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PURIFICATION AND CHARACTERIZATION OF XYLANASES FROM *CRYPTOCOCCUS* SP. X-1

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ABSTRACT

A strain of xylanolytic yeast, Cryptococcus *sp X-1, was isolated. Two xylanases were purified from the culture medium by chromatography on DEAE-cellulose and gel filtration on Sephacryl S-300. They lacked activity on carboxymethyl-cellulose. An enzyme of 48 kDa (Xln I) had a K_m of 4 mg/ml and a V_{max} of 28.31 U/mg protein. The pH and temperature optima for the activity were 4 and 50°C respectively. The second enzyme of 23 kDa (Xln II) had a K_m of 7.69 mg/ml and V_{max} value of 603.86 U/mg protein. The enzyme was highly acidic with a maximum activity at pH 2. The optimum temperature was 50°-55°C. Both Xln I and Xln II were stable at 40°C and were inhibited by 10 mM Cu^{2+} and 1% SDS. Xln I and Xln II were classified as debranching type endo-xylanases.*

INTRODUCTION

Xylan is a major component of hemicellulose and is found in large amount of many hard woods and agricultural wastes[1]. It consists of a backbone containing β-1,4-linked xylosyl residues and side chains, including arabinosyl, glucuronosyl, 4-*O*-methyl glucuronosyl, and acetyl residues[2]. Due to its abundant form of biomass second to cellulose, the conversion of xylan to useful products, therefore, presents part of the effort to strengthen the overall economics of the processing of lignocellulose biomass, and also to develop new ways of energy production from renewable resources. Complete breakdown of xylan requires the action of several hydrolytic enzymes, including xylanase (EC 3.2.1.18), β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), *a*-glucuronidase (EC 3.2.1.1), acetyl xylan esterase (EC 3.1.1.6) and *p*-coumaroyl and feruloyl esterases[3-4]. Xylanases are the key enzymes for the breakdown of xylan since they first convert xylan backbone to xylose or short-chain oligomers of xylose. Additional uses for xylanases are found in food processing[2, 5], pretreatment of animal feed[6], wood biopulping and biobleaching of pulp[7, 8]. For the latter applications, enzyme preparations lacking cellulase activity are required[9]. β-xylanases and β-xylosidases are widely distributed among fungi and bacteria and the properties of the enzymes produced have been studied. These microrganisms produce multiple xylanases with various specific activities and are mainly endo-type enzymes which act by a random attack mechanism[10-11]. Reports on their occurrence in yeast and yeast-like microorganisms are very rare[12-14] and studies of xylanase genes from these organisms are few. Among the yeasts, information on xylanase is largely derived from *Cryptococcus albidus*[13]. It secretes β-xylanase, a 48 kDa protein which hydrolyzes xylan into oligosaccharides, mainly to xylobiose and xylotriose. The gene coding for xylanase has been cloned and sequenced[15].

In the present paper we report the isolation of a high-xylanase-producing strain of *Cryptococcus* sp. X-1 that is free from cellulase activity. Two xylanases generated by the yeast were purified and their properties were studied.

MATERIALS AND METHODS

Isolation of Yeast strain

A yeast strain which produced clear zone on xylan medium was isolated. It was identified as *Cryptococcus* sp according to Manual of Clinical Microbiology[16].

Medium and Cultivation

For screening of xylanase producing yeast, a medium containing 0.67% yeast nitrogen base, 1% oat spelts xylan (Sigma) and 2% agar was used. For the production of xylanase, *Cryptococcus* sp. X-1 was grown on a reciprocal shaker at 30°C in a medium containing 0.67% yeast nitrogen base and 1% soluble β-1,4-xylan isolated from oat spelts. Growth of yeast was followed by measuring the absorbance at 420 nm of the cell suspension using cell-free medium as a reference solution. A culture of the microorganism grown in the same medium containing 1% glucose instead of xylan was used as inoculum. Cells harvested from 48 h culture were removed by centrifugation and the supernatant (crude xylanase preparation) was used for xylanase purification.

