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Purification and Characterization of Xylanase from *Bacillus* sp. A-6

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A xylanase was purified from the culture supernatant of *Bacillus* sp. A-6 by using ultrafiltration and ion exchange chromatography on the column of SP-Sepharose using 5 mM acetate buffer, pH 5.0. The xylanase was eluted from the column at the concentration less than 0.05 M NaCl. The eluted xylanase was shown to be a single protein band in SDS-PAGE. Zymogram analysis indicated that the protein band in SDS-PAGE had the enzyme activity to hydrolyze oat spelt xylan. The molecular weights of the xylanase were 15,000 based on SDS-PAGE and 14,100 based on gel filtration chromatography. Thin layer chromatography showed that the xylanase hydrolyzed oat spelt xylan into xylobiose and high-molecular-weight xylooligosaccharides. The relative activities of the heated xylanase decreased to 80% at 40°C after 7 hr and less than 40% at 60°C after 1 hr.

Key words: Xylanase, *Bacillus*, oat spelt xylan, purification

Introduction

Xylan, the main component of hemicellulose in the cell wall of plant, is widely distributed along with cellulose in plant kingdom and is a polysaccharide which consists of xylose linked with β-1,4 glycosidic bonds which form main backbone structure and other minor substituent sugars [2]. Thus, xylanase plays the most important role in degradation of xylan by disintegrating the main backbone structure of xylan into xylose and/or xylooligosaccharides [3, 21].

Isolation of xylanase-producing microorganism and biochemical characterization of xylanase have been extensively studied [3, 4, 12, 19]. Xylanases are produced by fungi, bacteria, yeast, marine algae, protozoans etc., but the principal commercial source is filamentous fungi [11, 17]. Driven by industrial demands for enzymes that can operate under process conditions, a number of extremophilic xylanases have been isolated, in particular those from thermophiles, alkaliphiles and acidophiles, while little attention has been paid to cold-adapted xylanases [5].

Xylanase is widely utilized for pulp biobleaching, fruit beverage clarification, bakery production, and livestock feed supplementation [3, 12]. Microbial xylanases have been applied particularly to livestock feed supplementation in livestock industry [1, 6, 20]. The grain by-products frequently used as raw materials for livestock feed contain non-starch polysaccharides (NSP), such as xylan and β-glucan, which are indigestible for monogastric animals including pigs and chickens and young ruminant animals with immature digestive organs. The NSPs absorb water and form adhesive mucous materials in the intestine to prevent digestive enzymes from accessing to nutrients and to slow down proceeding of digesta in the intestine, which causes decrease in feed uptake and intestinal disorder. In order to ameliorate the digestion problem, livestock feed can be supplemented with the NSP-digesting enzymes, such as xylanase and β-glucanase, so the viscosity of digesta in the intestine is lowered and nutrient utilization efficiency is improved [1]. Furthermore, low manure output and decreased nitrogen excretion may reduce environmental pollution [8].

Mesotrophic *Bacillus* spores are available commercially as probiotics for human use as dietary supplements and animal feed to prevent gastrointestinal infection [10]. The mechanisms for which *Bacillus* probiotics can inhibit the infection include immunomodulation and synthesis of antimicrobials. *Bacillus* may also contribute to digestive function of animals by producing xylanase after germination in the intestine and to prevention of environmental pollution by enhancing degradation of livestock manure.

In the previous study [14], xylanase-producing mesotrophic *Bacillus* sp. A-6 was isolated from rice bran. The objectives of this study were to purify xylanase from the culture of *Bacillus* sp. A-6 and examine its physicochemi-

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cal and enzymatic characteristics.

Materials and Methods

Culture for production of xylanase

Bacillus sp. A-6 isolated from rice bran in the previous study [14] was cultured at 30°C for 24 hr in 250 mL of xylan medium. The composition of the xylan medium is oat spelt xylan 5 g, tryptone 10 g, beef extract 5 g, Tween 80 1 g, $(NH_4)_2SO_4$ 1.4 g, KH_2PO_4 2 g, urea 0.3 g, $CaCl_2$ 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $FeSO_4 \cdot 7H_2O$ 5.0 mg, $MnSO_4 \cdot H_2O$ 1.6 mg, $ZnSO_4 \cdot 7H_2O$, 1.4 mg, $CoCl_2$ 2.0 mg, and distilled water 1 l. The pH of the medium was adjusted to 9.0. The inoculum (1%) obtained after culturing sequentially twice in 25 mL of the xylan medium at 30°C for 24 hr was transferred into the medium. The culture was incubated at 30°C for 24 hr in a shaking incubator at 100 rpm.

