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Overproduction, Purification, and Characterization of *Bacillus stearothermophilus* Endo-xylanase A (XynA)

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By using a T7 expression system, a large amount of *Bacillus stearothermophilus* endo-xylanase A (XynA) could be produced in *Escherichia coli* cells. The overproduced enzyme formed inclusion bodies, and so the protein could be more easily purified to homogeneity. The molecular weight of the purified enzyme was estimated to be 22 kDa by SDS-polyacrylamide gel electrophoresis and 43 kDa by Sephacryl S-200 gel filtration, suggesting that the native enzyme was a homodimer. The pI value was determined to be 8.4. The Michaelis constants for birchwood xylan and oat spelts xylan were calculated to be 3.83 mg/ml and 5.03 mg/ml, respectively, and the V_{max} values for both xylans were 2.86 μmole/min. The purified enzyme was most active at 55°C and pH 8.0, and stable up to 60°C and in the near neutral pH range. From the zymogram, *Bacillus stearothermophilus* was found to have at least three xylanases and the purified one was the smallest among them.

Xylan is a major component in the cell walls of monocots and hardwoods, representing up to 30% of the dry weight of these plants (28). This polymer is second only to cellulose in natural abundance and represents a major reserve of reduced carbon in the environment. Unlike cellulose, xylan is a complex polymer consisting of a β-D-1, 4-linked xylopyranoside backbone substituted with arabinosyl, acetyl, glucuronosyl, mannosyl, and glucosyl side chains. Therefore, the complete enzymatic hydrolysis of xylan requires the cooperative actions of a range of xylanolytic enzymes including endo-xylanase, β-xylosidase, α-arabinofuranosidase, α-glucuronidase, esterase, mannosidase, and glucosidase (29).

Recently, we have isolated a *Bacillus stearothermophilus* strain capable of producing enzymes necessary for xylan biodegradation (25), and two distinct genes for β-xylosidases (21), two for α-arabinofuranosidases (7), and two for acetylxylan esterases (13, 15) in addition to a gene for endo-xylanase (3) were cloned in *E. coli* from the isolated strain. Furthermore, the endo-xylanase gene (*xynA*) (4) and one (*xylA*) (20) of the β-xylosidase genes were sequenced and one β-xylosidase (19), one α-arabinofuranosidase (8), and two esterases (12, 14) were purified from *E. coli* harboring the recombinant plasmids which contained each of the above genes cloned from *B. stearothermophilus*.

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Before the *xynA* gene was cloned from *B. stearothermophilus*, an endo-xylanase was purified from the strain, and the molecular weight of the enzyme was estimated to be 170,000 Da. by SDS-PAGE (1). As the molecular mass of the endo-xylanase A (XynA) was calculated to be 23,283 Da. by DNA sequence of the *xynA* gene (4), the XynA was thought to be different from the endo-xylanase purified from *B. stearothermophilus* and the purification of the enzyme was attempted. But the endo-xylanase A was produced only in very small quantities from *E. coli* harboring the recombinant plasmid containing the *xynA* gene so the purification of the xylanase was very difficult.

The present work describes overproduction, purification, and characterization of the endo-xylanase A using a newly developed T7 expression system (26).

MATERIALS AND METHODS

Bacterial Strain and Plasmids

E. coli BL21 (DE3) (*hsdS*, g*al*, (λcI*ts*857, *ind*1, Sam7, *nin*5, *lac*UV5-T7 gene 1)) (26) having a copy of the T7 RNA polymerase gene on the chromosome was used as the host for the overexpression of the endo-xylanase gene (*xyn*A). To construct the overproducing plasmid pMGT7, the pT7/T3α-19 (26) vector containing the T7 promoter and the pMG119B26 (4) carrying the entire *xynA* gene, were used.

