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Purification and Characterization of Xylanase I from *Trichoderma koningii* ATCC 26113

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A xylanase (xylanase I) was purified 11.9-fold from the culture filtrate of *Trichoderma koningii* ATCC 26113 by the column chromatography on Sephadex G-75, SP-Sephadex C-50, DEAE-Sephadex A-50 and Sephadex G-50 with an overall yield of 8.2%. The molecular mass determined by gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis was found to be a monomeric polypeptide of *ca.* 35 kDa. The isoelectric point of the enzyme was estimated to be 9.3. The optimal reaction pH and temperature are 5.8 and 55°C, respectively. The enzyme is stable up to 60°C, while 78% of its activity is lost after the incubation for 10 min at 70°C. The enzyme hydrolyzes xylan with relatively high activity, as well as carboxymethyl cellulose and Avicel. The K_m values of the enzyme for oat-spelt xylan, larchwood xylan and Avicel were 3.5, 1.6 and 10.1 mg/m*l*, respectively. The enzyme hydrolyzed oat-spelt xylan to xylose, xylobiose, xylotriose and arabinoxylobiose, while it degraded larchwood xylan to xylose, xylobiose and xylotriose as the major products. The hydrolysis patterns indicate that xylanase I is endo-enzyme.

KEY WORDS ☐ *Trichoderma koningii*, xylanase I, endo-xylanase

Hemicelluloses are present in association with lignin and cellulose in plant cell walls. A major component of hemicelluloses is xylan, which has recently received increased attention as a renewable resource in the exploitation of plant biomass. It is composed of β-1,4-linked D-xylose polymer with side chains of β-1,3-linked L-arabinofuranose and β-1,2-linked D-glucopyranose, or its 4,*O*-methyl ether. Some xylans are acetylated at carbons 2 and 3 of the xylopyranoside residues. The number and kinds of branch residues are characteristic for different plant groups. Since the structure of xylan is variable, a cooperative action of several different enzymes is required in complete hydrolysis of xylan.

Hydrolysis of xylan could be commercially significant, since some industrial processes, for example, in the pulp and paper industry, release large quantities of xylan in their effluents. The xylose obtained by enzymatic treatment of xylan may be subsequently converted into liquid fuel, single-cell protein, solvents and other chemical products by using fermentative microorganisms.

Many xylanases have been purified from a wide range of microorganisms, such as *Bacillus* (4, 24), *Clostridium* (3, 18), *Streptomyces* (15, 20, 28), *Cryptococcus* (5, 21), *Aspergillus* (12, 13, 25) and other genera. Particularly, endoxylanases from *Trichoderma*, which is one of the best characterized cellulolytic and xylanolytic fungi, have been extensively studied (2, 7, 8).

Trichoderma koningii is an important mycelial fungus and plays a significant role in degradation of xylan. In addition to its multiple endoglucanases and β-glucosidases, *T. koningii* has been shown to possess extracellular endoxylanase activity (32). To increase our understanding of the enzymes from *T. koningii* involved in xylan hydrolysis, we undertook to purify and characterize a xylanase from this fungus.

In the present paper, we report the purification of a xylanase, designated as xylanase I, from *Trichoderma koningii*, its physical and chemical properties and mode of action.

MATERIALS AND METHODS

Microorganism and growth conditions

Trichoderma koningii ATCC 26113 was used for xylanase production. Spore suspensions, prepared from the slant cultures of *T. koningii* in potato dextrose agar medium, were inoculated in Mandels' medium (19) containing (g/*l*): Avicel, 5.0; carboxymethyl cellulose(CMC), 5.0; Bacto-peptone, 1.0; $(NH_4)_2SO_4$, 1.4; KH_2PO_4, 2.0; urea, 0.3; $MgSO_4 \cdot H_2O$, 0.3; (mg/*l*): $FeSO_4 \cdot H_2O$, 5.0; $MnSO_4 \cdot H_2O$,

1.6; $ZnSO_4 \cdot H_2O$, 1.4; $CoCl_2$, 2.0; and (ml/l): Tween 80, 2.0; Antifoam A emulsion, 5.0. The final concentration of conidia was about 5×10^5 conidia/ml. They were grown in 1000 ml flasks containing 250 ml of medium and incubated at 28°C on a reciprocal shaker for 8 days.