Purification of xylanase from the culture

The culture was centrifuged at 2,100 × g for 30 min and the supernatant obtained after the centrifugation was concentrated into 25 mL using a Stirred Cell (Millipore, USA) installed with the ultrafiltration membrane (PBCC, NMWL: 5,000, Millipore). The concentrated culture supernatant was dialyzed in 5 mM sodium acetate, pH 5.0 and introduced into a column (1.5 cm×10 cm) of SP-Sepharose (GE) which was equilibrated with the same buffer. The adsorbed xylanase was eluted with a linear gradient of 0~0.2 M NaCl. The volumes of the fractions in the chromatography were 10 mL. Absorbances at 280 nm and xylanase activities of the fractions were measured. The fractions showing xylanase activities were collected and frozen.

Measurement of xylanase activity

Xylanase activity in the sample was assayed using oat spelt xylan as substrate. The reducing sugar released from the xylan was determined by 3,5-dinitrosalicylic acid method [15]. The substrate solution consists of 0.5% oat spelt xylan in 50 mM sodium phosphate, pH 7.0. The substrate solution (0.5 mL) was mixed with xylanase sample (0.125 mL) and incubated at 40°C for 30 min. And then 3,5-dinitrosalicylic acid reagent (0.75 mL) was added to the reaction solution, which was then heated at 100°C for 15 min. Absorbance at 550 nm was meausured. Xylose (0~10 mM) was used as standard solution. One unit of xylanase activity was defined as the amount of enzyme producing reducing sugar equivalent to 1 mmol xylose per min.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [13] was used to determine purity and molecular weight of xylanase. The molecular weight markers (Bio-Rad) for SDS-PAGE consisted of myosin (200,000), β-galactosidase (116,250), phosphorylase b (97,400) bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), trypsin inhibitor (21,500), lysozyme (14,000), and aprotinin (6,500).

Zymogram analysis

Zymogram analysis of xylanase was performed in SDS-PAGE using polyacrylamide gel containing 0.1% oat spelt xylan. The polyacrylamide gel was washed in 25% isopropanol for 1 hr and then with 0.1 M phosphate, pH 7.0 for 10 min 4 times and incubated at 40°C for 30 min. The gel was stained with 0.1% Congo red and washed with 1 M NaCl and then 0.5% acetic acid [18].

Fast protein liquid chromatography

Fast protein liquid chromatography (FPLC) was performed using a gel filtration column, Protein Pak Glass Column (Waters) to determine molecular weight of the xylanase. The eluent was 0.05 M Tris-HCl, pH 7.5, 0.1 M KCl and flow rate was 0.6 mL/min. The void volume (Vo) and elution volumes (Ve) of the column were determined by using Blue dextran and molecular weight markers, respectively. The molecular weight markers (Sigma-Aldrich Co.) were cytochrome C (12,400), carbonic anhydrase (29,000), and bovine serum albumin (66,000).

Protein determination

Protein concentration was determined using BCA Protein Assay Reagent (Pierce). Bovine serum albumin was used for calibration curve.

Thin layer chromatography

Thin layer chromatography (TLC) was used to analyze sugar composition of oat spelt xylan hydrolyzed with the purified xylanase. 0.5 mL of 0.5% oat spelt xylan in 50 mM phosphate, pH 7.0 was added with 0.125 mL of the xylanse (4.1 mg/mL) and then the mixture was incubated at

40°C for 1, 1.5, and 2 hr. The xylan hydrolyzates were separated on TLC plate of silica using a developing solution consisting of n-butyl alcohol and water (85:15). The TLC plate was sprayed with 0.5% aniline, 0.5% diphenylamine, and 0.5% phosphoric acid and heated at 100°C for 10 min.

Heat stability

The purified xylanase (4.1 mg/mL) in 50 mM sodium phosphate, pH 7.0 was heated at 40, 50, and 60°C in water bath for up to 7 hr. The heated samples were taken every hour in order to measure the xylanase activities. The relative activities were calculated by dividing the xylanase activities of the heated samples with that of the unheated sample.

Results and Discussion

Bacillus sp. A-6 was cultured in 250 mL of the xylan medium at 30°C for 24 hr. The supernatant obtained after centrifugation of the culture was concentrated into 25 mL by using the ultrafiltration membrane in order to facilitate dialysis. Although the molecular weight cut-off of the ultrafiltration membrane was 5,000, yield of the ultrafiltration concentrate was 70% (Table 1), which indicated some loss of xylanase during ultrafiltraton. Considering the molecular weight of the xylanase, the enzyme should be completely retained by the ultrafiltration membrane and the yield should be almost 100%. Specific activity of the ultrafiltration concentrate was 17.8 U/mg, which indicated purification of 1.3 fold.

