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Purification, Characterization and Chemical Modification of the Xylanase from Alkali-tolerant *Bacillus* sp. YA-14

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The xylanase from alkali-tolerant *Bacillus* sp. YA-14 was purified to homogeneity by CM-cellulose, Sephadex G-50, and hydroxyapatite column chromatographies. The molecular weight of the purified enzyme was estimated to be 20,000 Da by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme slightly hydrolyzed carboxymethyl cellulose and Avicel, but did not hydrolyze soluble starch, dextran, pullulan, and *p*-nitrophenyl-β-D-xylopyranoside. The maximum degree of hydrolysis by enzyme for birchwood xylan and oat spelts xylan were 47 and 40%, respectively. The Michaelis constants for birchwood xylan and oat spelts xylan were calculated to be 3.03 mg/ml and 5.0 mg/ml, respectively. The activity of the xylanase was inhibited reversibly by $HgCl_2$, and showed competitive inhibition by N-bromosuccinimide, which probably indicates the involvement of tryptophan residue in the active center of the enzyme. The Xylanase was identified to be xylose-producing endo-type xylanase and did not show the enzymatic activities which cleave the branch point of the xylan structure.

Endo-1,4-β-D-xylanase (β-1,4-xylan xylanohydrolase, EC 3.2.1.8) catalyzes hydrolysis of β-1,4-xylan at random, and is formed in large amounts by many soil microorganisms. The use of xylanase in the utilization of waste biopolymers is under extensive study. There are many reports on the production and purification of xylanases from microorganisms (1-6). But the number of amino acid residues essential for activity and their role in the catalytic site have not been investigated. In our laboratory, several alkalophilic *Bacillus* strains were isolated from soil under alkaline condition (7-9), and they produced various useful enzymes such as cyclodextrin glycosyltransferase, β-galactosidase (10-13). The properties and expression of the gene coding for these enzymes were investigated (5, 14-20). Among these strains, *Bacillus* sp. YA-14 (10) produced large amount of xylanase into the culture broth, and the xylanase gene was cloned (16) and sequenced (21).

In the present paper, we purified endo-1,4-β-xylanase from *Bacillus* sp. YA-14 and the active site of the xylanase was investigated by the chemical modification experiments.

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Key words: xylanase, purification, chemical modification, alkali-tolerant *Bacillus* sp.

MATERIALS AND METHODS

Chemicals

Xylan from larchwood was purchased from Fluka Co. Protein molecular weight standards for SDS-PAGE, CM-cellulose, Sephadex G-50 were obtained from Sigma Chemical Co., St. Louis, Mo. Hydroxyapatite was purchased from Bio-Rad Laboratories, Richmond, Calif. β-mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), phenylmethylsulfonylfluoride (PMSF), *p*-chloromercuribenzoate (pCMB). N-bromosuccinimide (NBS), oat spelts xylan, and birchwood xylan were purchased from Sigma. Co.

Bacterial Culture

Bacillus sp. YA-14 was cultured at 37°C in 5,000 ml of XPM I medium containing 1% Bacto-tryptone, 1.5% soybean meal, 0.5% glucose, and 1.0% NaCl. After 20 hrs of incubation, the culture supernatant was obtained by centrifugation at 8,000 g for 15 min and used as a crude enzyme solution.

Enzyme Assay

Xylanase: Xylanase activity was determined by measuring the amount of the reducing sugar liberated from xylan as described earlier (22).

Arabinofuranosidase: *p*-Nitrophenyl-α-L-arabinofuranoside (pNPA) was used as the substrate. Assays contained 1 ml of 2 mM pNPA (in 10 mM Tris-HCl, pH 8.0), 0.5 ml of enzyme of appropriate dilutions. After incubation for 15 min at 50°C, 1 ml of 1 M sodium carbonate was added and released *p*-nitrophenol was determined from measurements of A_{405}.

Acetyl esterase: Assays for acetyl esterase contained 1 ml of 4 mM *p*-nitrophenylacetate, 1 m*l* of 10 mM Tris-HCl buffer (pH 8.0), and 0.5 m*l* of enzyme at appropriate dilutions. The substrate was dissolved in 50% (v/v) methanol and prepared immediately prior to use. After 15 min of incubation at 40°C, released *p*-nitrophenol was determined from measurements of A_{405}.

