Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 338 (2003) 219–230
CARBOHYDRATE RESEARCH
www.elsevier.com/locate/carres

Purification and characterization of an endo-β-(1→6)-galactanase from *Trichoderma viride*☆

Kazuo Okemoto,[a] Takamasa Uekita,[a] Yoichi Tsumuraya,[a,*] Yohichi Hashimoto,[a] Takeshi Kasama[b]

[a]*Department of Biochemistry and Molecular Biology, Faculty of Science, Saitama University, 255 Shimo-okubo, Saitama 338-8570, Japan*
[b]*Instrumental Analysis Research Center for Life Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan*

Received 1 May 2001; received in revised form 7 October 2002; accepted 12 October 2002

Abstract

An endo-β-(1→6)-galactanase from Onozuka R-10, a commercial cellulase preparation from *Trichoderma viride*, was purified 57-fold. Apparent M_r values of the purified enzyme, estimated by denaturing gel electrophoresis and gel filtration, were 47,000 and 17,000, respectively. The enzyme was assayed with a galactan from *Prototheca zopfii*, which has a high proportion of β-(1→6)-linked galactosyl residues. It exhibited maximal activity toward the galactan at pH 4.3. The enzyme hydrolyzed specifically β-(1→6)-galactooligosaccharides with a degree of polymerization higher than 3 and their acidic derivatives with 4-*O*-methyl-glucosyluronic or glucosyluronic groups at the nonreducing terminals. The methyl β-glycoside of β-(1→6)-galactohexaose was degraded to reducing galactooligomers with a degree of polymerization 2–5 as the products at the initial stage of hydrolysis, and galactose and galactobiose at the final stage, indicating that the enzyme can be classified as an endo-galactanase. The extent of hydrolysis of the carbohydrate portion of a radish root arabinogalactan-protein (AGP) increased when α-L-arabinofuranosyl residues attached to β-(1→6)-linked galactosyl side chains of the AGP were removed in advance. The enzyme released galactose, β-(1→6)-galactobiose, and 4-*O*-methyl-β-glucuronosyl-(1→6)-galactose as major hydrolysis products when allowed to act exhaustively on the modified AGP.

Keywords: Arabinogalactan-protein; Carbohydrase; Endo-β-(1→6)-galactanase; *Trichoderma viride*

1. Introduction

Arabinogalactan-proteins (AGPs) are a class of high-M_r proteoglycans typically consisting of $<10\%$ protein and $>90\%$ carbohydrate. They are found in all tissues of higher plants, either as water-soluble components of the extracellular matrix and the cell wall,[1–3] or immobilized on the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor.[4,5] The protein component of AGPs is typically rich in Hyp/Pro, Ala, and Ser. The carbohydrate component consists primarily of L-arabinose (Ara) and galactose (Gal) and has a highly branched framework consisting of a β-(1→3)-galactan backbone, substituted at *O*-6 with β-(1→6)-linked galactosyl side chains. Most of the L-arabinosyl residues are attached at *O*-3 of some of the galactosyl residues in the side chains. Lesser amounts of other sugars, such as glucosyluronic acid (GlcA), 4-*O*-methyl-glucosyluronic acid (4-Me-GlcA), L-rhamnose, and L-fucose are occasionally attached to the side chains, usually as nonreducing terminal residues.[1–3] Various lines of evidence have pointed to roles of AGPs in several aspects of growth and development, including cell proliferation, cell expansion, somatic embryogenesis, pollen tube growth, and cell death.[3]

The use of specific enzymes is required for a better understanding of the fine structure of the carbohydrate moiety of AGPs. The α-L-arabinofuranosidases (EC 3.2.1.55) from fungi and plants studied in[6–8] were

☆ Sugars in this paper are of the D series unless designated otherwise.

* Corresponding author. Tel.: +81-48-8583401; fax: +81-48-8583384
E-mail address: tsumura@molbiol.saitama-u.ac.jp (Y. Tsumuraya).

0008-6215/03/$ - see front matter
PII: S0008-6215(02)00405-6

found to be effective in removing L-arabinosyl residues of AGPs, thus exposing the β-3,6-galactan core to further degradation by β-galactosidases and β-galactanases. Our previous study[9] showed that an exo-β-(1→3)-galactanase (no EC number) from *Irpex lacteus* specifically cleaves β-(1→3)-galactosidic linkages in the β-3,6-galactan core of a radish root AGP. Following the exo-action of this enzyme capable of bypassing the branching points, the side chains can then be liberated as various oligomers with a Gal at the reducing terminal position, which originated from the linear backbone chain of the AGP. An enzyme exhibiting similar behavior has also been purified from a culture of *Aspergillus niger*.[10] An endo-β-(1→6)-galactanase (no EC number) isolated from a culture of *A. niger* is known to hydrolyze specifically β-(1→6)-linked galactosyl side chains of a grape AGP, resulting in release of Gal and β-(1→6)-galactobiose, while the β-(1→3)-galactan backbone remains resistant.[11] These fungal enzymes, as well as some enzymes of plant origin, have proven to be useful tools for controlled degradation in structural studies of AGPs.[9,12] In the present work, we describe the purification of an endo-β-(1→6)-galactanase from a commercially available enzyme source. Its action on various galactooligomers and a radish AGP in a combination with the action of an exo-β-(1→3)-galactanase is studied.

2. Experimental

2.1. Materials

Onozuka R-10, a commercial cellulase preparation (Lot No. 201057) from *Trichoderma viride*, was purchased from Yakult Pharmaceutical Ind. Co., Ltd. (Tokyo, Japan). Exo-β-(1→3)-galactanase was purified from Driselase.[9] The β-galactosidase (grade VIII, from *Escherichia coli*) and endo-glycosidase H were obtained from Sigma–Aldrich Japan.

Radish root AGP (designated as AGP-IV in the reference) and its enzymatically modified product obtained by digestion with *Rhodotorula flava* α-L-arabinofuranosidase were prepared as reported previously.[13] A β-(1→3)-galactan and a β-(1→4)-galactan were prepared by Smith degradation of acacia gum and by partial acid hydrolysis of soybean arabinogalactan, respectively.[14] Sugar beet arabinan was prepared by the method of Tagawa and Kaji.[15] The β-(1→3)- and β-(1→6)-linked galactobioses and -trioses were prepared from larch wood arabinogalactan.[16] The β-(1→6)-galactotetraose was prepared from gum ghatti.[17] The β-(1→4)-galactooligosaccharides with a degree of polymerization (DP) 2–5 were prepared from soybean arabinogalactan.[14] β-GlcA-(1→6)-Gal and β-GlcA-(1→6)-β-Gal-(1→6)-Gal were prepared from acacia gum, and 4-Me-β-GlcA-(1→6)-Gal, 4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal, and 4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-β-Gal-(1→3)-Gal were from the sap of the lac tree, *Rhus vernicifera*.[18] 4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-β-Gal-(1→6)-Gal and α-L-Ara*f*-(1→3)-β-Gal-(1→6)-Gal were prepared from enzymatic hydrolyzates of the radish AGP and Smith degradation product of acacia gum, respectively, by incubation with exo-β-(1→3)-galactanase.[9] Methyl β-glycosides of β-(1→3)-linked galactotetraose and -pentaose, and of β-(1→6)-linked galactopentaose and -hexaose were supplied by Dr. P. Kováč, National Institutes of Health, Bethesda, MD, USA.

