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Purification and characterization of a β-glucuronidase from *Aspergillus niger*☆

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Abstract

A β-glucuronidase from Pectinex Ultra SP-L, a commercial pectolytic enzyme preparation from *Aspergillus niger*, was purified 170-fold by ion-exchange chromatography and gel filtration. Apparent M_r of the purified enzyme, estimated by denaturing gel electrophoresis and size-exclusion chromatography, were 68,000 and 71,000, respectively, indicating that the enzyme is a monomeric protein. It released uronic acids not only from *p*-nitrophenyl β-glucosiduronic acid (PNP-GlcA) but also from acidic galactooligosaccharides carrying either β-D-glucosyluronic or 4-*O*-methyl-β-D-glucosyluronic residues at the nonreducing termini through β-(1→6)-glycosidic linkages. The enzyme exhibited a maximal activity toward these substrates at pH 3.0. A regioisomer, 3-*O*-β-glucosyluronic acid-galactose, was unsusceptible to the enzyme. The enzyme did act on a polymer substrate, releasing uronic acid from the carbohydrate portion of a radish arabinogalactan–protein modified by treatment with fungal α-L-arabinofuranosidase. The enzyme produced acidic oligosaccharides by transglycosylation, catalyzing the transfer of uronic acid residues of PNP-GlcA and 6-*O*-β-glucosyluronic acid-galactose to certain exogenous acceptor sugars such as Gal, *N*-acetylgalactosamine, Glc, and xylose.

Keywords: Arabinogalactan–protein; *Aspergillus niger*; Galactooligosaccharides; β-Glucuronidase; Transglycosylation

1. Introduction

Arabinogalactan–proteins (AGPs) are a class of proteoglycans that are rich in galactosyl and L-arabinosyl residues. They appear to be present in all plant cells as soluble molecules in extracellular and cell-wall spaces or as membrane-associated forms, and seem to be involved in several aspects of plant development. It is known (see reviews, Refs. 1–3) that the glycan moieties of AGPs have a common structure consisting of β-(1→3)-linked galactan backbones to which side chains of (1→6)-linked β-galactosyl residues are attached through O-6. L-Arabinose (L-Ara) and lesser amounts of such other sugars as glucuronic acid (GlcA), 4-*O*-methyl-glucuronic acid (4-Me-GlcA), L-rhamnose, and L-fucose (L-Fuc) are attached to the side chains, usually as nonreducing terminal residues. In general, AGPs contain between 2 and 10% core protein rich in Hyp, Ser, and Ala.

Various hydrolytic enzymes that may participate in AGP breakdown and turnover have been reported. Working with radish and spinach plants, several α-L-arabinofuranosi-

☆ Sugars described in this paper belong to the D series unless otherwise noted.

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dases (EC 3.2.1.55), which can remove terminal arabinosyl residues from the side chains of AGPs, have been found.[4,5] β-Galactosidases (EC 3.2.1.23) catalyzing a stepwise elimination of galactosyl residues from the galactan framework of AGPs have also been found in these plant tissues.[5,6] Some phytopathogenic fungi also produce hydrolytic enzymes that degrade AGPs. These fungal enzymes include α-L-arabinofuranosidase,[7] *exo*-β-(1→3)-galactanase,[8,9] and *endo*-β-(1→6)-galactanase.[10] These enzymes have proven useful as tools for controlled degradation in structural studies of AGPs. However, no β-glucuronidase (β-GlcAase; EC 3.2.1.31) cleaving glycosyluronic groups attached to AGPs has been found so far. Structural analyses of AGPs have revealed that glucosyluronic or 4-*O*-methyl-glucosyluronic residues occasionally occupy nonreducing ends of (1→6)-linked β-galactosyl side chains through β-(1→6) linkages.[1,2] Indeed, side chains of mature-root AGP of radish carry 4-Me-GlcA at their nonreducing ends, and such acidic side chains account for more than 70% of the total side chains of the AGP.[8] Hence it is anticipated that either 6-*O*-β-glucosyluronic acid-galactose (β-GlcA-(1→6)-Gal) or 6-*O*-(4-*O*-methyl-β-glucosyluronic acid)-galactose (4-Me-β-GlcA-(1→6)-Gal), an aldoboiuronic acid characteristic of the acidic side chains of AGPs, can serve as a substrate for the assay of β-GlcAase acting specifically on AGPs and/or their degraded fragments. In this paper, we report the detection of such a β-GlcAase in a commercially available enzyme source. We have purified the enzyme and studied its properties and action on various uronic acid-containing oligosaccharides and AGPs, as well as its transglycosylation reaction.

2. Experimental

Materials.—Pectinex Ultra SP-L, a commercial pectolytic enzyme preparation (Lot No. G 770) from *Aspergillus niger*, was purchased from Novo Nordisk Ferment Ltd. (Chiba, Japan); β-galactosidase (grade VIII; EC 3.2.1.23) of *Escherichia coli* was from Sigma–Aldrich Japan (Tokyo, Japan); *p*-nitrophenyl β-glucopyranosiduronate (PNP-Glc*p*A) and PNP-β-glycosides of Gal*p* and galactopyranosiduronic acid (Gal*p*A), PNP-α-glycosides of L-Ara*f* and L-Fuc*p*, acacia gum, and azocasein were from Sigma–Aldrich. Radish root AGP (designated as AGP-IV) and its enzymatically modified product by digestion with *Rhodotorula flava* α-L-arabinofuranosidase were prepared as reported in Ref. 11. Sap of the lac tree, *Rhus vernicifera* (Chinese), was purchased from Fujii Lacquer Co. (Tokyo, Japan). Other chemicals were obtained as follows: DEAE-cellulose (DE52) and CM-cellulose (CM32) (Whatman Paper Ltd.); Sephadex G-100 (Amersham Pharmacia Biotech); CM-Toyopearl 650M (Tosoh, Tokyo, Japan).