Protein assay

Protein from the columns was measured by A_{280}. Protein concentration of the enzyme samples was determined by the Bradford's method (23) with bovine serum albumin as a standard.

Molecular Weight Determination

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli (24). The stacking gel and the resolving gel were 5% and 12.5% acrylamide, respectively. The molecular weight standards used were bovine serum albumin (66,000 Da), egg albumin (45,000 Da), trypsinogen (24, 000 Da), α-lactoglobulin (18,400 Da), and lysozyme (14, 300 Da).

Calculation of the Degree of Enzymatic Hydrolysis of Substrates

The degree of substrate hydrolysis by the xylanase was calculated from the following equation:

$$\text{Hydrolysis degree } (\%) = [A/(T-D)] \times 100$$

A; The amount of reducing power produced by the enzymatic action from the substrate estimated as xylose by the method of Somogyi-Nelson (25)

T; The amount of total sugar of the substrate estimated as xylose by the phenol-sulfuric acid method (26)

D; The direct reducing power of the substrate estimated as xylose by the method of Somogyi-Nelson

Purification of Xylanase

All purification steps were carried out at 4°C.

Precipitation with ethanol: Cold ethanol was added to the culture supernatant to 80% saturation. The precipitates formed were collected by centrifugation at 6,000 g for 10 min, and dissolved in 10 mM Tris-HCl buffer, pH 8.0. The insoluble materials remained were removed by centrifugation.

CM-Cellulose chromatography: The enzyme solution was applied to a CM-cellulose column (4 by 25 cm), previously equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The column was washed with the same buffer, and then with 10 mM Tris-HCl buffer (pH 8.0) containinig 0.5 M NaCl. Xylanase was eluted with a linear gradient of 0.1 to 0.25 M NaCl in 10 mM Tris-HCl buffer (pH 8.0). The flow rate and fraction size were 30 m*l*/hr and 9.0 m*l*, respectively.

Sephadex G-50 gel filtration: The active fractions from the CM-cellulose column were pooled, concentrated by ultrafiltration and applied to a Sephadex G-50 column (1.8 by 95 cm) which had been equilibrated with 10 mM Tris-HCl buffer containing 0.2 M NaCl. Elution was carried out by using the same buffer at a flow rate of 10 m*l*/hr, and 3 m*l* fractions were collected.

The First hydroxyapatite column chromatography: After being pooled and concentrated, the enzyme fractions were applied to a hydroxyapatite column (1.5 by 10 cm) pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.8). The column was eluted successively with the same buffer and with a linear gradient of 0 to 0.4 M sodium phosphate buffer of pH 6.8. Xylanase was eluted with a linear gradient of 0.1 to 0.25 M sodium phosphate.

The second hydroxyapatite column rechromatography: The active fractions from the hydroxyapatite column were pooled, concentrated by ultrafiltration, and applied to a hydroxyapatite column again. The elution conditions were the same as the above.

Thin layer chromatography (TLC)

The sugars released by enzymatic hydrolysis of xylan were separated by ascending TLC on DC-Fertigplatten Kieselgel 60 (Merck Co.) in the solventing system of ethylacetate/pyridine/water (10/3.5/2.5 by vol). Chromatograms were sprayed with a diphenylamine/aniline/phosphoric acid solution [solution A (4 g diphenylamine in 100 m*l* acetone)/solution B (4 m*l* aniline 96 m*l* acetone)/solution C (85% orthophosphoric acid) (5/5 /1 by vol)] and heated at 100°C for 10 min.

RESULTS

Enzyme Purification

The purification of the enzyme was carried out as described in MATERIALS AND METHODS. A 4,720 m*l* batch of *Bacillus* sp. YA-14 culture supernatant was used as the starting materials for enzyme purification. The supernatant containing 566.4 units of xylanase and 660. 8 mg of protein was chromatographed on CM-cellulose. Xylanase activity was found in the fraction of eluent with a linear gradient of 0.1 to 0.25 M NaCl. After pas-

sing through the Sephadex G-50 column, the enzyme fraction was purified through the first hydroxyapatite column and the active fraction was purified through the second hydroxyapatite column twice (Fig. 1). In this procedure, xylanase was purified 60.5 folds and recovered 1.9% of its supernatant activity (Table 1).