Chemicals and Enzymes

Oat spelts xylan, birchwood xylan, isopropyl-β-D-thiogalactopyranoside (IPTG), DEAE-Sepharose CL-6B, and Sephacryl S-200 were purchased from Sigma Co. (St. Louis, MO, USA), and the restriction enzymes used in this work and the T4 DNA ligase were obtained from New England Biolab (Beverly, MA, USA). All other materials used were of analytical grade.

Construction of a Plasmid for Overproduction of XynA

To construct the overproducing plasmid pMGT7, the pT7/T3α-19 vector containing the T7 promoter was digested with *Hin*dIII and *Eco*RI, and the 1.3-kilobase *Hin*dIII-*Eco*RI fragment carrying the entire *xynA* gene was excised from the pMG119B26 plasmid which contains the *xynA* gene. Both DNA fragments were ligated and the ligation mixture was used to transform competent *E. coli* BL21 (DE3) cells. The xylanase positive subclone was selected as described earlier (3).

Overexpression of *xynA*

Cells containing the pMGT7 plasmid were grown in LB broth supplemented with 100 μg/ml carbenicillin. When the culture reached A_{600} = 0.6-0.8, the cells were induced for production of T7 RNA polymerase by adding IPTG in a final concentration of 0.4 mM and incubated for an additional 2 h.

A portion (1.5 ml) of the culture was centrifuged and the cell pellet was suspended in 100 μl of 1×SDS gel-loading buffer (50 mM Tris-Cl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The suspension was heated to 100°C for 5 min and various amounts (5 to 30 μl) of the lysate were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12.5% polyacrylamide).

Purification of Endo-xylanase A from the Overproduction System

Cells carrying the pMGT7 plasmid were grown in 2 liters of LB broth supplemented with 100 μg/ml carbenicillin. When the culture reached A_{600} = 0.6-0.8, IPTG was added in the final concentration of 0.4 mM and the culture was then spun with a Beckman JA10 rotor at 6,000 rpm for 20 min at 4°C after incubating for 2 h at 37 °C. The collected cells were resuspended in 40 ml of Tris buffer (50 mM, pH 8.0) and then sonicated until they were lysed. Cell debris was spun down with a Beckman JA20 rotor at 10,000 rpm for 15 min. The separated cell debris was resuspended in 20 ml of Tris buffer (50 mM, pH 8.0) containing 6 M guanidine hydrochloride and spun at 12,000 rpm for 30 min at 4°C, and the supernatant was then diluted gradually to 1,200 ml with Tris buffer (50 mM, pH 8.0). The diluted protein suspension was centrifuged at 12,000 rpm for 30 min at 4°C, and the supernatant was diluted again to 2,200 ml with Tris buffer (pH 8.0). Then, 150 ml of DEAE-Sepharose CL-4B having been equilibrated with Tris buffer (pH 8.0), was added to the diluted protein solution to adsorb proteins. Most of the endo-xylanase A was present in the non-adsorbed supernatant and it was concentrated to 3 ml by ultrafiltration with an Amicon YM10 membrane (molecular cut-off, 10,000). The concentrated enzyme solution was run on a Sephacryl S-200 column (1.6 by 85 cm) equilibrated with the same buffer at a flow rate of 20 ml/hr (2 ml/tube).

Enzyme and Protein Assays

Endo-xylanase activity was determined by measuring the amount of reducing sugar liberated from oat spelts xylan as described earlier (3).

Protein from the column was measured by reading A_{280} (Beckman DU-64 spectrophotometer) and the protein concentration of the enzyme samples was determined by Lowry's method (16) using bovine serum albumin as a standard.

Measurement of Enzyme Properties

The effect of temperature on enzyme activity was assessed by incubating the reaction mixtures at different temperatures in the range from 20 to 70°C. Thermostability was monitored by incubating the enzyme at a fixed temperature and removing aliquots at intervals to test xylanase activity. The pH effect study was done in citrate-phosphate (pH 4-6), phosphate (pH 6-8), Tris (pH 8-9.5), glycine-NaOH (pH 9.5-10.5) buffers at 55°C.

