Plant Physiol. (1992) 100, 388–396
0032-0889/92/100/0388/09/$01.00/0

Received for publication December 31, 1991
Accepted April 1, 1992

α-L-Arabinofuranosidase from Radish (*Raphanus sativus* L.) Seeds

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ABSTRACT

An α-L-arabinofuranosidase has been purified 1043-fold from radish (*Raphanus sativus* L.) seeds. The purified enzyme was a homogeneous glycoprotein consisting of a single polypeptide with an apparent molecular weight of 64,000 and an isoelectric point value of 4.7, as evidenced by denaturing gel electrophoresis and reversed-phase or size-exclusion high-performance liquid chromatography and isoelectric focusing. The enzyme characteristically catalyzes the hydrolysis of *p*-nitrophenyl α-L-arabinofuranoside and *p*-nitrophenyl β-D-xylopyranoside in a constant ratio (3:1) of the initial velocities at pH 4.5, whereas the corresponding α-L-arabinopyranoside and β-D-xylofuranoside are unsusceptible. The following evidence was provided to support that a single enzyme with one catalytic site was responsible for the specificity: (a) high purity of the enzyme preparation, (b) an invariable ratio of the activities toward the two substrates throughout the purification steps, (c) a parallelism of the activities in activation with bovine serum albumin and in heat inactivation of the enzyme as well as in the inhibition with heavy metal ions and sugars such as Hg^{2+}, Ag^{+}, L-arabino-(1→4)-lactone, and D-xylose, and (d) results of the mixed substrate kinetic analysis using the two substrates. The enzyme was shown to split off α-L-arabinofuranosyl residues in sugar beet arabinan, soybean arabinan-4-galactan, and radish seed and leaf arabinogalactan proteins. Arabinose and xylose were released by the action of the enzyme on oat-spelt xylan. Synergistic action of α-L-arabinofuranosidase and β-D-galactosidase on radish seed arabinogalactan protein resulted in the extensive degradation of the carbohydrate moiety.

AGPs[1] are widely distributed in plant tissues (6, 8) and their organ-specific forms differing in sugar compositions and electrophoretic properties have received special interest relevant to development and differentiation of plant tissues (20). Working with radish plant, we have demonstrated considerable changes in the compositions, structures, and serological characteristics of the carbohydrate moieties of seed, leaf, and developing root AGPs (27–29). These findings led us to assume that the AGP and AG in germinating seeds might be metabolized and reutilized to support the synthesis of the organ-specific AGPs during the development of roots and other organs in the seedlings. Furthermore, after purifying a basic β-Galase from radish seeds, we have characterized the enzyme to be highly specific for (1→3)- and (1→6)-linked β-D-galactosyl residues, thereby participating in the degradation of the β-3,6-D-galactan backbone of AGPs as an exocleaving enzyme after digestion with a fungal α-L-Arafase (22).

In this paper, we report purification and characterization of an α-L-Arafase (α-L-arabinofuranoside arabinofuranohydrolase, EC 3.2.1.55) from radish (*Raphanus sativus* L.) seeds, which catalyzes the hydrolysis of aryl β-D-xylopyranosides, β-(1→4)-linked xylooligosaccharides, and β-D-xylopyranosyl residues in xylans. Several lines of evidence are presented to verify that a single enzyme is responsible for the specificity. Furthermore, synergistic action of the α-L-Arafase and β-Galase on a radish seed AGP provided suggestive data for their possible physiological roles in the enzymic degradation of the proteoglycans.

MATERIALS AND METHODS

Materials

Seeds of radish (*Raphanus sativus* L. var *hortensis* cv Aokubi) were purchased from Tokita Seeds and Plant Co., Ltd., Saitama, Japan. Porcine eyes were purchased from a local slaughter house.

Chemicals

PNP α-L-Ara*f* and PNP α-L-Ara*p* were synthesized (22). Phenyl β-D-Xyl*f* were synthesized by the method of Börjeson et al. (3). Other chemicals were obtained as follows: PNP β-glycosides of D-Xyl, D-Glc, D-Gal, D-Man, D-GlcNAc, D-GlcUA, PNP α-D-mannoside, PNP α-D-galactoside, phenyl β-D-Xyl*p*, 4-MU α-L-Ara*f*, 4-MU β-D-Xyl*p*, larch wood xylan, and 4-*O*-methyl-glucuronoxylan (Sigma Chemical Co.); L-arabino-(1→4)-lactone (Koch-Light Laboratories, Colnbrook, England); D-galactono-(1→4)-lactone, D-glucono-(1→5)-lactone, and other monosaccharides (Wako Pure Chemical Industries, Osaka, Japan); Sephadex G-15 and G-150, Sephacryl S-200, CM- and DEAE-Sepharose CL-6B, Con A-Sepharose 4B, and Octyl-Sepharose CL-4B (Pharmacia LKB Biotechnology, Tokyo, Japan); hydroxylapatite (GIGAPITE, Toa Gousei Kagaku, Tokyo, Japan); Bio-Gel P-2 (Bio-Rad Laboratories); CM-cellulose (CM-32, Whatman); and a carrier

[1] Abbreviations: AGP, arabinogalactan protein; AG, arabinogalactan; α-L-Arafase, α-L-arabinofuranosidase; β-D-Xylpase, β-D-xylopyranosidase; β-Galase, β-D-galactosidase; PNP α-L-Ara*f*, *p*-nitrophenyl α-L-arabinofuranoside; PNP α-L-Ara*p*, *p*-nitrophenyl α-L-arabinopyranoside; PNP β-D-Xyl*p*, *p*-nitrophenyl β-D-xylopyranoside; 4-MU, 4-methylumbelliferyl; ONP, *o*-nitrophenyl; 2-ME, 2-mercaptoethanol; p-CMB, *p*-chloromercuribenzoate; RP-HPLC, reversed-phase HPLC; PC, paper chromatography; Rha, rhamnose.

ampholyte, Servalyte pH 3-6 (Serva Fine Biochemica, Heidelberg, FRG).