The ultrafiltration concentrate was loaded into the column of SP-Sepharose and the column was extensively washed with 5 mM sodium acetate, pH 5.0, until absorbanace at 280 nm of the eluent decreased below 0.005. The column was eluted by using a gradient from 0 to 0.2 M NaCl. The chromatogram in Fig. 1 showed that the xylanase was eluted from the column at the concentration below 0.05 M NaCl. There were no other protein peaks which were eluted later in the chromatography.

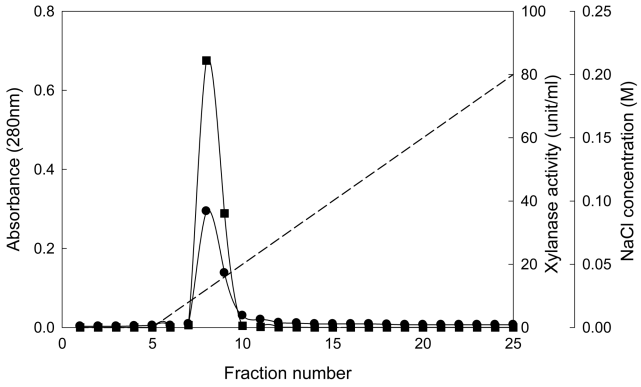

Fig. 1. Ion exchange chromatography of ultrafiltration concentrate from culture supernatant of *Bacillus* sp. A-6 on a column of SP-Sepharose. The column (1.5×10 cm) was washed with 5 mM actetate, pH 5.0 and elution was carried out with a linear gradient from 0.0 to 0.2 M NaCl in the same buffer. Fractions of 10 mL were collected. ●, absorbance at 280 nm; ■, xylanase activity (unit/mL); ---, NaCl concentration.

Ion exchange chromatography on the SP-Sepharose column resulted in dramatic increase of the specific activities of the xylanase samples from 17.8 to 3,130 U/mg, from which purification of 225 fold was calculated. Total protein decreased from 324 to 0.81 mg (Table 1), which suggested that most proteins in the ultrafiltration concentrate were washed from the column with 5 mM sodium acetate, pH 5.0 before elution with the NaCl gradient in the chromatography (Fig. 1).

SDS-PAGE analysis of the ultrafiltration concentrate showed several major polypeptides with molecular weights of 150,000, 23,000, and 10,000 and numerous minor polypeptides (Fig. 2 lane B). The fraction obtained from ion exchange chromatography was shown to contain a single polypeptide with the molecular weight of 15,000 (Fig. 2 lane C). The zymogram using oat spelt xylan as substrate of the fraction showed the clear zone at the same site of the polypeptide (Fig. 2 lane D) suggesting that the single polypeptide in SDS-PAGE gel is xylanase.

FPLC on gel filtration column, Protein Pak Glass Column (Waters) was used to determine the molecular weight of the

Table 1. Purification of xylanase from *Bacillus* sp. A-6.

Step	Vol (mL)	Tota protein (mg)	Tota activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture supernatant	250	589	8,180	13.9	1.0	100
Ultrafiltration concentrate	25	324	5,750	17.8	1.3	70
Ion exchange chromatography	30	0.81	2,530	3,130	225	31

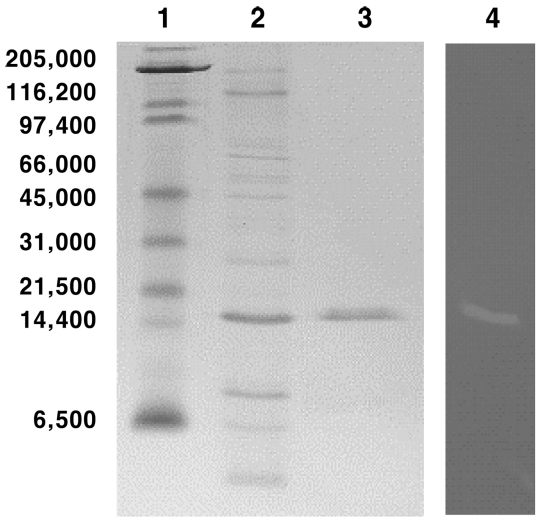

Fig. 2. SDS-PAGE and zymogram of xylanase fractions from purification steps. lane 1, protein molecular weight markers; lane 2, ultrafiltration concentrate; lane 3, purified xylanase; lane 4, zymogram of purified xylanase.

