Plant Physiol. (1989) 90, 567–574
0032-0889/89/90/0567/08/$01.00/0

Received for publication August 24, 1988
and in revised form January 22, 1989

A β-Galactosidase from Radish (*Raphanus sativus* L.) Seeds

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ABSTRACT

A basic β-galactosidase (β-Galase) has been purified 281-fold from imbibed radish (*Raphanus sativus* L.) seeds by conventional purification procedures. The purified enzyme is an electrophoretically homogeneous protein consisting of a single polypeptide with an apparent molecular mass of 45 kilodaltons and pI values of 8.6 to 8.8. The enzyme was maximally active at pH 4.0 on *p*-nitrophenyl β-D-galactoside and β-1,3-linked galactobiose. The enzyme activity was inhibited strongly by Hg^{2+} and 4-chloromercuribenzoate. D-Galactono-(1→4)-lactone and D-galactal acted as potent competitive inhibitors. Using galactooligosaccharides differing in the types of linkage as the substrates, it was demonstrated that radish seed β-Galase specifically split off β-1,3- and β-1,6-linked D-galactosyl residues from the nonreducing ends, and their rates of hydrolysis increased with increasing chain lengths. Radish seed and leaf arabino-3,6-galactan-proteins were resistant to the β-galase alone but could be partially degraded by the enzyme after the treatment with a fungal α-L-arabinofuranosidase leaving some oligosaccharides consisting of D-galactose, uronic acid, L-arabinose, and other minor sugar components besides D-galactose as the main product.

β-Galactosidases (EC 3.2.1.23) are widely distributed in various plant tissues (8). The enzymic properties, multiple forms, and specificities relevant to structural studies on glycoproteins have been investigated (2, 8, 19). Recently, interest in this enzyme has been focused on its *in vivo* functions concerning the degradation of such galactose-containing cell wall polysaccharides as galactan-pectin polymers and xyloglucan in relation to cell growth (16), fruit ripening (25), and seed germination (9). Further, characterization of a thylakoid bound β-Galase[1] in wheat leaf chloroplasts implicated the role for the intermediary degradation of mono- and digalactosylglycerol abundant in thylakoid membranes during senescence of chloroplasts (4).

Working with organs that develop after germination of radish seeds, we have recently reported the formation of organ-specific AGPs in primary and mature roots and leaves, which were clearly distinguishable from the seed proteoglycans in chemical composition, structure, and serological properties (21, 30, 31).

In the present paper, we report the purification and characterization of a radish seed β-Galase whose specificity is highly restricted to β-1,3- and β-1,6-linked D-galactosyl residues, thereby participating in the degradation of the backbone structure of radish AGPs.

MATERIALS AND METHODS

Plant Material

Seeds of the radish (*Raphanus sativus* L. var *hortensis* cv Aokubi) were purchased from Tokita Seed and Plant Co., Ltd., Saitama, Japan. The seeds were sterilized with 3% (w/v) $Ca(OCl)_2$, rinsed with distilled water, and imbibed at room temperature for 24 h on a moistened filter paper in a plastic tray covered with an aluminum foil.

Chemicals

PNP-α-L-arabinofuranoside and -pyranoside were synthesized (12). PNP-β-glycosides of D-Xyl, D-Gal, D-GlcUA, D-GlcNAc, D-Fuc, PNP-α-D-Man, ONP-β-D-Gal, 4-MU-β-D-Gal, acacia gum, and larch wood AG were from Sigma Chemical Co. Other chemicals were obtained as follows: methyl-β-D-Gal, D-galactono-(1→4)-lactone, X-Gal, and other mono- and disaccharides (Wako Pure Chemical Co., Ltd., Osaka, Japan); Sephadex G-25, G-50, G-100, and G-150 (Pharmacia-Japan, Tokyo); hydroxylapatite and Bio-Gel P-2 (Bio-Rad). CM-32 (Whatman); LiChrosorb NH_2 (5 μm) (Merck).

Radish leaf AGP (designated as R-II) and seed AG and AGP were prepared by the methods as reported previously (21, 30). Digestion with *Rhodotorula flava* α-L-Arafase (32) provided enzymically modified radish AG and AGPs (29, 30).

β-1,3-Galactan was obtained in a yield of 11% from acacia gum by thrice Smith degradation (29), and its properties are as follows: $[\alpha]_D^{20}$ + 35.5° (*c* = 1.0, water); D-Gal content 96% (w/w); Kjeldahl N < 0.1% (w/w); mol wt, 25,000. The molar proportions (mol %) in the mode of linkages of the polymer were Gal-(1→ (8): →3)-Gal-(1→ (85): →3,6)-Gal-(1→ (7), and a chemical shift (104.69 ppm) on ^{13}C-NMR confirmed the β-anomeric configuration of the D-galactosidic linkage.

Soybean arabinan-galactan was isolated according to Labavitch *et al.* (17). A β-1,4-galactan was obtained by heating the arabinan-galactan with diluted H_2SO_4 (pH 1.1) for 1 h at 100°C.