Chemicals

Oat-spelt xylan, larchwood xylan, laminarin, polygalacturonic acid, *p*-nitrophenyl-β-D-xyloside (PNPX) and *p*-nitrophenyl-β-D-glucoside (PNPG) was purchased from Sigma (U.S.A.), Avicel from Fluka (Swiss), and CMC from Wako (Japan). All the other chemicals used were of the highest quality generally available.

Enzyme assay

An aliquot of xylanase was incubated for 20 min (xylan, CMC, laminarin and polygalacturonic acid) or for 1 h (Avicel) at 40°C in 0.5 ml of 50 mM sodium acetate buffer (pH 5.0) containing 1% (wt/vol) xylan, 0.5% (wt/vol) CMC, 0.5% (wt/vol) Avicel, 0.5% (wt/vol) laminarin and 1% (wt/vol) polygalacturonic acid. The amount of reducing sugars liberated was measured according to the method proposed by Somogyi-Nelson (26). One unit of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 μmol of reducing sugars per min.

β-Xylosidase and β-glucosidase activity were determined by measuring the amount of *p*-nitrophenol (PNP) released from PNPX and PNPG, respectively. The reaction mixture was composed of 2 mM substrate, 50 mM sodium acetate buffer (pH 5.0) and an appropriate amount of enzyme in a final volume of 0.5 ml. After the incubation at 40°C for 30 min, 1 ml of 1 M sodium carbonate solution was added to the mixture. The mixture was then diluted with 5 ml of distilled water and the absorbance at 420 nm was measured. One unit of enzyme activity was defined as the amount of the enzyme that catalyzed the release of 1 μmol of PNP per min.

Enzyme purification

After 8 day-incubation, the fungal mycelia were filtered through a filter (Whatman No. 1 filter) and the culture solution was precipitated with solid ammonium sulfate between the limits of 20 and 80% saturation. The precipitate was resuspended in 50 mM sodium acetate buffer (pH 5.0; buffer A) and dialyzed against the same buffer at 4°C. The dialyzed solution (50 ml) was applied to a column of Sephadex G-75 equilibrated with buffer A and eluted with the same buffer at a flow rate of 22 ml/h. The active fractions were pooled and fractionated by SP-Sephadex C-50 column equilibrated with buffer A. The enzymes were eluted with 800 ml of buffer A followed by a linear concentration gradient of 0~0.25 M NaCl in the same buffer. The xylanase fractions were collected and concentrated to 18 ml by ultrafiltration through a Diaflo membrane, type 5 (Amicon, U.S.A.). The solution was exchanged for 20 mM sodium phosphate buffer (pH 6.8; buffer B) by repeated dilution and ultrafiltration. The enzyme was fractionated further on a DEAE-Sephadex A-50 column and eluted with buffer B. Final purification was achieved by twice-repeated Sephadex G-50 column and the proteins were eluted from the column with buffer A. Fractions containing xylanase activity were pooled, concentrated and kept at −20°C.

Determination of protein concentration

Protein concentration was determined according to the method proposed by Bradford (6), with bovine serum albumin (Sigma, U.S.A.) as a standard protein.

Molecular mass determination

The apparent molecular mass of the purified enzyme was determined by SDS-polyacrylamide gel electrophoresis according to the method proposed by Laemmli (16). A 10% polyacrylamide gel was run and stained with Coomassie brilliant blue R-250. As standard marker proteins, phosphorylase b (97.4 kDa), glutamate dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa) and trypsin inhibitor (20.1 kDa), purchased from Boehringer Mannheim (Germany), were used.

Isoelectric point estimation

Analytical isoelectric focusing was performed using a Pharmacia Phast system (Pharmacia Fine Chemicals, Sweden). Broad-range pH 3 to 10 IEF gel and calibration kit proteins were used. Pharmacia protein mixture contained amyloglucosidase (3.5 kDa), soybean trypsin inhibitor (4.55 kDa), β-lactoglobulin A (5.2 kDa), bovine carbonic anhydrase B (5.85 kDa), human carbonic anhydrase B (6.55 kDa), horse myoglobin (acidic; 6.85 kDa), horse myoglobin (basic; 7.35 kDa), lentil lectin (acidic; 8.15 kDa), lentil lectin (middle; 8.45 kDa), lentil lectin (basic; 8.65 kDa) and trypsinogen (9.3 kDa), as pI marker proteins.