Enzyme Assays

Activity of endo-1,4-β-xylanase was measured by following release of reducing sugars from oat spelts xylan. The assay mixture of 0.5 ml total volume containing enzyme and 2.5 mg substrate in 50 mM sodium phosphate buffer pH 6.0 was incubated for appropriate time at 40° -50°C. Released reducing sugars were determined as xylose after termination the reaction with dinitrosalicylic acid reagent and boiling for 10 min[17]. Blanks to collect the values for reducing power of the substrate and the enzyme solution were discounted in each case. β-Xylosidase was determined in a mixture consisting of 1 mg of *p*-nitrophenyl-β-D-xylopyranoside in 0.5 ml of 0.05 M acetate buffer (pH 5.4) incubated at 30° C. Reaction was terminated by addition of 1 ml saturated solution of Na_2B_4O (pH 9.4). Released *p*-nitrophenol was determined spectrophotomically at 410 nm.

1 unit of β-xylanase is defined as the amount of enzyme which is capable of liberating from xylan 1 μmole equivalents of xylose in 1 min. 1 unit of β-xylosidase is defined as the amount of enzyme which liberates from the corresponding substrates 1 μmole *p*-nitrophenol in 1 min.

Purification of xylanase

Unless otherwise indicated, all operations were performed at 4°C. A 48 h culture of *Cryptococcus* sp. X-1 on xylan medium was centrifuged to remove the cells. Powdered ammonium sulfate was added to the supernatant (80% saturation). After stirring for one hour, the precipitate was collected by centrifugation at 8,000 g for 15 min. The precipitated enzyme was dissolved in 50 mM sodium phosphate buffer pH 7.0. The solution was dialyzed against a solution of the same buffer.

The dialyzed sample was loaded on a column (2.6 cm x 30 cm) of DEAE-cellulose (Whatman) previously equilibrated with 50 mM phosphate buffer pH 7.0. The resin was washed with the same buffer until the A_{280} returned to the baseline, then eluted with a linear gradient of NaCl (0-1 M) dissolved in the same buffer at a flow rate of 9 ml/h. Active fractions were pooled and concentrated by lyophilization.

The lyophilized sample was redissolved in 3 ml of 50 mM sodium phosphate buffer pH 7.0 and loaded on a column (2.2 cm x 30 cm) of Sephacryl S-300. The column was eluted with the same buffer at a rate of 10 ml/h.

Gel electrophoresis

SDS gel electrophoresis was performed according to Laemmli[18]. Low molecular weight standards (Pharmacia) including phosphorylase B (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used for estimation of molecular weight of enzymes. Proteins were visualized by staining with Coomassie brilliant blue.

Paper Chromatography

Products of hydrolysis of xylan formed upon the action of β-xylanase were separated by chromatography on Whatman no.1 paper in the system ethyl acetate/acetic acid/water (18:7:8, by vol.). Reducing sugars were detected with the silver nitrate reagent.

Determination of kinetic constants

Enzyme was incubated with varying concentration of soluble oat spelts xylan (0.625 - 5 mg/ml). The initial velocity was the slope of the linear portion of reducing sugar formation versus time as measured by linear regression.

Protein sequencing

1-2 μg of sample was dissolved in buffer (125 mM Tris-HCl pH 6.8, 0.1%SDS, 10% glycerol), and placed in the sample wells of a stacking gel and overlayed with Lysilendopeptidase solution (20:1 w/w). After the electrophoresis, sample in the gel was electrotransfered to membrane and sequenced by PSQ-10 SIMADZU protein sequencer[19].

RESULTS

Isolation and characterization of a yeast *Cryptococcus* sp.

We have screened for yeasts that digest xylan from fruits and oil palm pulp. A strain of xylan degrading yeast was isolated. It formed a light pink colony with surrounding clear zone on xylan plate. Figure 1 shows the haloes formed on the xylan plate with strain X-1. The xylan producing yeasts, *Pichia stipitis*, CBS 5775 and CBS 5776, were used as references. The morphology of the vegetative cells of strain X-1 grown in YPD medium for 2 days at 30°C was oval and reproduced by multilateral budding. True mycelium and pseudomycelium were not observed. The color of the colony was yellow to pink. The chemical and morphological characteristics of strain X-1 are shown in the Table 1. and this strain was suggested to be a *Cryptococcus* sp. Activities of β-xylanase and β-xylosidase were predominantly detected in culture fluid and cell homogenate respectively. The time course of production of extracellular activities of β-xylanase during growth on xylan medium was examined. The production of xylanase was associated with growth and the highest enzyme activity of 1.89 units/mg protein appeared in the growth medium at 48 h. Xylanase production was not observed when xylose or glucose was used as a carbon source. The enzyme was inducible upon the addition of xylan.