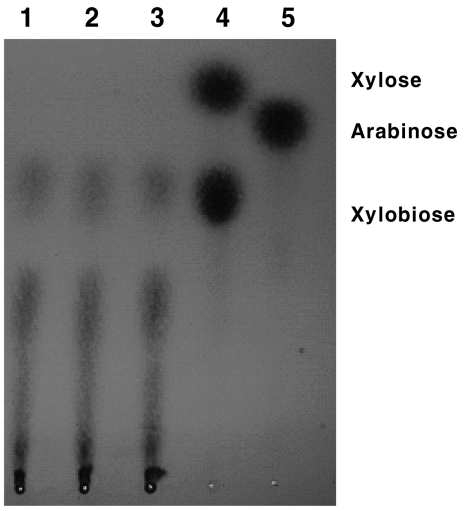

Fig. 3. Thin layer chromatogram of xylan hydrolysates treated with the purified xylanase from *Bacillus* sp. A-6. The xylan hydrolyzates were analyzed after treating oat spelt xylan with the xylanase for 1~2 hr. lane 1, 1 hr; lane 2, 1.5 hr; lane 3, 2 hr; lane 4, xylose and xylobiose; lane 5, arabinose.

purified xylanase in the physiological solution of 0.05 M Tris-HCl, pH 7.5, 0.1 M NaCl (Fig. 3). The ratio of elution volume (Ve) to void volume (Vo) for the xylanase was 2.25. Comparing with the ratios of Ve/Vo for molecular weight markers, the molecular weight of the xylanase was derived to be 14,100, which was similar with 15,000 in SDS-PAGE. These results showed that the xylanase existed in a monomeric protein in the physiological solution.

Thin layer chromatography in Fig. 4 of oat spelt xylan

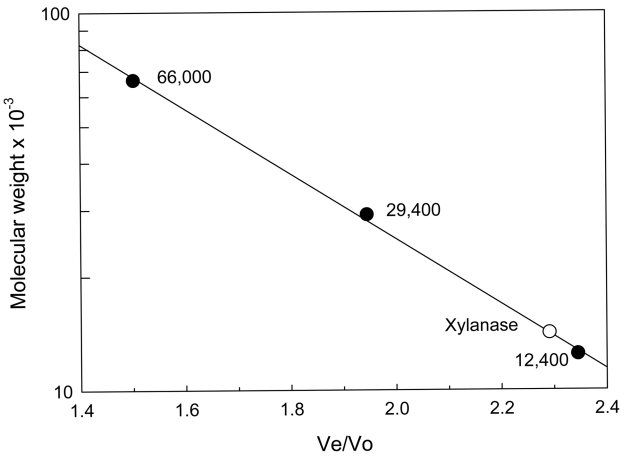

Fig. 4. Molecular weight determination of the purified xylanase using gel filtration chromatography in FPLC. ●: bovine serum albumin (66,000), carbonic anhydrase (29,000), cytochrome C (12,400), ○: xylanase.

which was hydrolyzed with the xylanase for 1, 1.5, and 2 hr showed that the xylan hydrolyzate contained xylobiose and high-molecular-weight xylooligosaccharides, but not xylose and arabinose. There was no difference between the TLC patterns of the xylan hydrolyzates with different incubation times.

Heat stability of the purified xylanase was determined by heating the purified xylanase at 40, 50, and 60°C in a water bath for 1~7 hours and measuring relative activities of the heated samples of the xylanase (Fig. 5). The relative activities were still more than 80% after heating at 40°C for 7 hr. The relative activities remained at 50% at 50°C for 7 hr, but drastically decreased to less than 40% after 1 hr at 60°C, indicating inactivation of the xylanase. These results indicated that the purified xylanase from *Bacillus* SP-6 is relatively heat-labile.