Kinetic studies

For the determination of K_m and V_{max} values of the purified xylanase for several substrates (oat-spelt xylan, larchwood xylan, Avicel), the enzyme activity was assayed at various concentrations of the substrates. The substrate concentrations were ranged over 0.3125~5 mg/ml for oat-spelt and larchwood xylan, and 1.25~20 mg/ml for Avicel. K_m and V_{max} values were estimated from the Lineweaver-Burk plot.

Analysis of reaction products by HPLC

The reaction products of enzymatic hydrolysis of xylan were analyzed by HPLC. Hydrolyzates were centrifuged in a microcentrifuge (Eppendorf, Germany) at 12,000×rpm for 30 sec in order to remove any solids and the supernatants were filtered through 0.45 μm-filter (Millipore, U.S.A.).

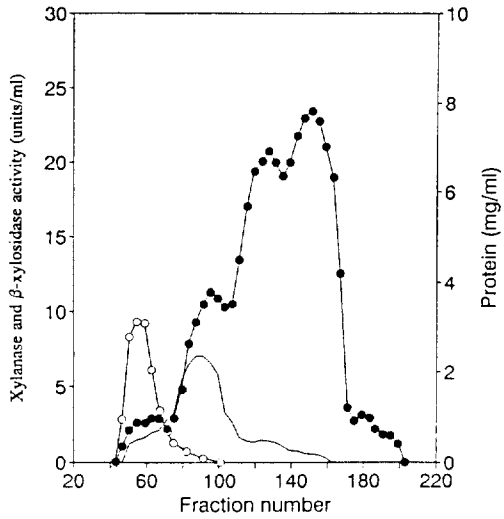

Fig. 1. *Gel filtration chromatography of crude extract from T. koningii on Sephadex G-75.*
The column (3.7×110 cm) was eluted with 50 mM acetate buffer (pH 5.0) at a flow rate of 22 ml/h. Fractions of 6 ml were collected. ●, Xylanase; ○, β-xylosidase; ---, protein concentration.

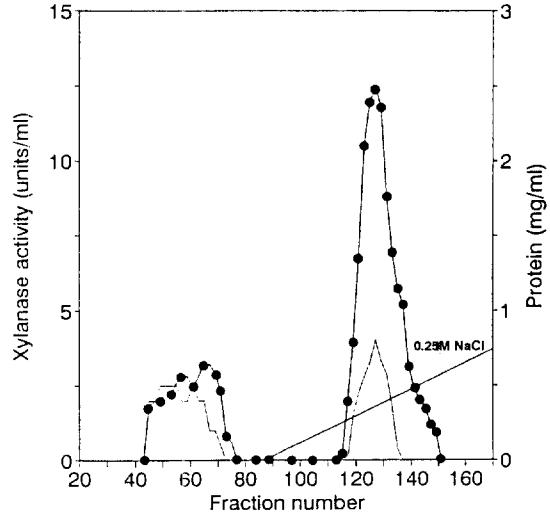

Fig. 2. *Ion exchange chromatography of xylanase on SP-Sephadex C-50.*
The column (2.2×50 cm) was eluted with 50 mM acetate buffer (pH 5.0), followed by a NaCl gradient, 0~0.25 M, at a flow rate of 30 ml/h. Fractions of 6 ml were collected. ●, Xylanase; ---, protein concentration.

These filtrates were analyzed on the μ-Bondapak NH_2 column (Waters, U.S.A.) using 76% acetonitrile as eluant at a flow rate of 1.5 ml/min. The sugar products were detected with a Waters R 401 differential refractometer.

Amino acid composition

20 μg of the purified enzyme was dried in vacuo and hydrolyzed with 1% phenol in 6 N HCl *in vacuo* and oxygen-free nitrogen condition at 105°C for 24h. To the thoroughly dried hydrolyzates, 10 μl of redrying agent [ethanol:water:triethylamine (TEA)=2:2:1 (V/V/V)] was added and the sample was dried again. Then 20 μl of the reagent solution [ethanol:water:TEA:phenylisothiocyanate (PITC)=7:1:1:1 (V/V/V/V)] was added to the sample, reacted for 20 min at room temperature, and dried. To the dried sample, 250 μl of sample diluent (5% acetonitrile in sodium phosphate buffer, pH 7.4) was added and analyzed by HPLC system containing a model 6000 A pump and a Pico-Tag column (Waters, U.S.A.). Samples were eluted by a linear concentration gradient of solvent A (2% sodium acetate, 0.05% TEA, 6% acetonitrile) and solvent B (60% acetonitrile) at a flow rate of 1.0 ml/min.