[1] Abbreviations: β-Galase, β-galactosidase; PNP-β-D-Gal, *p*-nitrophenyl β-D-galactoside; ONP-β-D-Gal, *o*-nitrophenyl β-D-galactoside; 4-MU-β-D-Gal, 4-methylumbelliferyl β-D-galactoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; AG, arabinogalactan; AGP, arabinogalactan-protein; α-L-Arafase, α-L-arabinofuranosidase; 2-ME, 2-mercaptoethanol.

For preparation of β-1,4-linked galactooligosaccharides, soybean arabinan-galactan (1.5 g) was hydrolyzed in 0.5 N H_2SO_4 (600 mL) for 1 h at 100°C, and the hydrolysate was fractionated on a 3.6 × 39 cm charcoal column. Stepwise elution with increasing ethanol concentrations (2.5–30%, v/v) followed by paper chromatographic separation using Whatman 3MM paper afforded galactobiose (36 mg), triose (42 mg), tetraose (38 mg), and pentaose (18 mg), respectively. These oligosaccharides were identified by methylation analyses for mode of glycosidic linkages and paper chromatography using authentic specimens as standard (22). The anomeric configuration of the biose was determined to be β-anomer by a chemical shift (105.1 ppm) in ^{13}C-NMR spectrometry (6).

β-1,3-Linked and β-1,6-linked galactobioses and -trioses were prepared from larch wood AG by the method of Aspinall *et al.* (3).

Methyl β-D-glycosides of β-1,3-linked galactotetraose and -pentaose, and of β-1,6-linked galactopentaose and -hexaose were kindly supplied by Dr. P. Kovac, National Institutes of Health, Bethesda, MD, USA.

Analytical Methods

Total carbohydrate, reducing sugar, and protein were determined colorimetrically (21, 29). GLC analysis of alditol acetates was done in a Shimadzu gas chromatograph GC-6A by the method of Albersheim *et al.* (1). Methylation analysis of poly- and oligosaccharides was performed as described previously (29). Separation and identification of sugar were made by descending paper chromatography using Whatman No. 1 or 3 MM paper and a solvent of 1-butanol/pyridine/water (6:4:3, v/v/v). The spot of sugar was detected by silver nitrate reagent.

Amino Acid Analysis

Duplicate samples (90 μg each) of the purified enzyme was hydrolyzed with 4 N methanesulfonic acid (0.1 mL) in evacuated sealed tubes for 24 h at 110°C, and analyzed for amino acid composition in a Hitachi 655 HPLC system adapted for *o*-phthalaldehyde method.

HPLC

HPLC analysis for sugar was carried out at 30°C on a 4.6 × 250 mm LiChrosorb NH_2 column in a Shimadzu high performance liquid chromatograph LC-6A fitted with a Shodex RI SE-31 refractometer (Showa Denko Co.). Sugars were resolved by an isocratic elution using 60% (v/v) acetonitrile containing 1% (v/v) methanol.

Aliquots (200 μg) of methyl β-D-glycoside of β-1,6-linked galactopentaose were digested in a reaction mixture (50 μL) containing 100 mM acetate buffer (pH 4.0), and enzyme (5 mU) at 37°C for 1, 2.5, 5, 10, and 15 min. The digests (20 μL) were analyzed for the products after passage through a small column of mixed Dowex 50W (H^+) and Dowex 1 (HCO_3^-).

PAGE

Native and denaturing PAGE were made by the methods of Reisfeld *et al.* (26) and Laemmli (18), respectively. Protein band in gel was made visible by 0.25% (w/v) Coomassie brilliant blue R-250 in 7.5% (v/v) acetic acid-50% (v/v) methanol. The enzyme activity in a gel was stained by the method of Singh and Knox (28) using X-Gal. β-Galase was extracted with 20 mM phosphate buffer (pH 7.0) (200 μL) from 2-mm wide discs obtained by slicing the electrophoresed 7.5%, pH 4.3 gel, and assayed for the activity toward PNP-β-D-Gal and α-L-Arafase-treated radish leaf AGP.

Determination of Molecular Mass

An apparent molecular mass of β-Galase was calculated by a linear regression analysis from the data obtained by denaturing PAGE using 12.5% gel and the following marker proteins: phosphorylase b (97.4 kD), bovine serum albumin (66.2 kD), ovalbumin (42.7 kD), carbonic anhydrase (31.0 kD), soybean trypsin inhibitor (21.5 kD), lysozyme (14.4 kD). Estimation of apparent molecular mass was performed on a 1 × 48 cm Sephadex G-100 column which had been equilibrated with 20 mM phosphate buffer, pH 6.8, containing 0.1 M KCl and calibrated by using human transferrin (80 kD), bovine serum albumin, ovalbumin, and bovine α-lactalbumin (14.4 kD).

Electrofocusing

Electrofocusing of β-Galase was performed in a LKB 8101 column (110 mL) using a carrier ampholite of a pH range of 3.5 to 10 at 2°C. Fractions (1 mL) were collected and monitored for the enzyme activity and at 280 nm.

