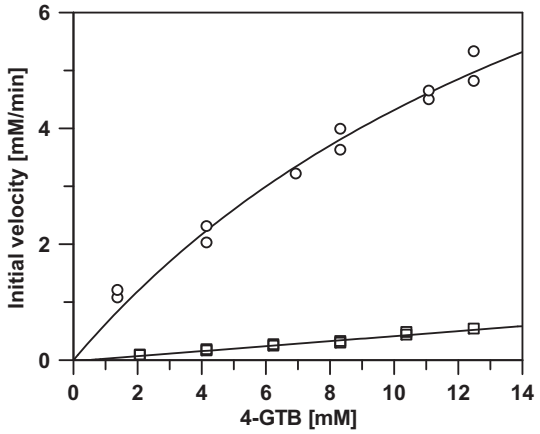


Figure 3. Michaelis–Menten curve for hydrolysis of 4-GTB by β-1,4-galactanases. Measurements with AAGAL are marked with circles and MGGAL with squares. The figure was generated using the software package GraFit.[18]

k_{cat}/K_m of 62 M^{-1} s^{-1} is obtained from the slope and the K_m value must be at least 60 mM according to the following reasoning: the linear dependency for MGGAL in the v versus [S] plot should only be true for $K_m \gg$ [S]. Therefore a conservative estimate for a minimum value of K_m can be 5 × max [S] equal to 60 mM. Since K_m is at least 60 mM, then k_{cat} must be at least 3.7 s^{-1}. No hydrolysis of 4-GTB by BLGAL was observed, even at enzyme concentrations as high as 5.5 mg/mL.

2.5. Structural interpretation of results

The two fungal enzymes, AAGAL (GenBank ID: AAA32692.1) and MGGAL (GenBank ID: AAQ77827.1), have a sequence identity of 43% as calculated by ClustalW[19] (Fig. 4), and can be expected to have very similar active sites. Eight key residues involved directly or indirectly in catalysis (Glu136, Glu246, Trp297, Arg45, Ser213, His81, Asn135, and Gly40), and reported to be functionally conserved[21] or to have functional counterparts[22] for Clan GH-A, are all found in both AAGAL and MGGAL. The Trp115 and Asp117 residues implicated in substrate binding at the −2 subsite for BLGAL (GenBank ID: AAO31370.1, PDB ID: 1UR0)[3] are conserved in both AAGAL and MGGAL, while Lys120 of BLGAL is only conserved in MGGAL and cannot make similar interactions in AAGAL. The putative BLGAL residues[3,15] interacting with the substrate at subsite −1, Asn164, Tyr234 and Ala116, are conserved in both AAGAL and MGGAL. This similarity is consistent with the fact that their k_{cat}/K_m values are only fivefold different—corresponding to less than 1 kcal/mol difference in transition state affinities for the two enzymes. Such a difference is comparable to those observed for disruptions of single hydrogen bonds in other enzyme carbohydrate complexes,[23] and can be rationalized by small structural differences.

The bacterial β-1,4-galactanase BLGAL, unlike the two fungal β-1,4-galactanases MGGAL and AAGAL, does not cleave 4-GTB and 2-NPG while showing activity on dye-modified galactan. Using the

Figure 4. Sequence alignment. Eight functionally conserved key galactanase residues in Clan GH-A (Glu136, Glu246, Trp297, Arg45, Ser213, His81, Asn135 and Gly40, AAGAL numbering)[21] are coloured grey. The residues lining the subsites −1 (Asn164, Tyr234, Ala116) and -2 (Trp115, Asp117, Lys120) based upon the BLGAL structure (BLGAL numbering) are marked with a dotted square and a solid square, respectively. Loop 8 is colored black in the BLGAL sequence. Secondary structure elements are annotated as in.[3] This figure was made with ClustalW[19] and Stride.[20]

known crystal structures for AAGAL and BLGAL[3,14] (PDB IDs: 1FOB, 1UR0), we conclude that this is due to the presence of an especially long loop 8 in the structure of BLGAL, which is much shorter in the structure of AAGAL and in the sequence of MGGAL. This long loop contains two important residues Trp347 and Trp363 that, together with the conserved Trp115, constitute aromatic platforms at the substrate binding subsites −2, −3, and −4 (Fig. 5). The loop enables BLGAL to bind 4-GTB non-productively by the use of subsites −3 and −4 or −2 and −3, most likely subsites −2 and −3, where galactobiose has been shown bound in the crystal structure.[3] The lack of cleavage of 2-NPG could be due to non-productive binding, or because a single subsite is insufficient for binding.