Purity and Molecular Weight of the Xylanase

Purified xylanase gave a single protein band on SDS-polyacrylamide gel electrophoresis as shown in Fig. 2. The molecular weight of purified xylanase determined by SDS-polyacrylamide gel electrophoresis was 20,000 Da (Fig. 2).

Substrate Specificity

The substrate specificity of the xylanase was examined by testing its ability to hydrolyze various substrates at 1% as listed in Table 2. Xylan from oat spelts was hydrolyzed to the level of 61% compared to that of birchwood xylan. In addition, carboxymethyl cellulose (CMC) and Avicel were hydrolyzed slightly. Soluble starch, dextran, pullulan, and *p*-nitrophenyl-β-D-xylopyrano side (pNPX) were not able to act as a substrate during incubation for 20 min at 40°C.

Xylan Hydrolysis

The hydrolysis of xylans from birchwood and oat spelts were examined with 1% substrate at 40°C and pH 8.0. Samples were removed at intervals during incubation and analyzed. Time course of xylan hydrolysis is shown in Fig. 3. The maximum hydrolysis degree of xylans from birchwood and oat spelts were 47 and 40%, respectively.

Effect of Substrate Concentration on Enzyme Activity

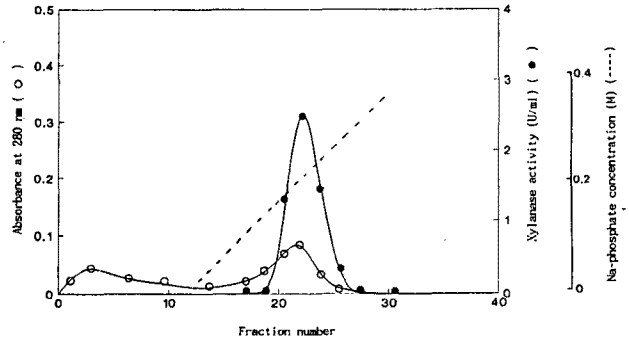

Fig. 1. Hydroxyapatite column rechromatography for the xylanase. The column measured 1.5×10 cm.
The flow rate was 10 ml/hr and fractions of 3 ml were collected.

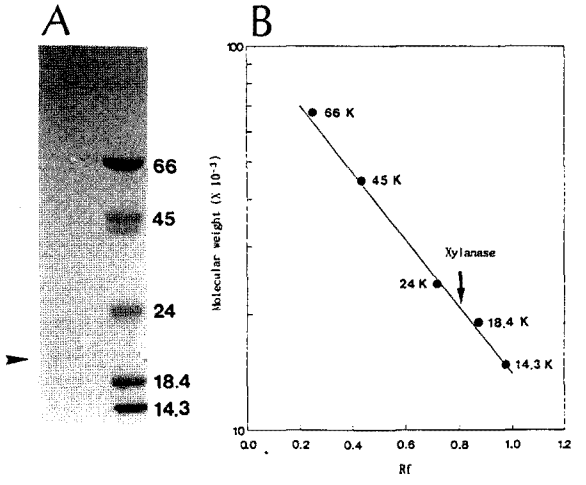

Fig. 2. SDS-polyacrylamide gel electrophoresis pattern (A) and molecular weight determination (B) of prepared xylanase.
Standard protein molecular weight markers: 66 KD, Bovine albumin; 45 KD, Egg albumin; 24 KD, Trypsinogen; 18.4 KD, β-Lactoglobulin; 14.3 KD, Lysozyme.

Table 2. Substrate specificity of xylanases from *Bacillus* sp. YA-14

Substrate (1% (w/v))	Relative degree of hydrolysis (%)
Xylan	
from birchwood	100
from oat spelt	61
CMC	5
Avicel	1
Soluble starch	0
Dextran	0
Pullulan	0
pNPX*	0

*pNPX; *p*-nitrophenyl-β-D-xylopyranoside.