Other chemicals were obtained as follows: Sephadex G-50 and G-100 (Pharmacia Biotech); Bio-Gel P-2 (extra fine), P-10, and P-100 (Bio-Rad Laboratories); DEAE-cellulose (DE52) and CM-cellulose (CM32) (Whatman Paper Ltd.); Bakerbond CBX and QUAT (Yamazen Corp., Osaka, Japan); various *p*-nitrophenyl (PNP) glycosides (Sigma–Aldrich and Nacalai Tesque, Kyoto, Japan); azocasein (Sigma–Aldrich).

2.2. Preparation of algal galactan

Prototheca zopfii ATCC 16533, a unicellular organism belonging to Chlorophyceae, was grown on an agar (2%, w/v) plate containing 3% (w/v) Sabouraud dextrose broth (pH 6.8; Difco Laboratories) for 5 d at 25 °C under fluorescent lamp as described by Manners et al..[19] The cells (dry weight, 15 g) were disrupted by grinding with carborundum. According to the method of Roy et al.,[20] a polysaccharide fraction was extracted from the mixture with hot water, precipitated with EtOH, deproteinized by shaking with 1,1,2-trichloro-1,2,2-trifluoroethane, dialyzed against water, and freeze-dried (yield, 2.9 g). The crude polysaccharides were digested with alpha-amylase (1.3 mg; Type I-A, from porcine pancreas, Sigma–Aldrich) in 100 mL of 20 mM phosphate buffer, pH 6.8, containing 6 mM NaCl for 1.5 h at 37 °C.[20] The mixture was inactivated by heating, dialyzed against water, and freeze-dried (1.6 g). The polysaccharides were chromatographed on a column (3.6 × 28 cm) of DEAE-cellulose (HCO_3^-; Serva Feinbiochemica GmbH & Co., Germany) and monitored for total sugar (see below) and absorbance at 280 nm. After washing the column with water, a galactan fraction was eluted as a broad peak in a range of 50 to 250 mM during a linear gradient (0–0.5 M, 2.2 L) of $NaHCO_3$. The galactan fraction (700 mg) was loaded on a column (2.6 × 120 cm) of Sephadex G-50 and eluted with 1% (v/v) HOAc. The fractions that emerged near the void volume of the column were recovered (590 mg) and used for enzyme assays.

2.3. Carbohydrate analyses

The following colorimetric methods were used: total sugar was determined by the phenol–H_2SO_4 method[21] using Gal as the standard; reducing sugar by the method of Nelson[22] and Somogyi[23] using Gal as the standard; uronic acid by a modified carbazole–H_2SO_4 method[24] using GlcA as the standard. Poly- and oligosaccharides were hydrolyzed by heating in 1 M H_2SO_4 for 4 h at 100 °C and in 2 M trifluoroacetic acid for 1 h at 121 °C, respectively. Sugars were analyzed by paper chromatography on Whatman No.1 or 3MM paper in solvent A, 6:4:3 (v/v/v) 1-butanol–pyridine–water or solvent B, 5:2:3 (v/v/v) 1-butanol–HOAc–water. Sugar spots on the chromatograms were detected with alkaline silver nitrate. Thin layer chromatography (TLC) on Silica gel $60F_{254}$ (Merck) was performed with 7:1:2 (v/v/v) 1-propanol–EtOH–water and sugars were detected by charring with H_2SO_4.

Gas–liquid chromatography (GLC) of sugars as alditol acetate derivatives was performed with a Shimadzu gas chromatograph GC-6A according to the method of Albersheim et al.[25] Carboxyl groups in polysaccharides were reduced by the method of Taylor and Conrad.[26] Methylation of native and carboxyl-reduced samples was performed by the Hakomori method[27] and the products were analyzed by GLC[28] and gas chromatography-mass spectrometry (GC/MS). For GC/MS, a TSQ70 triple quadrupole mass spectrometer (Finnigan, San Jose, CA, USA) equipped with a Varian 4300 gas chromatograph was used. GC/MS was carried out with a column (0.25 mm i.d. × 30 m) of 5% phenylmethylpolysiloxane fused capillary (DB-5; J & W Scientific Inc., Molsom, CA, USA) with the temperature increasing from 180 to 270 °C (4 °C/min).

To identify the uronic acid component in the galactan produced by *P. zopfii*, the polysaccharide (1 mg) was hydrolyzed, and the hydrolyzate was labeled with *p*-aminobenzoic acid ethyl ester (ABEE) according to the method of Matsuura and Imaoka.[29] The ABEE-derivatized monosaccharides were analyzed by reversed-phase high-performance liquid chromatography (HPLC) with a Shimadzu LC-10AS system fitted with a fluorescence detector model RF-$10A_{XL}$ and a column (4.6 mm i.d. × 150 mm) of Develosil ODS-UG5 (Momura Chemical Co., Aichi, Japan). The column was eluted with 0.2 M potassium borate buffer (pH 9.0), containing 6% MeCN at a flow rate of 1.0 mL/min and at 45 °C. Fluorescence in the eluate was monitored at 305 nm (excitation) and 360 nm (emission).[30]

The M_r of polysaccharides was estimated by gel filtration on a 2.5 × 60-cm Sephadex G-100 column, equilibrated and eluted with 1% HOAc. The column system was calibrated with pullulans (Shodex Standard P-82; Showa Denko, Tokyo, Japan), blue dextran (V_o), and Gal (V_i).

2.4. Enzyme assays

The activity of the endo-β-(1→6)-galactanase was determined using a reaction mixture (0.1 mL) consisting of the enzyme, 0.5% (w/v) algal galactan, 0.01% (w/v) bovine serum albumin (BSA), and 50 mM acetate buffer, pH 4.3 (hereafter referred to as the standard assay mixture). After incubation for 5 to 20 min at 30 °C, liberated sugars were determined reductometrically. One unit of enzyme activity liberates 1 μmol of reducing sugars (as Gal equivalent) per min. Other activities for various polysaccharides were tested similarly in 50 mM acetate buffer, pH 4.6, for 4 h at 30 °C.

Glycosidase activities were assayed by measuring the amount of *p*-nitrophenol released from 1 mM substrate of the respective *p*-nitrophenyl glycosides by incubation in 0.2 mL of 50 mM acetate buffer, pH 4.6, for 4 h at 37 °C. The reactions were terminated by the addition of 0.2 M Na_2CO_3 (0.8 mL), and they were monitored at 420 nm. Caseinolytic activity was assayed with azocasein as the substrate as described previously.[9]

The enzyme assays were performed, at least, in duplicate and mean values were recorded.