A radish mature leaf AGP (designated R-II) and radish seed AG and AGP were prepared as reported previously (27, 28). Soybean arabinan-4-galactan was isolated (22). Sugar beet arabinan was prepared by the method of Tagawa and Kaji (23). The purified arabinan was determined to be composed of Ara, Gal, and Rha in a molar proportion of 94:5.5:0.5. Chemical shifts (108.0 and 108.5 ppm) on ^{13}C-NMR confirmed the α-anomeric configuration of the L-arabinofuranosyl residues (13). Oat-spelt xylan (Fluka Chemie AG, Buchs, Switzerland) contained Ara and Xyl in a molar proportion of 28 and 69%, together with small amounts of Rha, Man, Gal, and Glc. Wheat arabino-(1→4)-β-D-xylan was obtained from Biosupplies Australia, Parkville, Victoria, Australia.

β-(1→4)-Linked xylooligosaccharides in the partial hydrolyzate of larch wood xylan (5 g) were fractionated on a charcoal column (24) and monitored for phenol-sulfuric acid reaction. Chromatography of sugars on a 3.2- × 80-cm Bio-Gel P-2 column using water as solvent afforded xylobiose (202 mg), -triose (162 mg), -tetraose (76 mg), and -pentaose (16 mg), each of which was identified upon analysis for sugar compositions and ^{13}C-NMR spectrometry (9).

D-Xylose dehydrogenase was prepared from porcine lenses by the method of Wissler and Logemann (30). A purified β-Galase of radish seeds was prepared as described previously (22). *Pseudomonas* D-galactose dehydrogenase was obtained from Boehringer-Mannheim.

Analytical Methods

Colorimetric analyses of total and reducing sugar and PC of sugars were done as described previously (28). Proteins were determined by the method of Bradford (4), using BSA as a standard. Analysis of alditol acetates was carried out 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 (28). ^{13}C-NMR spectra (100.6 MHz) were recorded using a Brucker AM400 spectrometer in D_2O at 70°C (for arabinan) for 25°C (for xylooligosaccharides) with 1,4-dioxane (67.40 ppm downfield from tetramethylsilane) as the internal standard.

RP-HPLC analysis of protein was done on a 2.1- × 30-mm column of Aquapore PH-300 or RP-300 (Applied Biosystems Inc.) in a Hewlett-Packard model HP1090 HPLC. Aliquots (15 μg) of the samples were chromatographed by a linear acetonitrile gradient (0–80%, v/v) in 0.99% (v/v) TFA and monitored at 206, 275, and 290 nm.

Electrofocusing of the enzyme was performed in a LKB 8101 column (110 mL) using a carrier ampholyte of a pH range of 3.0 to 6.0 at a constant voltage of 300 V for 96 h and at 4°C. Fractions (1 mL) were collected and monitored for the enzyme activities and pH.

Determination of Mol Wt

The apparent mol wt of the purified enzyme (10 μg) was determined on SDS-PAGE according to the method of Laemmli (16) using *Escherichia coli* β-galactosidase (116,000), phosphorylase *b* (97,400), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and myoglobin (17,000) as the mol wt standards. Protein in the gel was stained with Coomassie brilliant blue R-250. The mol wt of the denatured enzyme (50 μg) was also determined using an HPLC system equipped with tandem columns (7.8 × 300 mm, each) of TSKgel G3000SW$_{XL}$ and G4000SW$_{XL}$ (Tosoh) preequilibrated with 10 mM phosphate buffer, pH 6.8, containing 6 M guanidine-HCl. The effluents were monitored at 215, 275, and 290 nm, and the column system was calibrated using the mol wt standard protein mixture as above. The apparent mol wt of the native enzyme was estimated on a 1.0- × 84-cm Sephadex G-150 column which had been equilibrated with 20 mM phosphate buffer, pH 6.8, containing 300 mM NaCl and calibrated by using human transferrin (80,000), BSA, ovalbumin, and bovine α-lactalbumin (14,400).

Enzyme Assays

Assays for the activity toward PNP or ONP glycosides were performed in a reaction mixture (total volume, 200 μL) composed of the enzyme, 1 mM substrate, and 50 mM acetate buffer, pH 4.5. Unless otherwise stated, BSA (20 μg) was added to the mixture. After incubation for 15 min at 37°C, the reaction was terminated by the addition of 200 mM Na_2CO_3 (800 μL), and the amount of *p*- or *o*-nitrophenol or ONP liberated was determined at 420 nm. One unit of the enzyme activity is defined as the amount of the enzyme capable of liberating 1 μmol of the aglycons per min. PNP α-L-Ara*f* and PNP β-D-Xyl*p* were used as the standard substrate for routine enzyme assay. With phenyl glycosides, the amount of phenol liberated under the same assay condition as above was determined by the method of Asp (2). The amount of 4-methylumbelliferone liberated from 4-MU glycosides was determined fluorimetrically (22). The activity for xylooligosaccharides was assayed in a reaction mixture (100 μL) composed of the enzyme, 1 mM substrate, 100 mM acetate buffer, pH 4.5, and BSA (20 μg) at 37°C. The quantity of free D-Xyl in the enzyme digest was estimated by the D-xylose dehydrogenase method (30). Porcine D-xylose dehydrogenase was found to oxidize β-(1→4)-linked xylobiose and -triose in their relative ratios of 25 and 17 for xylobiose and xylotriose, respectively, taking that for D-Xyl as unity (100%). Thus, correction was made for the substrate blank of each sugar.