The effect of substrate concentration on endo-xylanase activity was studied by measuring the activity in various concentrations of oat spelts and birchwood xylans ranging from 0.5 to 2.0 mg/ml in Tris buffer (pH 8.0). Incubations were performed at 55°C.

Gel Electrophoresis and Analytical IEF

SDS-PAGE (12.5% polyacrylamide) was performed according to the method of Smith (24) and isoelectric focusing (IEF) was carried out with ampholytes of pH 3 to 10 (Bio-Rad Co.) as described previously (19). Proteins were stained with Coomassie brilliant blue R and densitometric analysis of a gel was carried out with a scanner from Shimadzu (Japan).

In order to detect xylanase activity, the customary SDS-PAGE of the samples was simultaneously carried out on the same polyacrylamide gels, but water was replaced by 0.5% Birchwood xylan in water. Immediately after electrophoresis, the gel was washed four times each for 30 min in Tris buffer (the first two washes contained 25% isopropanol) and the gel was then incubated in the same buffer for 30 min at 55°C. The gel was introduced into a 0.1% solution of Congo red and gently rocked for 15 min. Excess dye was decanted, and the gel was washed with 1 M NaCl until excess stain was totally removed from the active band. After the final rinse in 5% acetic acid, the background turned dark blue facilitating photographic documentation.

Preparation of Proteins for Zymogram from *B.*

stearothermophilus

B. stearothermophilus was grown at 45°C for 16 h in 200 ml of maximal xylanase production medium (25) and the culture was then spun with a Beckman JA10 rotor at 6,000 rpm for 30 min at 4°C. To obtain extracellular proteins, the supernatant fluids collected were concentrated 50 fold by ultrafiltration with an Amicon YM10 membrane (molecular cut-off, 10,000). Intracellular proteins were prepared from whole-cell sonicated extracts as follows; Collected cells were washed twice with Tris buffer (pH 8.0) and resuspended in 5 ml of the same buffer. The suspension was sonicated until cells were lysed and spun down with a Beckman JA20 rotor at 10,000 rpm for 15 min to remove cell debris.

RESULTS AND DISCUSSION

Subcloning and Overexpression of the *xynA* Gene

The xylanase positive subclone, which had formed a clear zone around the colony on the LBX agar medium (LB agar medium containing 50 μg/ml ampicillin and 0.5% oat spelts xylan), was found to have the overproducing plasmid pMGT7. In the liquid culture of the recombinant *E. coli* BL21 (DE3)/pMGT7, the growth rate of cells slowed down after IPTG was added, and the cells overproduced endo-xylanase A of about 22 kDa (Fig. 1). On the basis of the results of densitometer scanning of the gel, it was estimated that the overproduced endo-xylanase A made up to about 10% of the total cellular protein.

In the best overexpression systems, the proteins overexpressed were reported to yield up to 50% of the total cell protein (11). In our case, the 10% yield of the endo-xylanase A might be due to the weak ribosome-binding sequence of the *xynA* gene because the free energy of binding of the 3' end of *E. coli* 16S rRNA to the SD sequence of *xynA* was -18.8 kcal/mol (4), which was much higher than that of an average *E. coli* SD sequence, -11.7 kcal/mol (17).

Purification of Endo-xylanase A from the Overproduction System

The overproduced endo-xylanase A was in the form of inclusion bodies and present in the cell debris when the cell was broken. As the first purification step, the endo-xylanase A in the cell debris was dissolved in 6 M guanidine hydrochloride. In the second step, DEAE-Sepharose CL-4B resin was used and we found that most of the other proteins were adsorbed to the ion-exchanger but the endo-xylanase A was not adsorbed (Table 1). The next purification step was Sephacryl S-200 gel filtration chromatography, which was repeated twice in immediate succession. The protein purity after the first gel filtration was 86% and that after the second was 98%, as determined by densitometric analysis (Fig. 2). The enzyme activity was observed to comigrate with the protein band (Fig. 2, lane I), and the specific activity of the purified enzyme was 233.9 U/mg (Table 1).

