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Purification and Characterization of an Exo-1,5-α-L-Arabinanase from *Aspergillus sojae*

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Abstract: An exo-1,5-α-L-arabinanase was purified as an electrophoretically homogenous protein from a liquid culture of *Aspergillus sojae.* The molecular mass of the purified enzyme was estimated to be 41 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 43 kDa by gel filtration chromatography. The isoelectric point of the enzyme was 3.7. The maximum velocity of carboxymethyl (CM)-linear arabinan degradation by the exo-arabinanase was attained at 50°C and at pH 5.0. The purified enzyme was stable in a range from pH 6.0 to 8.0 and up to 45°C. The activity of the enzyme was significantly inhibited by Ag^{+} (1 mM) and Cr^{2+} (1 mM), and stimulated by SDS (5 mM). The K_m value for the 1,5-arabinan from beet was 5.8 mg/mL. The sequence of amino-terminus (25 residues) of the exo-arabinanase from *A. sojae* exhibits extensive identity (69%) with that of *Penicillium chrysogenum.* After the hydrolysis of 1,5-arabinan from beet, the major product was arabinobiose, and no liberation of arabinose was observed in the reaction mixture.

Key words: *Aspergillus sojae,* exo-1,5-α-L-arabinanase, 1,5-arabinan

L-Arabinosidases, *i.e.* α-L-arabinofuranosidase (EC 3.2.1.55) and endo-1,5-α-L-arabinanase (EC 3.2.1.99) play important roles in the degradation of hemicellulosic and pectic substances, *e.g.* arabinoxylan, arabinans and arabinogalactan.[1)] In the fermentation and food industries, those enzymes not only are used to saccharize raw materials like wheat and soybean, but also contribute to preventing haze formation in fruit juice. For the application of L-arabinosidases to the various industries (*e.g.* food, feed, paper and pulp), we have been investigating the enzymatic characteristics and possible roles of hemicellulases from *koji* mold.[2)] Previously, we purified and cloned an α-L-arabinofuranosidase from *Aspergillus sojae,* and indicated that the enzyme might be a new type of α-L-arabinofuranosidase.[3,4)] In this connection, we purified an arabinan-degrading enzyme from *A. sojae* and found, incidentally, the enzyme liberated arabinobiose from 1,5-arabinan in exo-fashion. Although various researchers reported endo-type arabinanases from aspergilli while producing arabinobiose,[5–7)] little is known of exo-type arabinanase.

Here, we describe the purification and properties of an exo-1,5-α-L-arabinanase from *A. sojae* for the first time, and compare its properties with those of the known enzymes.[8–11)]

MATERIALS AND METHODS

Biological materials. *A. sojae* no. 3 (ATCC 200440) was used in this study. The strain was cultured on potato dextrose agar at 30°C and the stock culture was kept in a refrigerator at 4°C.

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Chemicals. CM-linear arabinan, 1,5-arabinan from beet, arabinobiose, arabinotriose and arabinotetraose were purchased from Megazyme International Ireland Ltd., Bray, Co. Wicklow, Ireland. Arabinogalactan from larch wood, gum arabic from acacia tree, pectin from citrus fruits, polygalacturonic acid from orange, and ρ-nitorophenyl synthetic substrates were obtained from Sigma-Aldrich Japan K.K., Tokyo, Japan. Arabinoxylan from straw was prepared according to the method of Fukumoto *et al.*,[12)] and arabinogalactan from soybean was prepared according to the method of Morita.[13)] Arabinan from beet pulp was prepared according to the method of Tagawa and Kaji.[14)]