2.5. Enzyme purification

All operations were carried out at 0–4 °C. A mixture of Onozuka R-10 (15 g) and cellulose powder (6 g) in 180 mL of 0.5 M acetate buffer, pH 5.0, was stirred for 20 min and then centrifuged at 35,000 × *g* for 15 min. The supernatant was brought to 80% saturation with $(NH_4)_2SO_4$, left for 2 h, and centrifuged. The precipitate was dissolved in 10 mM Tris–HCl buffer, pH 8.0, and dialyzed overnight against the buffer. The crude enzyme solution was applied onto a 2.4 × 16-cm DEAE-cellulose (DE52) column equilibrated with the buffer. After washing the column with the buffer, the enzyme was eluted with a linear KCl gradient (0–0.4 M) in the buffer (total volume, 2.5 L). Fractions (17.5 mL) were collected into tubes containing 0.5 mL of 0.5 M acetate buffer, pH 5.0, to prevent inactivation of the enzyme, and monitored for enzyme activity. The active fractions were collected, brought to 80% saturation with $(NH_4)_2SO_4$, left standing overnight, and centrifuged. The precipitate was dissolved in a small volume of 10 mM acetate buffer, pH 4.5, and dialyzed against the buffer. The dialysate was applied onto a 2.4 × 16-cm CM-cellulose (CM32) column equilibrated with the buffer. After the column was washed with the buffer, the enzyme was eluted with a linear KCl gradient (0–0.4 M) in the buffer (total volume, 1.4 L; 10 mL/fraction). Pooled fractions were concentrated to 8 mL, using an Amicon ultrafiltration apparatus fitted with a YM-3 membrane. The enzyme solution was applied onto a 2.0 × 140-cm Sephadex G-100 column equilibrated and eluted with 50 mM acetate buffer, pH

5.0 (3 mL/fraction). The active fractions were combined, concentrated, and electrofocused using a LKB 8101 column (110 mL) and a carrier ampholyte (Ampholine; LKB Japan, Tokyo, Japan) of pH range of 5–8, at a constant voltage of 600 V for 48 h. Fractions of 1 mL were collected. The active fractions emerging over a pH range of 5.2–5.8 were pooled, concentrated, and desalted by passage through a 2.0 × 51-cm Bio-Gel P-10 column equilibrated and eluted with 20 mM acetate buffer, pH 5.0. The active fractions containing the purified endo-β-(1→6)-galactanase, were pooled, concentrated, and stored at −20 °C. The two ion-exchange chromatography steps on DE52 and CM32 columns could be replaced with a Bakerbond QUAT column (1.5 × 21 cm) and a CBX column (1.5 × 11 cm), respectively, which were run in a Yamazen BPLC chromatograph system. This alternative procedure facilitated enzyme purification since a larger amount of Onozuka R-10 (30 g) could be used due to the higher binding capacity of these resins for enzyme proteins. It also allowed shortening the operation time for the chromatographic analyses.

2.6. Protein analyses

Enzyme protein was determined by the method of Bradford.[31] Proteins in radish AGP and algal galactan were determined by the method of McGrath.[32] BSA was used as the standard for both procedures.

Aliquots (9 μg) of the native enzyme were analyzed by polyacrylamide gel electrophoresis (PAGE) on three parallel (10%, pH 9.5) gels prepared by the method of Davis.[33] Protein in one gel was stained with Coomassie Brilliant Blue R-250. Carbohydrate in another gel was detected fluorometrically with a dansyl hydrazine reagent.[34] Protein in the third gel was extracted from sliced gel discs (2-mm wide) by incubation with 50 mM acetate buffer (pH 4.3, 0.5 mL) and assayed for enzyme activity. Apparent M_r of the purified enzyme (4 μg) was determined by SDS-PAGE according to the method of Laemmli[35] using a 12.5% gel and an M_r marker protein mixture containing phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

The M_r of the native enzyme was determined on a 2.0 × 140-cm Bio-Gel P-100 column equilibrated and eluted with 50 mM acetate buffer, pH 5.0, containing 0.1 M NaCl and calibrated with marker proteins containing transferrin, BSA, ferritin, ovalbumin, α-lactalbumin, and cytochrome *c*. The M_r of the denatured enzyme was determined by HPLC using a column (7.6 × 500 mm) of Asahipak GS-510 (Asahi Chemical Ind., Tokyo, Japan) equilibrated and eluted with 6 M guanidine-HCl (1 mL/min).

The purified enzyme was examined for its binding characteristics on a HPLC affinity column, LA-Con A (4.6 × 150 mm; Hohnen Corp., Tokyo, Japan), carrying immobilized concanavalin A, which was eluted with 50 mM Tris–HCl buffer, pH 7.0, containing 0.15 M NaCl and each 1 mM of $MnCl_2$, $CaCl_2$, and $MgCl_2$ at a flow rate of 0.5 mL/min and at 30 °C. After washing the column with the buffer, the enzyme was eluted with 50 mM Tris–HCl buffer, pH 7.0, containing 0.2 M methyl α-mannoside. Fractions were collected and assayed for enzyme activity.

The enzyme (7 μg) was incubated with endo-glycosidase H (0.1 milliunits) in 50 mM citrate–phosphate buffer (pH 5.2, 60 μL) for 24 h at 37 °C, and analyzed on SDS-PAGE.

Amino acid analysis was carried out using a Jasco 880 HPLC system adapted for dabsylated amino acid derivatives. Samples were hydrolyzed with 6 M HCl in evacuated sealed tubes for 24 h at 110 °C and the hydrolyzate was derivatized with a dansylation kit (Amino-chrome; Ciba Corning Diagnostics Ltd., Halstead, UK). Cysteine was analyzed after peroxyformic acid oxidation.[36] The amino-terminal sequence analysis of the enzyme was performed with a Shimadzu PSQ-2 Protein Sequencer.

2.7. Mode of action

The mode of action of the purified galactanase was analyzed by incubation of methyl β-glycoside of β-(1→6)-galactohexaose (250 μg) with the enzyme (0.1 μg) in 50 mM acetate buffer (pH 4.3, 50 μL) for 0–120 min at 30 °C. At appropriate time intervals, a portion was withdrawn and inactivated by heating. The reducing sugars (but none of the methyl glycosides) liberated were coupled at their reducing terminals with ABEE as described above. The ABEE-derivatized sugars were analyzed by HPLC with a column (4.6 mm i.d. × 250 mm) of TSKgel Amide-80 (Tosoh, Tokyo, Japan). The column was eluted with a linear gradient of MeCN–water, from 74:26 to 58:42 (v/v), for 40 min at a flow rate of 1.0 mL/min and at 40 °C. The ABEE-sugars were monitored by a UV detector model SPD-10A at 304 nm.[29]

2.8. Enzymatic degradation of AGP

A sample (60 mg, based on sugar content) of α-L-arabinofuranosidase-treated radish AGP was digested with endo-β-(1→6)-galactanase (5 μg) in 50 mM acetate buffer (pH 4.3, 10 mL) for 24 h at 30 °C under toluene. When the liberation of reducing sugar reached a plateau, the digestion products were heated in a boiling water bath for 5 min and chromatographed on a 2.5 × 60-cm Sephadex G-100 column eluted with 1% HOAc (2.2 mL/fraction). Fractions were monitored for total sugar, uronic acid, and protein contents. The degradation products were separated into two fractions, high-

and low-M_r components, which were collected separately and concentrated by evaporation with repeated addition of water to remove HOAc: yield, 28 and 32 mg for high- and low-M_r components, respectively. The high-M_r component was analyzed for sugar composition and type of glycosidic linkages. The low-M_r component was chromatographed on a 1.5×5-cm DEAE-cellulose (HCO_3^-; Serva) column. Neutral sugars (23 mg) were eluted with water and acidic sugars (7 mg) were eluted as single peak after a linear $NaHCO_3$ gradient (0–0.5 M, 200 mL). The acidic fraction was desalted by passage through a small column of Dowex 50W (H^+). Both fractions were then further fractionated by chromatography on a 2.5×95-cm Bio-Gel P-2 column eluted with 1% HOAc.