The purification of the endo-xylanase A was made relatively simple by the formation of the inclusion bodies which are characteristic for many overproduced proteins (11, 18). Since proteins in inclusion bodies had been re-

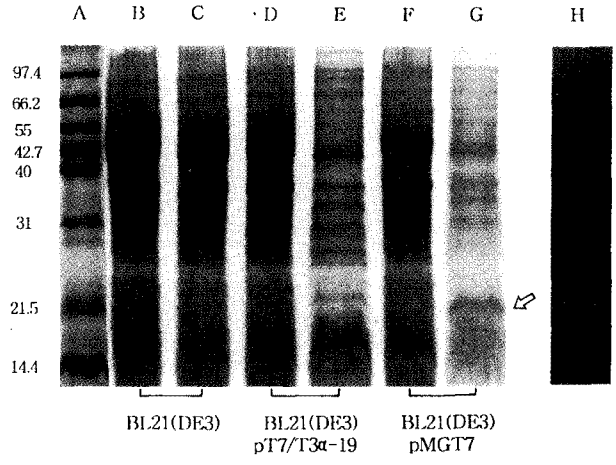

Fig. 1. Overexpression of endo-xylanase A.

SDS-PAGE was performed on a 12.5% polyacrylamide gel. Lane A, protein molecular weight markers; lanes B, D, and F, proteins from the cells grown at 37°C in LB (for lanes D and F, 100 μg/ml ampicillin added); lanes C, E, and G, as for lanes B, D, and F, except that IPTG was added to 0.4 mM when cell density reached $OD_{550} = 0.5$, and then harvested after additional 3 h; lane H, xylanase zymogram analysis of the lane G. The *E. coli* strain and the plasmid in the cell are indicated at the bottom. A sample from each cell lysate was run on SDS-PAGE (12.5% polyacrylamide). Numbers in the left margin indicate mass (in kDa) of molecular weight markers. An arrow indicates the endo-xylanase A.

Table 1. Summary of purification of endo-xylanase A.

Step		Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell lysate		40	128	250	0.5		1
Guanidine hydrochloride extracts		2,020	3,460	150	23.1	100	46.2
DEAE-Sepharose CL-4B		1,950	2,940	24	122.5	85	245
Sephacryl S-200	1st	150	1,475	7.6	194.1	43	388.2
	2nd	60	725	3.1	233.9	21	467.8

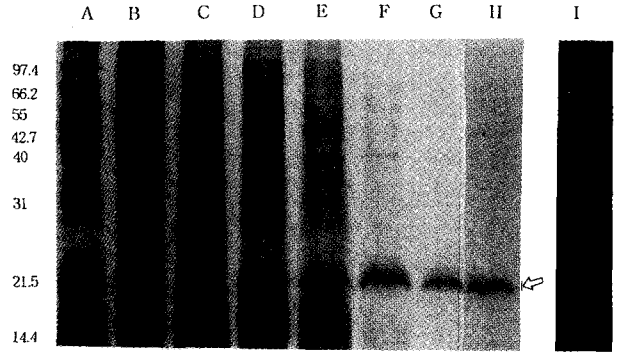

Fig. 2. SDS-PAGE monitoring of the major steps of endo-xylanase A purification.

SDS-PAGE was performed on a 12.5% polyacrylamide gel. Lane A, protein molecular weight markers; lane B, crude lysate of host cell, BL 21(DE3); lane C, crude lysate of host cell harboring pT7/T3α-19 plasmid; lane D, crude lysate of host cell harboring pMGT7 plasmid; lane E, diluted supernatant of guanidine hydrochloride extract of cell debris from cells carrying pMGT7; lane F, unadsorbed supernatant after DEAE-Sepharose CL-4B treatment; lane G: the first Sephacryl S-200-purified fractions; lane H, the second Sephacryl S-200-purified fractions; lane I, xylanase zymogram analysis of the lane H. An arrow indicates the endo-xylanase A.