RESULTS AND DISCUSSION

Purification of xylanase I

Fig. 1 shows the elution profile of xylanase and β-xylosidase on Sephadex G-75 chromatography. The xylanase activity was slightly separated into three main peaks. The first peak with the lowest xylanase activity among three peaks exhibited a higher activity for CMC than for xylan. Therefore, it is suggested that the enzyme corresponding to the first peak is cellulase. Fractions of the second and third peak containing relatively high xylanase activity were pooled and applied to the SP-Sephadex C-50 column. The main xylanase was eluted at about 0.13 M NaCl and separated from minor xylanase proteins (Fig. 2). Further purification was performed on DEAE-Sephadex A-50 column. Xylanase proteins did not adsorbed to the column and eluted as a single peak. On Sephadex G-50 column, the xylanase was partially resolved into two peaks (Fig. 3). Fractions corresponding to the first peak (X 1) was rechromatographed by Sephadex G-50. The xylanase activity was detected in a symmetrical peak, designated as xylanase I (Fig. 4). As shown in Table 1, the enzyme was purified 11.9-fold over the culture filtrate with an overall yield of 8.2%.

Molecular properties

The molecular weight of the purified enzyme was determined by SDS-polyacrylamide gel electrophoresis with standard proteins (Fig. 5). Fig. 6 shows a plot of the relative mobilities of standard proteins versus their molecular weights. From this curve, the molecular weight of the

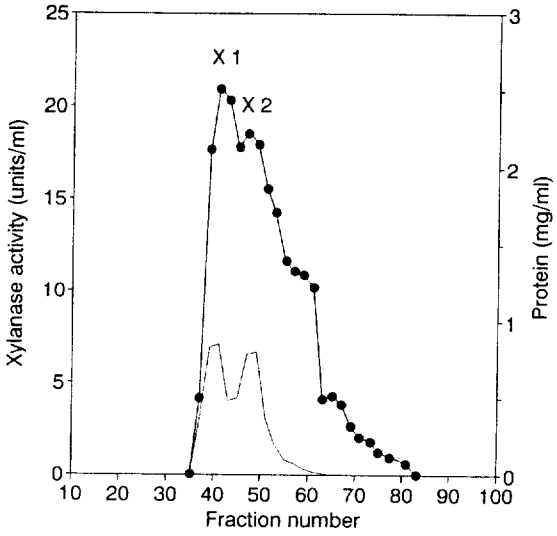

Fig. 3. *Gel filtration chromatography of xylanase on Sephadex G-50.*
The column (2.3×100 cm) was eluted with 50 mM acetate buffer (pH 5.0) at a flow rate of 8.3 ml/h. Fractions of 3 ml were collected. ●, Xylanase; ---, protein concentration.

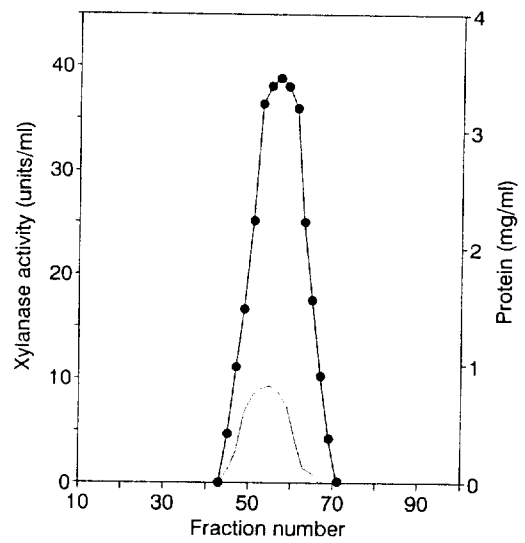

Fig. 4. *Gel filtration chromatography of xylanase I on Sephadex G-50.*
The column (2.3×100 cm) was eluted with 50 mM acetate buffer (pH 5.0) at a flow rate of 8.3 ml/h. Fractions of 2 ml were collected. ●, Xylanase; ---, protein concentration.

