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Article

Purification and Characterization of α-L-Arabinosidase from *Trichoderma* sp. SY

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***Trichoderma* sp. SY most effectively produces an extracellular α-L-arabinofuranosidase (AF) using arabinose as a carbon source. AF grown on cellulose as a carbon source was purified 28-fold with 4.4% yield by DEAE exchange and HQ/20 cation exchange chromatographies The purified enzyme was found to be homogeneous on SDS-PAGE with molecular weight of 89 kDa. It exhibited a high level of activity with *p*-nitrophenyl α-L-arabinofuranoside, showing K_m and V_{max} values of 0.15 μM and 239.85 U · mg^{-1}, respectively and did not require any metal ion for activity. It also released p-nitrophenol from *p*-nitrophenol conjugated β-D-xylopyranoside, and β-D-galactopyranoside not from β-D-glucopyranoside.**

Key words: Trichoderma, *α-L-arabinofuranoside, arabinan*

Polysaccharides containing arabinose are commonly found in hemicelluloses such as arabinan and arabinoxylan. Arabinan has a backbone of 1,5-α-L-arabinofuranoside polymer that is substituted with other α-arabinofuranose via α-1,2 and/or α-1,3 linkage.[1] and arabinoxylan is a polymer of β-1,4-xylose branched with α-arabinofuranose through O-3 position, or both O-2 and O-3 positions of xylose.[2] The release of arabinose from these polymers is catalyzed by two type of enzymes, α-L-arabinofuranosidases (AFs) and arabinanases.[3] AFs are exo-type enzymes, which act at the terminal nonreducing group of arabinose-containing polysaccharides and release α-arabinofuranosyl group, and can hydrolyze 1,3 and 1,5 α-arabinosyl linkages of arabinan. On the other hand, arabinanases are endotype enzymes hydrolyzing 1,5-α-L-arabinofuranosidic linkage by endo-fashion.

Fungus *Trichoderma* is well-known for the production of many kinds of cellulose and hemicellulose degradation enzymes, among which *T. reesei* is one of the most efficient cellulase and hemicellulase producers.[4] Several enzymes including cellulases, xylanases, β-xylosidase, β-mannase, and α-glucuronidase have been characterized, some of which are currently used industrially. Previously, we isolated *Trichoderma* sp. SY from soil and characterized two major hemicellulose degradation enzymes, xylanase and β-xylosidase.[5,6] Here, we report the purification and characterization of AF, which is crucial for complete degradation of xylan, from *Trichoderma* sp. SY.

Materials and Methods

Fungal cultures. Previously isolated fresh *Trichoderma sp*. SY[5] well-grown on PDA medium was inoculated into a standing culture of 1-*l* flask containing 200 m*l* of the liquid medium. For the production of AF, fungi were grown at 25°C for 10 days in HMT-medium.[5]

Enzyme purification. Ten-day-old culture filtrates were collected after filtration and centrifugation (12,000 rpm for 20 min at 4°C), and concentrated using ultrafiltration membrane (10 kD, cut-off, Millipore, Billerica, MA,USA). The concentrate was dialyzed in 10 mM Tris/HCl buffer (pH 7.5) at 4°C for 12~14 h. The sample was loaded onto a DEAE-cellulose chromatography column (2 cm × 10 cm) previously equilibrated with the dialysis buffer. The binding proteins were eluted by a 30-min linear gradient of the dialysis buffer and 0.5 M NaCl at a flow rate of 2.0 m*l* · min^{-1}. The fractions showing α-arabinosidase activity were collected, dialyzed in 10 mM sodium acetate buffer (pH 4.0), and resolved again in HQ/20 cation exchange Fast Performance Liquid Chromatography (FPLC) column (4.6 × 100 mm, Porus, Persptive Biosystmes, Inc., Cambridge, MA, USA).

Characterization of enzyme. Molecular weights of the purified enzymes were determined by SDS-PAGE at 7.5%. The optimum pH was determined using 50 mM each sodium citrate (pH 3.0-4.0), potassium acetate (pH 4.5-5.5), potassium phosphate (pH 6.0-8.0), and Tris/HCl (pH 8.5-10.0) buffers. The optimum temperate was determined by incubating the purified enzymes at 20~75°C. α-Arabinofuranosidase activity was determined by assaying the amount of *p*-nitrophenol (pNP) released from the substrate *p*-phenyl-α-L-arabinofuranoside at pH 4. One unit of the enzyme was defined as the amount required to produce 1 μg of *p*-nitrophenol min^{-1} · m*l*$^{-1}$ at 37°C.