Purification of the xylanase

The results of enzyme purification are summarized in Table 2. The crude preparation by ammonium precipitation of β-xylanase was chromatographed on DEAE-cellulose column (Fig.2). There were two active fractions of enzyme which were not retained on the column (further referred to as F1 and F2). These two fractions were desalted, concentrated and analyzed on SDS-PAGE. Figure 4 (lane 4-5) shows the protein patterns after staining with Coomassie

Table 1 Physiological and Biochemical Characteristics of Strain X-1.

Fermentation:	–
Production of ammonia from urea:	+
Capsule	–
Pellicle	+
Hyphae	–
Growth at 37°C	+
Assimilation of these carbon compounds:	
D-Glucose	D-Xylose
L-Arabinose	Trehalose
Maltose	Raffinose
Lactose	Starch
Cellobiose	Inositol
D-Galactose	Dulcitol
Sucrose	

Abbreviations: +, positive; –, negative

Table 2 Summary of Purification of Xylanase from *Cryptococcus* sp. X-1.

Fraction	Total volume (ml)	Total Protein (mg)	Total activity	Specific activity (units/mg)	Yield (%)	Purification factor
Culture filtrate	1,200	300	670.8	2.24	100	1
Ammonium precipitate	52	68.64	33.38	0.49	4.98	0.22
DEAE-cellulose	48	85.44	271.64	3.18	40.49	1.42
Sephacryl S-300						
peak 2 (Xln I)	10	3.76	4.35	1.16	0.65	0.52
peak 3 (Xln II)	10	4.13	15.78	3.82	2.35	1.71

Table 3 Specific activity (U/mg protein) of xylanases on different substrates.

Substrate	Crude enzymes	Xln I	Xln II
Oat spelts xylan	1.07	2.4	3.8
Birchwood xylan	1.11	ND	ND
Arabinan	0	0	0
Arabinogalactan	0	0	0
Carboxymethylcellulose	0	0	0
p-NP-α-L-Arabinofuranoside	0.2	0.158	ND
p-NP-β-D-xylopylanoside	0.004	0	0

ND, Not determined. The enzyme assays were made as described in Materials and methods and in Morales *et al.* (29)

Table 4 Effects of various chemicals on xylanases activity.

Reagent	Relative activity (%)	
	Xln I	Xln II
Control	100	100
5% Ethanol	102	98
1% SDS	2.6	4
10 mM KCl	110.6	110
10 mM NaCl	111.9	110.5
10 mM $CaCl_2$	117.4	108.7
10 mM $MgCl_2$	118.9	120
10 mM $ZnSO_4$	114.8	112.2
10 mM $CuSO_4$	31.5	30.3
10 mM EDTA	73.6	80

The enzyme was preincubated with each reagent in 50 mM acetate buffer pH 4.0 for 10 min and residual activity was assayed. The activities are expressed as percentage of the activity in the absence of reagent (control).