Biochemical assays. The activity of arabinanase was measured on the basis of release of reducing sugar from CM-linear arabinan (the carboxymethyl degree of substitution is about 0.05, Megazyme International Ireland Ltd.). An assay mixture containing 0.25 mL of 0.5% CM-linear arabinan solution in 0.1 M sodium acetate buffer (pH 5.0) and 0.25 mL of diluted enzyme solution was incubated at 40°C for 10 min. The reducing sugar released was measured as arabinose by the Somogyi-Nelson method.[15)] One unit of enzyme activity was defined as the amount of enzyme which liberate 1 μmol equivalent of L-arabinose per minute from CM-linear arabinan. Native polyacrylamide gel electrophoresis (native-PAGE) was performed according to the method of Davis.[16)] Sodium dodecyl sulfate (SDS)-PAGE was performed according to the method of Laemmli.[17)] The protein in the gel was stained with Coomassie Brilliant Blue. The molecular weight of the purified enzyme was determined by gel filtration chromatography (GFC) using TSK gel G3000SWXL (Tosoh Co., Tokyo) and SDS-PAGE. Protein sequencing was done by an Applied Biosystems (Foster City, CA, USA) model 494 protein sequencer system. The protein content of the

enzyme was measured according to the method of Bradford[18] with bovine serum albumin as the standard. Gel isoelectric focusing was done on a thin-layer gel (Bio-Rad Labs., Richmond, CA, USA). The method used for p*I* determination was based on the procedure reported by Låås *et al*.[19]

For the hydrolysis of 1,5-arabinan from beet, 0.4 units of the purified enzyme was mixed 10 mg of 1,5-arabinan in 10 mL of 0.05 M sodium acetate buffer (pH 5.0). The mixture was then incubated at 40°C for 24 h. At definite times, aliquots (1 mL) of the reaction mixture were collected, and analyzed by thin-layer chromatography (TLC). The products of TLC using LHP-KD silica gel HPTLC plate (Whatman International Ltd., Maidstone UK) were separated with *n*-butanol : ethanol : water (5 : 3 : 2) solvent, and the sugars were detected by aniline-diphenylamine reagent.[20]

RESULTS

Purification of arabinan degrading enzyme.

A. sojae No. 3 was cultured in a 500 mL Erlenmeyer flask containing a medium that consisted 2.0 g of arabinan from beet pulp, 0.6 g of peptone, 0.2 g of yeast extract, 1.0 g of KH_2PO_4 and 200 mL of tap water (pH 5.3) at 30°C for 72 h under static conditions. The culture broth was filtered through 4 layers of cheese cloth. The filtrate (940 mL) was fractionated by ammonium sulfate precipitation (40–90% saturation), and dialyzed against 50 mM sodium acetate buffer (pH 5.0). The enzyme solution (47 mL) was loaded on a HiPrep 16/10 Q-Sepharose FF column (Amersham Biosciences Co., Piscataway, USA, 1.6×10 cm), which was equilibrated with 50 mM sodium acetate buffer (pH 5.0). After washing the column with the buffer, the enzyme was eluted with a linear gradient of NaCl from 0 to 0.5 M in the buffer at a rate of 60 mL/h. The active fraction (52 mL) was concentrated by ultrafiltration (Centriprep YM-10; Millipore Co., Bedford, USA), loaded onto a HiPrep Sephacryl S-100 HR column (Amersham Biosciences, 1.6×60 cm) equilibrated with 50 mM sodium acetate buffer (pH 5.0) containing 0.3 M NaCl, and eluted with the buffer. The active fraction (50 mL) was dialyzed against 1 mM potassium phosphate buffer (pH 6.8) and concentarated by Centriprep YM-10. The fraction was loaded onto a Bio-scale CHT2-I column (Bio-Rad Labs., 0.7×5.2 cm) equilibrated with 1 mM sodium phosphate buffer (pH 6.8). The column was washed with the equilibrating buffer, and the enzyme was eluted with a linear gradient from 1 mM to 0.2 M of sodium phosphate buffer (pH 6.8) at a rate of 10 mL/h. The active fraction (8 mL) was pooled and stored in an ice bath.

Table 1 shows a summary of the steps used to purify of the arabinanase. The purified enzyme had a specific activity of 17.5 U/mg protein, and the recovery of the activity was about 3% based on the culture broth.

Molecular characteristics of purified enzyme.