Table 1. Purification of xylanase from *Bacillus* sp. YA-14

Step	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture supernatant	4,720	566.4	660.8	0.85	100.0	1.0
Ethanol precipitation	145	330.8	87.0	3.80	58.4	4.5
CM-cellulose	220	66.0	4.4	15.0	11.7	17.6
Sephadex G-50	33	34.8	2.0	17.4	6.1	20.5
Hydroxyapatite						
1st	33	21.1	1.0	21.1	3.7	24.8
2nd	7	10.8	0.21	51.4	1.9	60.5

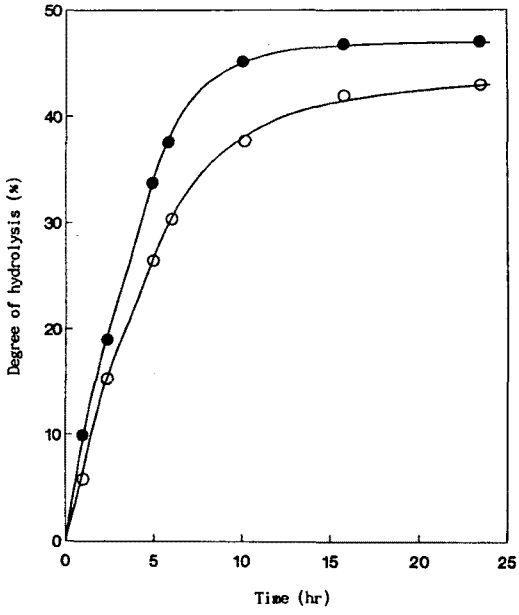

Fig. 3. Time course of hydrolysis of birchwood xylan (●) and oat spelts xylan (○) by xylanase.

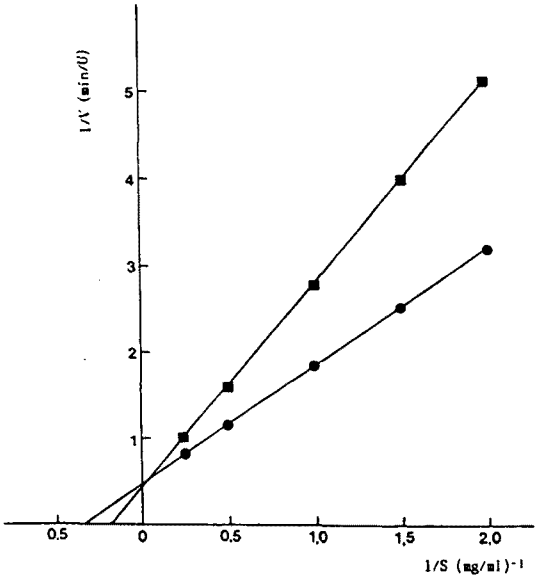

Fig. 4. Lineweaver-Burk plot for the xylanase.
●, Birchwood xylan; ■, Oat spelts xylan.

Reaction velocities of the hydrolysis of xylans from birchwood and oat spelts at various concentrations were measured, and the Michaelis constants (K_M) were estimated from Lineweaver-Burk plot as shown in Fig. 4. The K_M values calculated for birchwood xylan and oat spelts xylan were 3.03 mg/ml and 5.0 mg/ml, respectively. The values of maximal velocity (V_{max}) for birchwood xylan and oat spelts xylan were calculated to be 2.17 μmol/min and 2.27 μmol/min, respectively.

Table 3. Effect of metals on the xylanase activity

Metal ions (1 mM)	Relative activity (%)
None	100
$AgNO_3$	89
$AlCl_3$	97
$MnSO_4$	90
$FeSO_4$	87
$MgCl_2$	85
$ZnSO_4$	90
$CaCl_2$	98
$HgCl_2$	20
$CuSO_4$	94
KCl	94
$NiCl_2$	90
NaCl	101
$BaSO_4$	72
$PbSO_4$	96
$CoCl_2$	101

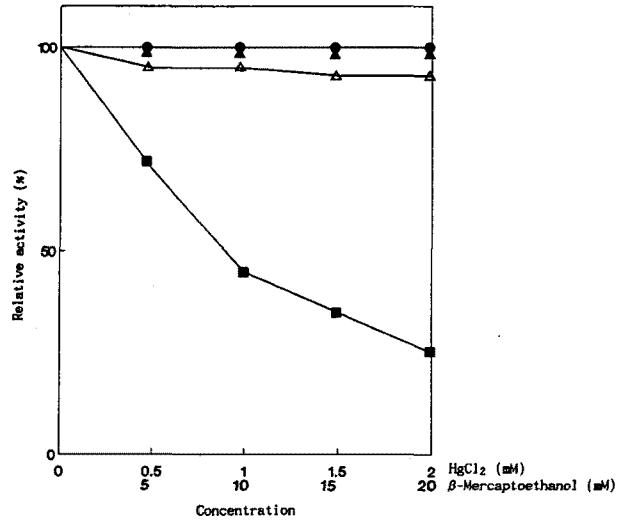

Fig. 5. Effect of β-mercaptoethanol on the $HgCl_2$ inhibition of xylanase activity.
●, β-mercaptoethanol; ■, $HgCl_2$; ▲, pretreatment of β-mercaptoethanol before $HgCl_2$ inhibition; △, addition of β-mercaptoethanol after $HgCl_2$ inhibition.