Carbohydrate analyses.—The following colorimetric methods were applied to assay the respective sugar: total sugar content by the phenol–H_2SO_4 method[12] using Gal as the standard unless otherwise noted; uronic acid by a modified carbazole–H_2SO_4 method[13] using GlcA as the standard; free uronic acid by the reductometrical method of Milner and Avigad[14] using GlcA or 4-Me-GlcA as the standard. Sugars were analyzed by paper chromatography on Whatman No.1 or 3MM paper in A, 6:4:3 (v/v/v) 1-butanol–pyridine–water or B, 5:2:3 (v/v/v) 1-butanol–AcOH–water as solvent systems. Sugar spots on the chromatograms were visualized with alkaline $AgNO_3$. Thin-layer chromatography (TLC) on Silica gel 60 (E. Merck) was performed with 2:1:1 (v/v/v) 1-butanol–AcOH–water as the solvent. 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.[15] Methylation was performed by the Hakomori method,[16] and the products were analyzed by GLC.[17] The carboxyl groups of glucosyluronic or 4-*O*-methyl-glucosyluronic residues in native and permethylated acidic oligomers were reduced with $LiAlH_4$ in THF for 1 h at 50 °C,[18] and the resulting Glc or 4-*O*-methyl-Glc, and its methylated derivatives were analyzed. The ^1H NMR spectra (400 MHz) of oligosaccharide alditols were recorded using a Bruker AM400 spectrometer in D_2O at 70 °C with sodium

2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate as the internal standard. Separation of sugars was carried out by high-performance anion-exchange chromatography (HPAEC) using a Dionex DX-500 fitted with a pulsed amperometric detector. A sample of sugars was chromatographed on a 4×250-mm Dionex CarboPac PA-1 column, at a flow rate of 1.25 mL/min. The elution protocol comprised linear gradients of NaOH (20–35 mM, 10 min and 35–100 mM, 0.5 min), followed by a linear gradient of NaOAc (0–250 mM) in 100 mM NaOH (9.5 min), and an isocratic elution with a mobile phase of the final composition (5 min), respectively. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) was performed using a Kompact MALDI IV instrument (Shimadzu) operated at an acceleration energy of 20 kV, in reflectron mode, and with positive-ion detection. 2,5-Dihydroxybenzoic acid was used as a matrix in 10% EtOH at a concentration of 10 mg/mL. Each sample was mixed with 0.5 μL of the matrix solution and a 0.5% NaCl solution. Oligosaccharide masses were determined from the masses of pseudomolecular ions (sodium adduct, $[M + Na]^+$).

Preparation and characterization of acidic oligosaccharides

Preparation of oligosaccharides from Acacia gum. Acidic oligosaccharides containing GlcA were isolated from partial acid hydrolyzates of acacia gum.[19] Acacia gum (10 g) was hydrolyzed in 0.5 M H_2SO_4 (1 L) for 2 h in a boiling-water bath. The hydrolyzate was made neutral with $BaCO_3$, chromatographed on a column (3×30 cm) of DEAE-cellulose (HCO_3^-, Serva Feinbiochemica GmbH & Co., Germany), and monitored for total sugars[12] and uronic acid.[13] Neutral sugars were eluted with water (500 mL) and acidic sugars were eluted at around 150 mM, during a linear gradient (0–500 mM, 1 L) of $NaHCO_3$. The acidic sugars (1.1 g) were fractionated on a column (3.6×40 cm) of charcoal (chromatography grade, Wako Pure Chemical Industries, Osaka, Japan). Stepwise elution with increasing EtOH concentrations (0–30%, v/v) followed by paper chromatographic separation using Whatman 3MM paper with the solvent B yielded oligosaccharides **1** (480 mg) and **2** (120 mg).

Preparation of oligosaccharides from sap of the lac tree. Acidic oligosaccharides containing 4-Me-GlcA were prepared by the modified method of Oshima and Kumanotani.[20] A crude polysaccharide fraction (31 g) prepared from sap (500 mL) of the lac tree by precipitation with acetone was partially purified on a column (4.5×30 cm) of CM-cellulose (H^+, Seikagaku Corp., Tokyo, Japan) equilibrated and eluted with water (yield, 28 g). The polysaccharide (10 g) was hydrolyzed in 0.2 M H_2SO_4 (1 L) for 2 h in a boiling-water bath. From the hydrolyzate, acidic oligosaccharides **3** (400 mg), **4** (160 mg), and **5** (100 mg) were isolated by a method similar to that used for the oligosaccharides from acacia gum.

Preparation of oligosaccharide from a bacterial polysaccharide. An acidic heteropolysaccharide containing allose as a characteristic sugar constituent was prepared from a culture of a MeOH-assimilating bacterium, *Pseudomonas viscogena* TS-1004.[21] The polysaccharide (500 mg) was hydrolyzed by heating in 0.5 M H_2SO_4 (100 mL) for 4 h in a boiling-water bath. The hydrolyzate was separated by preparative paper chromatography, yielding an aldobiouronic acid, **6** (20 mg).

Characterization of oligosaccharides. The oligosaccharide **1**, $[\alpha]_D$ −7.4° (*c* 0.5, water), gave a single spot on paper chromatograms with the solvents A and B and gave uronic acid and Gal on acid hydrolysis. The carboxyl groups of the saccharide were reduced[18] and analyzed by paper chromatography and GLC after acid hydrolysis, which yielded Glc and Gal in an equimolar ratio. The analytical data obtained for **1** concerning the mode of glycosidic linkages by methylation, the anomeric configurations from ^1H NMR, and the mass numbers from MALDI-TOFMS are summarized in Table 1. On MALDI-TOFMS, the oligosaccharide was observed at m/z 379.4 as sodium adduct, which is in good agreement with the expected mass of an aldobiouronic acid, GlcA·Gal (M_r 356.3). From these data, the oligosaccharide **1** was identified as β-Glc*p*A-(1→6)-Gal*p* (lit.[22], $[\alpha]_D$ +13°). It had 87% purity when the sugar content was assayed by the phenol–H_2SO_4 method[12] with an equimolar mixture of GlcA and Gal as the standard. Similarly, **2**, $[\alpha]_D$ −7.3° (*c* 0.5, wa-

ter), was identified as β-Glc*p*A-(1→6)-β-Gal*p*-(1→6)-Gal*p* (lit.[22], $[\alpha]_D$ −30°). It had 94% purity when sugar content was assayed colorimetrically with a mixture of GlcA and Gal in a molar ratio of 1:2 as the standard.