The purified enzyme (25 μg) showed a single protein band after native PAGE (Fig. 1A). The molecular mass of the native enzyme was estimated to be 43 kDa by GFC. On SDS-PAGE gel, the purified enzyme gave a single protein band, and the molecular mass was estimated to be 41 kDa (Fig. 1B). The isoelectric point (p*I*) of the purified enzyme was estimated to be 3.7 by thin-layer polyacrylamide gel isoelectrophoretic analysis. The amino acid sequence at the amino-terminal of the purified enzyme (100 pmol) was AETPTTFSEVTIFSPPSDYVILPTL-.

Table 1. Summary of purification of exo-1,5-α-L-arabinanase from *A. sojae*.

Step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Culture broth	940	244	1231.4	0.2	100
$(NH_4)_2SO_4$ precipitation (40–90 % saturation)	47	259	248.0	1.0	106
Q-Sepharose FF	52	60	41.6	1.4	25
Sephacryl S-100	50	23	3.0	7.7	9
Hydroxyapatit	8	7	0.4	17.5	3

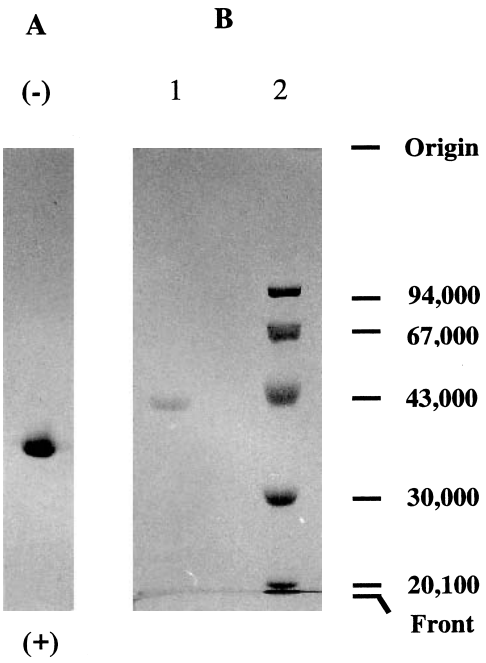

Fig. 1. Native and SDS-polyacrylamide gel electrophoresis of exo-1,5-α-L-arabinanase.

(A) Native-PAGE. The purified enzyme (25 μg) was electrophoresed according to the method of Davis[16] using 10% polyacrylamide gel. (B) SDS-PAGE. Lane 1, purified enzyme (5 μg); lane 2, LMW standard proteins (Amersham Biosciences Co.). Purified enzyme and standard proteins were treated with SDS at 100°C for 2 min, after which electrophoresis was carried out at 10 mA for 3 h with 0.1% SDS.

Enzymatic properties.

The optimum pH and temperature for the activity of the purified enzyme using CM-linear arabinan as a substrate were 5.0 and 50°C, respectively. The purified enzyme was stable at 30°C for 18 h in the pH range 6.0–8.0. The activity was stable up to 45°C at pH 5.0, and was completely lost after incubation at 65°C for 10 min without CM-linear arabinan. The purified enzyme solution (0.02 U) was preincubated in a mixture containing various chemicals at 30°C for 10 min and the residual arabinanase

Table 2. Substrate specificity of exo-1,5-α-L-arabinanase from *A sojae*.

Substrate	Major linkage*	Relative activity (%)
CM-linear arabinan	α-1,5-Ara*f*	67.0
1,5-arabinan from beet	α-1,5-Ara*f*	82.0
Arabinan from beet pulp	α-1,5-Ara*f*, α-1,3-Ara*f*	36.8
Arabinogalactan		
from soybean	α-1,5-Ara*f*, α-1,3-Ara*f*, β-1,4-Gal*p*	0
from larch wood	α-1,3-Ara*f*, β-1,3-Gal*p*, β-1,6-Gal*p*	0
Gum arabic		
from acacia tree	β-1,3-Gal*p*, β-1,6-Gal*p*	0
Pectin from citrus fruits	α-1,4-Gal*p* UA	0
Polygalacturonic acid		
from orange	α-1,4-Gal*p* UA	0
Arabinoxylan from straw	α-1,3-Ara*f*, α-1,2-Ara*f*, β-1,4-Xyl*p*	0
Arabinobiose	α-1,5-Ara*f*	0
Arabinotriose	α-1,5-Ara*f*	52.6
Arabinotetraose	α-1,5-Ara*f*	100
PNP α-L-arabinofuranoside		0
PNP β-L-arabinopyranoside		0
PNP α-D-xylopyranoside		0
PNP β-D-xylopyranoside		0
PNP α-D-galactopyranoside		trace**
PNP β-D-galactopyranoside		0
PNP α-D-glucopyranoside		0
PNP β-D-glucopyranoside		0