Enzyme Assay

The activity toward PNP- or ONP-glycosides was assayed in a reaction mixture (200 μL) containing 50 mM acetate buffer (pH 4.0), 2.5 mM substrate and enzyme. After incubation for 10 min at 37°C, the reaction was terminated by addition of 200 mM Na_2CO_3 (800 μL) and monitored at 420 nm. One unit was defined as the amount of enzyme capable of liberating 1 μmol of *p*- or *o*-nitrophenol per min. 4-Methylumbelliferone liberated was determined fluorimetrically in a Hitachi 850 fluorospectrometer at 375 nm (exciter) and 475 nm (emitter).

The quantity of D-Gal released from oligomers was estimated by D-galactose dehydrogenase method of Finch *et al.* (13). β-Galase was incubated at 37°C in a reaction mixture (100 μL) consisting of 100 mM acetate buffer (pH 4.0) and 5 mM substrate. Aliquots (20 μL) of the sample were withdrawn at time intervals and assayed for D-Gal released by measuring A_{340} of NAD^+ reduced. Unexpectedly, β-1,6-linked galactobiose and -triose were oxidized by *Pseudomonas* D-galactose dehydrogenase (Boehringer-Mannheim), in relative activity ratios of D-Gal (100):galactobiose (89):galactotriose (73), and thus correction was made for the substrate blank in the enzyme assay.

The degradation of galactans and AGPs was assayed reductometrically using D-Gal as standard.

To analyze the anomeric configuration of D-Gal released, aliquots (45 mU) of β-Galase were incubated with β-1,3-galactan (500 μg) in 50 mM acetate buffer (pH 4.0), in a total volume of 100 μL for 5 min at 37°C. The reaction was

terminated by dipping the mixture in acetone-dry ice, and the frozen sample was lyophilized. Another reaction mixture was boiled for 2 min, left at room temperature for 6 h, and lyophilized. D-Gal in the samples were trimethylsilylated in 20 μL of pyridine/hexamethyldisilazane/trimethylchlorosilane (1:0.2:0.1, v/v/v) and analyzed on GLC (30).

Cytochemical Localization

Sterilized radish seeds were imbibed on a moistened filter paper in a 2 × 9 cm Petri dish in the dark at room temperature for 24 h. Peeled seeds were dissected into cotyledons and radicles which were then fixed in 100 mM phosphate buffer (pH 7.2), containing 2% (v/v) glutaraldehyde in an ice bath for 2 h. Semi-thin sections (10 μm thick) of frozen tissues were cut on a cryostat (Cryocute, Reichert-Jung), and mounted on a 0.1% (w/v) polylysine-coated silde glass. β-Galase was localized in the tissues by indigogenic method (28). For a control, sections were incubated in a standard X-Gal-containing medium to which 10 mM D-galactono-(1→4)-lactone was added. Micrographs were taken on a Kodak Panatoic-X film in a Olympus VANOX model AHB-LB microscope.

RESULTS

Glycosidase Activities in Crude Seed Extracts

The imbibed seeds (10 g, dry weight) were homogenized with three times their weight of 20 mM Na-phosphate buffer (pH 7.2), containing 130 mM NaCl, 2 mM EDTA, and 20 mM 2-ME (buffer A) for 10 min, and centrifuged at 20,000*g* for 30 min. The sediment was treated again as above, and the combined supernatants were filtered through a Whatman No. 2 filter paper on a Buchner funnel. To the filtrate, solid $(NH_4)_2SO_4$ was added to 90% saturation, and left for 30 min. The resulting precipitate was collected by centrifugation as before, dissolved in 20 mM acetate buffer (pH 5.0) (buffer B, 10 mL), and passed through a 2.5 × 17 cm Sephadex G-25 column equilibrated with the buffer. Fractions containing proteins were pooled and assayed for glycosidase activities using PNP-glycosides as described in "Materials and Methods." The activities of α-mannosidase (287 mU/mL), β-Galase (250 mU/mL), and β-*N*-acetylglucosaminidase (245 mU/mL) were predominant in the crude extract, and significant activities of α-L-Arafase (26 mU/mL), β-xylosidase (12 mU/mL), and β-glucuronidase (2 mU/mL) were detected which were possibly involved in the degradation of the seed AGP. Further, paper chromatographic analysis and determination of reducing sugar revealed a complete hydrolysis of β-1,3-linked galactobiose, and a partial degradation of lactose and β-1,4-linked galactobiose after incubation with the seed extract (β-Galase activity, 12 mU) for 18 h at 37°C. PAGE on a pH 4.3 gel of the seed extract gave a single β-Galase component responsible for the hydrolysis of both PNP-β-D-Gal and α-L-Arafase-treated AGP (data not shown).