One portion (25 mg) of the high-M_r component obtained by digestion with endo-β-(1→6)-galactanase was subjected to further degradation with exo-β-(1→3)-galactanase[9] in order to analyze its structural features. The component was digested with the enzyme (0.6 units) in 50 mM acetate buffer (pH 4.6, 3 mL) for 24 h at 37 °C. The digestion products were fractionated on a Sephadex G-100 column as above, resulting high- and low-M_r components with respective yields of 6 and 18 mg. The low-M_r component was chromatographed in a manner similar to the above on a DEAE-cellulose column, yielding neutral (11 mg) and acidic (6 mg) sugars. These sugars were separated on a Bio-Gel P-2 column.

Oligosaccharides thus isolated were identified by procedures essentially the same as those used for the products obtained by digestion with exo-β-(1→3)-galactanase alone,[9] i.e., determination of their mobility on paper chromatography with solvents A and B in comparison with standards, sugar composition, susceptibility to *E. coli* β-galactosidase, and mode of glycosidic linkages.

3. Results and discussion

3.1. Characterization of algal galactan

The galactan produced by *P. zopfii* used in this study is a branched structure with main chains of (1→6)-linked β-Gal*p* residues, together with a small amount (~10%) of side chains composed of single nonreducing terminal Gal*f* residues.[19,20] This galactan was therefore anticipated to be a good substrate to measure enzyme activity hydrolyzing (1→6)-linked β-Gal*p* sequences when surveying various enzyme sources. But a more complete characterization of the galactan as well as its degraded products may be required in order to adapt it for this purpose. The purified galactan was found to have the following properties: $[\alpha]_D$ +13.6° (*c* 0.5, water); M_r, 85,000; total sugar content, 91% (w/w); uronic acid, 4% (w/w); protein 7% (w/w); Gal as the major (97 mol%) sugar constituent together with small amounts of rhamnose, ribose, L- or D-Ara, and mannose as neutral sugars. Galacturonic acid (GalA) was the sole uronic acid component found when the hydrolyzates of the galactan were analyzed by HPLC as their ABEE derivatives. Methylation followed by GL/MS analysis of the carboxyl-reduced galactan confirmed the involvement of Gal*p* residues linked through *O*-6, and *O*-3,6 at branching points, together with minor amounts of residues linked at *O*-3 and nonreducing terminals (Table 1). The galactan appeared to have a more complex architecture than the model proposed previously[19,20] and contained Gal*f* residues linked through *O*-3 as well as nonreducing terminals. Neither the location of the GalA residues in the galactan chains, nor whether they occur in the form of pyranose or furanose rings could be unambiguously determined by comparison of the methylation data between the native galactan (data not shown) and the carboxyl-reduced specimen (Table 1).

Table 1
Methylation analysis of native algal galactan and the high-M_r component remaining after digestion with purified endo-β-(1→6)-galactanase

Sugar component [a]	Mode of linkage	Proportion (mol%)	
		Native galactan [b]	Galactanase-digested product [b,c]
2,3,4,6-Me_4-Gal	Gal*p* 1→	9	22
2,3,5,6-Me_4-Gal	Gal*f* 1→	9	7
2,4,6-Me_3-Gal	→3Gal*p* 1→	6	11
2,5,6-Me_3-Gal	→3Gal*f* 1→	7	4
2,3,6-Me_3-Man	→4Man*p* 1→	1	1
2,3,4-Me_3-Gal	→6Gal*p* 1→	49	29
2,4-Me_2-Gal	→3,6Gal*p* 1→	19	26

[a] Determined as *O*-acetyl alditol derivatives.
[b] Determined after carboxyl reduction.
[c] A high-M_r product (yield, 49%) in the reaction mixture which was isolated by chromatography on a Sephadex G-100 column.

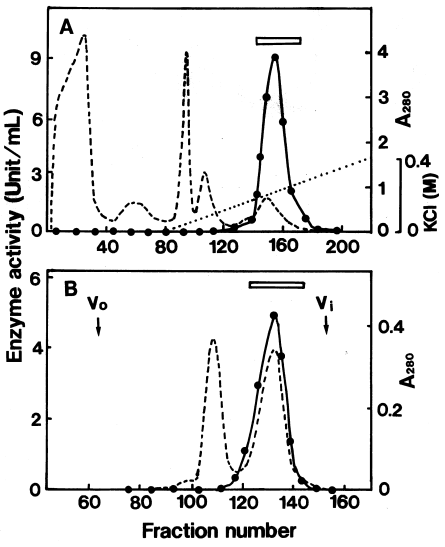

Fig. 1. Column chromatography of endo-β-(1→6)-galactanase. A, CM-cellulose; B, Sephadex G-100, in which the column was calibrated by using blue dextran (V_o) and Glc (V_i). ●—●, enzyme activity; - - -, absorbance at 280 nm; ···, concentration of KCl. The bars indicate the fractions pooled.

Upon exhaustive digestion of the algal galactan with a galactanase purified from a commercial cellulase specimen (see below), Gal and one oligomer migrated as standard β-Gal-(1→6)-Gal together with several unknown oligomers were detected as low-M_r products on paper chromatography with solvents A and B: the limit of hydrolysis of the galactan was 13% based on reductometric estimation using Gal as the standard. On the other hand, a high-M_r component remained after the enzymatic digestion product was recovered by chromatography with a Sephadex G-100 column at a yield of 49% (based on weight) of the initial galactan. The content of uronic acid increased to 9% (w/w) in this fraction. In the high-M_r component, the proportion of *O*-6-linked Gal*p* residues decreased, while the proportion of nonreducing terminal, branching and *O*-3-linked Gal*p* residues increased correspondingly (Table 1). These results suggest that the enzyme liberates oligosaccharides from linear consecutive β-(1→6)-linked galactosyl moieties of the galactan, leading to accumulation of the branching points and nonreducing terminal Gal residues in the high-M_r fraction. In contrast, the galactan is hardly hydrolyzed by exo-β-(1→3)-galactanase,[9] and was resistant to *E. coli* β-galactosidase. These results support the idea that the galactan is a suitable substrate for enzyme assays of endo-β-(1→6)-galactanases.

Reaction under standard assay conditions using varying amounts of the polysaccharide, resulted in the following kinetic data by the Lineweaver–Burk plot: $K_m = 3.0$ mg/mL and $V_{max} = 340$ μmol Gal/min/mg protein. In order to make the galactan a better substrate for the galactanase, it was partially hydrolyzed by heating with 0.1 M H_2SO_4 for 90 min in a boiling water. After neutralization, the modified polysaccharide was recovered and examined for its susceptibility to enzymatic hydrolysis. However, this attempt failed to increase the limit of hydrolysis, since again about half of the polysaccharide remained as a high-M_r fraction inaccessible to the enzyme. The modified polysaccharide did however become more accessible to the enzyme in that the rate of initial hydrolysis was about 3 times higher than that for the native galactan. This may be attributed to the removal of a large portion of Gal*f* residues which obstruct enzyme action in the native galactan.