Table 1. *Purification steps of xylanase I from T. koningii.*

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
Culture filtrate	986	4390	4.5	100	1.0
Dialyzed ammonium sulfate precipitate	379	4056	10.7	92.0	42.4
Sephadex G-75	68	3102	45.6	70.7	10.1
SP-Sephadex C-50	47	2738	58.3	62.4	13.0
DEAE-Sephadex A-50	29	1795	62.0	40.9	13.8
First Sephadex G-50	10	426	42.6	9.7	9.5
Second Sephadex G-50	6.7	359	53.6	8.2	11.9

enzyme was estimated to be 35 kDa. From Fig. 4, it is suggested that the molecular weight of native enzyme obtained from Sephadex G-50 gel filtration chromatography is in the range of 30~40 kDa. Therefore, the results of molecular weight estimation by SDS-PAGE and Sephadex G-50 clearly indicate that the xylanase is a monomeric enzyme. The enzyme has appreciably higher molecular weight than that of endoxylanase 1 reported by Wood and McCrae (32). The isoelectric point of the purified enzyme was 9.3 (Fig. 7). This value is much higher than that of endoxylanase 1 reported from Wood and McCrae (32). Isoelectric point above 9 has been reported for xylanases from *Trichoderma pseudokoningii* (2), *Bacillus circulans* (10), *Cryptococcus flavus* (23), *Trichoderma viride* (30) and *Aeromonas caviae* (31).

Effects of pH and temperature on the enzyme activity

The enzyme exhibited the highest activity at pH 5.8, and more than 90% of maximal activity was observed in the broad range of 4.3~7.2 (Fig. 8). The optimum temperature was 55°C. The Arrhenius plot of the xylanase indicates an activation energy of 4.27 Kcal/mol from 40 to 55°C (Fig. 9). Below 40°C, there is an abrupt change to 10.49 Kcal/mol. This sharp change at 40°C might result from the change of the rate-limiting from one step to another. In thermal stability, the enzyme had 98% of its activity after 3h pre-

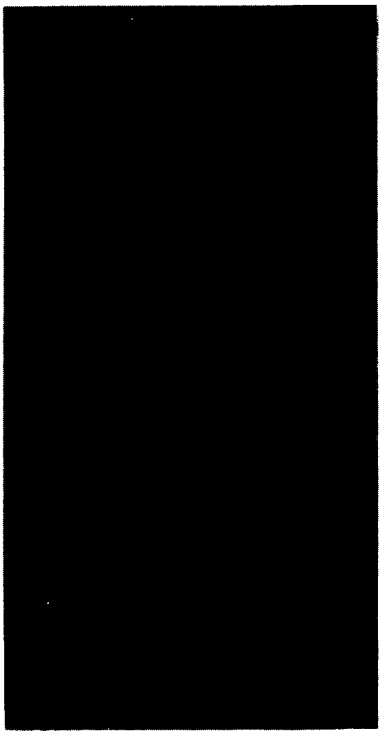

Fig. 5. *SDS-polyacrylamide gel electrophoresis of xylanase I.*
Samples were electrophoresed in 10% gel. Lane A, molecular weight markers: 1, phosphorylase b (97,400); 2, glutamate dehydrogenase (55,400); 3, lactate dehydrogenase (36,500); 4, trypsin inhibitor (20,100). Lane B: xylanase I.

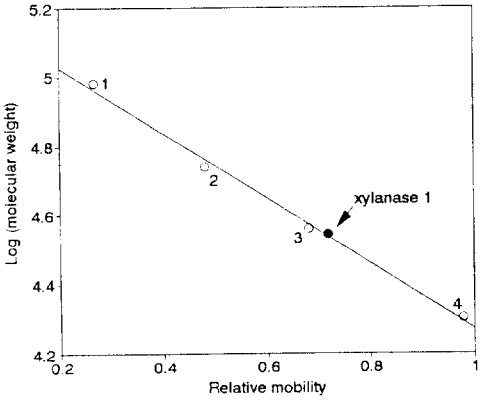

Fig. 6. *Molecular weight estimation of xylanase I by SDS-PAGE.*
Relative mobility was plotted against log molecular weight of standard proteins. The enzyme was indicated by the closed circle. Protein markers are the same as those of Fig. 5.

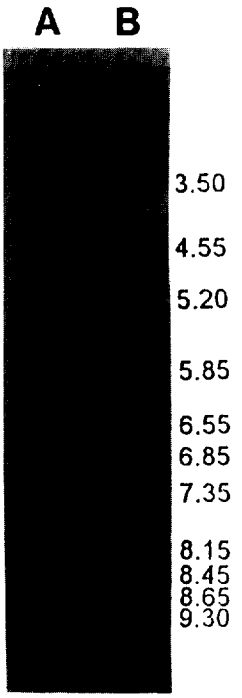

Fig. 7. *Analytical isoelectric focusing of xylanase I on Pharmacia Phast gel using a pH range of 3~10.*
Lane A contains xylanase I. Lane B contains Pharmacia protein mixture. pI values at right are approximate.