The acidic oligosaccharides **3**, **4**, and **5** gave single spots on paper chromatography and single peaks on HPAEC: the mobilities and the elution times of **3** and **4** were identical to those of the standard oligosaccharides, 4-Me-β-Glc*p*A-(1→6)-Gal*p* and 4-Me-β-Glc*p*A-(1→6)-β-Gal*p*-(1→6)-Gal*p*,[8] respectively. Each oligosaccharide (90 μg) was digested in a mixture (8 μL) containing purified β-GlcAase (0.25 μg) and 15 mM acetate buffer, pH 4.6, for 8 h at 37 °C, followed by addition of β-galactosidase (2 μg) in 100 mM phosphate buffer (2 μL), pH 7.5, and an incubation for additional 12 h. According to analyses of the hydrolyzates on paper chromatography and HPAEC, the sugar constituents of the saccharides were 4-Me-GlcA and Gal in molar ratios of 1:1, 1:1.7, and 1:2.6 for **3**, **4**, and **5**, respectively. Based on analytical data (Table 1), the oligosaccharides were identified as follows: **3**, 4-Me-β-Glc*p*A-(1→6)-Gal*p*; **4**, 4-Me-β-Glc*p*A-(1→6)-β-Gal*p*-(1→6)-Gal*p*; **5**, 4-Me-β-Glc*p*A-(1→6)-β-Gal*p*-(1→6)-β-Gal*p*-(1→3)-Gal*p*.

Similar analyses allowed us to identify the aldobiouronic acid **6**, $[\alpha]_D$ +14.5° (*c* 0.5, water), as β-Glc*p*A-(1→3)-Gal*p* (lit.[23], $[\alpha]_D$ +12°).

Enzyme assays.—The activity of β-GlcAase was determined using a reaction mixture (0.1 mL) consisting of the enzyme, 0.1% (2.8 mM) β-GlcA-(1→6)-Gal, and 50 mM acetate buffer, pH 4.6. After incubation for 5–20 min at 37 °C, reactions were terminated by dipping the reaction tubes into ice-water bath and

Table 1
Characterization of oligosaccharides

Oligosaccharide	R_{Gal} (solvent B)	Mode of glycosidic linkages [a] (molar ratio)		^{1}H NMR		$[M+Na]^+$
				Chemical shift [b] (ppm) ($J_{1,2}$ Hz) (integral proton)	Assignment	(*m*/*z*) (M_r of identified saccharide)
1	0.43	Glc*p*A1→	(1.0)	4.49 (7.9) (1)	Glc*p*A1→β	379.4 (356.3)
		→6Gal-ol [c]	(1.1)			
2	0.20	Glc*p*A1→	(1.0)	4.45 (7.8) (1)	→6Gal*p*1→β	541.3 (518.4)
		→6Gal*p*1→	(0.8)	4.50 (7.9) (1)	Glc*p*A1→β	
		→6Gal-ol	(1.1)			
3	0.76	4-Me-Glc*p*A1→	(1.0)	3.48 (3)	–OCH_3	393.0 (370.3)
		→6Gal-ol	(1.2)	4.46 (7.8) (1)	4-Me-Glc*p*A1→β	
4	0.39	4-Me-Glc*p*A1→	(1.0)	3.48 (3)	–OCH_3	555.9 (532.4)
		→6Gal*p*1→	(1.0)	4.42 (7.6) (1)	→6Gal*p*1→β	
		→6Gal-ol	(1.1)	4.46 (7.9) (1)	4-Me-Glc*p*A1→β	
5	0.28	4-Me-Glc*p*A1→	(1.0)	3.48 (3)	–OCH_3	717.8 (694.6)
		→6Gal*p*1→	(1.8)	4.39–4.48 [d] (3)		
		→3Galol [c]	(0.9)			
6	0.53	Glc*p*A1→	(1.0)	4.50 (7.6) (1)	Glc*p*A1→β	379.1 (356.3)
		→3Gal-ol	(0.8)			

Oligosaccharides were isolated from the hydrolysates of acacia gum (**1**, **2**), from a polysaccharide of the lac tree (**3**, **4**, **5**), and from a *P. viscogena* polysaccharide (**6**).

[a] Samples were methylated after reduction with sodium borohydride. Methyl esters of the methylated acidic oligosaccharides were then reduced with lithium aluminum hydride[18] and remethylated.

[b] Analyzed as oligosaccharide alditols.

[c] O-6 (or O-3)-linked galactitol as a reduction of O-6 (or O-3)-linked reducing terminal Gal.

[d] Overlapped peaks attributable to β-linked anomeric protons of 4-Me-GlcA and Gal.

mixing immediately with the copper reagent of the method of Milner and Avigad.[14] Liberated GlcA was determined reductometrically by this method. One unit of enzyme activity (as used in the tables and figures) liberates 1 μmol of GlcA per min. When 4-Me-GlcA-containing oligosaccharides were used, activity was calculated based on the liberation of 4-Me-GlcA.

Enzyme activity was also determined by measuring the amount of *p*-nitrophenol released from PNP-GlcA. These assays were done at 37 °C, using a mixture (0.3 mL) containing the enzyme, 3.3 mM substrate, and 50 mM acetate buffer, pH 4.6. Reactions were terminated by the addition of 0.2 M Na_2CO_3 (1.2 mL), and were monitored at 420 nm for the liberated *p*-nitrophenol. Here one unit of the enzyme activity is capable of liberating 1 μmol of *p*-nitrophenol per min. In the transglycosylation reaction, the quantities of liberated GlcA were also determined reductometrically.[14]

Other glycosidases were assayed by measuring the amount of *p*-nitrophenol liberated from the corresponding PNP-glycosides as substrate under the same conditions. Cazeinolytic activity was assayed with azocasein as the substrate as described previously.[8]

These enzyme assays were done, at least in duplicate, and mean values were recorded.