Effects of chemicals. Various metal ions (Ca^{+2}, Cu^{+2}, Fe^{+2}, and Mg^{+2}) and chemicals [EDTA and dithiothreitol (DTT)] were added by adding these to the enzyme solution at the final concentration of 5 or 10 mM to examine the effects on the α-arabinosidase activity. After incubation at 37°C for 30 min, the residual activity was determined.

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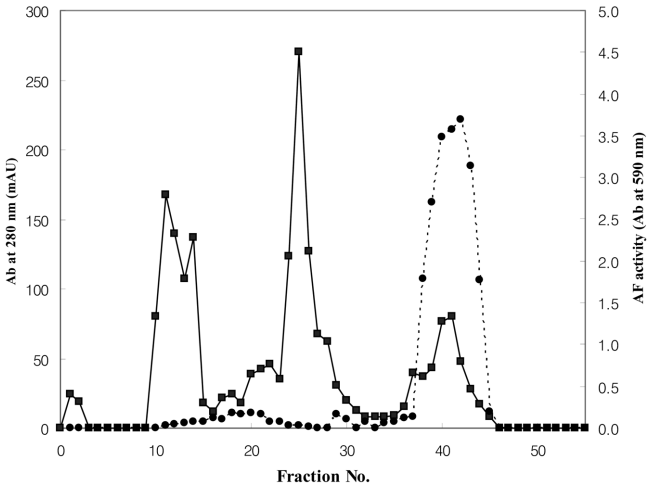

Fig. 1. Cation-Exchange HPLC fractionation of AF in the anion-exchange fraction. Absorbance at 280 nm (—), AF activity (-----).

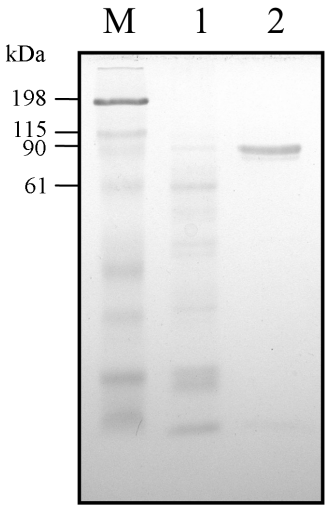

Fig. 2. SDS-PAGE analysis of the purified AF. Lane 1, Culture filtrates; Lane 2, Purified AF.

Results and Discussion

Production of AF. The fungus *Trichoderma* sp. was grown on several carbon sources such as arabinose, birchwood xylan, cellulose, glucose, oat spelt xylan, and oat meal to determine the effective carbon source for the production of AF. The production of AF from *T.* sp SY was highly induced by arabinose, resulting in about 13-fold and 4-fold increases compared to using cellulose and oat spelt xylan, respectively. Because oat spelt xylan contains arabinose, arabinose could have acted as an inducer for the production of the AF. In *T. reesei* and *Aspergillus niger*, L-arabitol also induced the production of AF as effectively as arabinose.[7,8] On the other hand, glucose acted as a repressor for the production of AF. Glucose was known as a catabolite repressor in the production of other cellulose and hemicellulose degradation enzymes from various fungi. Catabolic repression of glucose is mediated by carbon catabolite repressor *Cre1* in fungi including *Aspergillus* and *Neurospora*[9], However, in *T. reesei*, *Cre1* was shown to be partially involved in the negative regulation of two the hydrolytic genes, cellobihyrolase 1 and xylanase 1 whereas not involved in the regulation of cellobihyrolase 2 and xylanase 2.[10] Regulation of AF gene expression by glucose was also mediated by *Cre1* in *A. niger*, yet.[11]

Purification of AF. α-Arabinosidase activity reached maximum at 10 days of culturing and remained constant up to 14 days. Therefore, 10-day-old cultures were harvested, concentrated by ultrafiltration membrane, and fractionated by DEAE-cellulose chromatography. The fractions containing AF activity were collected and resolved again through HQ/20 cation exchange Fast Performance Liquid Chromatography. AF activity was associated with the major peak of UV absorbance (Fig. 1). SDS-PAGE analysis of the purified AF showed that AF was purified to almost homogeneity with molecular weights estimated to be 89 kDa (Fig. 2). Molecular weights of AFs from fungi and bacteria range from 30 Da to 240 kDa with those higher than 200 kDa is composed of more than two subunits.[3] The AF was purified 28-fold with a yield of 4.4% (Table1).