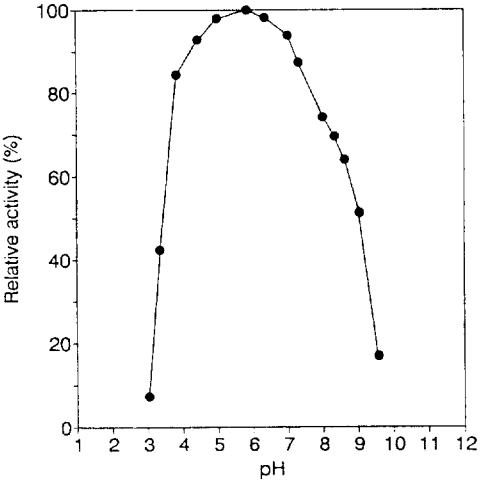

Fig. 8. *Effect of pH on the activity of xylanase I.*
The optimal pH of xylanase was measured at various pH values. The buffer solutions were the following: pH 3.0~3.8, citrate phosphate buffer; pH 4.0~5.8, sodium acetate buffer; pH 6.0~8.0, sodium phosphate buffer; pH 8.2~9.5, Tris-HCl buffer.

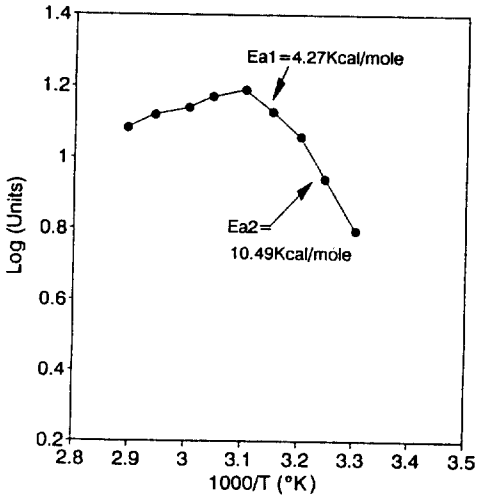

Fig. 9. *Arrhenius plot of xylanase I.*
The optimal temperature was measured by incubating the enzyme at the defined temperatures.

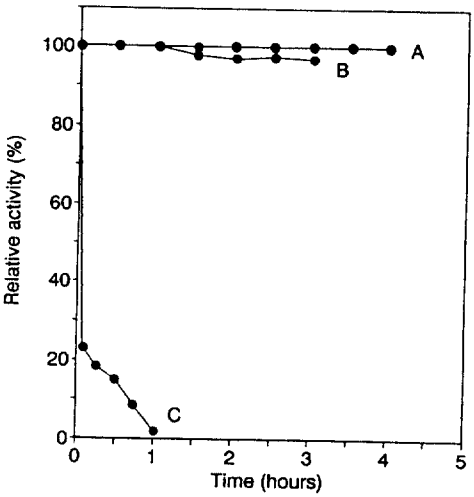

Fig. 10. *Thermal stability of xylanase I.*
The enzyme was preincubated in acetate buffer (pH 5.0) at 50°C (A), 60°C (B) and 70°C (C) in the absence of substrate. The enzyme activity was measured at intervals as described in Materials and Methods section.

incubation at 60°C in the absence of substrate, whereas it lost 78% of its activity after the incubation for 10 min at 70°C (Fig. 10).

Effects of various reagents on enzyme activity

The activity of the enzyme was affected by certain chemicals. At 10 mM, Hg^{2+}, Mg^{2+}, Mn^{2+} and EDTA inhibited enzyme activity, by 86, 37, 32 and 4%, respectively. The enzyme, which was inactivated by Hg^{2+}, was completely reactivated by the addition of 10 mM cysteine. There was substantial stimulation of enzyme activity in the presence of dithiothreitol, Ag^{2+} and Ba^{2+}. The inhibition of enzyme activity by Hg^{2+} was similar to that observed for other xylanases (1, 3, 22, 27). The inhibition by Hg^{2+} and reactivation by the addition of cysteine reveals that thiol-containing amino acids may be involved in the active site of the enzyme.