Enzyme purification.—All operations were done at 0–4 °C. Fractions collected in each step of the purification were monitored for activity toward β-GlcA-(1→6)-Gal and PNP-GlcA. The enzyme solution (100 mL) of Pectinex Ultra SP-L was mixed with cellulose powder (5 g) in 10 mM acetate buffer (pH 4.6, 100 mL), stirred for 20 min, and then centrifuged at 12,000*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 a small volume of 10 mM Tris–HCl buffer, pH 7.0, and dialyzed overnight against the buffer. The crude enzyme solution was applied onto a 2.2 × 30-cm DEAE-cellulose (DE52) column equilibrated with the buffer. After the column had been washed with the buffer, the enzyme was eluted with a linear KCl gradient (0–80 mM) in the buffer (total volume, 1.7 L). Fractions (10 mL, 50 mL/h) were collected and monitored for enzyme activity. The active fractions (200 mL) were collected, brought to 80% saturation with $(NH_4)_2SO_4$, and centrifuged. The precipitate was dissolved in a small volume of 10 mM acetate buffer, pH 4.6, and dialyzed against the buffer. The dialyzate was applied onto a 2.5 × 13-cm CM-cellulose (CM32) column equilibrated with the buffer. After the column had been washed with the buffer, the enzyme emerged from the column with a linear KCl gradient (0–0.1 M) in the buffer (total volume, 900 mL; 5 mL/fraction, 60 mL/h). Pooled fractions (250 mL) containing the enzyme were concentrated to 11 mL, using an Amicon ultrafiltration apparatus fitted with a YM-3 membrane. The enzyme solution was applied onto a 2.2 × 95-cm Sephadex G-100 column equilibrated and eluted with the buffer (1.6 mL/fraction, 16 mL/h). The active fractions (37 mL) were combined, concentrated, and applied onto a 1.6 × 8-cm CM-Toyopearl 650M column equilibrated with 10 mM acetate buffer, pH 4.6. The enzyme was eluted from the column with a linear KCl gradient (0–0.1 M) in the buffer (total volume, 300 mL; 2.2 mL/fraction, 25 mL/h). The active fractions (37 mL) were concentrated, exchanged into 10 mM acetate buffer, pH 4.6, by repeated addition of the buffer during the concentration, and stored at −20 °C.

Estimation of molecular weight.—The apparent M_r value of β-GlcAase was determined by SDS-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli[24] using a gradient (5–20%) gel (PAGEL, ATTO, Tokyo, Japan) and M_r standard kits (Low Range, Bio-Rad Laboratories, and HMW kit E, Amersham Pharmacia Biotech). Protein in the gel was stained with Coomassie Brilliant Blue R-250. The M_r of the native enzyme was determined by high-performance liquid chromatography (HPLC) using a Shimadzu LC-10A fitted with a column (7.8 × 300 mm) of TSKgel $G3000SW_{XL}$ (Tosoh, Tokyo, Japan), equilibrated and eluted with 50 mM sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl, at a flow rate of 0.5 mL/min at 30 °C, and monitored at 280 nm. The column was cali-

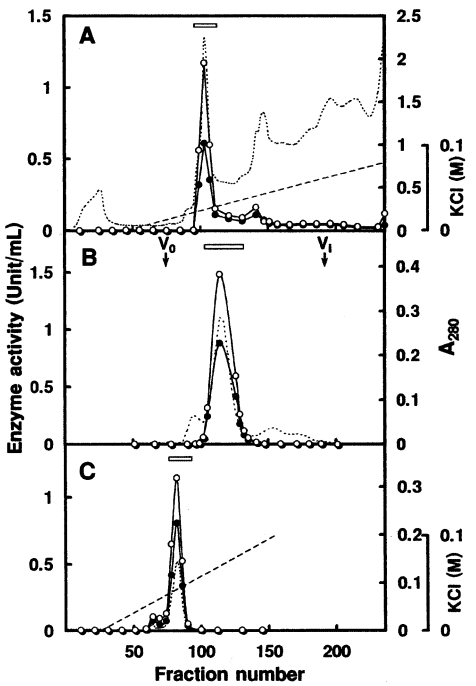

Fig. 1. Column chromatography of β-GlcAase. (A) DEAE-cellulose; (B) Sephadex G-100, in which the column was calibrated by using blue dextran (V_0) and Glc (V_i); (C) CM-Toyopearl. ●–●, enzyme activity for β-GlcA-(1→6)-Gal; ○–○, enzyme activity for PNP-GlcA; ···, absorbance at 280 nm; ---, concentration of KCl. The bars indicate the fractions pooled.

brated with an M_r standard kit (Gel Filtration Standard, Bio-Rad Laboratories) containing thyloglobulin (670,000), γ-globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B-12 (1350). The effluents were fractionated at 0.5 min intervals, and assayed for enzyme activity toward β-GlcA-(1→6)-Gal, PNP-GlcA, and PNP-β-GalA. The M_r of the enzyme was also determined by MALDI-TOFMS using sinapic acid as a matrix at a concentration of 10 mg/mL in 10% EtOH containing 0.1% trifluoroacetic acid.

Other methods.—Proteins were determined by the method of Bradford,[25] using bovine serum albumin as a standard. The isoelectric point (pI) was determined by electrofocusing an LKB 8101 column (110 mL) using a carrier ampholyte (Ampholine, Amersham Pharmacia Biotech) of pH range 3.5–10. The amino-terminal sequence analysis of the enzyme was performed with a Shimadzu PSQ-2 Protein Sequencer.