Kinetic studies have been performed on β-1,4-galactanases from other species than those investigated here. Most of this work was however done on heterogenous polymeric substrates and can therefore not be used for comparison. Kinetic studies have been carried out with the bacterial *Pseudomonas fluorescens* sub. *cellulosa* (now known as *Cellvibrio japonicus*) (GenBank ID: CCA62990.1) β-1,4-galactanase using 2,4-dinitrophenyl β-1,4-galactobioside,[12] yielding K_m and k_{cat} values of 2.3 mM and 32 s^{-1}, respectively. *C. japonicus* β-1,4-galactanase, like BLGAL, contains a tryptophan residue (Trp 347) in subsite −3 but lacks Trp363 in subsite −4. A large part of the loop containing subsite −3 and −4 is missing in *C. japonicus* β-1,4-galactanase (Fig. 4), so subsite −3 might well be absent or may be very different, thereby explaining why *C. japonicus* β-1,4-galactanase is unlikely to have the same kinetic properties as BLGAL.

3. Conclusion

Kinetic measurements were performed on two well defined aryl glycoside substrates 2-NPG and 4-GTB, yielding values of K_m and k_{cat} and k_{cat}/K_m for 4-GTB of 18.6 ± 5.5 mM, 6.2 ± 1.3 s^{-1} and 333 M^{-1} s^{-1} for AAGAL, while a k_{cat}/K_m value for MGGAL of 62 M^{-1} s^{-1} was found. BLGAL did not hydrolyze 4-GTB. Kinetic studies with 2-NPG at concentrations above 4 mM yielded non-linear progress curves, suggesting very effective transglycosylation

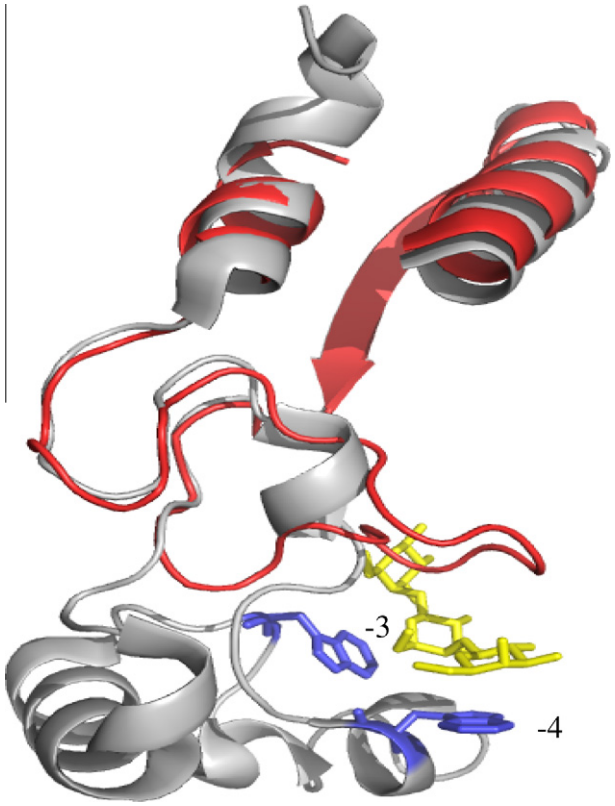


Figure 5. Comparison of the loop region 7/8 of BLGAL (gray) and AAGAL (red) in the published crystal structures.[3,14] The ligand galactotriose (bound in the BLGAL structure) is shown in yellow. Trp347 and Trp363 in subsite −3 and −4 are shown in blue. This figure was generated with Pymol[24] using coordinates with PDB codes 1UR0 and 1FOB for BLGAL and AAGAL, respectively.

for AAGAL and MGGAL, while BLGAL showed no activity. Transglycosylation was confirmed by thin layer chromatography. The lack of BLGAL activity against 2-NPG and 4-GTB is probably a result of an extra long loop 8 so far only observed in the structure of BLGAL.

4. Experimental

4.1. Chemicals

4.1.1. General and previously described chemicals

All chemicals were purchased from Sigma Aldrich A/S unless otherwise stated. 2,4-Dinitrophenyl β-D-galactopyranoside and 4-nitrophenyl 4-deoxy-4-thio-β-D-galactopyranoside were synthesized as described previously.[25,26]