Characterization of AF. The purified AF showed at least 80% of the maximum activity between pH 2.5~4.5 , reaching maximum at pH 3.5 (Fig. 3). The optimum temperature was 55°C. Activities of AFs from fungi such as *Aspergillus*, *Cochliobolus*, *Penicillium* and *Scelerotinia*[12-15] reach optimum at acidic pH range (3.5 to 4.0) while those of bacteria at neutral pH range (5.0 to 7.0).[16-18] The enzymes could release *p*-nitrophenol from *p*-nitrophenol-conjugate α-L-arabinopyranoside, β-D-xylopyranoside, and β-D-galactopyranoside, while could not use *p*-nitrophenol β-D-glucopyranoside. In addition, AF

Table 1. Purification of α-Arabinosidase from *Trichoderma* sp. SY

	Total protein (mg)	Total α-Arabinosidase (U)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
1. Culture filtrate	70.8	2.06	0.03	100	1
2. U. F. Retentate	17.6	0.84	0.05	40.8	1.64
3. DEAE-Cellulose eluant	1.32	0.26	0.20	12.6	6.77
4. Cation column eluant	0.02	0.09	0.82	4.4	28.12

*α-Arabinofuranosidase activity was determined by assaying the amount of *p*-nitrophenyl (*p*NP) released from the substrate *p*-phenyl-α-arabinofuranoside. One unit of the enzyme was defined as the amount required to produce 1 μg *p*-nitrophenol min^{-1} · ml^{-1} at 37°C.

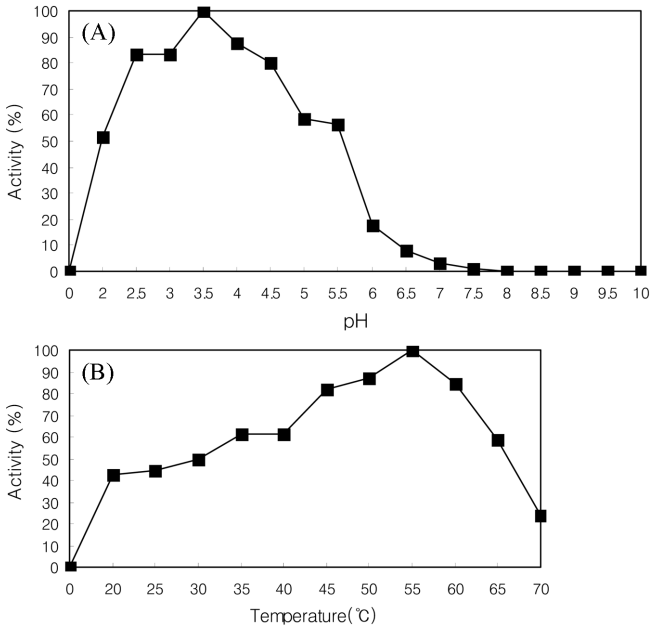

Fig. 3. Enzymatic activity of AF depending in temperature (A) and pH (B).

Table 2. Relative activity toward different substrates

Substrate	%
p-NP-α-L-Arabinofuranoside	100.0
p-NP-α-D-Xylopyranoside	63.5
p-NP-α-D-Galactopyranoside	58.4
p-NP-α-D-Glucopyranoside	3.4

Table 3. Effects of metal ions and chemicals on AF activity

	Concentration (mM)	Relative enzyme activity (%)
None	-	100.0
$CaCl_2$	5	101.8
$CuSO_4$	5	85.2
$FeSO_4$	5	90.5
$MgCl_2$	5	71.4
DTT	10	93.4
EDTA	10	70.8

released arabinoses from wheat arabinoxylan wherease it did not release any reducing sugars from sugar beet arabinan. A similar result was also found in AF from *A. awamori* which was also highly specific for arabinoxylan but did not show any activity toward arabinans and *p*-NP-α-L-arabinofuranoside.[19] The activity of AF was partially inhibited by Cu^{+2}, Fe^{+2}, and Mg^{+2}. Copper ion is also known to inhibit other AFs.[20,21] The reducing agent dithiothreitol (DTT) did not have any effect on the enzymatic activity, which indicates that the AF activity did not need a disulfide bond to carry out the hydrolysis reaction. The addition of chelating agent EDTA resulted in about 30% decrease of AF activity, which suggests that metal ions are involved in the catalytic function of the enzyme (Table 3). The kinetic parameters of the purified enzymes towards *p*-nitrophenyl-α-L-arabinofuranoside were examined. The K_m and V_{max} values of the enzymes were determined to be 0.15 μM and 239.85 $U \cdot mg^{-1}$, respectively indicating that the AF is highly specific for *p*-nitrophenyl-α-L-arabinofuranoside.

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