3. Results

Enzyme purification.—We looked for enzyme activity hydrolyzing β-GlcA-(1→6)-Gal in various commercially available enzyme preparations. For instance, the β-GlcAase specimen (type H-5, Sigma–Aldrich) of snail (*Helix pomatia*) hardly hydrolyzed β-GlcA-(1→6)-Gal under a condition where PNP-GlcA was rapidly hydrolyzed. But Pectinex Ultra SP-L, a pectolytic enzyme preparation, was found to exhibit high activity and selected as a suitable candidate for purification of the enzyme.

A β-GlcAase in Pectinex Ultra SP-L was purified by $(NH_4)_2SO_4$ fractionation and several subsequent column chromatographic operations. Elution profiles of the enzyme from DEAE-cellulose, Sephadex G-100, and CM-Toyopearl columns are illustrated in Fig. 1. Upon chromatography on DEAE-cellulose (Fig. 1(A)), the enzyme was adsorbed on the column and a large portion of it was recovered in the low concentration range of the KCl gradient. Final chromatography on CM-Toyopearl (Fig. 1(C)) gave a single peak of enzyme activity, the elution of which coincided with that of the protein. The amounts of protein and β-GlcAase activity, and the ratio of the activity for β-GlcA-(1→6)-Gal to that for PNP-GlcA in each step of the purification are summarized in Table 2. The enzyme was purified 174-fold in a yield of 12%. A nearly constant ratio (0.75:1) and exactly overlapping peaks of activities hydrolyzing β-GlcA-(1→6)-Gal and PNP-GlcA upon chromatographic separation were retained throughout the purification (Fig. 1 and Table 2). The purified enzyme was confirmed to be free from β-galactosidase, α-L-arabinofuranosidase, α-L-fucosidase, and proteolytic enzyme. Indeed, the enzyme preparation had β-galacturonidase activity at 8.5% of that for β-GlcAase when assayed with 3.3 mM of PNP-β-GalA.

Purity and M_r.—The enzyme migrated as a single protein band with an apparent M_r of 68,000 on SDS-PAGE (Fig. 2). Upon size-exclusion HPLC on a calibrated TSKgel $G3000SW_{XL}$ column under non-denaturing conditions, the enzyme was eluted as a single peak with an apparent M_r of 71,000, indicat-

Table 2
Purification of β-GlcAase from Pectinex Ultra SP-L

Step	Total volume (mL)	Total protein (mg)	Total activity [a] (units)	Specific activity (units/mg protein)	Activity ratio [b]	Purification (fold)	Yield (%)
Ammonium sulfate fractionation	208	1120	141 (175)	0.13 (0.16)	0.81	1 (1)	100 (100)
DEAE-cellulose	27.4	58.4	53.2 (71.8)	0.91 (1.23)	0.74	7 (8)	38 (41)
CM-cellulose	11.0	3.2	43.6 (57.2)	13.6 (17.9)	0.76	105 (112)	31 (33)
Sephadex G-100	16.1	1.9	34.6 (46.7)	18.2 (24.6)	0.74	140 (154)	25 (27)
CM-Toyopearl	12.4	0.73	16.5 (21.4)	22.6 (29.3)	0.77	174 (183)	12 (12)

Starting with 100 mL of the enzyme preparation.
[a] Activity determined with β-GlcA-(1→6)-Gal as the substrate (activity with PNP-GlcA in parentheses).
[b] Ratios for the activities toward β-GlcA-(1→6)-Gal and PNP-GlcA.

ing a single polypeptide chain. The elution profile of the protein on the chromatography agreed with the activity profiles toward β-GlcA-(1→6)-Gal, PNP-GlcA, and PNP-β-GalA. A similar M_r for the enzyme was obtained by MALDI-TOFMS ($[M+H]^+$, m/z 71,000). The isoelectric point (pI) of the enzyme was pH 5.0. The amino-terminal sequence (10 residues) of the enzyme was QIVVSQSQPE–.

Properties.—The optimum pH for the enzyme on both β-GlcA-(1→6)-Gal and PNP-GlcA was 3.0, based on the pH–activity curves using glycine–HCl buffer (pH 2.0–4.0) and acetate buffer (pH 3.2–6.2) at a final concentration of 50 mM. The enzyme was stable within pH ranges of 4.5–9.0 and 2.0–9.0, where we measured remaining activities after incubation with buffers of pH 2.0–10.0 for 3 h at 37 °C and for 24 h at 4 °C, respectively. The remaining activity was also measured after exposure of the enzyme to various temperatures (30–75 °C) for 20 min at pH 4.6. It retained full activity up to 60 °C and was completely inactivated at 75 °C. The pH and thermal stabilities of the enzyme were identical toward β-GlcA-(1→6)-Gal and PNP-GlcA.

The effects of metal ions and various sugars on enzyme activity were examined using PNP-GlcA as the substrate. Of metal ions, Hg^{2+} was found to be an inhibitor: 23% inhibition of the activity was observed after incubation with 1 mM Hg^{2+} for 10 min at 37 °C. Other metal ions including Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, and Mg^{2+} influenced the activity within a range of ±5% at 1 mM. D-Glucono-1,5-lactone, D-galactono-1,4-lactone, and L-arabino-1,4-lactone influenced the activity within ±10% at 5 mM. A slightly higher inhibitory effect was observed for D-glucaric acid 1,4-lactone, which inhibited 30% of the enzyme activity at 5 mM.

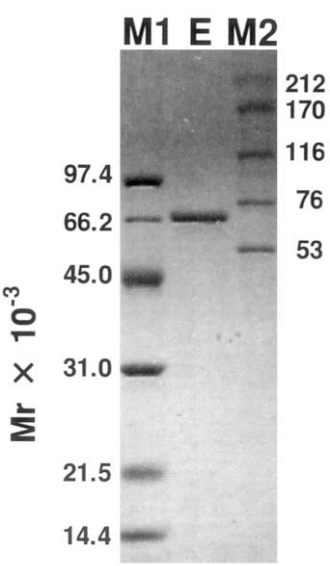

Fig. 2. SDS-PAGE analysis of the purified β-GlcAase. M1 and M2, M_r markers; E, the purified enzyme (3 μg).