Degradation of Polysaccharides

Aliquots (50 μg) of AG, AGP, and other L-Ara*f*-containing polysaccharides were digested with the purified enzyme (30 milliunits) in 100 mM acetate buffer, pH 4.5 (100 μL), at 37°C under toluene for 24 h, followed by the addition of the additional enzyme (30 milliunits) to the reaction mixture and incubation for an additional 24 h. Reducing sugar released was determined at time intervals. Sugar in the digest was identified by PC after desalting with mixed bed resins of Dowex 50W (H^+) and Dowex 1 (HCO_3^-). Sugars (L-Ara and D-Xyl) released from oat-spelt xylan were estimated reductometrically and by the D-galactose dehydrogenase method (19).

To examine the synergistic action of radish seed α-L-Ara-

fase and β-Galase on radish seed AGP, the substrate (40 μg) was incubated with α-L-Arafase (25 milliunits) and β-Galase (50 milliunits) in 100 mM acetate buffer, pH 4.5 (100 μL) at 37°C. After 24 h, a mixture of the two enzymes with the same units as above was added to the reaction mixture and incubated for an additional 48 h. For the controls, α-L-Arafase (total units, 50 milliunits) or β-Galase (total units, 100 milliunits) acted separately on the substrate under the same conditions. The amounts of reducing sugar liberated were determined at time intervals reductometrically using L-Ara or a mixture of an equal amount of L-Ara and D-Gal as the standard.

Methylation Analysis

Sugar beet arabinan (4.8 mg) was digested to the hydrolysis limit (28.1%) with purified α-L-Arafase (total of 240 milliunits) in 100 mM acetate buffer, pH 4.5 (500 μL) for 48 h under condition as above. The digested arabinan (yield, 3.4 mg) was lyophilized after passage through a 1- × 87-cm Sephadex G-15 column preequilibrated with water. The native arabinan and the digested product were methylated (28), and the partially methylated sugars resulting from hydrolysis were analyzed by GLC.

Enzyme Purification

All procedures were performed at 4°C or below, unless otherwise stated. Fractions collected in each step of the purification were monitored for the activities toward PNP α-L-Ara*f* and PNP β-D-Xyl*p*.

Dry radish seeds (800 g) were ground in a blender for 5 to 10 s, and the seed coats that were peeled off were removed in a draft of air. Cotyledons and embryonic tissues were homogenized with three times their weight of 20 mM phosphate buffer, pH 7.2, containing 130 mM NaCl, 2 mM EDTA, and 20 mM 2-ME (buffer A) in a blender at the highest speeds for 10 min, and the homogenate was centrifuged at 12,000*g* for 30 min. The sediment was suspended in buffer A and treated as above.

Chilled petroleum ether (800 mL) at −20°C was added in drops to the combined oily supernatants (800 mL) while stirring, and the yellowish upper layer was discarded after standing for 1 h. To the aqueous layer, acetone chilled at −20°C was added to 80% (v/v) and left standing for 1 h. The precipitate formed was collected by centrifugation at 12,000*g* for 30 min, dissolved in buffer A (500 mL), and centrifuged again. The resulting clear supernatant was brought to 30% saturation with $(NH_4)_2SO_4$, left for 30 min, and centrifuged at 12,000*g* for 30 min. Pulverized $(NH_4)_2SO_4$ was added to the supernatant at 60% saturation, left for 18 h, and centrifuged as above. The precipitate formed was dissolved in 10 mM Tris-HCl buffer, pH 7.2 (200 mL, buffer B), and dialyzed against buffer B.

After centrifugation, the dialyzate was applied to a 4- × 16-cm CM-32 column preequilibrated with buffer B. After elution with buffer B, fractions were collected and monitored for the enzyme activities. A large portion of the enzyme that emerged in flow-through fractions was collected and subjected to chromatography on a 2.6- × 33-cm DEAE-Sepharose CL-6B column preequilibrated with buffer B. After the column was washed with buffer B, the enzyme was eluted with a linear KCl gradient (0–500 mM) in buffer B (total volume, 900 mL). The active fractions were combined and dialyzed against 10 mM acetate buffer, pH 5.0 (buffer C), for 18 h. The dialyzate was applied onto a 2- × 24-cm CM-Sepharose CL-6B column preequilibrated with buffer C. The enzyme emerged from the column with a linear KCl gradient (0–700 mM) in buffer C (total volume, 400 mL).

The active fractions were combined, concentrated to 7 mL using a PM-10 membrane in an Amicon ultrafiltration apparatus, and chromatographed through a 2.2- × 143-cm Sephacryl S-200 column preequilibrated with 20 mM phosphate buffer, pH 6.8, containing 300 mM KCl. The active fractions were combined, equilibrated with 1 mM phosphate buffer, pH 6.8, by ultrafiltration using a PM-10 membrane, and passed through a 1.2- × 7.3-cm hydroxylapatite (GIGAPITE) column preequilibrated with 1 mM phosphate buffer, pH 6.8. The column was washed with 1 mM phosphate buffer, pH 6.8, until A_{280} in the effluents decreased below 0.01. The enzyme was desorbed from the column with 10 mM phosphate buffer, pH 6.8 (buffer D). The fractions containing the enzyme were combined, made up to 14% (w/v) $(NH_4)_2SO_4$, and applied onto a 1.8- × 8-cm Octyl-Sepharose CL-4B column that had been equilibrated with 14% (w/v) $(NH_4)_2SO_4$ in buffer D. After the column was washed with 14% (w/v) $(NH_4)_2SO_4$ in buffer D, elution of the enzyme was conducted by linearly decreasing $(NH_4)_2SO_4$ concentrations in buffer D (100 mL). The active fractions were collected, dialyzed against buffer D containing 0.05% (w/v) NaN_3, concentrated to 10 mL using PM-10 membrane, and stored at 4°C.