Some strains of *Bacillus* which had ability to hydrolyze xylan were reported to produce two groups of xylanases [7, 9, 16, 21]. One group was basic (pI 8.3 to 10.0) with low molecular weight (16,000 to 22,000) by SDS-PAGE, and the other group was acidic with high molecular weight (43,000 to 50,000). Xylanases purified from *Bacillus circulans* WL-112 had molecular weights of 85,000 and 15,000 which yielded xylose and xylobiose, respectively. The range of optimum pH of both enzymes was 5.5-7.0 [7]. The xylanase from the culture supernatant of *Bacillus* sp. A-6 was adsorbed to SP-Sepharose, a cation exchange gel, in 5 mM sodium acetate, pH 5.0, during the purification, but not to DEAE-Sephacel, an anion exchange gel, in

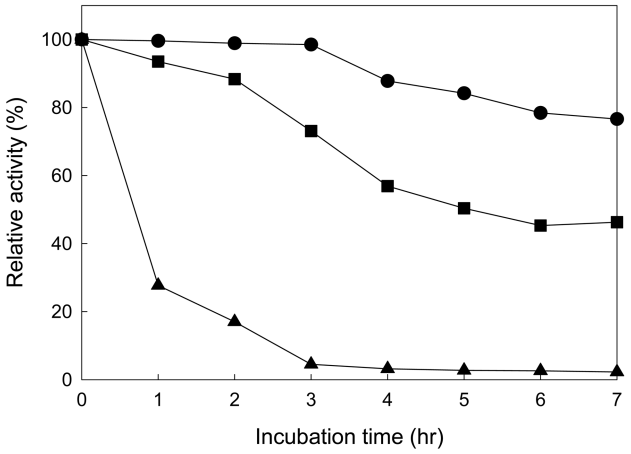

Fig. 5. Thermal stability of the purified xylanase. The xylanase solutions in 0.05M sodium phosphate, pH 7.0 were held at the various temperatures (▲, 60°C; ■, 50°C; ●, 40°C) for 7 hr.

10mM Tris-HCl, pH 8.0, in an initial study for purification (results were not shown), which suggested that the xylanase should be a basic protein. The molecular weights of the xylanase were 15,000 and 14,100 in SDS-PAGE and gel filtration column, respectively. The xylanase hydrolyzed oat splet xylan into xylobiose and xylooligosaccharides. These results suggested that the xylanase in this study might belong to the first group which consists of basic low-molecular-weight proteins. Further studies are necessary to investigate the presence of acidic high-molecular-weight xylanase in the culture supernatant of *Bacillus* sp. A-6.

Many studies concerning purification and characterization of xylanase from *Bacillus* gave focus on thermostable alkalophilic enzymes which may be used for paper bleaching [1, 6, 20]. However, the objective of isolation and characterization of the xylanase and xylanase-producing *Bacillus* sp. A-6 [14] was to develop probiotics which may help degradation of NSP in the animal intestine and reduction of manual output [3, 12]. The xylanase purified in this study was a heat-sensitive enzyme, of which the activity decreased to less than 40% at 60°C after 1 hour, indicating inactivation of the xylanase. Thus, the enzyme itself does not seem to be effective as a feed additive, because it may be inactivated during the manufacture of feed, where the enzyme is often heat-treated during spray-drying of enzyme and pelletizing of feed.

The previous study [14] showed that the optimum pH and temperature for the enzyme activities were 7.0 and 50°C, respectively. The optimum temperature of *Bacillus* sp. A-6 for enzyme production was 30°C. The relatively low temperature and neutral pH for optimal enzyme activity and the low temperature for optimal enzyme production may enable *Bacillus* sp. A-6 to promote degradation of xylan during fermentation of feed and degradation of animal feces at ambient temperature. Thus, the mesotrophic *Bacillus* sp. A-6 producing the xylanase may be applied as probiotics to promote animal production and to prevent environmental pollution.

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국문초록

Bacillus sp. A-6의 Xylanase의 정제와 특성

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Bacillus sp. A-6의 배양액의 상등액으로부터 한외여과와 5 mM sodium acetate, pH 5.0 용액으로 평형화된 SP-Sepharose column을 사용한 이온교환 크로마토그래피에 의해 xylanase를 정제하였다. Column에 흡착된 xylanase는 0.05 M NaCl 이하의 농도에서 용출되었다. 용출된 xylanase가 SDS-PAGE에서 단일 펩티드 밴드로 분리되어 순수함을 확인하였으며 oat spelt xylan을 기질로한 zymogram에서 xylan을 분해하는 밴드로 나타났다. Xylanase의 분자량은 SDS-PAGE에서 15,000이었고 겔여과 크로마토그래피에서 14,100 이었다. 박층막 크로마토그래피에서 xylanase가 oat spelt xylan을 xylobiose와 xylooligosaccharide로 분해함을 보였다. Xylanase를 가열할 때에 상대활성도가 40°C에서 7시간 후에 80%로 감소하였으며 60°C에서는 1시간 후에 40% 이하로 감소하였다.