3.2. Enzyme purification

Galactanase capable of hydrolyzing the algal galactan was searched for in various commercially available enzyme preparations. One cellulase specimen, Onozuka R-10, was found to exhibit high activity and was thus selected as a source for purification. A galactanase from the enzyme specimen was purified by $(NH_4)_2SO_4$ fractionation and several subsequent column chromatographic operations. The enzyme was adsorbed on a DEAE-cellulose column and eluted as a single peak around 0.1 M KCl. Elution profiles of the enzyme during subsequent purification steps using CM-cellulose and Sephadex G-100 columns are illustrated in Fig. 1. Isoelectric focusing gave a single peak of enzyme activity, the elution of which coincided with that of the protein. The galactanase was purified 57-fold in a yield of 3% (Table 2). When QUAT and CBX columns were used as an alternative purification procedure, elution profiles similar to those of DEAE- and CM-cellulose columns, respectively, and a similar yield of the enzyme were obtained. The purified enzyme was confirmed to be free from proteolytic activity and glycosidase activities when assayed for *p*-nitrophenyl glycosides of both α- and β-forms of Gal, Glc, GlcNAc, GalNAc, and Man, and of β-Xyl, α-L-Fuc, and α-L-Ara*f*. The purified enzyme also did not hydrolyze the following polysaccharides: dextran, arabinan, starch, laminarin, β-(1→4)- and β-(1→3)-galactans, larch wood arabinogalactan, acid-insoluble polygalacturonic acid.[37]

3.3. Purity and M_r

On native PAGE, the purified enzyme migrated as a single protein band at the same position as the enzyme activity (Fig. 2A). The band gave a positive reaction when carbohydrate was stained fluorometrically, indicating the glycoprotein nature of the enzyme. This characteristic was further confirmed by binding of the enzyme on a concanavalin A column and its elution with methyl α-mannoside. However, the enzyme was resistant to digestion with endo-glycosidase H, and its M_r did not decrease when analyzed on SDS-PAGE.

Table 2
Purification of endo-β-(1→6)-galactanase from 15 g of Onozuka R-10

Step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)	Yield (%)
Crude extract	300	735	2090	2.8	1.0	100
Ammonium sulfate fractionation	190	542	1730	3.2	1.1	83
DEAE-cellulose	168	44	1170	27	10	56
CM-cellulose	485	8.2	524	64	23	25
Sephadex G-100	146	3.0	364	121	43	17
Isoelectric focusing	11	0.4	64	160	57	3

The enzyme behaved as a single glycoprotein with an apparent M_r of 47,000 on SDS-PAGE (Fig. 2B). The determination of apparent M_r on a calibrated Bio-Gel P-100 column gave a much lower M_r of 17,000 for the native enzyme. A similar value (M_r 17,500) was also obtained on size-exclusion HPLC on a calibrated Asahipak column under denaturing conditions. The observed discrepancy of M_r values of the enzyme between SDS-PAGE and size exclusion chromatographies could be due to the glycosylated nature of the enzyme and/or to interaction with the gel matrices. A similar lack of agreement has been observed for an endo-β-(1→6)-galactanase isolated from *A. niger*, in which M_r values of 60,000 (SDS-PAGE) and 29,000 (gel-filtration) were obtained.[11]

The isoelectric point (pI) of the enzyme is pH 5.4. It contains high proportions of Ser, Ala, Glx, Gly, Thr, and Leu (Table 3). The amino-terminal sequence (11 residues) of the enzyme is DTTLSIDPTSN-.

3.4. Properties of the enzyme

The activity of the purified galactanase tended to decrease when stored in dilute solutions: for example, enzyme solutions with less than 25 ng/mL at pH 4.5 considerably decreased in activity after having been left standing overnight at 4 °C. But the addition of BSA in the enzyme solutions overcame this loss of activity. The standard assay for enzyme activity was therefore carried out in the presence of 0.01% BSA. Repeated freezing and thawing of dilute (less than 0.5 μg/mL) enzyme solutions also led to their inactivation.

The optimum pH for the enzyme on algal galactan was 4.3, based on the pH-activity curves using acetate buffer (pH 2.8–6.0) and cacodylate buffer (pH 5.5–7.5) at final concentration of 50 mM. Without BSA in the reaction mixtures the same value was obtained. The enzyme was found to be stable within pH ranges of 3.0–11.0 and 4.0–9.5, when remaining activities were measured after incubation with buffers of pH 2.5–11.0 in the presence or absence of BSA, respectively, for 24 h at 4 °C. The remaining activity was measured after exposure of the enzyme to various temperatures (25–70 °C) for 10 min at pH 4.3 without BSA. This treatment resulted in 30% and 100% loss in activity at 50 °C and at 60 °C, respectively. Addition of BSA did not protect the enzyme from thermal inactivation

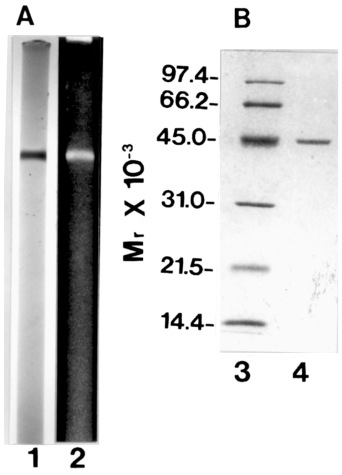

Fig. 2. Native PAGE (A) and SDS-PAGE (B) of the purified endo-β-(1→6)-galactanase. Lanes: (1) protein staining; (2) carbohydrate staining; (3) M_r marker proteins; (4) the enzyme.

Table 3
Amino acid composition of endo-β-(1→6)-galactanase

Amino acid	mol%	Amino acid	mol%
Asx	5.1	Tyr	3.8
Glx	9.9	Val	6.0
Ser	13.2	Met	0.8
Gly	10.7	Cys [a]	2.3
His	2.0	Ile	4.0
Arg	4.9	Leu	8.0
Thr	8.2	Phe	4.8
Ala	10.3	Trp	n.d. [b]
Pro	3.1	Lys	2.9

[a] Determined as cysteic acid.
[b] Not determined.

Table 4
Action of endo-β-(1→6)-galactanase on various oligosaccharides

Substrate	Hydrolysis [a] and relative rate of hydrolysis (%) [b]
Algal galactan	100
β-(1→3)-Linked oligosaccharides	
Galactobiose	−
Galactotriose	−
Methyl β-galactotetraoside	−
Methyl β-galactopentaoside	−
β-(1→4)-Linked oligosaccharides	
Galactobiose	−
Galactotriose	−
Galactotetraose	−
Galactopentaose	−
β-(1→6)-Linked oligosaccharides	
Galactobiose	−
Galactotriose	59
Galactotetraose	67
Methyl β-galactopentaoside	85
Methyl β-galactohexaoside	114
Galactobiitol	−
Galactotriitol	4
Galactotetraitol	48
β-GlcA-(1→6)-Gal	−
4-Me-β-GlcA-(1→6)-Gal	−
β-GlcA-(1→6)-β-Gal-(1→6)-Gal	2
4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal	1
4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-β-Gal-(1→6)-Gal	+
4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-β-Gal-(1→3)-Gal	8
α-L-Ara*f*-(1→3)-β-Gal-(1→6)-Gal	−

[a] The enzyme (28 ng) was incubated in a mixture (25 μL) containing various substrates (each 5 mg/mL) and 50 mM acetate buffer, pH 4.3, for 10 h at 30 °C. The reaction was terminated by heating in a boiling water bath for 3 min and analyzed for products on TLC or paper chromatography. Unreactive and degraded oligomers are shown as − and +, respectively.

[b] The enzyme (5–120 ng) was incubated under standard assay conditions containing above degraded substrates (galactan, 5 mg/mL; each oligomer, 5 mM). At suitable time intervals, initial hydrolysis rates were determined reductometrically: values were corrected by the respective time-zero blanks. Relative rates were calculated based on that for algal galactan as unity (100%).