RESULTS

Enzyme Purification

Treatment of radish seed extract with petroleum ether followed by acetone precipitation was effectively applied to eliminate large parts of the oil and yellowish pigment(s) in the seed homogenate and thus facilitated subsequent $(NH_4)_2SO_4$ fractionation and column chromatographic operations. Chromatography of the $(NH_4)_2SO_4$ fraction on CM-32 at pH 7.2 allowed further removal of the remaining yellowish pigment(s) that disturbed the enzyme assay. Upon chromatography on CM-32, a large portion of α-L-Arafase could be recovered in flow-through fractions that were practically free from β-glucuronidase and a basic β-Galase. Elution profiles of the enzyme from CM-Sepharose, Sephacryl S-200, and Octyl-Sepharose columns are depicted in Figure 1. The enzyme emerged as a single and sharp peak of the enzyme activities from the Sephacryl S-200 column (Fig. 1B). Chromatography on hydroxylapatite was effective in removing a trace of β-Galase. The purified enzyme obtained after chromatography on an Octyl-Sepharose column, as shown in Figure 1C, was confirmed to be completely free from other glycosidases such as α- and β-Galases, β-glucosidase, β-glucuronidase, β-*N*-acetylhexosaminidase, and α- and β-mannosidases in the crude extract (22). The amounts of protein and α-L-Arafase activity and the ratio of the activity

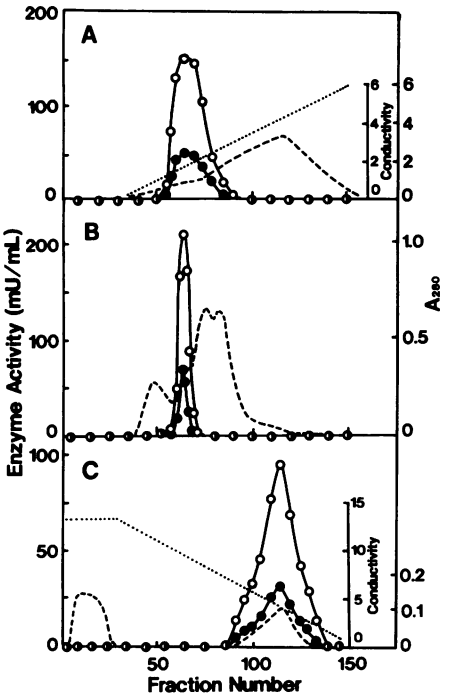

Figure 1. Elution profiles of radish seed α-L-Arafase. A, CM-Sepharose; B, Sephacryl S-200; C, Octyl-Sepharose. O, Activity toward PNP α-L-Araf; ●, activity toward PNP β-D-Xylp; – – – –, A_{280}; · · · · ·, conductivity (mmho). Fraction volumes were 3, 3.5, and 1 mL/tube for A to C, respectively.

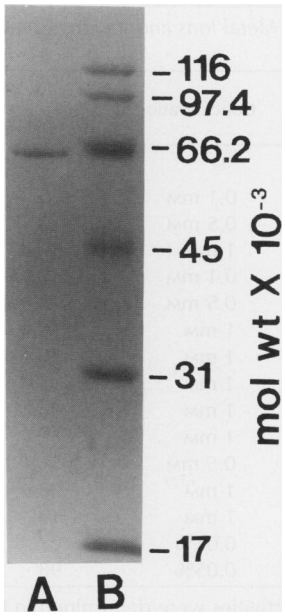

Figure 2. Electrophoretic patterns of radish seed α-L-Arafase on SDS-PAGE. A, Purified α-L-Arafase (10 μg); B, mol wt markers.

for PNP α-L-Araf to that for PNP β-D-Xylp in each step of the purification are summarized in Table I. The enzyme activities decreased markedly in the last two steps and could be restored by the addition of BSA, resulting in an approximately 1.5-fold increase in the activities. Finally, a radish seed α-L-Arafase was purified 1043-fold in a yield of 7.5% based on the specific activity obtained in the second step of purification. A nearly constant ratio (3:1) and exactly overlapping peaks of α-L-Arafase and β-D-Xylpase activities upon chromatographic separation were retained throughout the purification (Fig. 1, Table I).

Purity and Mol Wt

Upon RP-HPLC using Aquapore PH-300 or RP-300 column, the purified enzyme was eluted as a single peak from the columns as measured at 206, 275, and 290 nm, indicative of a high degree of purity of the preparation. The enzyme migrated as a single protein band with an apparent mol wt of 64,000 on SDS-PAGE (Fig. 2). Size-exclusion chromatography on tandem columns of TSKgel G3000SW_{XL} and G4000SW_{XL} under denaturing condition revealed the enzyme to be a single polypeptide chain with an apparent mol wt of 57,000. Similarly, an apparent mol wt of 63,000 for the native enzyme was obtained on a calibrated Sephadex G-150 column.

The enzyme was adsorbed on a Con A-Sepharose column equilibrated with Tris-HCl buffer, pH 7.5, containing 5 mM $MgCl_2$ and 5 mM $CaCl_2$. A complete elution of the enzyme

Table I. *Purification of Radish Seed α-L-Arafase*

Step	Total Protein	Total Activity	Specific Activity	Activity Ratio[a]	Yield	Purification
	mg	*units*	*units/mg*		%	*-fold*
Extract	27,980	–[b]	–	–	–	–
Ammonium sulfate	9,390	58.8	0.0063	3.22	100	1
CM-32	1,310	50.3	0.038	3.12	86	6
DEAE-Sepharose	514	29.5	0.057	3.22	50	9
CM-Sepharose	32	17.3	0.54	3.03	29	86
Sephacryl S-200	7.2	12.3	1.71	3.12	21	271
Hydroxylapatite	1.2	6.0	5.00	3.03	10	794
Octyl-Sepharose	0.67	4.4	6.57	3.03	7.5	1,043

[a] A ratio for the activities toward PNP α-L-Araf and PNP β-D-Xylp. [b] The activity was not determined because of the presence of yellow pigment(s) that disturbed the enzyme assay.