4.1.2. Synthesis of 4-nitrophenyl-1,4-β-D-thiogalactobioside

4-Nitrophenyl 4-deoxy-4-thio-β-D-galactopyranoside[25] (80 mg, 252 mmol, 20 mM), 2,4-dinitrophenyl β-D-galactopyranoside (130 mg, 390 mmol, 30 mM) and the mutant Abg E170G (~1 mg/ml) were incubated for 1 h at room temperature in phosphate buffer (80 mM, pH 7.5) as reported previously for similar systems.[16,17] The reaction was monitored by TLC (7:2:1 EtOAc/MeOH/H_2O), which revealed a complete consumption of donor and the formation of a new UV-active compound. Additional 2,4-dinitrophenyl β-D-galactopyranoside was then added to a final concentration of 55 mM and the reaction was maintained at room temperature for 24 h. TLC indicated complete consumption of donor but no further consumption of acceptor. The pH of the reaction mixture was adjusted to ~2.5 using a concentrated solution of acetic acid, and the dinitrophenol (DNP) was extracted with CH_2Cl_2. The thiogalactoside was initially purified by solid phase extraction, then acetylated and worked up by standard procedures (Ac_2O/pyridine) and characterized by ^{1}H NMR.

^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20 (m, 2H, aryl), 7.06 (m, 2H, aryl), 5.46 (dd, 1H, $J_{2,1}$ 7.5 Hz, $J_{2,3}$ 9.6 Hz, H-2). 5.42 (dd, 1H, $J_{4',3'}$ 3.4 Hz, $J_{4',5'}$, 1 Hz, H′-4), 5.14 (dd, 1H, $J_{3,2}$ 9.6 Hz, $J_{3,4}$ 4.4 Hz, H-3), 5.16 (dd, 1H, $J_{2',3'} = J_{2',1'}$ 10.0 Hz, H′-2), 5.10 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1), 5.00 (dd, 1H, $J_{3',4'}$ 3.4 Hz, $J_{3',2'}$ 10 Hz, H-3′), 4.51 (d, 1H, $J_{1',2'}$ 10 Hz, H′-1), 4.46 (dd, 1H, $J_{6a,6b}$ 12.2 Hz, $J_{6a,5}$ 7.7 Hz, H-6a), 4.36 (dd, 1H, $J_{6b,6a}$ 12.2 Hz, $J_{6b,5}$ 3.5 Hz, H-6b), 4.19–4.06 (m, 3H, H-5, 2 × H-6), 3.84 (m, 1H, H′-5), 3.66 (dd, 1H, $J_{4,3}$ 4.4 Hz, $J_{4,5}$ 2.0 Hz, H-4), 2.20–1.95 (m, 21H, $-COCH_3$).

4.2. Enzymes

4.2.1. Cloning, expression, and purification of AAGAL, BLGAL, and MGGAL

Cloning and expression of AAGAL (Gen bank ID: AAA32692.1), BLGAL (Gen bank ID: AAO31370.1) and MGGAL (Gen bank ID: AAQ77827.1) are described in detail in US patent 5474922, 6,331,426 B1 and 6,329,185 B1. The molecular weights of AAGAL, BLGAL and MGGAL as calculated from the gene sequences were 36758.7 Da, 43591.4 Da and 34610 Da, respectively. AAGAL and BLGAL were purified as previously described[3,27] to a final concentration of 5.8 mg/mL and 4.1 mg/mL, respectively. The gene encoding MGGAL was expressed in *Aspergillus oryzae* essentially as described for *Humicola insolens* galactanase.[14] The culture broth was centrifuged (10,000×g, 20 min) and the supernatant filtered through a Nalgene 0.2 μm filtration unit in order to remove residual *Aspergillus* host cells. The filtrate was concentrated approx. 15 times by ultrafiltration on a Filtron omega cassette (cut-off = 3000 Da). Ammonium sulfate was added to give a 1.2 M final concentration and the filtrate was applied to a Phenyl Toyopearl 650S column (from TosoHaas) equilibrated in 20 mM succinic acid/NaOH, 1.2 M ammonium sulfate, pH 6.0 (buffer A). After washing with equilibration buffer the column was eluted with a linear ammonium sulfate gradient (1.2–0 M) over four column volumes. The eluted fractions showing galactanase activity were adjusted to 1.2 M ammonium sulfate and applied to a SOURCE Phenyl column (from GE Healthcare) equilibrated in buffer A. After washing with equilibration buffer the column was step-eluted with 20 mM succinic acid/NaOH, pH 6.0. The peak containing the galactanase activity was collected and applied to a Superdex 75 column (GE Healthcare) equilibrated in 10 mM dimethylglutaric acid, 100 mM H_3BO_3, 2 mM $CaCl_2$, 100 mM NaCl, pH 6.0 and the column was eluted with the same buffer. Fractions containing galactanase activity and where only one band was seen on a Coomassie stained gel were pooled and concentrated to 2.0 mg/mL.