Table 2. *Activity of xylanase I against various substrates.*

Substrate	Relative activity (%)
Oat-spelt xylan	100
Avicel	44.7
Carboxymethyl cellulose	41.2
Soluble starch	17.0
Polygalacturonic acid	10.8
Laminarin	5.1
p-Nitrophenyl-β-D-xyloside	0
p-Nitrophenyl-β-D-glucoside	0

Substrate specificity

As shown in Table 2, the enzyme was able to hydrolyze xylan as well as CMC and Avicel. The xylanase I also showed slight activity toward soluble starch, polygalacturonic acid and laminarin, but no activity toward PNPX and PNPG. Thus they had no xylosidase and glucosidase activities. Substrate cross-specificity is often found between xylanases and cellulases that hydrolyze polysaccharides which are structurally very similar. Endoxylanases with activity against cellulose are observed among both prokaryotes, such as *Clostridium acetobutylicum* (18) and *Bacillus acidocaldarius* (29), and eukaryotes, such as *Aspergillus niger* (11), *Irpex lacteus* (14) and *Trichoderma reesei* (17). It is not clear whether or not xylanolytic and cellulolytic systems are under separate orcommon regulatory control. Therefore, additional genetic and biochemical studies are needed to clarify the regulation process of xylanases and cellulases in microorganisms.

Kinetic properties

K_m and V_{max} values of the enzyme for several substrates were determined using Lineweaver-Burk plot. K_m and V_{max} values of xylanase I for oat-spelt xylan, larchwood xylan and Avicel were 3.5, 1.6 and 10.1 mg/ml; 35.7, 40.4 and 12.5 units/mg protein, respectively. K_m values of the enzyme for oat-spelt and larchwood xylan were 2.9 and 6.3 fold higher than that for Avicel, respectively.

Analysis of hydrolysis products

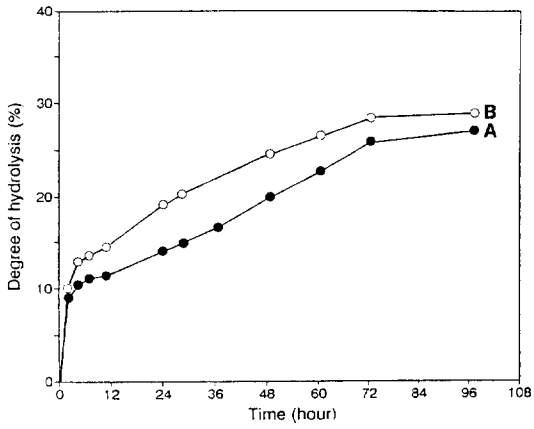

Fig. 11. *Time course of hydrolysis of oat-spelt (A) and larchwood xylan (B) by xylanase I.*
The reaction mixture contained 10 m*l* of each xylan (1% wt/v), 50 mM sodium acetate buffer (pH 5.0), and 0.8 *μl* of enzyme protein. At the times indicated, aliquots were taken and the degree of hydrolysis was estimated from the liberation of reducing sugars.

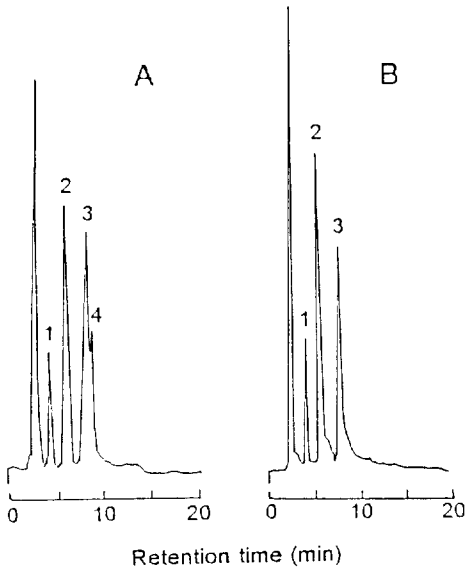

Fig. 12. *HPLC of hydrolysis products of oat-spelt (A) and larchwood xylan (B) by xylanase I.*
The reaction mixture was sampled after 96 h-incubation in the experiment shown in Fig. 11. 1, Xylose; 2, xylobiose; 3, xylotriose; 4, arabinoxylobiose.