Table II. *Effects of Metal Ions and Various Compounds on the Enzyme Activities*

Metal Ion or Compound	Concentration	Relative Activity[a]	
		α-L-Arafase	β-D-Xylpase
		%	%
Hg^{2+}	0.1 mM	59	52
	0.5 mM	12	11
	1 mM	5.8	9.1
Ag^{+}	0.1 mM	62	60
	0.5 mM	4.4	7.2
	1 mM	0.4	0.3
IAA	1 mM	70	73
DTT	1 mM	95	100
2-ME	1 mM	98	101
Urea	1 mM	89	87
SDS	0.5 mM	54	44
EDTA	1 mM	98	103
p-CMB	1 mM	19	16
Triton X-100	0.01%	127	150
	0.05%	98	103

[a] The enzyme activities were determined in the absence of BSA.

from the column was obtained with 500 mM D-Glc or 200 mM methyl α-D-mannoside, indicating its glycoprotein nature.

Properties

The purified enzyme was characterized regarding its dual activities capable of hydrolyzing PNP α-L-Araf and PNP β-D-Xylp. The isoelectric point of the enzyme was determined to be pH 4.70 on an LKB column. The elution profile revealed that the enzyme was focused as a single and completely overlapped peak of the activities with a ratio of 3:1.

The enzyme activities were assayed by using citrate-phosphate buffer (pH 3.0–3.5), acetate buffer (pH 3.5–5.5), phosphate buffer (pH 5.5–7.0), and Tris-HCl buffer (pH 7.0–9.0) at a final concentration of 50 mM. The pH activity curves in the hydrolysis of the PNP glycosides and sugar beet arabinan indicated that the enzyme was maximally active for each substrate at pH 4.5. Assay for the remaining activities after exposure of the enzyme to buffers of various pHs at 50 mM for 24 h at 4°C proved the enzyme to be stable within a pH range of 5.0 to 8.0.

The remaining activities toward PNP α-L-Araf and PNP β-D-Xylp were measured after incubation of the enzyme at various temperatures (30–70°C) for 10 min at pH 4.5. The enzyme was fully active on both substrates up to 55°C but equally lost about 90% of the activities at 70°C.

The effects of metal ions and various compounds on the enzyme activities are summarized in Table II. Of metal ions, Hg^{2+} and Ag^{+} were found to be the most potent inhibitors. For instance, 88 and 89% of α-L-Arafase and β-D-Xylpase activities, respectively, were inhibited after incubation with 0.5 mM Hg^{2+} for 5 min at 37°C. The addition of 1 and 5 mM DTT to the Hg^{2+}-inactivated enzyme and incubation for 15 min at 37°C restored 58 and 79% for PNP α-L-Araf and 57 and 76% for PNP β-D-Xylp, respectively, based on the original activities. Both activities were also inhibited strongly by p-CMB. These results suggest that the SH-group is essential for the enzyme activities. Other metal ions including Ca^{2+}, Cu^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+}, and Fe^{3+} and compounds such as 2-ME, DTT, L-Cys, and EDTA influenced the activities within a range of ±10% at 1 mM. The activities decreased as the purification proceeded, probably because of the reduction in protein concentrations, and could be equally regained by the addition of BSA or 0.01% Triton X-100. With increasing concentrations (0.1–1000 μg/mL) of BSA added to the reaction mixtures, the activities toward PNP α-Araf and PNP β-D-Xylp gradually increased and plateaued at their maxima (1.65-fold activation) at 100 μg/mL. Thus, the routine assays for the enzyme activities were carried out in the presence of BSA (100 μg/mL), and no significant effect of BSA on the inhibition with metal ions and compounds was observed.

Substrate Specificity and Kinetics

The relative activities and kinetic parameters in the hydrolysis of aryl glycosides of L-Araf and D-Xylp and xylooligosaccharides are summarized in Table III. PNP and 4-MU α-L-Arafs can serve as the best substrates among the substrates tested, providing an unambiguous basis for the assertion that the enzyme is essentially an α-L-Arafase. The enzyme hydrolyzes aryl β-D-Xylps at rates comparable to those for aryl α-L-Arafs, and the relative rates for β-D-Xylps decrease depending upon their aglycons in the order of ONP > PNP > phenyl > 4-MU. PNP α-L-Arap and phenyl β-D-Xylf were totally unsusceptible to the enzyme. β-(1→4)-Linked xylobiose and -triose were also found to be good substrates, as assayed by the D-xylose dehydrogenase method. Liberation of D-Xyl from xylotetraose and -pentaose was also confirmed by PC. Aryl α-L-Arafs as the substrates are clearly distinguishable from the relevant glycosides of β-D-Xylp, based on their higher K_m and V_{max} values. The V_{max} values for PNP α-L-Araf and PNP β-D-Xylp increased 1.75- and 1.78-fold, respectively, in the presence of BSA, whereas the K_m values for both substrates remained unaffected.

Mixed substrate kinetic analysis was performed to determine whether the purified preparation contained (a) two enzyme proteins that copurified, each specific for a discrete glycoside or (b) a single enzyme protein capable of hydrolyz-

Table III. *Substrate Specificity and Kinetic Parameters*

Substrate	Relative Activity	K_m	V_{max}
	%	mM	$\mu mol \cdot min^{-1} \cdot mg^{-1}$
PNP α-L-Araf	100	9.76 (9.72)[a]	73.0 (41.7)
4-MU α-L-Araf	73	13.91	69.3
PNP β-D-Xylp	33	0.95 (0.95)	5.56 (3.13)
ONP β-D-Xylp	64	0.65	6.82
Phenyl β-D-Xylp	23	0.45	2.45
4-MU β-D-Xylp	13	1.07	1.93
Xylobiose	46[b]	ND[c]	ND
Xylotriose	81[b]	ND	ND

[a] The numbers in parentheses indicate the K_m and V_{max} values that were determined in the absence of BSA. [b] Determined by D-xylose dehydrogenase method. [c] Not determined.