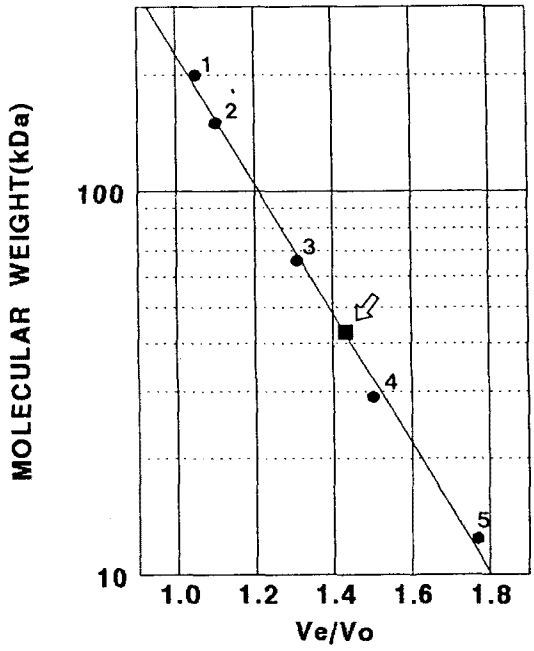

Fig. 3. Molecular weight estimation of endo-xylanase A by gel filtration.

Sephacryl S-200 column (1.6 by 85 cm) was used. The standard proteins used were as follows; 1, β-amylase (200 kDa); 2, alcohol dehydrogenase (150 kDa); 3, bovine serum albumin (66 kDa); 4, carbonic anhydrase (29 kDa); 5, cytochrome C (14.2 kDa). An arrow indicates the endo-xylanase A.

ported not to be active, we extracted the endo-xylanase A from the inclusion bodies and cell debris by using denaturing and renaturing conditions (9, 18). The denaturing agent of guanidine hydrochloride was successfully performed in this work.

Physicochemical Properties of Endo-xylanase A

The molecular weight of endo-xylanase A was estimated by SDS-PAGE to be approximately 22 kDa (Fig. 2). This value corresponded closely to that calculated by the DNA sequence of the *xynA* gene (4). The native endo-xylanase A was thought to be homodimer of 22 kDa subunits because the molecular mass estimated by Sephacryl S-200 gel filtration was about 43 kDa (Fig. 3). The pI of the endo-xylanase A was determined to be about 8.4 (Fig. 4). Wong *et al.* (29) proposed that the xylanases from *Bacillus* sp. can be divided into two major types. One is basic (pI, 8.3 to 10.0), with a low molecular mass (16 to 22 kDa), and the other is acidic (pI, 3.6 to 4.5), with a high molecular mass (43 to 50 kDa). The purified endo-xylanase A in this study fitted the first type.

Effect of Substrate Concentration on Enzyme Activity

Reaction velocities of the hydrolysis of birchwood and oat spelts xylans each at various concentrations were measured, and the Michaelis constants (K_M) were estimated from a Lineweaver-Burk plot (Fig. 5). The K_M values calculated for birchwood xylan and oat spelts xylan were 3.83 mg/ml and 5.03 mg/ml, respectively. The values of maximal velocity (V_{max}) for birchwood xylan and oat spelts xylan were calculated to be the same as 2.86 μmole/min. These results indicate that the endo-xylanase A has higher affinity for birchwood xylan than oat spelts xylan.

Effect of Reaction pH on Enzyme Activity and Stability

The activities and stabilities of the endo-xylanase A at various pH values were measured by using oat spelts xylan as the substrate. The reaction pHs were adjusted to the range of pH from 4.0 to 10.5 with several different buffers (Fig. 6 and Fig. 7) and other experimental conditions were the same as those for the standard assay. Most of the xylanases from alkaliphiles described earlier had pH optima in the near neutral range (6, 10, 22, 23, 27), and the pH optimum of the endo-xylanase A purified in this study was also in the near neutral pH (pH 8.0). The endo-xylanase A was most stable at pH 7.0 and unstable at alkaline pH ranges.