The degree of hydrolysis of oat-spelt and larchwood xylan by the purified enzyme was estimated (Fig. 11) and the hydrolysis products

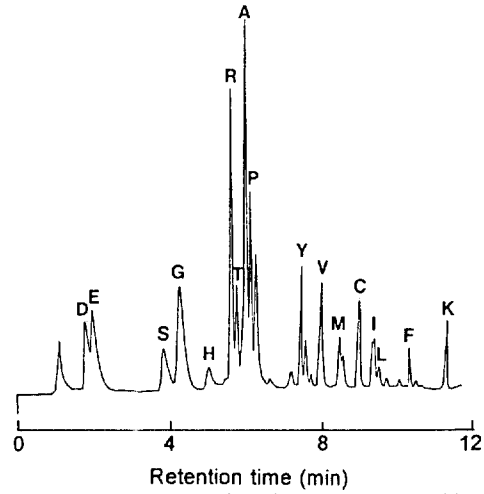

Fig. 13. *HPLC analysis of amino acid composition of xylanase I.*
Abbreviation: D, Asp; E, Glu; S, Ser; G, Gly; H, His; R, Arg; T, Thr; A, Ala; P, Pro; Y, Tyr; V, Val; M, Met; C, Cys; I, Ile; L, Leu; F, Phe; K, Lys.

Table 3. *Amino acid composition of xylanase I.*

Amino acid residue	Number of residue	Abundancy (%)
Asp+Asn	25	8.8
Glu+Gln	39	13.9
Se	27	9.6
Gly	24	8.5
His	4	1.4
Arg	12	4.2
Thr	12	4.2
Ala	35	12.4
Pro	11	4.0
Tyr	5	1.8
Val	20	7.1
Met	8	2.8
Cys	16	5.7
Ile	22	7.8
Leu	11	0.3
Phe	11	4.0
Lys	10	3.5
Total	282	100

were analysed by HPLC (Fig. 12). As shown in Fig. 11, the rate of hydrolysis increased rapidly during the first 3.5 h and then decreased. Xylanase I hydrolyzed 27 and 29% of oat-spelt and larchwood xylan, respectively. The hydrolysis products of oat-spelt xylan by the enzyme after 96 h-incubation were xylose, xylobiose, xylotriose and arabinoxylobiose, whereas the hydrolysis

products of larchwood xylan were xylose, xylobiose and xylotriose. This enzyme did not release arabinose from the arabinoxylan (i.e., oat-spelt xylan). According to Dekker's classification of xylanases (9), the enzyme belongs to the group of "nondebranching xylanases". At an early stage of each reaction, higher oligomers than xylotetraose were accumulated, and as the reaction proceeded, lower oligomers increased. These results obtained above clearly indicate that xylanase I is endo-enzyme.

Amino acid composition

The amino acid composition of xylanase I was analyzed by HPLC (Fig. 13). The number of amino acid residues per molecule of the enzyme was calculated, based on a molecular weight of 35 kDa and from the average contents of amino acids (Table 3). The amino acid composition shows a high percentage of serine, glycine, alanine, valine and isoleucine, while it reveals a low content of histidine, tyrosine and methionine. The xylanase showed similarity to xylanases from *A. niger* (11, 12) and *Bacillus pumilus* (24) in that they have a high content of serine and glycine.

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초 록: *Trichoderma koningii* ATCC 26113으로부터 Xylanase I의 순수분리 및 특성
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Trichoderma koningii ATCC 26113의 배양액으로부터, Sephadex G-75, SP-Sephadex C-50, DEAE-Sephadex A-50 및 두 번의 Sephadex G-50 chromatography를 통해 한 종류의 xylanase (xylanase I)를 순수 분리하였다. 본 효소의 분자량은 35,000으로 측정되었으며 등전점은 9.3이었다. 효소의 활성에 있어 최적 반응온도와 pH는 각각 5.8과 55°C이었다. 본 효소는 60°C까지의 온도에서 안정하였으나, 70°C에서 10분 반응 후 그 활성도의 78%를 손실하였다. 효소는 Hg^{2+}에 의해 불활성화되었으나 cysteine의 첨가로 그 활성을 회복하였다. 본 효소는 xylan을 높은 활성으로 분해할 뿐 아니라 carboxymethyl cellulose와 Avicel도 분해하였다. Oat-spelt xylan, larchwood xylan 및 Avicel에 대한 효소의 K_m값은 3.5, 1.6, 10.1 mg/ml이었다. 본 효소는 oat-spelt xylan을 분해하여 xylose, xylobiose, xylotriose 및 arabinoxylobiose를 생산하였으며 larchwood xylan에 작용하여 xylose, xylobiose 및 xylotriose를 생성하였다. xylan에 대한 효소의 작용양상으로 보아 xylanase I은 endo 유형의 효소로 판단된다.