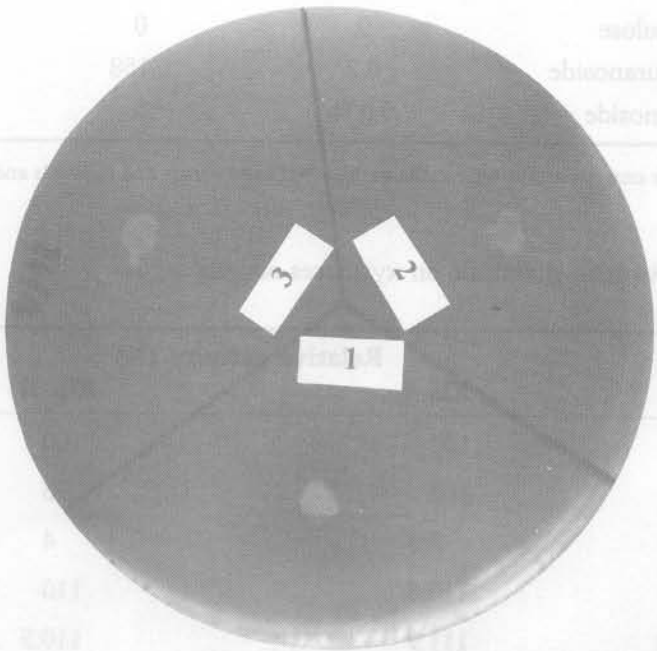

Fig. 1 Xylanolytic Ability of Strain X-1

1, *Cryptococcus* sp X-1; 2, *Pichia stipitis* (CBS 5775); 3, *Pichia stipitis* (CBS 5776). Each yeast was streaked onto the xylan plate and was incubated at 30°C for 2 days.