3.5. Substrate specificity

Table 4 summarizes the activities of the galactanase for galactooligosaccharides and their acidic derivatives differing in type of glycosidic linkages and in chain length. The enzyme hydrolyzed β-(1→6)-linked galactooligomers longer than DP 3, while, β-(1→3)- and β-(1→4)-linked oligomers did not serve as substrates. The respective rates of hydrolysis increased with increasing DP, as evidenced by a higher relative rate of hydrolysis for β-(1→6)-galactotetraose than that for triose. However, reduction of the reducing end groups of β-(1→6)-galactooligomers with sodium borohydride resulted in a marked decrease in the rates of hydrolysis.

The enzyme also slowly hydrolyzed β-(1→6)-linked galactooligomers substituted at nonreducing terminals with single GlcA or 4-Me-GlcA residues. It seems that β-Gal-(1→6)-Gal substituted with uronosyl residues satisfies the structural requirement to serve as a substrate for the enzyme, but only with low hydrolysis efficiency. In addition to acidic β-(1→6)-galactotriose, acidic galactotriose composed of β-(1→6)- and -(1→3)-glycosidic linkages was also hydrolyzed. Among hydrolyzates of these acidic oligomers, neither free GlcA nor 4-Me-GlcA was detected. On the other hand, β-(1→6)-galactobiose substituted at *O*-3 of nonreducing terminals with α-L-Ara*f* residues was not a substrate.

3.6. Mode of action of the enzyme

3.6.1. Galactooligosaccharides. To determine the mode of action of the enzyme, hydrolysis products of methyl β-glycoside of β-(1→6)-galactohexaose produced by the galactanase were monitored by HPLC (as ABEE derivatives; Fig. 3). Reducing galactooligosaccharides corresponding to galactobiose to -pentaose together with a small amount of Gal were detected at the initial stage (3 and 30 min incubation) of reaction. Among these oligomers, the appearance of galactopentaose, for example, indicates the cleavage of the fifth β-(1→6)-galactosidic linkage from nonreducing terminals of the substrate molecule, resulting in liberation of galactopentaose and methyl β-galactoside. At the final stage (120 min incubation), Gal and galactobiose were accumulated as the sole hydrolysis products. This indicates that the enzyme is an endo-acting galactanase.

The products formed from galactooligomers by the action of the enzyme were also analyzed on TLC (Fig. 4). β-(1→6)-Galactotriose and -tetraose were degraded to Gal and galactobiose as the final products, similar to methyl β-glycoside of β-(1→6)-galactohexaose. The enzyme seems to cleave β-(1→6)-galactotetraose preferentially at the central β-(1→6)-galactosidic linkage but also, with lower efficiency, the linkages at nonreducing and/or reducing terminal galactosyl residues, which produces galactobiose with a small amount of Gal. This action pattern is not at great variance with that of some other endo-type glycanases. For example, *Cryptococcus* endo-β-(1→4)-xylanase is known to cleave xylotriose, its smallest substrate, into xylobiose and xylose. While the degradation of xylotetraose produces not just xylobiose but also xylose, because some xylotetraose is initially cleaved into xylotriose and xylose.[38]

3.6.2. AGP. The relative rate of hydrolysis of native and α-L-arabinofuranosidase-treated radish AGPs by the galactanase was 41 and 101%, respectively, based on that of algal galactan as unity (100%). The limits of hydrolysis of these two substrates were 8 and 26% (as Gal equivalent against total sugar), respectively. Apparently, the native AGP became more susceptible to enzymatic hydrolysis after removal of a large portion of α-L-Ara*f* residues (amounting to 19% of total sugar in the native AGP[13]) attached to the side chains of the β-3,6-galactan backbone. This behavior is similar to that of an endo-β-(1→6)-galactanase from *A. niger* when acting on a grape AGP.[11]

Hydrolysis products of α-L-arabinofuranosidase-treated radish AGP by the galactanase were examined by paper chromatography with solvents A and B. Oligosaccharides corresponding to galactobiose and -triose together with higher oligomers were liberated at the initial stage of reaction. At the final stage Gal and galactobiose as well as acidic oligomers of DP 2 and 3 (see below) were accumulated with concomitant disappearance of the higher oligomers, consistent with the endo-type action of the enzyme for galactooligomers.

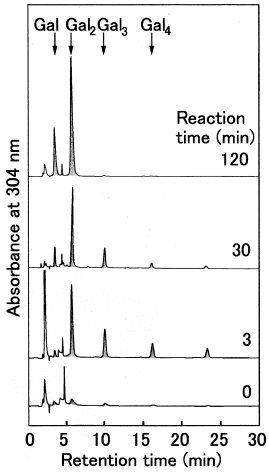

Fig. 3. Time course of methyl β-glycoside of β-(1→6)-galactohexaose hydrolysis by endo-β-(1→6)-galactanase. Reducing sugars (shaded peaks) released were derivatized with ABEE and analyzed by HPLC. Arrows indicate the elution positions of standard ABEE-derivatized Gal and β-(1→6)-galactooligomers with DP 2-4. The peak at about 23 min is assumed to be ABEE-derivatized β-(1→6)-galactopentaose. The unshaded peaks appearing at around 2–4 min are contaminants remaining after derivatization. The intensity of UV absorbance at reaction times 30 and 120 min is scaled down to 1/4 and 1/6, respectively, compared to that at 0 and 3 min.

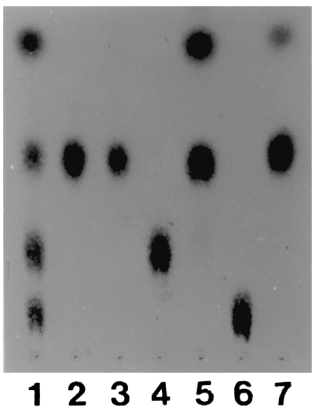

Fig. 4. Analysis by TLC of hydrolysis products produced by the action of endo-β-(1→6)-galactanase on β-(1→6)-galactooligomers. See legend for Table 4 for the reaction conditions. Lanes: (1) standard Gal and β-(1→6)-galactobiose, -triose and -tetraose (from top to bottom); (2) β-(1→6)-galactobiose; (3) β-(1→6)-galactobiose + enzyme; (4) and (5) β-(1→6)-galactotriose and its hydrolyzate; (6) and (7) β-(1→6)-galactotetraose and its hydrolyzate.