Table IV. *Mixed Substrate Kinetic Analysis*

Substrate		Enzyme Activity[a]		
PNP α-L-Araf (S_1)	PNP β-D-Xylp (S_2)	Measured	Calculated[b]	
			1 site	2 sites
mM		μmol·min^{-1}·mg^{-1}		
6.0	1.2	9.45	9.22	15.87
8.0	0.9	12.01	12.12	18.34
10.0	0.6	15.18	15.28	20.22

[a] The values were determined without the addition of BSA. [b] V_{max} (V_1) for PNP α-L-Araf = 38.0 μmol·min^{-1}·mg protein^{-1}; V_{max} (V_2) for PNP β-D-Xylp = 2.44 μmol·min^{-1}·mg protein^{-1}. The initial velocity of a reaction catalyzed by a single enzyme with one site active toward both substrates was calculated from this equation (14):

$$v_0 = \frac{V_1K_2[S_1] + V_2K_1[S_2]}{K_1K_2 + K_1[S_2] + K_2[S_1]},$$

where K_1 and K_2 are the Michaelis constants for substrates S_1 and S_2. For different and independent active sites for each substrate, initial velocity was calculated using the equation as follows:

$$v_0 = \frac{V_1K_2[S_1] + V_2K_1[S_2] + (V_1 + V_2)[S_1][S_2]}{K_1K_2 + K_1[S_2] + K_2[S_1] + [S_1][S_2]}$$

ing two glycosides either at a single flexible active site or at multiple sites, each active toward one of the glycosides. The data in Table IV show that the measured enzyme activity in each case is in good agreement with those calculated for one enzyme with a single active site acting on PNP α-L-Araf and PNP β-Xylp.

Effects of sugars related to the substrates on the activities for both substrates are summarized in Table V. L-Arabino-(1→4)-lactone served as a potent competitive inhibitor, with the lowest K_i value for both activities. D-Xylose, *p*-aminophenyl 1-thio-β-D-Xylp, and D-glucono-(1→5)-lactone were also found to inhibit competitively both activities. Other sugars with no appreciable inhibitory effect at 10 mM included D-Glc, D-Gal, D-Man, L-Fuc, D-Ara, L-Ara, D-galactono-(1→4)-lactone, L-arabinitol, D-xylitol, PNP α-L-Arap, phenyl β-D-Xylf, and *myo*-inositol.

Action on Arabinose-Containing Polysaccharides

In Figure 3 are depicted the curves showing the time course of hydrolysis of L-Araf-containing polysaccharidic substrates by the action of α-L-Arafase. The addition of more enzyme to reaction mixture after 24 h allowed further degradation of each substrate. The extents (percentage of the total L-Araf contents) of L-Ara released after 48 h were as follows: sugar beet arabinan (28.1%), soybean arabinan-4-galactan (41.5%), radish seed AG (63.2%), and radish leaf AGP (61.0%). Release of L-Ara as a sole product was confirmed by PC.

Table V. *Inhibition of the Enzyme Activities by Sugars and Sugar Derivatives*

Sugar	α-L-Arafase Activity[a]			β-D-Xylpase Activity[a]		
	Inhibition at 10 mM	K_i	Type of inhibition	Inhibition at 10 mM	K_i	Type of inhibition
	%	mM		%	mM	
D-Xylose	77.9	0.91	Comp[b]	72.9	2.23	Comp
L-Arabino-(1 → 4)-lactone	93.2	0.18	Comp	93.9	0.56	Comp
D-Glucono-(1 → 5)-lactone	85.8	0.28	Comp	75.1	0.60	Comp
p-Aminophenyl 1-thio-β-D-xylopyranoside	63.0	2.85	Comp	59.0	3.69	Comp

[a] The enzyme activities were determined in the absence of BSA. [b] Comp, Competitive inhibition. The type of inhibition and the K_i values for each inhibitor for the standard substrates were determined by the Lineweaver-Burk plot in the presence of two different concentrations of an inhibitor. For instance, reciprocal plots of the substrate concentrations (PNP α-L-Araf, 1–6 mM; PNP β-D-Xylp, 0.25–1.25 mM) versus the enzyme activities were made in the presence of L-arabino-(1 → 4)-lactone (the control, 0.5 mM, 1 mM for PNP α-L-Araf; the control, 0.1 mM, 0.25 mM for PNP β-D-Xylp).

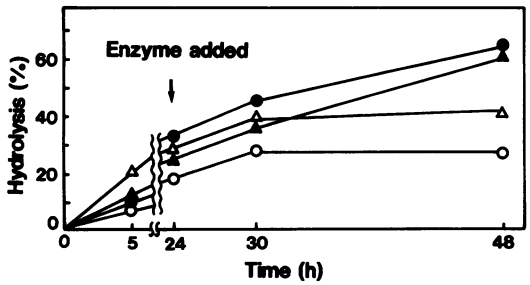

Figure 3. Action of α-L-Arafase on L-Ara-containing polysaccharides. O, Sugar beet arabinan; Δ, soybean arabinan-4-galactan (molar proportion, Ara:Gal:Man:Rha:Glc:Xyl = 54:22:13:7:3:1); ●, radish seed AG (molar proportion, Ara:Gal:Xyl:uronic acids = 81:14:2:3) (27); ▲, radish leaf AGP (molar proportion, Ara:Gal:Fuc:uronic acids = 29:69:6:5) (28). The amounts of L-Ara released were determined reductometrically and the extent (percentage) of the hydrolysis was calculated based on the Ara content of each substrate.