Purification of β-Galase

All the procedures were conducted at 0 to 4°C. Extraction of β-Galase was carried out by the procedure for preparation of the crude extract. The imbibed seeds (300 g, dry weight) were homogenized with buffer A (1,000 mL), and centrifuged at 12,000*g* for 30 min. The resultant sediment was extracted again with buffer A (500 mL). The combined supernatants were filtered to remove flocculent precipitates. To the clear yellowish filtrate, pulverized $(NH_4)_2SO_4$ was added to a final saturation of 30%, stirred and left for 4 h. The resultant precipitate was removed by centrifugation and discarded. The supernatant was brought to 60% saturation of $(NH_4)_2SO_4$, stirred, and left standing for 12 h. After centrifugation as before, the pellet was dissolved in buffer B, passed through a 6.5 × 53 cm Sephadex G-25 column equilibrated with the buffer B, and loaded onto a 5 × 17 cm CM-32 column equilibrated with the buffer B. The unadsorbed proteins were washed through the column with buffer B, and β-Galase was eluted from the column by a linear KCl gradient (0–500 mM) in the buffer (a total volume, 2,000 mL). The active fractions were pooled and passed through a Sephadex G-25 column equilibrated with 10 mM phosphate buffer (pH 6.8) (buffer C), and subjected to chromatography on a 4 × 10 cm hydroxylapatite column equilibrated with buffer C. The column was washed with buffer C (3 bed volumes) and then 400 mM KCl in the buffer until protein could no longer be detected. The column was then reequilibrated with buffer C, and β-Galase fractions emerged by a linear gradient of phosphate buffer, pH 6.8 (10–400 mM) were pooled, concentrated to 10 mL in an Amicon ultrafiltration apparatus fitted with a PM-10 membrane, and chromatographed on a 2.6 × 100 cm Sephadex G-150 column equilibrated with 20 mM phosphate buffer (pH 6.8) containing 100 mM KCl. The major fractions containing β-Galase were pooled, desalted on a Sephadex G-25 column, and focused in a LKB column using a carrier ampholite of a pH range of 3.5 to 10 at a constant voltage of 300 V for 96 h. β-Galase activity was located in fractions over a pI range of 4 to 9, and the most active fractions with the pI values of pH 8.6 to 8.8 were pooled, passed through a 2 × 50 cm Sephadex G-50 column equilibrated with buffer B, concentrated by ultrafiltration, and stored at −80°C. Fractions containing more acidic β-Galase(s) with pI values of 5 to 7 were pooled and treated as above. Figure 1 illustrates the elution profiles of β-Galase in each chromatographic step of purification. Finally, a major basic β-Galase which was thereafter designated as β-Galase was purified 281-fold, based on the specific activity of β-Galase in the 2nd step of purification because of yellow pigment(s) disturbing the enzyme assay in the crude extract (Table I). No sign of other glycosidases present in the crude extract could be detected in the final preparation after incubation for 1 h.

Purity

The purified enzyme (80 μg, each) was electrophoresed in three paralell 7.5% gels at pH 4.3, and gels after the run were stained separately for protein, the enzyme activity, and sugar. As shown in Figure 2, it migrated as a single protein band at the same position as that of a band stained by the indigogenic method using X-Gal (28). A faint red band was visualized in another electrophoresed gel after periodate-Schiff staining.

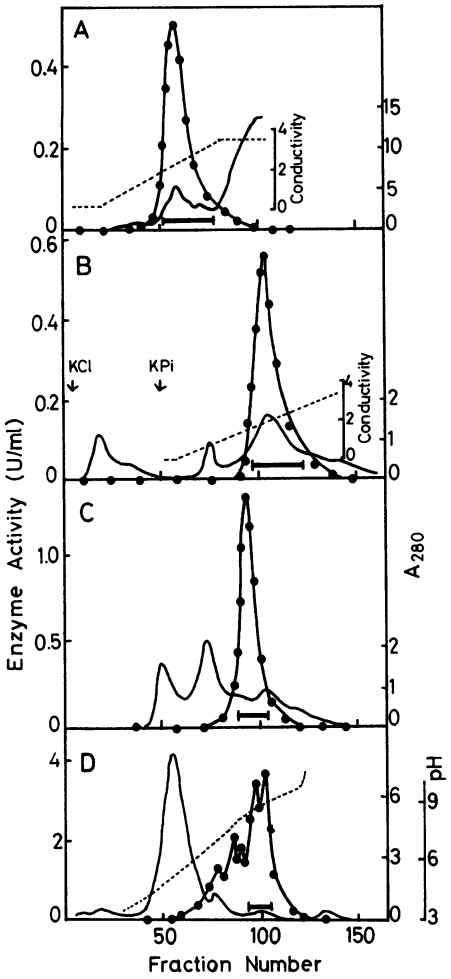

Figure 1. Column chromatography and electrofocusing of radish seed β-Galase. A, CM-32; B, hydroxylapatite; C, Sephadex G-150; D, electrofocusing. ●, enzyme activity; (——), A_{280}; (– – –), conductivity (mmho) or pH. Fraction volumes were 17, 7, 3, and 1 mL/tube for panels A to D, respectively. The bars indicate the fractions pooled.

Molecular Mass

The apparent molecular mass of β-Galase was 45 kD on SDS/PAGE and 60 kD on a calibrated Sephadex G-100 column, respectively.

Amino Acid Composition

Radish seed β-Galase was rich in aliphatic and hydroxy amino acids, particularly in proline (Table II). A high pI value of β-Galase suggests the presence of most of aspartic acid and glutamic acid as their amides.