*Ara*f*, arabinofuranose; Gal*p*, galacopyranose; Xyl*p*, xylopyranose; Gal*p* UA, galactopyranosyluronic acid. **trace, less than 1.0%. The enzyme was reacted with the various polysaccharides in a mixture containing 0.04 units of purified enzyme, and 2.5 mg of substrate in 0.5 mL of 0.05 M sodium acetate buffer (pH 5.0) at 40°C for 10 min. The enzyme was reacted with the various oligosaccharides or synthetic substrates in a mixture containing 0.04 units of purified enzyme, and 1.25 μmol of substrate in 0.5 mL of 0.05 M sodium acetate buffer (pH 5.0) at 40°C for 20 min. The hydrolysis of various substrates was measured by the Somogyi-Nelson method[15] with L-arabinose or D-galactose as a standard.

activities were then measured under the standard assay conditions. Addition of Zn^{2+}, Mn^{2+}, Hg^{2+} and Fe^{3+} at a concentration of 1 mM caused 14.8, 16.9, 12.8 and 16.3% inhibition of the enzyme activity, respectively, whereas Ag^{+} and Cr^{2+} at the same concentration resulted in 58.8 and 59.6% inhibition, respectively. None of the metal ions tested markedly stimulated the activity of the enzyme. Group-specific reagents—ditiothreitol, 2-mercaptoethanol, iodicacetate (all 1 mM) and ρ-chloromercuricbenzoate (PCMB) (0.1 mM)—exhibited no inhibitory effect on the enzyme activity. In contrast, SDS (5 mM) markedly stimulated the activity (121%) of the purified enzyme under the same conditions.

Table 2 shows the substrate specificity of the purified enzyme toward various substrates. The purified enzyme showed strong activity toward arabinotetraose, 1,5-arabinan from beet, CM-linear arabinan, and arabinotriose, and some activity toward arabinan from beet pulp. Analysis of the hydrolyzates of those substrates revealed that the purified enzyme released arabinobiose from each one. However, the enzyme showed no activity toward pectin from citrus, polygalacturonic acid from orange, arabinoxylan from straw, arabinogalactan from larch wood, gum arabic, arabinobiose, or synthetic substrates (ρ-nitorophenyl (PNP) α-L-arabinofuranoside, PNP β-L-arabinopyranoside, PNP α-D-xylopyranoside, PNP β-D-xylopyranoside, PNP α-D-glucopyranoside and PNP β-D-galactopyranoside). However, a weak activity was recognized toward PNP α-D-galactopyranoside.

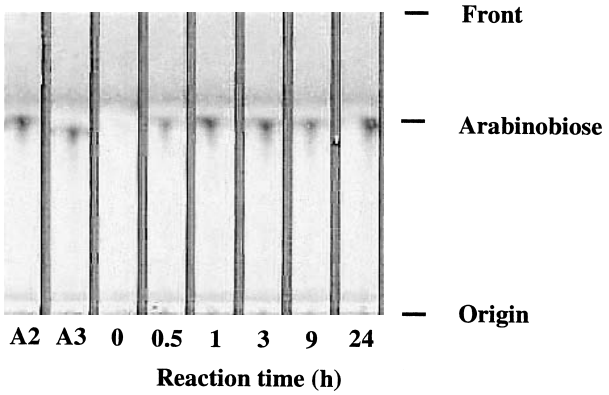

Fig. 2. Thin-layer chromatography of hydrolyzates of 1,5-arabinan from beet by exo-1,5-α-L-arabinanase.