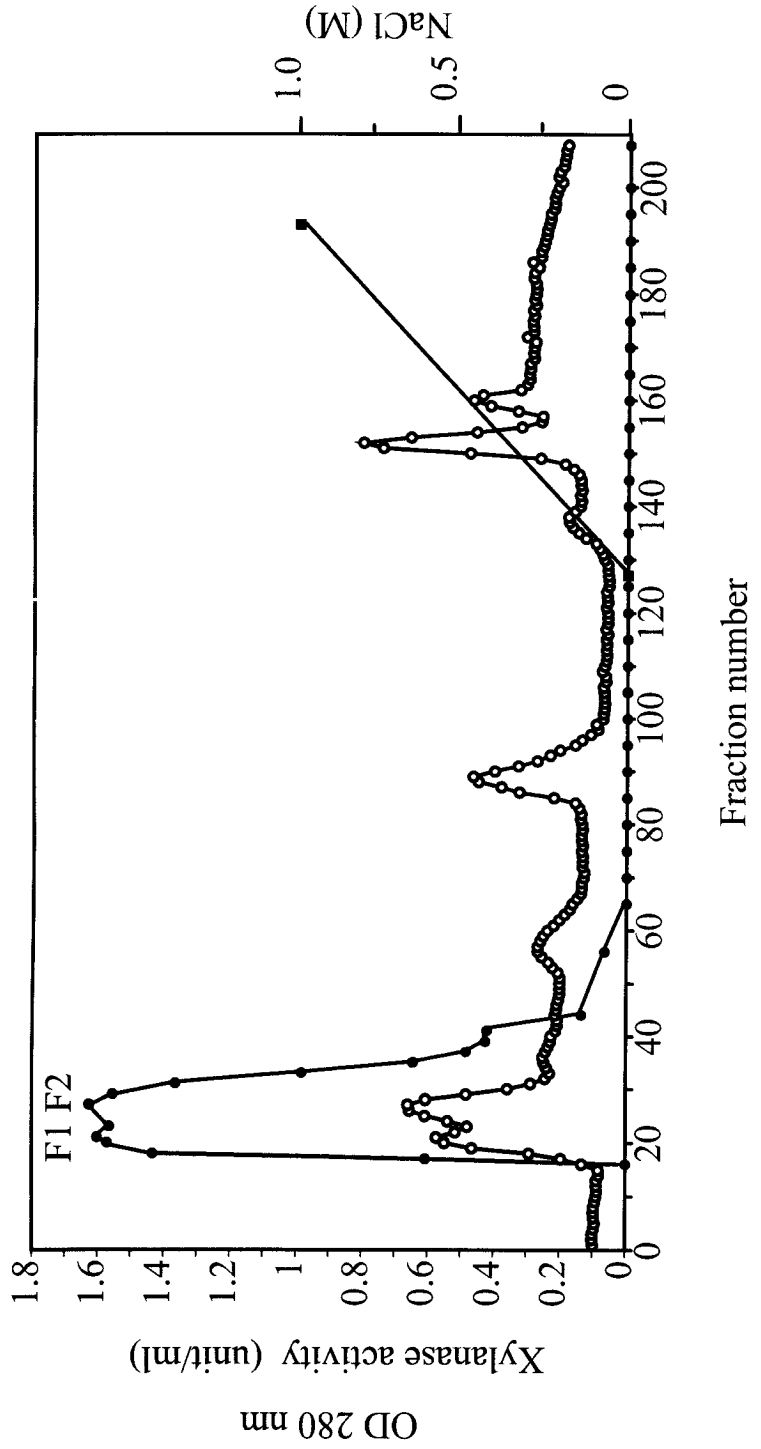

Fig. 2 Chromatography of Crude Xylanase Preparation on DEAE-cellulose
Crude enzyme preparation, columns, buffers, elution rates, fraction sizes and enzyme assays were as described in Materials and Methods.
Open circles denote absorbance at 280 nm, closed circles denote xylanase activity. Straight line denotes NaCl concentration