Effect of pH

PNP-β-D-Gal, and β-1,3-linked galactobiose was shown optimally to be hydrolyzed at pH 4.0, based on the pH-activity curves obtained by using citrate-phosphate buffer (pH 2.0–3.5), acetate buffer (pH 3.5–5.5), phosphate buffer (pH 5.5–7.0), Tris-HCl buffer (pH 7.0–8.5), and glycine-NaOH buffer (pH 9.0–10.0) at 50 mM (final concentration). β-Galase was found to be stable within a pH range of 3.5 to 8.5 by measuring the remaining activity after incubation with the buffers of pH 3.5 to 10 for 24 h at 4°C.

Effect of Temperature

β-Galase was exposed to various temperature (30–60°C) at pH 4.0 for 10 min, and the residual activity was assayed under the standard condition. It retained the full activity up to 40°C but was completely inactivated at 55°C.

Effect of Metal Ions and Various Compounds

Such heavy metal ions as Hg^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Ag^{+}, and 4-chloromercuribenzoate abolished almost completely the enzyme activity at 1 mM. Iodoacetic acid at 1 mM caused 52% inhibition. Inhibition with Hg^{2+} was not restored by addition of an excess of dithiothreitol or 2-ME. β-Galase lost completely the activity in the presence of 0.01% SDS. Enhancement of the activity (133%) was observed with 0.01% of Triton X-100. L-Ara, D-Ara, L-Fuc, D-Fuc, D-GalN, D-Gal, and D-GalUA inhibited 18, 15, 30, 20, 49, 60, and 65% of the enzyme activity at 10 mM, respectively. D-Galactal and D-galactono-(1→4)-lactone acted as potent competitive inhibitor with their respective K_i values of 150 and 17 μM.

Specificity

Aryl β-D-galatosides served as good substrate for β-Galase but their rates of hydrolysis were influenced by the nature of aglycones considerably. The relative rates of hydrolysis decreased in the following order: PNP-β-D-Gal (100), ONP-β-D-Gal (89), and 4-MU-β-D-Gal (48). PNP-β-D-Fuc was hydrolyzed at about one-seventh lesser rate than that for PNP-β-D-Gal. PNP-α-L-arabinopyranoside remained unhydrolyzed. Table III summarizes the relative rates for hydrolysis of galactooligosaccharides differing in the types of linkage and chain

Table I. *Purification of Radish Seed β-Galase*

Step	Total Volume	Total Protein	Total Activity	Specific Activity	Yield	Purification
	mL	*mg*	*units*	*units/mg*	*%*	*-fold*
Crude extract	1,510	66,700	—[a]	—	—	—
Ammonium sulfate fractionation	730	19,800	389	0.020	100	1
CM-cellulose (CM-32)	587	3,290	254	0.077	65.3	3.9
Hydroxylapatite	235	371	140	0.377	36.0	18.9
Sephadex G-150	88	65.1	101	1.55	26.0	77.5
Electrofocusing	29.5	9.4	52.8	5.62	13.6	281

[a] Not determined.

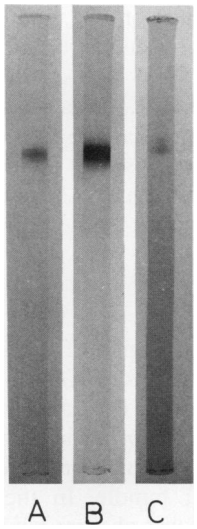

Figure 2. Electrophoretic patterns of radish seed β-Galase on PAGE. A, Staining for protein; B, staining for the enzyme activity; C, periodate-Schiff staining.

Table II. *Amino Acid Composition of Radish Seed β-Galase*

Amino Acid	mol%	Amino Acid	mol%
Asx	9.9	Met	2.7
Thr	6.2	Ile	4.8
Ser	8.4	Leu	8.1
Glx	8.8	Tyr	4.7
Pro	5.9	Phe	4.2
Gly	10.1	Lys	6.2
Ala	7.0	His	1.3
Half-Cys	0.9	Trp	2.4
Val	5.0	Arg	3.4

lengths, based on the rate for PNP-β-D-Gal as unity. β-1,3- and β-1,6-Linked oligomers were hydrolyzed at comparable rates to that for PNP-β-D-Gal, and their rates increased with increasing degrees of polymerization. In contrast, β-1,4-linked oligomers were virtually unsusceptible to β-Galase regardless to their degrees of polymerization although lactose was hydrolyzed appreciably. Reduction with $NaBH_4$ of the reducing group resulted in a marked decrease in the hydrolytic rates of β-1,3- and β-1,6-linked galactobioses and -trioses.

Kinetics

The following K_m (mM) and V_{max} (μmol/min/mg) values were determined for aryl glycosides and galactooligosaccharides by Michaelis-Menten plot: PNP-β-D-Gal, K_M = 0.46, V_{max} = 5.36; ONP-β-D-Gal, K_m = 1.19, V_{max} = 4.69; 4-MU-β-D-Gal, K_m = 1.27, V_{max} = 4.86; PNP-β-D-Fuc, K_m = 10.2, V_{max} = 8.71; β-1,3-glactobiose, K_m = 7.79, V_{max} = 10.26; β-1,3-galactotriose, K_m = 4.87, V_{max} = 8.51 to 12.77; lactose, K_m = 8.55, V_{max} = 5.65.