Effect of Metals

The enzyme solution (100 μl) was mixed with 100 μl of 10 mM Tris-HCl buffer (pH 8.0) containing 2 mM metal ions, pre-incubated at 40°C for 30 min, and then 300 μl of 1% xylan was added. The remaining activity was measured and the enzymatic activity in the absence of metal ions was taken as 100%. The activity of the xylanase was strongly inhibited by Hg^{2+}, but over 70% of relative activities were maintained when other metal ions were used as can be seen in Table 3.

Effect of β-Mercaptoethanol and EDTA on $HgCl_2$ Inhibition

Changes of the Hg^{2+} inhibition of the xylanase was investigated by treatment with the reducing agent, β-mercaptoethanol (Fig. 5). The enzyme activity rapidly decreased in concentrations of over 0.5 mM $HgCl_2$ and maintained 25% residual activity at the concentration of 2 mM $HgCl_2$. The enzyme maintained 100% its activity at the concentration of 2 mM $HgCl_2$ when the enzyme was treated with β-mercaptoethanol before being treated with $HgCl_2$. When the enzyme was treated with β-mercaptoethanol after the treatment of $HgCl_2$, 90% of its original activity was restored. As shown in Fig. 6, the enzyme activity which had decreased due to the treatment of $HgCl_2$, was rapidly restored by the addition of 2 mM EDTA.

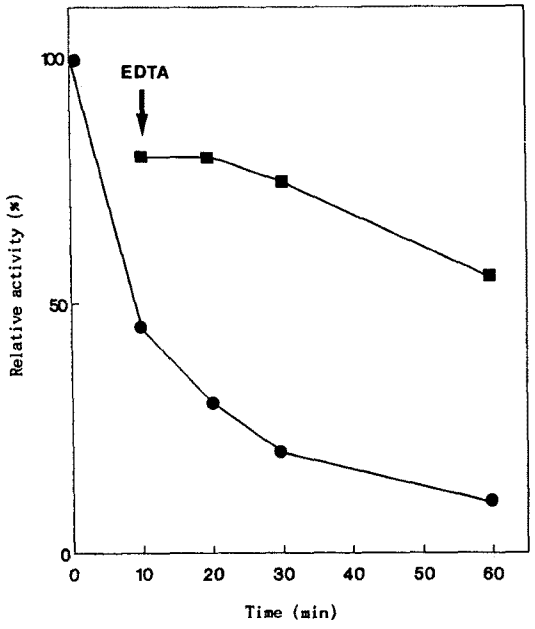

Fig. 6. Effect of EDTA addition on the $HgCl_2$ inhibition of xylanase activity.
●, 1 mM $HgCl_2$; ■, addition of 2 mM EDTA at 10 min after $HgCl_2$ inhibition.

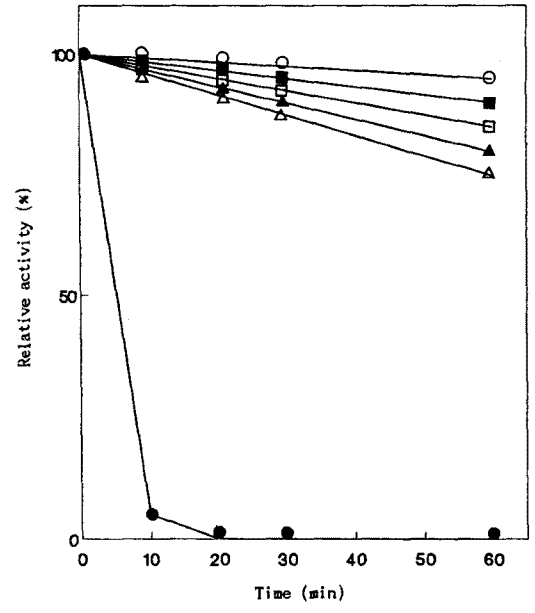

Fig. 7. Effect of inhibitors on the xylanase activity.
●, N-bromosuccinimide; ○, iodoacetate; ■, PMSF and phenylglyoxal; □, pCMB; ▲, hydrogen peroxide; △, I_2.