Effect of Temperature on Enzyme Activity and Stability

The optimum temperature for enzyme action was determined by varying the reaction temperature at pH 8.0. As shown in Fig. 8, the endo-xylanase A was most active at 55°C. The enzyme solution was heated for 5 h at temperatures ranging from 50 to 70°C and residual ac-

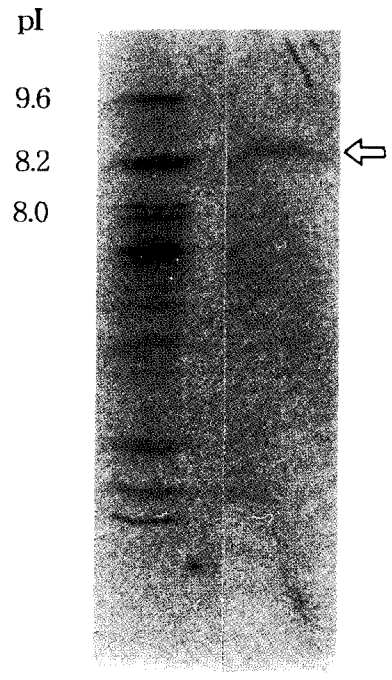

Fig. 4. Isoelectric focusing.
Focusing was carried out on 5% polyacrylamide gel containing biolyte 3/10 (pH 3-10) under constant voltage condition at 200 V for 1.5 h and then at 400 V for 1 h. The standard proteins used (Bio-Rad IEF standards) were as follows (from top to bottom); cytochrome C, pI 9.6; lentil lectin (3 bands), pI 8.2, 8.0, 7.8; human hemoglobin C, pI 7.5; human hemoglobin A, pI 7.1; equine myoglobin, pI 7.0; human carbonic anhydrase, pI 6.5; bovine carbonic anhydrase, pI 6.0; β-lactoglobulin B, pI 5.1; glucose oxidase, pI 4.2. An arrow indicates the purified endo-xylanase A.

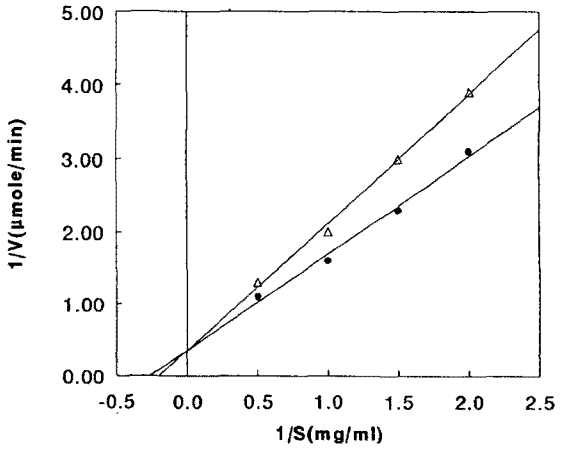

Fig. 5. Lineweaver-Burk plot of the enzyme on birchwood xylan (●) and oat spelts xylan (△).
Enzyme reaction was measured at 55°C in 5-10 min interval with various concentrations of substrate until the maximun velocity was reached.

tivities were measured at intervals of 1 h at 55°C by standard assay. The endo-xylanase A had about 80% resi-