Mode of Action

Degradation of methyl β-D-glycoside of β-1,6-linked galactopentaose was followed by HPLC analysis for the product at suitable time intervals. Reducing power was detected only in the galactose fraction during the hydrolysis of the substrate. Thus, the consecutive release of D-Gal from the nonreducing ends indicates that β-Galase operates in an exowise fashion.

Anomeric configuration of D-Gal liberated by the enzymic hydrolysis from acacia β-1,3-galactan was examined by GLC as trimethylsilylated derivatives. A higher ratio of β- (80) to α-anomer (20) was obtained in rapidly frozen sample of the hydrolyzate than that (65:35) of the mutarotated sample, providing evidence for the retention of β-anomeric configuration of the released D-Gal.

Table III. *Substrate Specificity of Radish Seed β-Galase*

Substrate	Relative Rate[a]
	%
PNP-β-D-Gal	100
Methyl-β-D-Gal	13
β-1,3-Linked oligosaccharides	
Galactobiose	38[b]
Galactotriose	40 ~ 61[c]
Methyl β-D-galactotetraoside	59
Methyl β-D-galactopentaoside	65
Galactobiitol	1
Galactotriitol	11
β-1,6-Linked oligosaccharides	
Galactobiose	66
Galactotriose	83
Methyl β-D-galactopentaoside	62
Methyl β-D-galactohexaoside	90
Galactobiitol	2
Galactotriitol	17
β-1,4-Linked oligosaccharides	
Galactobiose	1[b]
Galactotriose	0
Galactotetraose	0.1
Galactopentaose	1
Lactose	13

[a] The initial rates of hydrolysis were determined by incubating the substrates (5 mM) with the enzyme (7 or 20 mU) as described in "Materials and Methods." [b] The rate of hydrolysis was calculated by multiplying the value for the released D-Gal by ½. [c] The rate of hydrolysis was assumed to be within a range of the values obtained by multiplying the observed value by 2/3 and 1.

Degradation of Galactans and AGPs

The relative initial rates and limits of hydrolysis were examined for galactans, AG, AGPs and their enzymically modified products. As summarized in Table IV, the modified leaf AGP was degraded rapidly at one-half rate for PNP-β-D-Gal while β-1,3-galactan and the modified seed AGP were attached more slowly. After incubation for 30 h, β-Galase released about 30% of D-Gal from acacia β-1,3-galactan whereas soybean arabinan-galactan and a β-1,4-galactan thereof was essentially resistant. Native leaf and seed AGPs were also unsusceptible to β-Galase even after prolonged incubation. Hence, the pretreatment with α-L-Arafase enabled these AGPs highly susceptible to β-Galase, thus releasing 29 and 28% of D-Gal.

Gel filtration of the digests derived from modified leaf and seed AGPs on a Bio-Gel P-2 column revealed the formation of small amounts of oligomers in addition to D-Gal and the

Table IV. *Action of Radish Seed β-Galase on Galactans, AG, and AGPs*

Substrate	Relative[a] Rate	Limit of Hydrolysis[b]
	%	
PNP-β-D-Gal	100	
β-1,3-Galactan	17	30
Radish leaf AGP		
Native	0.3	5
α-L-Arafase-treated	49	29
Radish seed AGP		
Native	0.2	4
α-L-Arafase-treated	17	28
Radish seed AG		
Native	0.1	0.3
α-L-Arafase-treated	—[c]	14
Soy bean arabinan-galactan	0.1	1
β-1,4-Galactan	0.1	1

[a] The initial rates were determined by incubation of the substrates (PNP-β-D-Gal, 1 mM; polymers, 5 mg/mL) with the enzyme (16 mU) in a reaction mixture (100 μL) containing 100 mM acetate buffer (pH 4.0), at 37°C, and the reducing sugars liberated were estimated reductometrically at suitable time intervals. Relative rates were calculated based on that for PNP-β-D-Gal as unity. [b] The reaction was carried out under the condition as mentioned above except for the concentration of the substrates (1 mg/mL) for 24 h followed by further incubation with an equal amount of the enzyme for more 6 h. Limit of hydrolysis was determined after the liberation of reducing sugars reached a plateau, and expressed as D-Gal equivalent against the total sugar in the reaction mixture. [c] Not determined.

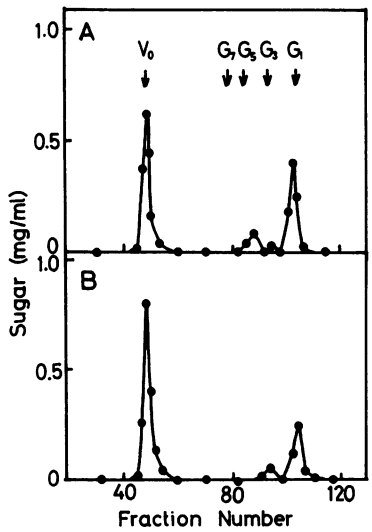

Figure 3. Elution profile of the degradation products from α-L-Arafase-treated radish AGPs from a Bio-Gel P-2 column. A, Leaf AGP; B, seed AGP. The digests were chromatographed on a 1 × 84 cm Bio-Gel P-2 column equilibrated with 1% (v/v) acetic acid. Fractions (0.45 mL) were collected at a flow rate of 6 mL/h and monitored for sugar by phenol-H_2SO_4 method. V_0, the elution peak of blue dextran. G_1 to G_7, maltooligosaccharides with DP of 1, 3, 5, and 7.