Substrate specificity and kinetics.—Besides PNP-GlcA, Gal and galactooligosaccharides, differing in types of linkage and chain length, substituted with either β-(1→6)-linked GlcA or 4-Me-GlcA residues at their non-reducing termini served as good substrates for the enzyme, resulting in release of GlcA or 4-Me-GlcA (Fig. 3). Liberation of GlcA or 4-Me-GlcA as the sole hydrolysis product from these acidic oligosaccharides was confirmed by HPAEC. We calculated the rate parameters of β-GlcAase, Michaelis constants and maximum velocities, for hydrolysis of PNP-GlcA and various acidic galactooligosaccharides (Table 3). The rates of hydrolysis for galactooligomers substituted with β-(1→6)-linked GlcA or 4-Me-GlcA residues were comparable to that for PNP-GlcA, irrespective of the presence or absence of *O*-methyl groups at C-4 of glycosyluronic residues. β-GlcA-(1→3)-Gal, a regioisomer of these substrates, was highly resistant to enzymatic hydrolysis. Among oligosaccharide substrates, 4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal (**4**) gave the highest activity. However, the order of the hydrolysis rates at 5 mM of substrate concentration seems to not depend on chain length and type of linkages in aglycone moieties. Apparent K_m values for these oligosaccharides tend to decrease with increasing chain length, and the values for GlcA-containing oligosaccharides are lower than those for corresponding 4-Me-GlcA-containing oligosaccharides. This indicates that *A. niger* β-GlcAase has a higher affinity for unsubstituted β-glucosyluronates than for those substituted with 4-*O*-methyl groups. However, apparent V_{max} values tend to increase with increasing chain length, except for the acidic tetrasaccharide (**5**), whose aglycone consists of mixed galactosidic linkages.

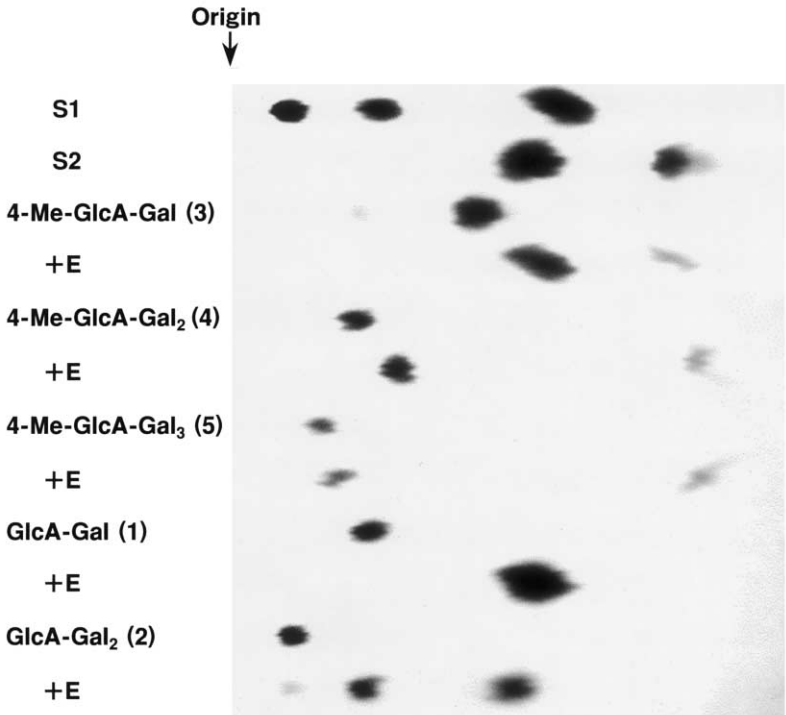

Fig. 3. Analysis by paper chromatography of hydrolysis products produced by the action of β-GlcAase on various GlcA- or 4-Me-GlcA-containing acidic galactooligosaccharides. The numbers in parentheses are listed in Section 2. S1, standard Gal, β-(1→6)-galactobiose and -triose (from right to left); S2, standard 4-Me-GlcA and GlcA (from right to left); 4-Me-GlcA-Gal and (+E) its enzymatic hydrolysate, and so on. Oligosaccharides (60 μg, each) were incubated with 0.25 μg of the enzyme in 20 μL of 10 mM acetate buffer, pH 4.6, for 14 h at 37 °C. The reaction mixtures were, then, directly spotted on a paper sheet and developed with solvent B.

Table 3
Action of β-GlcAase on PNP-GlcA and various uronic acid-containing oligosaccharides

Substrate	Relative rate of hydrolysis (%)	K_m (mM)	V_{max} (μmol/min/mg protein)
PNP-GlcA	100	0.23	20.9
β-GlcA-(1→6)-Gal	76	0.71	15.9
β-GlcA-(1→6)-β-Gal-(1→6)-Gal	67	0.53	19.8
4-Me-β-GlcA-(1→6)-Gal	62	3.6	24.4
4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-Gal	117	1.9	50.8
4-Me-β-GlcA-(1→6)-β-Gal-(1→6)-β-Gal-(1→3)-Gal	106	1.4	15.8
β-GlcA-(1→3)-Gal	2.0	nd [a]	nd

The enzyme (0.25 μg) was incubated in mixtures as described in Section 2, but at 5 mM of substrate concentration. At suitable time intervals, the amounts of released *p*-nitrophenol or uronic acids were determined, and are expressed as relative rates calculated by taking that of PNP-GlcA as unity (100%). The K_m and V_{max} values were calculated from the Lineweaver–Burk plot using varying amounts of substrates.

[a] Not determined.