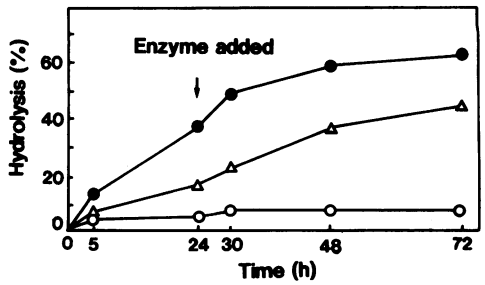

Figure 4. Synergistic actions of α-L-Arafase and β-Galase on radish seed AGP. ●, A simultaneous action of α-L-Arafase and β-Galase; Δ, α-L-Arafase alone; O, β-Galase alone. Molar proportion of radish seed AGP (Ara:Gal:Xyl:Glc:uronic acids = 45:42:3:0.2:10) (27). The amounts of sugars released were determined reductometrically and the extent (percentage) of hydrolysis was calculated based on the total sugar content of the AGP.

Methylation analysis of α-L-Arafase-digested sugar beet arabinan demonstrated the proportions of nonreducing terminal and branching α-L-arabinofuranosyl residues to be decreased with a concomitant increase of the (1→5)-linked α-L-arabinofuranosyl residues that constituted the backbone chains (Table VI).

As shown in Figure 4, α-L-Arafase and β-Galase were allowed to act separately or simultaneously on radish seed AGP followed by the addition of each enzyme or both to the reaction mixtures after 24 h of incubation. Only 8.1% of D-Gal was released after incubation with β-Galase (total units, 100 milliunits) for 72 h, indicating that the β-3,6-D-galactan structure of the native AGP was highly resistant to β-Galase alone. α-L-Arafase (total units, 50 milliunits) was found to be able to release 43.1% of L-Ara*f* from the substrate after 72 h of incubation. A simultaneous action of α-L-Arafase (total units, 50 milliunits) and β-Galase (total units, 100 milliunits) enabled 63.1% of reducing sugar as a mixture of L-Ara and D-Gal to be released from the AGP under the same reaction conditions as above, and the products were identified upon PC.

After incubation of oat-spelt xylan with α-L-Arafase for 48 h, the extent of hydrolysis reached 26.3% based on the total sugar content, yielding a mixture of L-Ara and D-Xyl, of which 43% was determined to be L-Ara by the D-galactose dehydrogenase method (19). Wheat arabino-(1→4)-xylan and 4-*O*-methyl-glucuronoxylan also underwent hydrolysis by α-L-Arafase, liberating 2.5 and 5.1% of D-Xyl equivalent, respectively.

Table VI. *Comparison in Glycosidic Linkages of Sugar Beet Arabinan and α-L-Arafase-Digested Arabinan*

Mode of Linkage	Proportion	
	Arabinan	Digested arabinan
	mol·100 mol^{-1}	
L-Ara*f*-(1→	29.9	7.4
D-Gal*p*-(1→	0.7	1.8
→3)-L-Ara*f*-(1→	1.7	2.0
→5)-L-Ara*f*-(1→	38.6	71.8
→3,5)-L-Ara*f*-(1→	23.1	5.3
→2,5)-L-Ara*f*-(1→	1.1	0.3
→3)-D-Gal*p*-(1→	1.5	2.1
→4)-D-Gal*p*-(1→	1.0	5.5
→6)-D-Gal*p*-(1→	1.3	1.3
→3,6)-D-Gal*p*-(1→	1.1	2.5

DISCUSSION

An α-L-Arafase capable of hydrolyzing both PNP α-L-Ara*f* and PNP β-D-Xyl*p* with a constant ratio of 3:1 in the initial velocities at 1 mM of each substrate concentration has been purified from dormant radish seeds. Analyses by SDS-PAGE, RP-HPLC, and size-exclusion HPLC confirmed the apparent homogeneity of the preparation. Two or three minor protein bands migrating closely with a major one were located in the gel after native PAGE at pH 6.6. All the enzyme components in the gel were stainable after incubation of the gel with 4-MU glycosides of α-L-Ara*f* and β-D-Xyl*p* as the substrates, respectively, which is indicative of the presence of minor isoforms of the enzyme (data not shown).

The apparent mol wt of radish seed α-L-Arafase was estimated to be 64,000, 57,000, and 63,000 by the SDS-PAGE, size-exclusion HPLC, and gel filtration methods, respectively. These values are comparable with those of α-L-Arafases from such plant sources as lupin cotyledons (I, 70,000; II, 120,000) (18), *Scopolia japonica* suspension-cultured cells (62,000) (26), carrot-cultured cells (I, 94,000; II, 110,000) (15), and soybean cotyledons (87,000) (11). The isoelectric point value (4.7) of radish seed α-L-Arafase indicates its acidic nature, similar to that (isoelectric point, 4.7) of carrot-cultured cells (15), whereas a basic enzyme (isoelectric point, 8.0) from *S. japonica*-cultured cells (26) has been reported. No significant stimulation with Ca^{2+} or Zn^{2+}, as found with soybean and carrot enzymes (11, 15), was observed with radish seed α-L-Arafase.

The enzyme hydrolyzes both PNP α-L-Ara*f* and 4-MU α-L-Ara*f* at pH 4.5 (K_m 9.8 and 13.9 mM and V_{max} 73.0 and 69.3 μmol/min/mg, respectively). These kinetic parameters are in a similar order of magnitude to those for other plant α-L-Arafases (15, 18, 26) except that for a soybean enzyme (0.53 mM) (11).