A2, arabinobiose; A3, arabinotriose. The reaction mixture contained 10 mg of 1,5-arabinan from beet and 0.4 units of the purified enzyme in 10 mL of 50 mM sodium acetate buffer (pH 5.0), and was incubated at 40°C. At definite times, aliquots (1 mL) of the reaction mixture were collected and heated at 100°C for 5 min. The selected samples were deionized with AG 501-x 8 resin (Bio-Rad Labs.), and after that the samples were analyzed by HPTLC.

Hydrolysis of arabinan.

In hydrolysis of 1,5-arabinan from beet, the major product in the early stage of the reaction (1 h) was arabinobiose, that was corresponding to the standard arabinobiose. In the later stage of the reaction (24 h), mainly arabinobiose was accumulated, but other oligosaccharides and arabinose were not liberated from 1,5-arabinan (Fig. 2). Furthermore, the Lineweaver-Burk plot showed that the K_m and V_{max} values for 1,5-arabinan from beet were 5.3 mg/mL and 89.1 μmol/min/mg protein, respectively.

DISCUSSION

A. sojae excreted an arabinanase into the culture broth, and the enzyme was purified to homogeneity to analyze its molecular characteristics. In the purification steps, the $(NH_4)_2SO_4$ precipitation step was a useful method for purification of the enzyme, because the specific activity of the enzyme was 5-fold over that of the culture broth (Table 1).

Table 2 shows the substrate specificity of the purified enzyme. The enzyme was strongly inhibited by the presence of arabinofuranosyl branches, which were attached arabinofuranosyl units linked to C3 of the main 1,5-arabinan chain; *i.e.* arabinan from beet pulp. The result indicated that the enzyme did not act on the α-1,3-linkage of arabinofuranose. Since arabinoxylan from straw and

Table 3. Properties of exo-1,5-α-L-arabinanases from various organisms.

Properties	*A. sojae* No. 3[*1]	*E. carotovora* IAM 1024[8]	*P. fluorescens*[9)*2]	*P. chrysogenum*[10]	*A. niger* (var. *aculeatus*)[11]
Molecular weight:					
SDS-PAGE	41 kDa	—	34 kDa	47 kDa	67 kDa
Gel filtration	43 kDa	—	—	—	—
Isoelectric point (p*I*)	3.7	—	—	—	2.85
Optimum pH	5.0	6.0	—	4.0	4.0
Optimum temperature (°C)	50	—	—	40	60
pH stability	6.0–8.0[*3]	5.0–11.0[*4]	—	3–8[*5]	—
Thermal stability (°C)	up to 45	—	—	up to 50	—
Inhibitor	Ag^+ (1 mM), Cr^{2+} (1 mM)	Hg^{2+} (0.2 mM)	—	Hg^{2+} (1 mM)	—
Reaction products:					
on 1,5-arabinan	arabinobiose	arabinotriose	arabinotriose	arabinobiose	arabinobiose
*K*m value (mg/mL)					
toward 1,5-arabinan	5.3	—	3.75	—	—

[*1]In this study. [*2]Recombinant arabinanase. [*3]Incubated at 30°C for 18 h. [*4]Incubated at 2°C for 96 h. [*5]Indicates less than 70% of residual activity when 1 mM inhibitor was used. —, not determined.

arabinogalactan from larch wood had α-1,3-arabinofuranose linkage as the major linkage in their structure, the enzyme did not act on them. Therefore, the purified enzyme acted specifically on the α-1,5-linkage of arabinofuranose. While, arabinogalactan from soybean contains 1,5-linked side chains of an average length of two arabinofuranosyl units to galactan,[21] the enzyme could not hydrolyze the 1,5-linkage of the side chain. An interesting point is that the enzyme did not act on arabinobiose, but acted on arabinotriose and arabinotetraose. Additionally, the enzyme degraded 1,5-arabinan with accumulation of arabinobiose as the final product (Fig. 2). The enzyme showed a weak activity toward PNP α-D-galactopyranoside, which is structural analogue of arabinofuranoside; however, the result remains to be tested.

From the results of substrate specificity experiments and the action pattern toward 1,5-arabinan, the enzyme was strongly suggested to be an exo-1,5-α-L-arabinanase producing arabinobiose.