Degradation of AGPs.—Table 4 summarizes the relative rates, extents of hydrolysis, and kinetic parameters of native and α-L-arabinofuranosidase-treated AGPs of the action of β-GlcAase using PNP-GlcA as a control. Obviously, the native AGP (carrying 4-Me-GlcA as the sole uronic acid constituent) was resistant to enzymatic hydrolysis, but became susceptible to hydrolysis after removal of a large portion of the α-L-arabinofuranosyl residues (amounting to 19% of total sugar in the native AGP) attached to the side chains of

the β-3,6-galactan backbone. 4-Me-GlcA was identified as the sole hydrolysis product on paper chromatography and on HPAEC. However, liberation of 4-Me-GlcA from the modified AGP ceased at about half of its total content even after extensive digestion. This limit of hydrolysis suggests the presence of 4-Me-GlcA-containing acidic side chains in the modified AGP inaccessible to the enzyme, partly due to steric hindrance caused by the variation in chain lengths of such side chains ranging from single to at least 20 residues.[8]

Transglycosylation reaction.—The action of β-GlcAase on PNP-GlcA was enhanced by addition of various monosaccharides into reaction mixtures when assayed for liberated *p*-nitrophenol. For instance, activity increased with the concentration of Gal. In the presence of 33 mM Gal (ten times the concentration of PNP-GlcA) activity reached more than twice that found in the absence of Gal (Table 5). On the other hand, a reciprocal relation was observed for the amount of GlcA released from the substrate, suggesting that the enzyme prefers hydroxyl groups of Gal as an acceptor molecule rather than water. Consistently, an apparent reduction of enzyme activity in the presence of Gal was observed when β-GlcA-(1→6)-Gal was used as an alternative substrate. In addition to Gal, similar results were

Table 4
Action of β-GlcAase on AGP and its enzymatically modified derivative

Substrate	Uronic acid content [a] (%)	Concentration	Relative rate of hydrolysis [b] (%)	Limit of hydrolysis [c] (%)	K_m [d] (mg/mL)	V_{max} [d] (μmol/min/mg protein)
PNP-GlcA		5 mM	100			
AGP	10	5 mg/mL	1.8	13	nd [e]	nd
α-L-Arabinosidase-treated AGP	14	5 mg/mL	36	48	0.12	32.3

[a] The content of 4-Me-GlcA against total weight of each polymer.
[b] A sample of radish root AGP or its α-L-arabinosidase-treated AGP was incubated with 0.5 μg of the enzyme in 0.1 mL of 50 mM acetate buffer, pH 4.6, at 37 °C. After appropriate time intervals, the amounts of 4-Me-GlcA released were determined, and are expressed as relative rates based on that of PNP-GlcA as unity.
[c] Determined when the amounts of 4-Me-GlcA liberated reached a plateau (after reaction for 21 h) under the condition mentioned above, and are expressed as the proportion against total content of 4-Me-GlcA in the reaction mixture.
[d] Kinetic parameters were determined by the Lineweaver–Burk plot using varying amounts of the polymer.
[e] Not determined.

Table 5
Transglycosylation action of β-GlcAase with Gal as an acceptor substrate

Substrate	Gal concentration (mM)	Relative activity [a]	
		p-Nitrophenol released (%)	GlcA released (%)
PNP-GlcA	0	100	100
	5	120	69
	10	150	56
	33	230	26
β-GlcA-(1→6)-Gal	0		100
	5		71
	10		68
	33		37

[a] The enzyme (0.15 μg) was pre-incubated in the presence of varying amounts of Gal for 10 min at 37 °C. The reactions were done by addition of PNP-GlcA (final 3.3 mM) or β-GlcA-(1→6)-Gal (final 2.8 mM) and the mixtures were incubated in 0.3 or 0.1 mL of 50 mM acetate buffer, pH 4.6, respectively, for 15 min at 37 °C. The amounts of liberated *p*-nitrophenol and GlcA from PNP-GlcA in separate mixtures and GlcA from β-GlcA-(1→6)-Gal were determined colorimetrically, and are expressed as relative rates calculated by taking those obtained without Gal as unity.

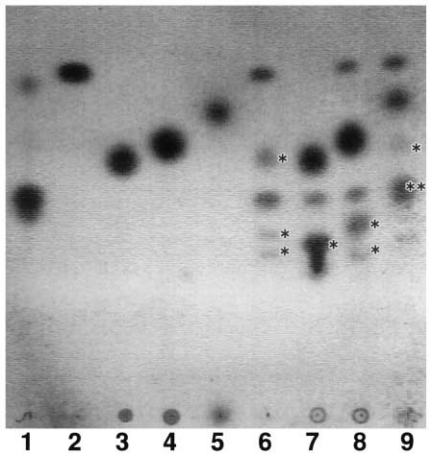

Fig. 4. Products formed by the transglycosylation reaction of β-GlcAase. The enzyme (0.25 μg) was incubated with 22 mM of PNP-GlcA with or without 33 mM of each monosaccharide in 20 μL of 10 mM acetate buffer, pH 4.6, for 75 min at 37 °C. The reaction products were analyzed on TLC. Lane 1, GlcA (the faster moving minor spot may be lactone form); lane 2, PNP-GlcA; lane 3, Gal; lane 4, Glc; lane 5, Xyl; lane 6, PNP-GlcA + enzyme; lanes 7, 8, and 9, products formed by the enzyme action toward PNP-GlcA in the presence of Gal, Glc, and Xyl, respectively. Note that the concentration of PNP-GlcA in the reaction mixtures was much higher than that used in Table 5. Besides GlcA as the hydrolysis product, putative transglycosylation products (asterisks) are visible in each lane, even in the reaction mixture incubated without monosaccharide. Double asterisks may be a transfer product overlapped with GlcA.

also obtained for *N*-acetylgalactosamine, Glc, and xylose (Xyl) in the following order of magnitude with respect to both stimulation of liberation of *p*-nitrophenol and reduction of liberation of GlcA: Gal ≒ *N*-acetylgalactosamine > Glc ≒ Xyl. The following monosaccharides did not exert an appreciable influence on enzyme activity at 5 mM in reaction mixtures containing 3.3 mM of PNP-GlcA: Man, Fru, Rib, L-Ara, D-Ara, L-Fuc, GlcA, GalA, GlcNAc.