partially degraded AGPs, respectively (Fig. 3). Analysis for the sugar constituents demonstrated the leaf oligomer to be composed of L-Ara, L-Fuc, and D-Gal in a molar ratio of 2:2:96, and uronic acid. The seed oligomer contained L-Ara and D-Gal in a molar ratio of 3:97, and uronic acid. Table V summarizes the data for methylation analysis of the residual polymers from leaf and seed AGPs. Alterations in the molar proportions of β-1,3- and β-1,6-D-galactosidic linkages implies a preferential attack of β-Galase on the nonreducing terminal β-1,-6-linked D-galactosyl residues in the unsubstituted side chains of the AGPs, from which a large part of L-arabinofuranosyl residues was removed by the action of α-L-Arafase.

Localization of β-Galase

Figure 4 shows micrographs of transverse sections of a radicle, which were stained for β-Galase activity with X-Gal as the substrate. Epidermal cells and intercellular space of cortical cells were clearly stained in a radicle section. Especially, a potent β-Galase activity was detected in endodermis surrounding a vascular bundle. In the cotyledon section, localization of β-Galase was obscure but distinct staining was visualized in the intercellular space of both palisade and spongy parenchymal tissues (data not shown). The enzyme activity was not observed in the control sections of a radicle and cotyledon, which were stained in the presence of 10 mM D-galactono-(1→4)-lactone.

DISCUSSION

Radish seed β-Galase was eluted apparently as a single component as judged by the elution profies in chromatographic steps of purification (Fig. 1, A, B, and C). However, the enzyme activity was detected in the fractions eluted over a wide range of pI values (pH 4–9) when the enzyme preparation of the fifth step was focused (Fig. 1D). In this work, we have investigated major basic β-Galase which was shown to be apparently homogeneous on PAGE after electrofocusing. Fractions focused in the pI range of 5 to 7 appear to contain minor β-Galase isozyme(s) which are distinct from the major one in their ability to hydrolyze β-1,4-galactobiose slowly besides PNP-β-D-Gal, β-1,3-galactobiose, and lactose. However, there still remains a possibility of the presence of other β-Galase isozyme(s) in the final preparation because of a broad elution profile of the activity on the electrofocusing.

The basic protein nature of the radish seed β-Galase is unique among plant β-Galases whose pI values were mostly in an acidic or neutral range (9, 16, 25).

Two different molecular masses of the β-Galase were obtained as determined by SDS/PAGE (45 kD) and gel filtration (60 kD). These values are comparable with those of β-Galases from such plant tissues as jack bean (75 kD) (19), nasturtium seeds (97 kD) (9), *Vigna sinensis* seeds (43.6, 66.8, 54.3, 79.4 kD) (5), and lupin cotyledons (74, 65, 54 kD) (20).

The kinetic parameters of radish seed β-Galase for the hydrolysis of aryl-β-D-Gal are in a similar order of magnitude to those of other plant enzymes (5, 9, 16, 19, 20, 25). Replacement of hydroxymethyl group at C-6 with methyl group in PNP-β-D-Fuc led to an increase in the K_m value and a marked decrease in the rate of hydrolysis. Further, the resistance of PNP-α-L-arabinopyranoside to radish seed β-Galase indicates a structural requirement of carbon atom at C-6 for serving as the substrate. A similar decrease in the hydrolytic rate of β-D-fucopyranoside has been reported for β-Galases from *Esche-*

Table V. *Alterations in Glycosidic Linkages of AGPs after Digestion with Radish Seed β-Galase*

Mode of Linkage	Radish Leaf AGP		Radish Seed AGP	
	α-L-Arafase[a]	α-L-Arafase[b] + β-Galase	α-L-Arafase[a]	α-L-Arafase[b] + β-Galase
		mol %		
Araf-(1→	2	2	1	1
Fucp-(1→	5	8	–[c]	–
Xylp-(1→	—	—	1	2
Glcp-(1→	—	—	1	1
Galp-(1→	13	14	26	24
→2)-Araf-(1→	6	4	1	1
→5)-Araf-(1→	—	—	16	13
→3,5)-Araf-(1→	—	—	+[d]	+
→2 or →4)-Xylp-(1→	—	—	+	+
→3)-Galp-(1→	12	24	14	18
→6)-Galp-(1→	41	29	17	15
→4)-Galp-(1→	—	—	2	2
→4)-Glcp-(1→	—	—	1	1
→3,6)-Galp-(1→	21	19	20	22

[a] α-L-Arafase-treated AGP. [b] After digestion with β-Galase of α-L-Arafase-treated AGP. [c] Not detectable. [d] Trace amount (<0.5%).