3.7. Successive degradation of AGP with endo-β-(1→6)- and exo-β-(1→3)-galactanases

To examine the mechanism of degradation of α-L-arabinosidase-treated radish AGP by endo-β-(1→6)-galactanase, products obtained after exhaustive digestion with the enzyme were separated on Sephadex G-100 into high- and low-M_r components (Fig. 5). The low-M_r fractions thus obtained were separated by ion-ex-

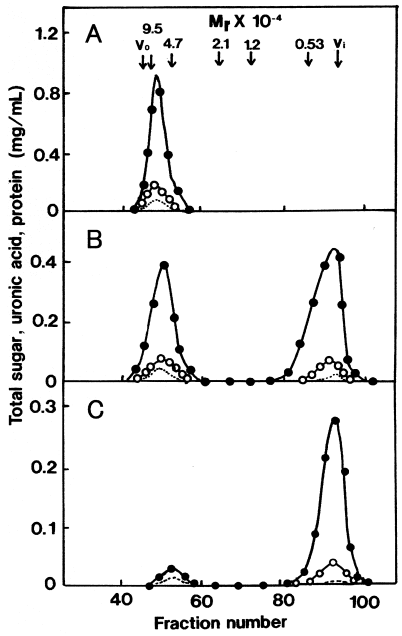

Fig. 5. Chromatography on Sephadex G-100 of α-L-arabinofuranosidase-treated AGP (A) and its digestion products with endo-β-(1→6)-galactanase (B) and with exo-β-(1→3)-galactanase (C). Chromatography was carried out as described under Section 2. ●—●, total sugar; ○—○ uronic acid; ···, protein. Fractions containing high-M_r (fraction numbers 43–58) and low-M_r (82–98) components in B were collected and analyzed for their structure. A part of the high-M_r component was subjected to further hydrolysis with exo-β-(1→3)-galactanase. Resulting high-M_r (fraction numbers 48–58) and low-M_r (85–98) components in C were collected and analyzed.

change chromatography followed by chromatography on a Bio-Gel P-2 column. Tables 5 and 6 summarize the analytical data for native and α-L-arabinosidase-treated AGPs and its high-M_r and low-M_r fractions obtained after successive digestion with two types of galactanases. Actually, all the oligosaccharides obtained in this study have been identified in those liberated from the modified AGP by digestion with exo-β-(1→3)-galactanase alone.[9]

About half of the sugar in α-L-arabinosidase-treated AGP was converted into oligosaccharides by the action of endo-β-(1→6)-galactanase (Fig. 5B, Table 5). Structural analysis of the high-M_r component indicated a decrease in the proportion of *O*-6-linked Gal residues and a concomitant increase of *O*-3- and *O*-3,6-linked Gal residues. These observations suggest that β-(1→6)-linked Gal sequences (partially substituted with 4-Me-GlcA residues at their nonreducing terminals) in side chains of the AGP are enzymatically removed, in such a way that short stubs remain attached to the backbone chains of β-(1→3)-linked Gal residues. The presence of short side chains that have survived the attack of the enzyme is also suggested by the fact that appreciable amounts of 4-Me-GlcA residues remained. Consequently, among neutral and acidic sugars (ratio 3:1), β-(1→6)-galactobiose and 4-Me-β-GlcA-(1→6)-Gal were identified as major products besides Gal (Table 6), which is consistent with the specificity of the enzyme towards various oligosaccharides (Table 4).

Subsequent action of exo-β-(1→3)-galactanase on the high-M_r component (limit of hydrolysis, 57% as Gal) again converted a large portion of sugar into oligosaccharides, leaving a core portion (5% of native AGP, based on sugar content) possibly consisting of a polypeptide chain with short β-3,6-galactan stubs attached to it (Fig. 5C, Table 5). The higher ratio (about 0.6:1) of protein versus sugar content in this fraction may support this idea, which had been proposed previously concerning the action of exo-β-(1→3)-galactanase alone.[9] However, about half of the protein originally presented in α-L-arabinosidase-treated AGP was fractionated into low-M_r components during suc-

Table 5
Characterization of native and α-L-arabinofuranosidase-treated AGPs, and high-M_r products obtained after successive digestion with endo-β-(1→6)- and exo-β-(1→3)-galactanases

	Native AGP [a]	α-L-Arabinosidase-treated AGP [a]	High-M_r product	
			With endo-β-(1→6)-galactanase	With exo-β-(1→3)-galactanase
Yield (%, based on sugar content)	100	81	41	5
M_r	88,000	70,000	64,000	48,000
Sugar composition (mol%)				
L-Ara	24	4	9	38
Gal	62	77	78	62
4-Me-GlcA	14 [b]	19 [b]	13 [b]	(13% of total sugar) [c]
Protein (%, based on sugar content)	6	7	11	62
Mode of glycosidic linkages (mol%)				
Ara*f* 1→	20 [b]	2 [b]	4 [b]	19 [c]
→2Ara*f* 1→	+ [d]	1	2	17
→5Ara*f* 1→	4	+	+	+
4-Me-Glc*p*A 1→	13	14	15	− [e]
Gal*p* 1→	3	7	19	32
→3Gal*p* 1→	7	6	19	3
→4Gal*p* 1→	−	−	−	+
→6Gal*p* 1→	23	54	13	20
→3,6Gal*p* 1→	30	16	28	9

[a] Data partially taken from.[9]
[b] Determined after carboxyl reduction.[26]
[c] Determined without carboxyl reduction. Uronic acid content determined colorimetrically in parentheses.
[d] Less than 1%.
[e] Not detectable.

cessive digestion with two galactanases, supporting again the possibility[9] of association of short polypeptides in radish AGP.

Structural analysis of the high-M_r fraction indicated a decrease in the proportion of *O*-3- and *O*-3,6-linked Gal residues, with concomitant increase of nonreducing terminal Gal and L-Ara*f* residues and *O*-2-linked L-Ara*f* residues. This suggests that the L-Ara*f* residues were located mainly at the inner part of the sugar chains of the native AGP and thus remained resistant even after the treatment with α-L-arabinosidase. Based on the action pattern of exo-β-(1→3)-galactanase capable of bypassing the branching of the β-3,6-galactan chains,[9] it is probable that the side chains remaining after the treatment with endo-β-(1→6)-galactanase were liberated as oligosaccharides with a Gal at the reducing terminal position, which originated from the backbone chain of β-(1→3)-linked Gal residues. Consistent with this, β-(1→6)-galactobiose and 4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal were found as major products together with a large amount of Gal in the hydrolyzate (Table 6). These products markedly differ from those released from α-L-arabinosidase-treated AGP by exo-β-(1→3)-galactanase alone, where β-(1→6)-galactooligomers of DP from 2 to at least 20, together with their corresponding acidic oligomers carrying 4-Me-GlcA at nonreducing terminals were obtained.[9] An acidic trisaccharide, namely 4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal, has been detected as a predominant product after digestion with exo-β-(1→3)-galactanase alone, indicating that a part of the side chains of 4-Me-β-GlcA-(1→6)-Gal attached to *O*-6 of Gal residues along the backbone chain survived the attack of endo-β-(1→6)-galactanase.

Table 6
Analyses of oligosaccharides liberated from α-L-arabinofuranosidase-treated AGP by successive digestion with endo-β-(1→6)- and exo-β-(1→3)-galactanases

Mono- and oligosaccharide	Yield [a]	Identified
Endo-β-(1→6)-galactanase digestion		
Neutral		
1 [b]	10	Gal
2	17	β-Gal-(1→6)-Gal
3	2	β-Gal-(1→6)-β-Gal-(1→6)-Gal
4	1	– [c]
Acidic		
1	1	–
2	7	4-Me-β-GlcA-(1→6)-Gal
3	1	4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal
4–5	1	–
Exo-β-(1→3)-galactanase digestion		
Neutral		
1	14	Gal
2	9	β-Gal-(1→6)-Gal
3	1	β-Gal-(1→6)-β-Gal-(1→6)-Gal
4	trace	–
Acidic		
1	0.2	–
2	3	4-Me-β-GlcA-(1→6)-Gal
3	8	4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal
4–5	0.8	–

[a] % of native AGP, based on sugar content: amounting to a total of 76%.
[b] Numbers represent respective DP.
[c] Not determined.