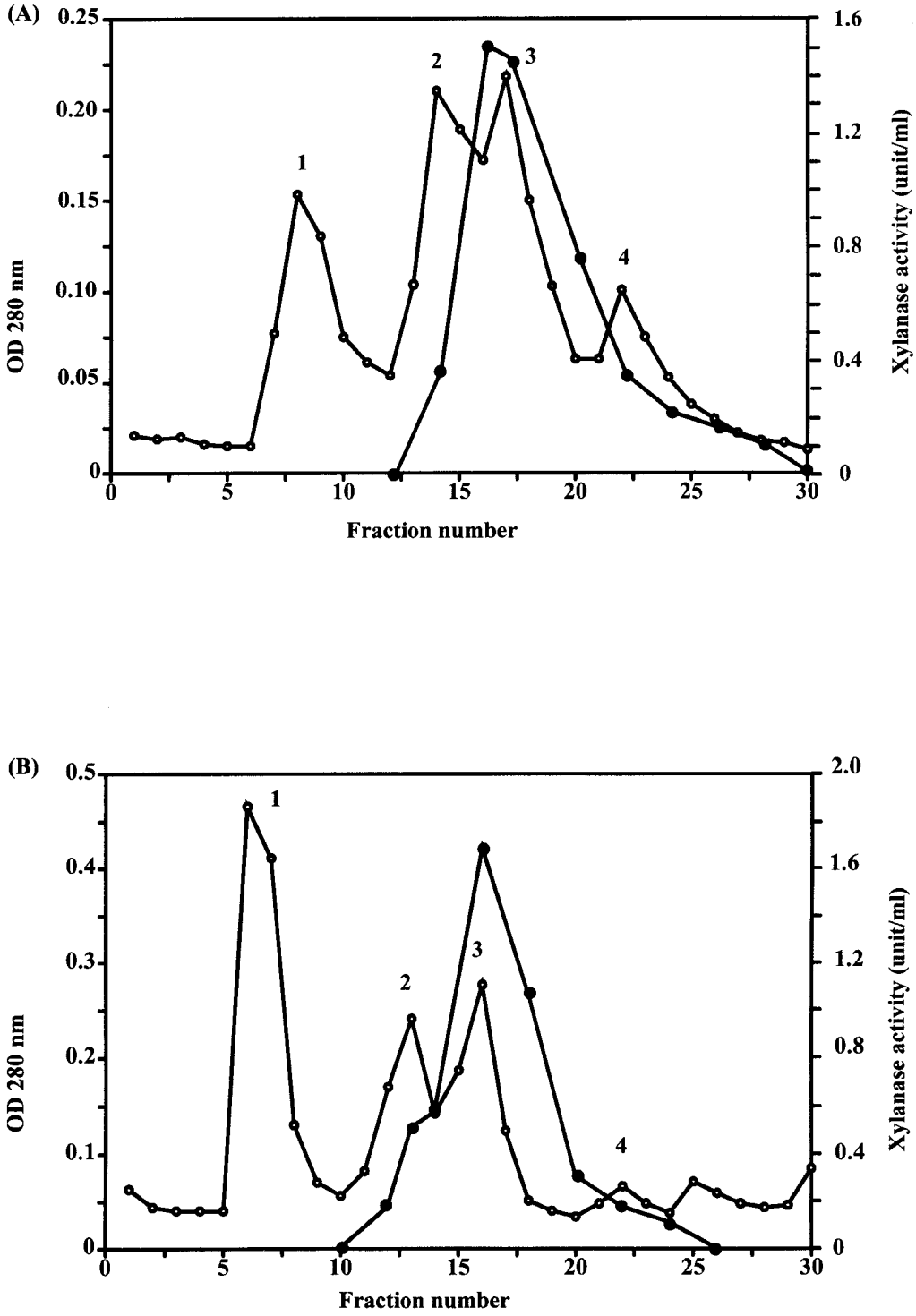

Fig. 3 Gel filtration Chromatography of pool fractions from DEAE-cellulose column

(A) pool fractions from F1 (B) pool fractions from F2

Crude enzyme preparation, columns, buffers, elution rates, fraction sizes and enzyme assays were as described in Materials and Methods. Open circles denote absorbance at 280 nm, closed circles denote xylanase activity.

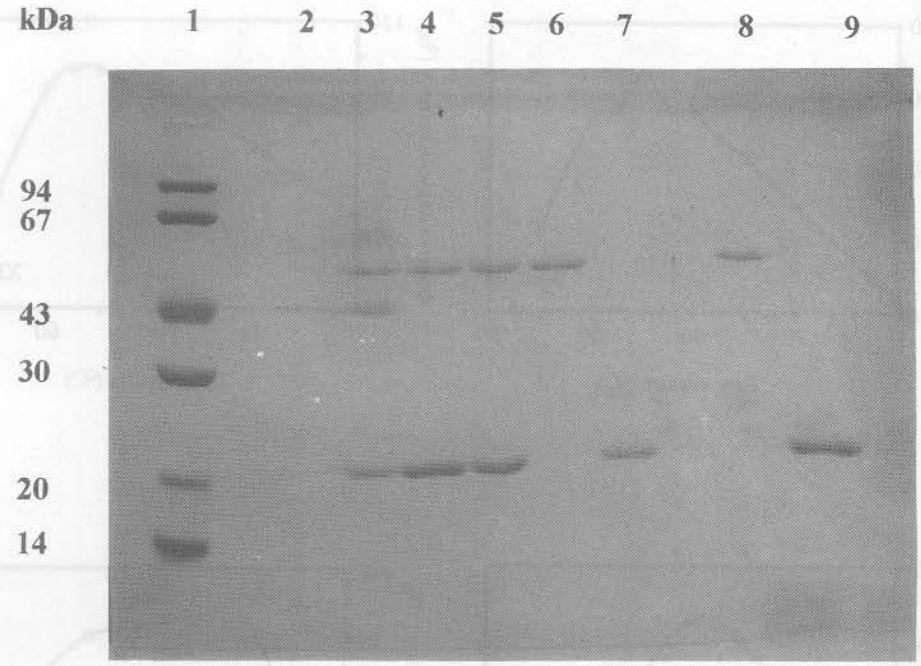

Fig. 4 SDS-PAGE of the Various Stages of Protein Purification.

The gel contained 8% polyacrylamide and was stained with Coomassie brilliant blue. Lane 1, molecular marker (Pharmacia): phosphorylase (MW 94 kDa), albumin (MW 67kDa), ovalbumin (MW 43 kDa), carbonic anhydrase (MW 30 kDa), trypsin inhibitor (MW 20.1 kDa), lactalbumin (MW 14.4 kDa); lane 2, crude enzyme; lane 3, ammonium-precipitated enzyme; lane 4, a pool from DEAE-cellulose (F1); lane 5, a pool from DEAE-cellulose (F2); lane 6, a pool from gel filtration (F1: peak 2); lane 7, a pool from gel filtration (F1: peak 3); lane 8, a pool from gel filtration (F1: peak 4); lane 9, a pool from gel filtration (F2: peak 2).