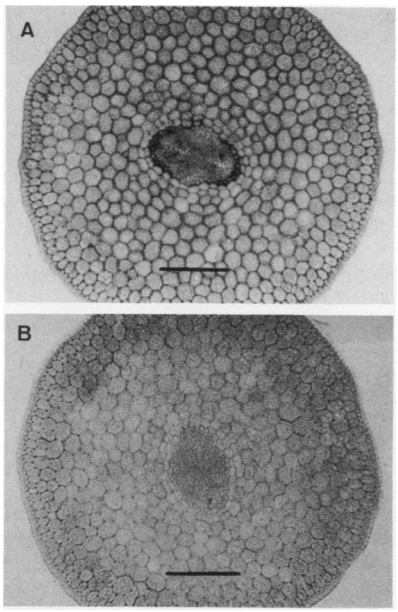

Figure 4. Localization of β-Galase in a radish radicle. A, transverse sections of a radicle. The enzyme activity was stained by indigogenic method. B, Transverse section of a radicle was stained in the presence of 10 mM D-galactono-(1→4)-lactone. Bar = 0.2 mm.

richia coli (33), *Diplococcus pneumoniae* (15), and *Hericella ericetorum* (8).

A characteristic feature of radish seed β-Galase in the substrate specificity was noted in its high preference to β-1,3- and β-1,6-linked D-galactosyl residues. For instance, their rates of hydrolysis for the β-1,3- and β-1,6-linked oligomers increased with increasing chain lengths suggesting the ability of β-Galase to act as an exo-β-galactanase capable of splitting off β-D-galactosyl residues from the nonreducing ends of β-3,6-galactan as the backbone structure of the AGPs. Indeed, it degraded methyl β-D-glycoside of β-1,6-linked galactopentaose in an exowise manner. β-1,4-Linked oligomers and β-1,4-galactan were virtuely unsusceptible to the enzyme while it attacked lactose that was an unnatural sugar in plant kingdom. Recently, Nakano *et al.* (23) have demonstrated the unsusceptibility of β-1,4-linked galactobiose and -triose to *E. coli* β-Galase which hydrolyzed lactose almost completely under the same reaction condition. Thus, it is not exceptional that the configuration at C-4 of the penultimate sugar residues in galactosides exerts a great influence upon the primary specificity of radish seed β-Galase.

Degradation of leaf and seed AGPs occurred only after the removal of α-L-arabinofuranosyl residues attached to β-D-galactosyl residues in the side chains by the action of α-L-Arafase. Methylation analysis of the partially degraded leaf and seed AGPs revealed a preferential cleavage of β-1,6-linked D-galactosyl residues in the unsubstituted side chains, which become susceptible to β-Galase after treatment with α-L-Arafase. D-Gal was identified as a major product by the exowise attack of β-Galase. The formation of a small quantity of oligosaccharides comprising D-Gal, uronic acid, and a small quantity of L-Ara suggested the possibility that β-Galase is able to hydrolyze β-1,6- and/or β-1,3-linked D-galactosidic linkages substituted at C-3 or C-6 with auxiliary sugar residues in backbone structure of the α-L-Arafase-treated AGPs.

Pressey (25) has reported the correlation of tomato softening with the increasing activity of β-Galase II in the ripening fruits, which catalyzes the exowise degradation of β-1,4-linked galactan isolated from the cell walls of immature tomatoes. A β-Galase purified from carrot cultured cells has been demonstrated to act on a galactan-pectin polymer, thereby participating in remodeling or loosening the structure of the cell walls (16). Data suggesting synergistic action of lupin cotyledonary α-L-Arafase and β-Galase have been presented for the degradation of lupin seed cell wall arabino-4-galactan and galactogalacturonan (20). Furthermore, degradation of xyloglucan in cotyledons of nasturtium seeds has been proposed to occur by sequential actions of a specific endo-β-1,4-glucanase, an α-xylosidase, and a β-Galase which is highly specific

for β-D-galactopyranosyl-(1→2)-α-D-xylopyranosyl-(1→ linkage in the polysaccharide but inactive on lupin seed β-1,4-linked galactan (9–11). In view of the high preference of β-1,3- and β-1,6-D-galactosidic linkages and an exo-galactanase activity of radish seed β-Galase, it is probable that the enzyme plays a role in degradative process of AGPs together with the concerted actions of α-L-Arafase(s) and β-glucuronidase(s).

Localization of β-Galases in plant tissues appears to be variable: its activity was located in chloroplasts (4), vacuoles (27), protein bodies (7), cell walls (7, 24), and intercellular fluid in which several isozymes of β-Galase and α-L-Arafase were detected (14). Indigogenic staining in the sections of radish radicle and cotyledon localized a large part of β-Galase in the intercellular space where positive reaction was observed (data not shown) with β-D-glucosyl Yariv antigen, a specific reagent to bind to AGPs. Physiological significance for the coexistence of β-Galase and its possible substrate remained to be explored.

ACKNOWLEDGMENTS

We wish to thank Dr. H. Nakano, Osaka Municipal Research Instuitute, and Dr. P. Kovac, National Institutes of Health for gifts of galactooligosaccharides.

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