The proportions of nonreducing terminal residues are higher that those of branching Gal residues in the methylation data (Table 5) for the carboxyl-reduced samples from native radish AGP to the high-M_r fraction obtained after endo-β-(1→6)-galactanase, and the degree of discrepancy tends to increase with each step of enzymatic digestion. This may reflect the fine structure of the AGP, the chain lengths and numbers of glycan chains along the single polypeptide backbone. For *Nicotiana* AGP, for example, 9 possible chains with approximately 95 sugar residues[39] and for a classical AGP many chains with 30–150 residues[40] have been proposed. If the structure of radish AGP is similar (although we do not have any conclusive data), stepwise removal of sugar residues up to about 95% as was reached after exo-β-(1→3)-galactanase digestion would leave only a few residues as stubs on the polypeptide backbone, which would imply a considerable increase in the proportion of nonreducing terminal residues. Our data thus seem to provide a clue for the glycan structures from the peripheral to core regions of radish AGP. We also note that the data on the mode of glycosidic linkages in the high-M_r fraction recovered after exo-β-(1→3)-galactanase digestion differs from our previous results[9] for the high-M_r fraction obtained after successive digestion of radish AGP with two enzymes, α-L-arabinosidase and exo-β-(1→3)-galactanase, in which the fraction was recovered in a yield of 7.3% (based on native AGP) with a higher proportion (51 versus 20 mol% in this study) of *O*-6-linked Gal residues. Apparently, this difference arises from the liberation of the Gal residues by the action of endo-β-(1→6)-galactanase prior to exo-β-(1→3)-galactanase digestion.

3.8. Conclusion

The enzyme purified from Onozuka R-10 may be classified as an endo-type galactanase specific for consecutive β-(1→6)-linked Gal residues longer than DP 3, which is able to liberate side chains of radish root AGP

as Gal and β-(1→6)-galactooligosaccharides with or without 4-Me-GlcA groups at nonreducing terminals. Subsequent application of exo-β-(1→3)-galactanase on the high-M_r product provided confirmative evidence for this action pattern of the endo-β-(1→6)-galactanase. The specificity and mode of action of this endo-β-(1→6)-galactanase appeared to be similar to those of the enzyme that isolated from *A. niger*.[11,12]

Acknowledgements

We would like to thank Dr. P. Kováč, National Institutes of Health, NIDDK, Bethesda, MD, USA, for gifts of methyl β-galactooligosaccharides. We also thank Professor T. Hiyama of this department for the peptide sequence analysis.

References

1. Clarke, A. E.; Anderson, R. L.; Stone, B. A. *Phytochemistry* **1979**, *18*, 521–540.
2. Fincher, G. B.; Stone, B. A.; Clarke, A. E. *Annu. Rev. Plant Physiol.* **1983**, *34*, 47–70.
3. Nothnagel, E. A. *Int. Rev. Cytol.* **1997**, *174*, 195–291.
4. Schultz, C.; Gilson, P.; Oxley, D. D.; Youl, J.; Bacic, A. *Trends Plant Sci.* **1998**, *3*, 426–431.
5. Svetek, J.; Yadav, M. P.; Nothnagel, E. A. *J. Biol. Chem.* **1999**, *274*, 14724–14733.
6. Kaji, A. *Adv. Carbohydr. Chem. Biochem.* **1984**, *42*, 383–394.
7. Hata, K.; Tanaka, M.; Tsumuraya, Y.; Hashimoto, Y. *Plant Physiol.* **1992**, *100*, 388–396.
8. Hirano, Y.; Tsumuraya, Y.; Hashimoto, Y. *Physiol. Plant.* **1994**, *92*, 286–296.
9. Tsumuraya, Y.; Mochizuki, N.; Hashimoto, Y.; Kováč, P. *J. Biol. Chem.* **1990**, *265*, 7207–7215.
10. Pellerin, P.; Brillouet, J.-M. *Carbohydr. Res.* **1994**, *264*, 281–291.
11. Brillouet, J.-M.; Williams, P.; Moutounet, M. *Agric. Biol. Chem.* **1991**, *55*, 1565–1571.
12. Saulnier, L.; Brillouet, J.-M.; Moutounet, M. *Carbohydr. Res.* **1992**, *224*, 219–235.
13. Tsumuraya, Y.; Ogura, K.; Hashimoto, Y.; Mukoyama, H.; Yamamoto, S. *Plant Physiol.* **1988**, *86*, 155–160.
14. Sekimata, M.; Ogura, K.; Tsumuraya, Y.; Hashimoto, Y.; Yamamoto, S. *Plant Physiol.* **1989**, *90*, 567–574.
15. Tagawa, K.; Kaji, A. *Carbohydr. Res.* **1969**, *11*, 293–301.
16. Aspinall, G. O.; Hirst, E. L.; Ramstad, E. *J. Chem. Soc.* **1958**, 593–601.
17. Aspinall, G. O.; Auret, B. J.; Hirst, E. L. *J. Chem. Soc.* **1958**, 4408–4414.
18. Kuroyama, H.; Tsutsui, N.; Hashimoto, Y.; Tsumuraya, Y. *Carbohydr. Res.* **2001**, *333*, 27–39.
19. Manners, D. J.; Pennie, I. R.; Ryley, J. F. *Carbohydr. Res.* **1973**, *29*, 63–77.
20. Roy, A.; Manjula, B. N.; Glaudemans, C. P. G. *Mol. Immunol.* **1981**, *18*, 79–84.
21. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
22. Nelson, N. *J. Biol. Chem.* **1944**, *153*, 375–380.
23. Somogyi, M. *J. Biol. Chem.* **1952**, *195*, 19–23.
24. Galambos, J. T. *Anal. Biochem.* **1967**, *19*, 119–132.
25. Albersheim, P.; Nevins, D. J.; English, P. D.; Karr, A. *Carbohydr. Res.* **1967**, *5*, 340–345.
26. Taylor, R. L.; Conrad, H. E. *Biochemistry* **1972**, *11*, 1383–1388.
27. Hakomori, S. *J. Biochem.* **1964**, *55*, 205–208.
28. Tsumuraya, Y.; Hashimoto, Y.; Yamamoto, S.; Shibuya, N. *Carbohydr. Res.* **1984**, *134*, 215–228.
29. Matsuura, F.; Imaoka, A. *Glycoconjugate J.* **1988**, *5*, 13–26.
30. Yasuno, S.; Murata, T.; Kokubo, K.; Yamaguchi, T.; Kamei, M. *Biosci. Biotech. Biochem.* **1997**, *61*, 1944–1946.
31. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248–254.
32. McGrath, R. *Anal. Biochem.* **1972**, *49*, 95–102.
33. Davis, B. J. *Ann. NY Sci.* **1964**, *121*, 404–427.
34. Eckhardt, A. E.; Hayes, C. E.; Goldstein, I. J. *Anal. Biochem.* **1976**, *73*, 192–197.
35. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
36. Schram, E.; Moore, S.; Bigwood, E. J. *Biochem. J.* **1954**, *57*, 33–37.
37. Takeuchi, Y.; Tsumuraya, Y. *Biosci. Biotechnol. Biochem.* **2001**, *65*, 1519–1527.
38. Biely, P.; Vršanská, M. *Methods Enzymol.* **1988**, *160*, 638–648.
39. Gane, A. M.; Craik, D.; Munro, S. L. A.; Howlett, G. J.; Clarke, A. E.; Bacic, A. *Carbohydr. Res.* **1995**, *277*, 67–85.
40. Majewska-Sawka, A.; Nothnagel, E. A. *Plant Physiol.* **2000**, *122*, 3–9.