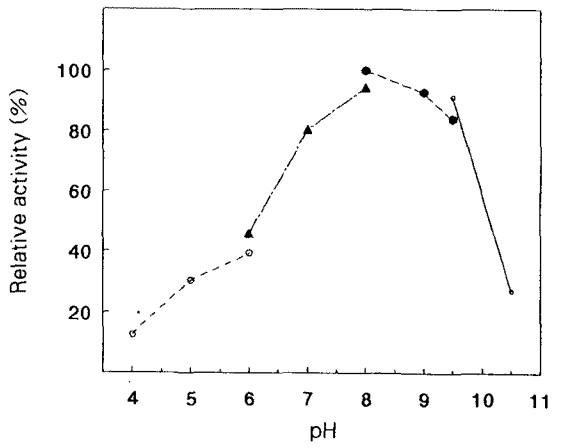

Fig. 6. Effect of pH on endo-xylanase A activity.
Buffers (50 mM) used were as follows: citrate-phosphate buffer (-○-) at pH 4, 5, and 6; phosphate buffer (-▲-) at pH 6, 7, and 8; Tris buffrer (-●-) at pH 8, 9, and 9.5; glycine-NaOH buffer (-⊖-) at pH 9.5 and 10.5. Substrate used for endo-xylanase A activity measurement was 2% oat spelts xylan in each buffer. Enzyme reaction was carried out at 55°C for 30 min. Relative activity was determined relative to the enzyme activity at pH 8.

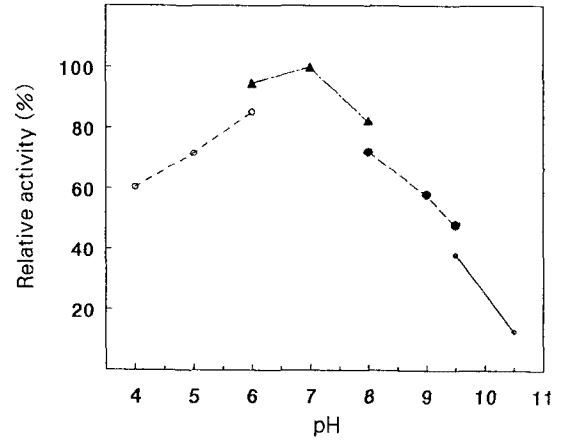

Fig. 7. Effect of pH on endo-xylanase A stability.
Enzyme solutions were exposed to the various pH buffers at 4°C for 12 h. Buffers used were as follows: citrate-phosphate buffer (-○-) at pH 4, 5, and 6; phosphate buffer (-▲-) at pH 6, 7, and 8; Tris buffrer (-●-) at pH 8, 9, and 9.5; glycine-NaOH buffer (-⊖-) at pH 9.5 and 10.5. Substrate used for endo-xylanase A activity measurement was 2% oat spelts xylan in each buffer. Enzyme reaction was carried out at 55° for 30 min.

dual activity after treatment for 5 h at 60°C and 100% at 50 and 55°C (Fig. 9).

Zymogram

The extracellular and intracellular proteins from *B. stearothermophilus* were run on two 12.5% SDS-polyacrylamide gels containing birchwood xylan. After electrophoresis, one gel was stained with Congo red to detect the activity bands and the other with Coomassie

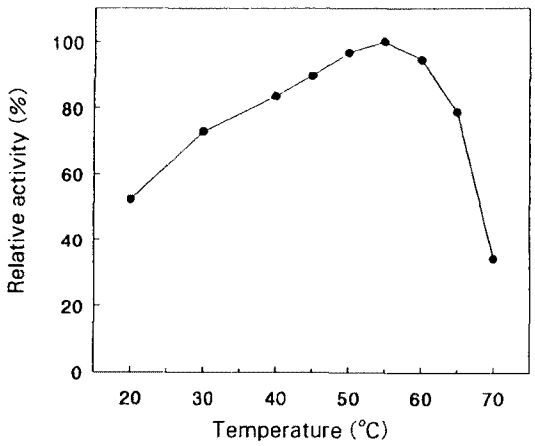

Fig. 8. Effect of temperature on endo-xylanase A activity.
Enzyme reaction was carried out for 30 min at various temperatures indicated in the figure. Relative activity was expressed as percentage, taking the enzyme activity at 55°C as 100%.