The dual activities of radish seed α-L-Arafase toward both aryl glycosides of α-L-Ara*f* and β-D-Xyl*p* were demonstrated to be due to a single enzyme protein whose identity was verified by evidence including (a) high purity of the enzyme preparation, as revealed by SDS-PAGE, RP-HPLC, and size-exclusion HPLC; (b) consistency of the activities in response to heat inactivation and activation with BSA; (c) inhibition by heavy metal ions, SH-reactive reagents, and such structurally related sugars as L-arabino-(1→4)-lactone, D-Xyl, and D-glucono-(1→5)-lactone; (d) mixed kinetic analysis that indicates one enzyme with a single active site is responsible for the hydrolysis of both of the substrates; and (e) the fact that the peaks of both activities coeluted in exactly the same elution volumes upon electrofocusing and gel filtration of the purified enzyme preparation, respectively. No plant α-L-Arafases so far purified have been examined for β-D-Xylpase activity (11, 15, 26) except lupin enzymes, for which PNP β-D-Xyl*p* does not serve as a substrate (18). α-L-Arafase activity was not examined for a sugarcane β-D-Xylpase (5). Only indirect evidence to support the presence of α-L-Arafases with dual activities has been reported by Holden and Rohringer (12), who localized the isozymes capable of attacking PNP β-D-Xyl*p* as well as PNP α-L-Ara*f* on nitrocellulose blots of two-dimensionally electrophoresed proteins in intercellular washing fluid of wheat leaves. Radish seed α-L-Arafase also attacked β-(1→4)-linked xylooligosaccharides and xylans in an exocleaving manner and could liberate L-Ara and D-Xyl from oat-spelt xylan. Thus, the enzyme could be regarded as an exoxylanase capable of splitting off D-Xyl from the nonreducing end of β-(1→4)-D-xylan.

A parallel enhancement of α-L-Arafase and β-D-Xylpase activities has been reported, both of which possibly participated in the degradation of cell wall arabinoxylan of GA_3-stimulated barley aleurone layer (7). It is interesting that *Trichoderma reesei* β-D-Xylpase has been reported to have α-L-Arafase activity that can hydrolyze PNP α-L-Ara*f* and that corresponds to about 70% of that for PNP β-D-Xyl*p* (21).

The dual activities of the enzyme might be related to its specificity in recognizing the configurations of hydroxyl groups at C-1, C-2, C-3, and C-4 in β-D-Xyl*p*, as well as those at C-1, C-2, and C-3, and the hydroxymethyl group at C-4 of α-L-Ara*f*, the original substrate, as summarized in Figure 5. The inhibitory potency of D-Xyl and D-glucono-(1→5)-lactone for both activities afforded another configurational basis for the specificity. This unique specificity of radish seed α-L-Arafase is clearly discernible from the lack of the specificity at C-4 of sweet almond emulsin β-glucosidase (10) and a jack bean β-*N*-acetylhexosaminidase (17), in that radish α-L-Arafase is unable to discriminate between the configurations of α-Ara*f* and β-D-Xyl*p*.

Structural analyses of the degraded product from sugar beet arabinan (23) implicate a preferential attack of α-L-Arafase on single nonreducing terminal α-L-arabinofuranosyl residues attached to the backbone chains at 0–3, leaving a product composed mainly of (1→5)-linked α-L-arabinofuranosyl residues that are much less susceptible to the enzyme. This mode of action is coincident with the results obtained with the *S. japonica* α-L-Arafase on sugar beet arabinan (25).

The action of α-L-Arafase on radish leaf AGP and seed AG plateaued at 61.0 and 63.2% hydrolysis of their total L-arabinofuranosyl residues, respectively. This limited hydrolysis might be ascribed to the presence of unattackable α-L-arabinofuranosyl residues substituted by α-L-fucopyranosyl residues or to branched structures in these substrates (27, 28). No appreciable amounts of D-Xyl could be detected in the α-L-Arafase digests of radish seed AG and AGP, possibly owing to its distribution as a minor (2–3% of total sugars) constituent integrated into the inner sites of the side chains (27) or to the low hydrolytic rate.

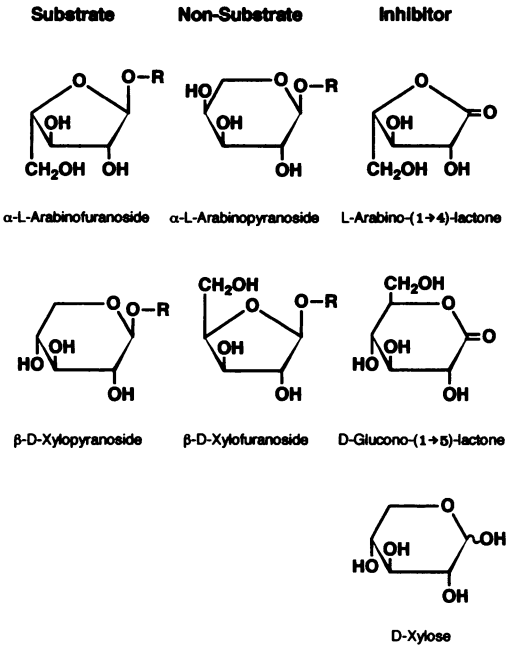

Figure 5. The structures of the substrates, related glycosides unsusceptible to the enzyme, and inhibitors. R, Aglycons.

Enzymic degradation of radish seed AG and AGP during germination has been suggested to occur by sequential action of endogenous exoglycosidases using a fungal α-L-Arafase and a β-Galase purified from radish seeds that had been permitted to imbibe (22). A simultaneous attack of the purified radish seed α-L-Arafase and β-Galase on radish seed AGP resulted in extensive degradation of the carbohydrate moieties, verifying that the model system involving the enzymes is valid. Obviously, prior removal of α-L-arabinofuranosyl residues attached to (1→6)-linked β-D-galactosyl residues constituting the side chains by α-L-Arafase enabled subsequent exocleaving attack of β-Galase on β-3,6-D-galactan as the backbone structure of the AGP. Incomplete hydrolysis of the AGP most likely resulted from the hindrance of the β-Galase action on the AGP by the substitution of β-D-galactosyl residues in the side chains with either 4-*O*-methyl-β-D-glucuronosyl residues at nonreducing termini or with the branched α-L-arabinofuranosyl residues resistant to α-L-Arafase.

ACKNOWLEDGMENT

We wish to thank Dr. K. Takio, the Institute of Physical and Chemical Research (Wako, Saitama), for carrying out HPLC analyses of the purified enzyme.

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