Effect of Chemical Modifier and Reducing Agent

Fig. 7 indicates the effect of various modifiers on xylanase activity. Xylanase activity was completely inhibited by N-bromosuccinimide during the incubation time of 20 min. Over 75% of the residual activity was maintained when other inhibitors were examined in the experiments. Whereas, reducing agents did not affect the xylanase activity as can be seen in Table 4.

Kinetics of Inhibition of Xylanase

In order to know the inhibition mode of the chemical modifiers, the activity of xylanase on xylan was tested

Table 4. Effect of reducing agents on the xylanase activity

Reducing agent (1 mM)	Relative activity (%)
None	100
β-mercaptoethanol (20 mM)	101
Na-Thiosulfate	94
Na-Sulfite	102
Na-Citrate	101
Cystein	105
L-Ascorbic acid	96

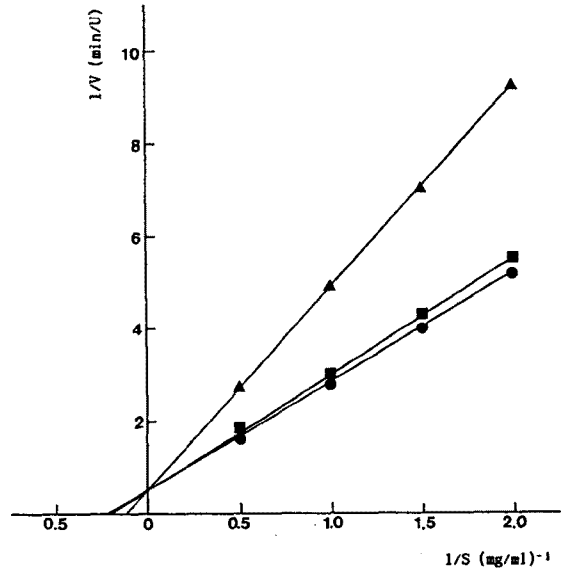

Fig. 8. Lineweaver-Burk plot for the xylanase activity at different concentrations of N-bromosuccinimide.
Enzyme activity is given as producing μmoles of xylose per min in the absence (●), presence of 0.5 (■) and 1.0 (▲) mM N-bromosuccinimide.

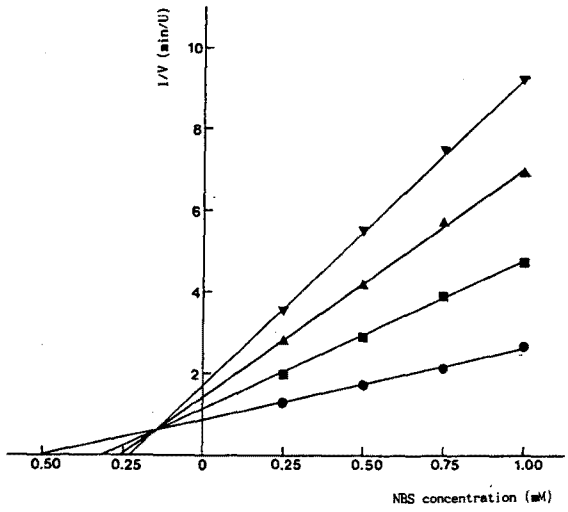

Fig. 9. Dixon plot for the determination of the inhibition constant (K_i) for N-bromosuccinimide on the xylanase activity.
Oat spelts xylan of concentrations; 20 (●), 10 (■), 6.7 (▲) and 5 (▲) mg/ml were used.