The products formed by transglycosylation were analyzed. When the enzyme was incubated with PNP-GlcA in the presence of Gal, Glc, or Xyl, several putative transglycosylation products were detected on TLC (Fig. 4). But the structures of these newly synthesized components are not yet known. The slowest moving component in the chromatogram, for example, produced with Gal, seems to be an acidic disaccharide, GlcA·Gal. A similar enzyme catalyzed transglycosylation occurred when β-GlcA-(1→6)-Gal was used as a donor substrate in the presence of monosaccharides. For example, addition of excess amounts of Glc to the reaction mixture led to formation of two new components, one of which is presumed to be GlcA·Glc (Fig. 5). Additionally, apparent suppression of the release of free GlcA as the hydrolysis product was observed, as mentioned above, in comparison with a control experiment in the absence of Glc.

4. Discussion

In this paper, we report the isolation of a β-GlcAase capable of liberating GlcAs linked to galactooligosaccharides through β-(1→6) linkages, by using as substrates β-GlcA-(1→6)-Gal, an aldobiouronic acid characteristic of the acidic side chains of AGPs, and PNP-GlcA, which is widely used in assays of various β-GlcAases. There seems to be no structural requirement in the enzyme's binding or action that distinguishes between β-glucosiduronates substituted with 4-*O*-methyl groups and unsubstituted β-glucosiduronates.

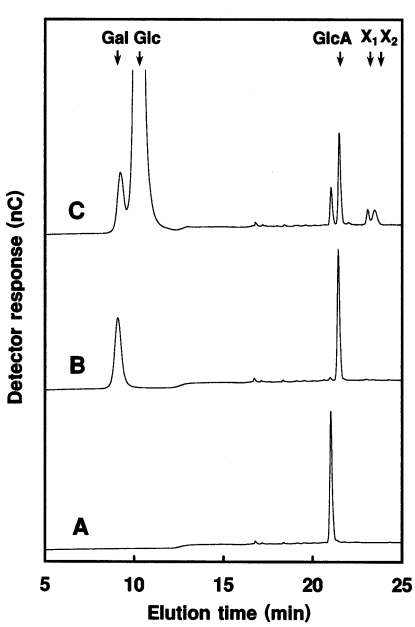

Fig. 5. Analysis by HPAEC of products formed by the transglycosylation reaction of β-GlcAase using β-GlcA-(1→6)-Gal as a donor and Glc as an acceptor substrate. The enzyme (0.15 μg) was incubated with β-GlcA-(1→6)-Gal (2.8 mM) in the presence (C) or absence (B) of Glc (33 mM) in 0.1 mL of 50 mM acetate buffer, pH 4.6, for 18 at 37 °C, and then subjected to HPAEC. A, β-GlcA-(1→6)-Gal alone. Arrows indicate the elution positions of monosaccharide standards and putative transglycosylation products (X_1, X_2).

It is thus able to release 4-Me-GlcA from acidic galactooligosaccharides involving the glycosyluronic residues at the nonreducing termini. With regard to specificity for the substrate, the enzyme hydrolyzed β-(1→6)-linked glycosyluronic groups in preference to β-(1→3) linkages. This specificity of *A. niger* β-GlcAase contrasts with mammalian liver β-GlcAase, which has the opposite preference, cleaving β-GlcA-(1→3)-Gal, a disaccharide isolated from chondroitin 4-sulfate, much faster than β-GlcA-(1→6)-Gal.[23] Other interesting aspects of this β-GlcAase in comparison with those isolated from mammalian sources are as follows: the enzyme isolated from *A. niger* consists of a monomeric polypeptide, while those from various mammalian sources are tetramers composed of identical subunits having M_r of approximately 75,000;[26] our enzyme is essentially insensitive to D-glucaric acid 1,4-lactone, which when added at micromolar concentrations is a potent inhibitor for mammalian β-GlcAases.[27]

The rates of hydrolysis of acidic galactooligosaccharides carrying either GlcA or 4-Me-GlcA by β-GlcAase did not depend on their chain lengths (Table 3). A similar lack of regularity in hydrolysis of oligosaccharides has previously been observed for α-GlcAases cleaving α-(1→2) linkages connecting nonreducing terminal 4-Me-GlcA to xylooligosaccharides, the building units of heteroxylans. For instance, it has been found that α-GlcAases from the xylanolytic fungus *Trichoderma reesei* and from *Thermoascus aurantiacus*, a thermophilic fungus, hydrolyze aldotriuronic acid [4-Me-α-GlcA-(1→2)-β-Xyl-(1→4)-Xyl] faster than aldobiouronic acid (4-Me-GlcA·Xyl) and aldotetrauronic acid (4-Me-GlcA·Xyl_3).[28,29] The specificity of the *T. reesei* enzyme is similar to that of the *A. niger* β-GlcAase studied here in that it liberates 4-Me-GlcA from high M_r heteroxylans at a much lower rate than from acidic xylooligosaccharides.

An interesting aspect of the action of β-GlcAase is its transglycosylation reaction, in which the enzyme releases uronic acids from PNP-GlcA and β-GlcA-(1→6)-Gal, and transfers them onto several particular monosaccharides supplied in the reaction mixture as acceptor substrates. The transfer action of β-GlcAase is known for a rat liver enzyme which catalyzes the transfer of GlcA residues from phenyl-β-GlcA to such acceptor sugars as Glc, Gal, Xyl, L-Ara, maltose, and cellobiose through β-(1→3)-glycosidic linkages.[30] Further study will be required to identify the structures of the newly synthesized oligosaccharides by the action of *A. niger* β-GlcAase.

Radish root AGP became susceptible to enzymatic hydrolysis after removal of most L-arabinosyl residues attached to the side chains of the AGP, resulting in liberation of maximally half of total 4-Me-GlcA residues in the AGP. Similar requirement for removal of L-arabinosyl residues prior enzyme action on AGPs has been known for several carbohydrases such as an *exo*-β-(1→3)-galactanase[8] and an *endo*-β-(1→6)-galactanase.[10] The result obtained indicates that *A. niger* β-GlcAase has potential utility for structural analysis and for, in the modification of AGPs.

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