Although exo-1,5-α-L-arabinanase has already been purified from *Erwinia carutovora* IAM 1024,[8] *Pseudomonas fluorescens*,[9] *P. chrysogenum*[10] and *Aspergillus niger* (var. *aculeatus*),[11] the details of the properties and molecular characteristics of the exo-1,5-α-L-arabinanase from *A. sojae* are reported here for the first time.

Various researchers purified and reported the properties and molecular characteristics of endo-1,5-α-L-arabinanases from aspergilli.[5–7] Although, the exo-1,5-α-L-arabinanase from *A. sojae* had similar the characteristics to endo-1,5-α-L-arabinanases of aspergilli, the action pattern and final products of the enzymes toward 1,5-arabinan were clearly different from that of the exo-1,5-α-L-arabinanase from *A. sojae*. Endo-1,5-α-L-arabinanase from aspergilli degraded 1,5-arabinan with accumulation of the arabinobiose and arabinotriose,[5,6] or arabinobiose and arabinose[7] as the final products, but the exo-1,5-α-L-arabinanase produced only arabinobiose from 1,5-arabinan. Furthermore, the *K*m value for the exo-1,5-α-L-arabinanase from *A. sojae* on 1,5-arabinan (5.3 mg/mL) indicated much higher values than those of endo-1,5-α-L-arabinanases from *A. niger*; those are 0.72[6]and 0.205 mg/mL,[22] respectively.

In Table 3, some properties of exo-1,5-α-L-arabinanase from various organisms are compared. Each enzyme showed remarkable difference regarding the molecular mass, pH stabilities and inhibitors. Although, the exo-1,5-α-L-arabinanase from *A. sojae* indicated some similar properties, *i.e.* optimum pH and reaction products on 1,5-arabinan, to those of *P. chrysogenum*[10] and *A. niger* (var. *aculeatus*),[11] it also showed remarkable difference concerning the molecular mass.

In the homology search, the molecular mass of the arabinanase from *A. sojae* (41 kDa) was remarkably different from that of *P. chrysogenum* (47 kDa)[10]; however, the amino-terminus sequence of the enzyme was compared with protein sequences in Swiss-Prot, TrEMBL, and PIR using the BLAST[23] protein database search program (http://www.au.expasy.org/tools/). The sequence of amino-terminus (25 residues) of the exo-1,5-α-L-arabinanase from *A. sojae* exhibits extensive identity (69%) with that of *P. chrysogenum* (TrEMBL Q7ZA77).

From that fact, the exo-1,5-α-L-arabinanase from *A. sojae* might have a different module structure from that of *P. chrysogenum*.[10] We are now studying the kinetic properties of the exo-1,5-α-L-arabinanase, and cloning of the enzyme gene from *A. sojae* to clarify the mode of liberation of arabinobiose from 1,5-arabinan.

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Aspergillus sojae のエキソ-1,5-α-L-アラビナナーゼの精製と諸性質の検討

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Aspergillus sojae の液体培養液からエキソ-1,5-α-L-アラビナナーゼを電気泳動的に均一な標品として精製した．精製酵素標品の分子質量は，ドデシル硫酸ナトリウム-ポリアクリルアミドゲル電気泳動（SDS-PAGE）によって 41 kDa，ゲル濾過クロマトグラフィーによって 43 kDa と決定された．本酵素の等電点は 3.7 であった．本エキソ-アラビナナーゼ活性は，カルボキシメチル-直鎖アラビナンに対し，50°C，pH 5.0 で作用させることで最大となった．活性は pH 6.0 から 8.0，45°C まで安定であった．精製酵素標品の活性は Ag^{+}（1 mM）および Cr^{2+}（1 mM）によって強く阻害され，SDS（5 mM）の添加により促進された．1,5-アラビナンに対する K_m 値は 5.8 mg/mL となった．本精製酵素標品の N 末端アミノ酸 25 残基は，*Penicillium chrysogenum* のそれと 69% という高い相同性を示した．精製酵素標品をビート由来の 1,5-アラビナンに作用させた場合のおもな生産物は，アラビノビオースであり，反応溶液中にアラビノースの遊離は認められなかった．