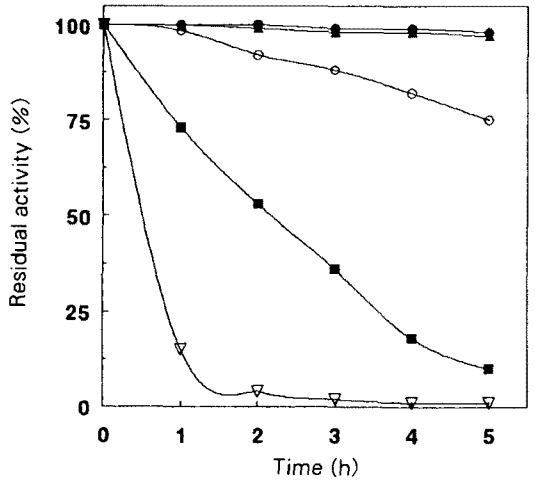

Fig. 9. Effect of temperature on endo-xylanse A stability.
Enzyme activity was measured at 55°C for 30 min allowing the enzyme solution stand at various temperatures in 1 h interval for 5 h. Residual activity was represented as the percentage of the untreated control.
●, 50°C; ▲, 55°C; ○, 60°C; ■, 65°C; ▽, 70°C.

brilliant blue R to detect protein bands. As shown in Fig. 10, in the extracellular fraction three xylanase activity bands could be detected, corresponding to the molecular weights of 94,000, 80,000, and 22,000, respectively, whereas only one band with a molecular weight of 94,000 was detected in the intracellular fraction. The purified endo-xylanase A appeared to be the smallest one among the three xylanases in the extracellular fraction. The multiplicity of xylanases in microorganisms appeared to be a general case (29) and in numerous microorganisms including *Clostridium stercorarium* (2), *Trichoderma reesei* QM9414 (5), and *Aeromonas* sp. (22),

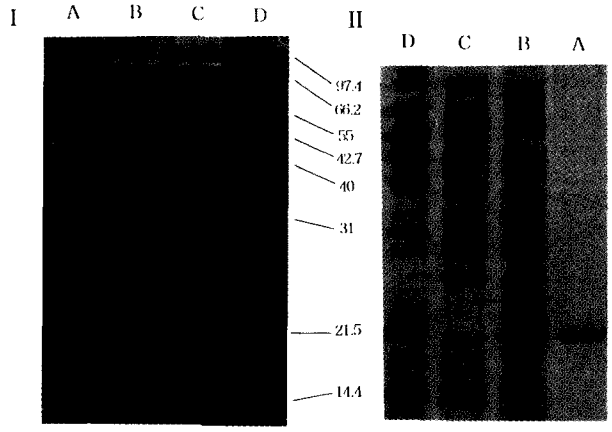

Fig. 10. Zymogram of xylanases from *Bacillus stearothermophilus* and purified endo-xylanase A.
SDS-PAGEs were performed on two 12.5% polyacrylamide gels containing soluble xylan. After electrophoresis one gel was stained with Congo red to detect the activity bands (I) and the other with Coomassie brilliant blue R to detect protein bands (II). Lane A, purified endo-xylanase A; lane B, extracellular proteins from *B. stearothermophilus*; lane C, intracellular proteins from *B. stearothermophilus*; lane D, protein molecular weight markers.

more than three xylanases have been reported.

In the previous study (1), we also purified an endo-xylanase from *B. stearothermophilus*. The xylanase was most active at 55°C and pH 9.0 , and stable up to 55°C and in the near neutral pH range. The properties of the enzyme was similar to that of XynA, but the xylanase showed the molecular weight of 170,000 Da. estimated by SDS-PAGE so the XynA was thought to be different from the endo-xylanase purifed from *B. stearothermophilus*.

The reason why the xylanase activity band corresponding to the molecular weight of 170,000 Da. was not detected is, however, not clear at present. Further study about the multiplicity of xylanases in *B. stearothermophilus* are thought to be needed.

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