4.3. Methods

4.3.1. Kinetic analysis with 2-nitrophenyl β-D-galactopyranoside and 4-nitrophenyl-1,4-β-D-thiogalactobioside

Rates were determined by measuring the absorbance continuously (every second) at 410 nm after mixing enzyme, buffer and substrate at room temperature for 60 s. For detecting hydrolytic activity at low 2-NPG concentrations (4 mM) the absorbance was monitored for up to 20 min. The initial velocities in mM/min were calculated from the slope of the absorbance versus time plot using the extinction coefficients determined for the reaction product 4-nitrophenol in 4 mM citrate pH 4.4 and pH 5.5 of ε_{410nm} = 0.0544 mM^{-1} and ε_{410nm} = 0.628 mM^{-1}, respectively, at room temperature (data not shown). The kinetic values were determined from the v versus [S] plot.

The conditions for the 2-NPG assay were 0.40 mg/mL MGGAL in 4 mM sodium citrate pH 5.5 and 12 mM substrate and 1.2 mg/mL AAGAL in 5 mM sodium citrate pH 4.4 and 16 mM substrate. For

the 4-GTB assay 0.40 mg/mL MGGAL in 4 mM sodium citrate pH 5.5 and 1.2 mg/mL AAGAL in 4 mM sodium citrate pH 4.4 and 16 mM substrate were used. For BLGAL 0.94 mg/mL BLGAL in 20 mM MES, 0.4 mM calcium chloride pH 6.5 and 16 mM substrate was used for the 2-NGP assays and 25 mM MES, 0.5 mM calcium chloride pH 6.5 for the 4-GTB assay. The buffers were chosen according to previously reported pH optima for AAGAL[27] and MGGAL (US patent 6329185) or conditions previously used for oligosaccharide degradation with BLGAL.[3] 4-GTB activity was also measured with 5.5 mg/mL BLGAL. All the measurements were done at 410 nm on a Perkin Elmer LAMBDA 800 UV/VIS spectrophotometer at room temperature, using a 10 μl ultra-micro cuvette with a path length of 1 cm from Hellma Analytics.

The 10 μl ultra-micro cuvette assay was tested and found to give reliable rate measurements. For testing, kinetic parameters obtained in a 1500 μl semi micro cuvette were compared with those obtained in a 10 μl ultra-micro cuvette for 0.8 U/mL *Aspergillus niger* β-1,4-galactosidase (Megazyme Ltd.) and 200–5000 μM 2-NPG, at room temperature.

4.3.2. Activity measurements against the polymeric substrate azo-galactan

The activity of the enzymes with the polymeric dyed substrate azo-galactan was estimated by an assay carried out according to the manufacturer's (Megazyme Ltd.) instructions described in AGALP 11/99 except for the buffers in the reaction mixture which were 4 mM sodium citrate pH 4.4 for AAGAL, 4 mM sodium citrate pH 5.5 for MGGAL and 25 mM MES pH 6.5 and 0.5 mM $CaCl_2$ for BLGAL. The protein concentrations used in the assay were 0.48 μg/mL AAGAL, 0.20 μg/mL MGGAL, and 1.4 μg/mL BLGAL. A_{590} was measured with a Perkin Elmer LAMBDA 800 UV/VIS spectrometer.

4.3.3. Thin layer chromatography

Three separate enzyme reactions were monitored: MGGAL (0.40 mg/mL MGGAL, 4 mM sodium citrate pH 5.5, 12 mM 2-NPG), BLGAL (0.94 mg/mL, 16 mM 2-NPG, 15 mM MES, 0.3 mM $CaCl_2$, pH 6.5, 3) and AAGAL (1.2 mg/mL AAGAL, 16 mM 2-NPG, 5 mM sodium citrate pH 5.5). The reactions were stopped by freezing in liquid nitrogen after 10 and 30 min of reaction time. Approximately 20 μl of each sample and standards (50 mM galactose, 10 mM galactobiose, 40 mM 2-NPG and 10 mM 2-nitrophenol) were transferred to a silica gel plate (Kiesel Gel 60, Merck) at room temperature by a capillary tube and eluted using ethyl acetate–MeOH–water (7:2:1 by vol) until the liquid front was 1–2 cm from the top of the plate. The UV active compounds were visualized by a UV lamp (UVGL-25 from UVP) at 365 nm. The plate was visualized by soaking briefly in 10 w/v% ammonium heptamolybdate tetrahydrate in 2 M sulfuric acid and air dried completely.[28] The plate was then heated for ~5 min at 105 °C to visualize the carbohydrates as blue spots.

Acknowledgments

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