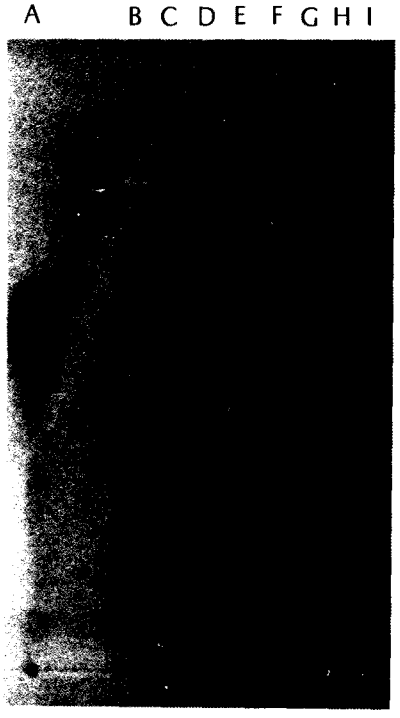

Fig. 10. Thin layer chromatogram of the hydrolysates from xylan by xylanase.
A, xylose; B, birchwood xylan non-digested; C, birchwood xylan digested for 1 hr; D, birchwood xylan digested for 6 hrs; E, birchwood xylan digested for 24 hrs; F, oat spelts xylan non-digested; G, oat spelts xylan digested for 1 hr; H, oat spelts xylan digested for 6 hrs; I, oat spelts xylan digested for 24 hrs.

with different concentrations of the chemical modifier, N-bromosuccinimide, and plotted the Lineweaver-Burk plot and Dixon plot. As shown in Fig. 8, V_{max} value of the xylanase on xylan was 2.00 μmol/min, regardless of the concentration of N-bromosuccinimide. It was clear that the inhibition mode of the N-bromosuccinimide was competitive and the inhibition constant (K_i) was 0.15 mM (Fig. 9).

Mode of Action of the Enzyme on Xylan

To each 0.4 ml of 1% birchwood xylan and oat spelts xylan, 3 units of the xylanase were added and the mixtures were incubated at 40°C for 24 hr. The reaction mixtures were chromatographed on the TLC plate. As shown in Fig. 10, larger oligosaccharides were detected during the early stage of reaction and as the reaction proceeded xylose was detected, regardless of the kinds of the substrates used. Thus the enzyme was found to be a xylose-producing endo-xylanase.

DISCUSSION

The purified xylanase was homogeneous on SDS-polyacrylamide gel electrophoresis and had low molecular weight (MW 20,000 Da by SDS-PAGE). Many fungal and bacterial 1,4-β-glucanases and xylanases show some degree of cross-specificity (27, 28, 29, 30, 31, 32) and xylan is hydrolyzed by two different types of carbohydrate degrading enzymes: cellulases possessing xylanase activity, such as those produced by, *Irpex lacteus* (29), and an extremely thermophilic *Bacillus* sp. (32), and xylanase with no cellulase activity (33). The purified xylanase from *Bacillus* sp. YA-14 also slightly degraded carboxymethyl cellulose and Avicel . The K_M value for oat spelts xylan is higher than that for birchwood xylan, and this results indicate that the xylanase from *Bacillus* sp. YA-14 has higher affinity for the birchwood xylan. This difference of the K_M values may be attributed to the difference in the detailed structure of the substrates.

The active site of the purified xylanase from alkalophilic *Bacillus* sp. YA-14 was investigated through a chemical modification experiment. Xylanase was reversibly inhibited by Hg^{2+}, but its activity was restored by adding the reducing agent, β-mercaptoethanol, or the chelating agent, EDTA. Therefore, xylanase showed reversible inhibition by Hg^{2+}. Xylanase was completely inhibited by N-bromosuccinimide. N-Bromosuccinimide is known to react with tryptophan residue specifically (34). Therefore it was assumed that the tryptophan residue existed in the active site of xylanase. Keskar *et al.* (35) also reported the presence of tryptophan and cystein residues at the active site of xylanase from thermotolerant *Streptomyces*.

Reilly (36) classified the xylanases into the following six groups; 1) β-xylosidase, 2) exo-xylanase, 3) four types of endo-xylanases; a) Those that cannot cleave L-arabi-

nosyl initiated branch points, but produce mainly xylobiose and xylose as a final products. b) Those that cannot cleave branch points, and produce mainly oligosaccharide fragments larger than xylobiose. c) Those that can cleave branch points and produce mainly xylobiose and xylose. d) Those that can cleave branch points and produce mainly xylooligosaccharides of medium sizes. Xylanase from *Bacillus* sp. YA-14 produced xylose during the late stage of the xylan hydrolysis reaction and did not show arabinofuranosidase nor esterase activities. This indicates that the xylanase cannot attack the branch point of the xylan structure. From this result, xylanase from *Bacillus* sp. YA-14 can be classified as an endoxylanase that cannot cleave branch point, but produce xylose.

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