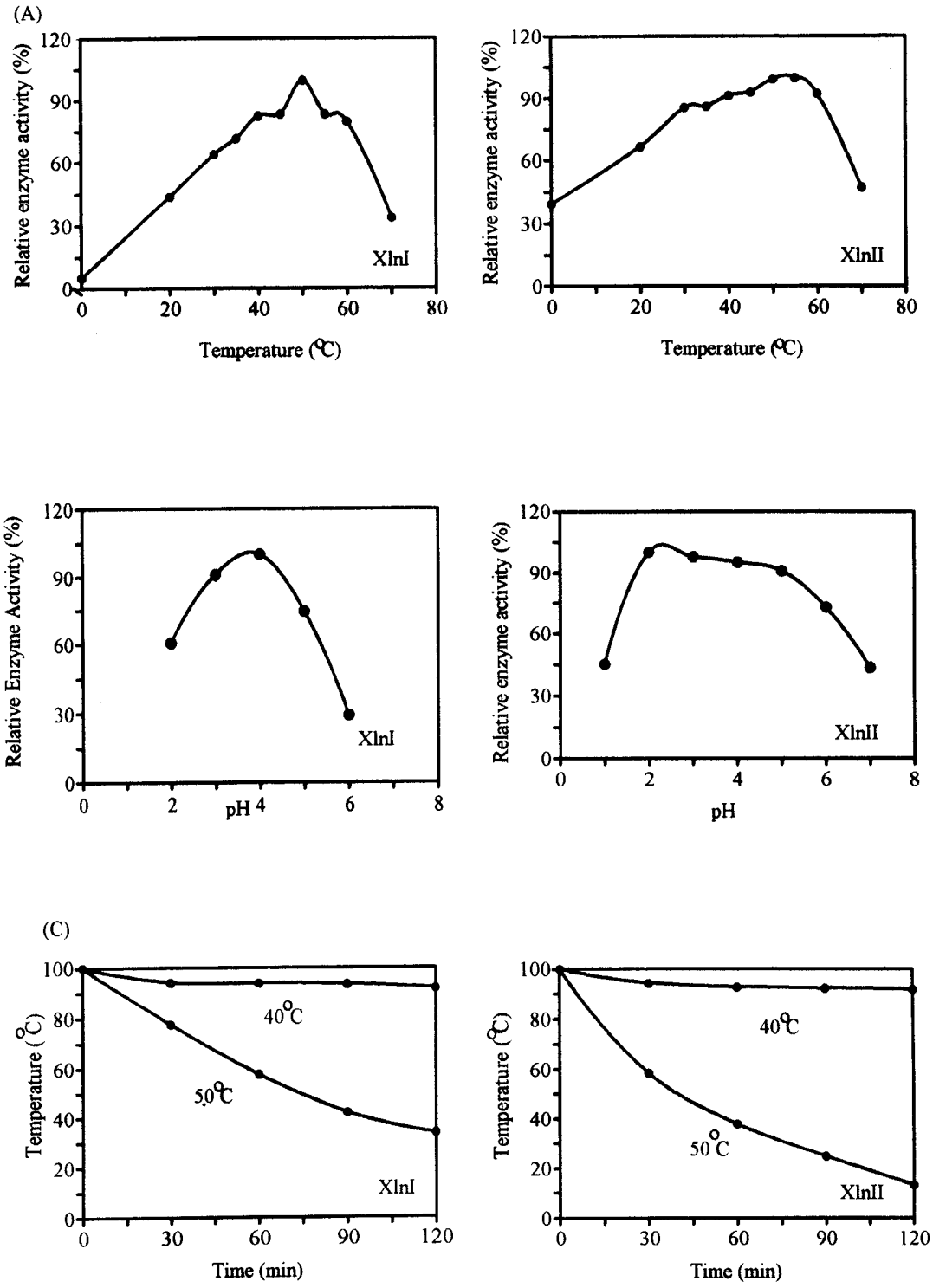

Fig. 5 Enzymatic Properties of Xylanase.

(A) optimum temperature of Xln I and Xln II

(B) optimum pH of Xln I and Xln II

(C) thermal stability of Xln I and Xln II, the enzyme solution was heated for 0.5-2 h, each indicated temperature and residual activity was measured

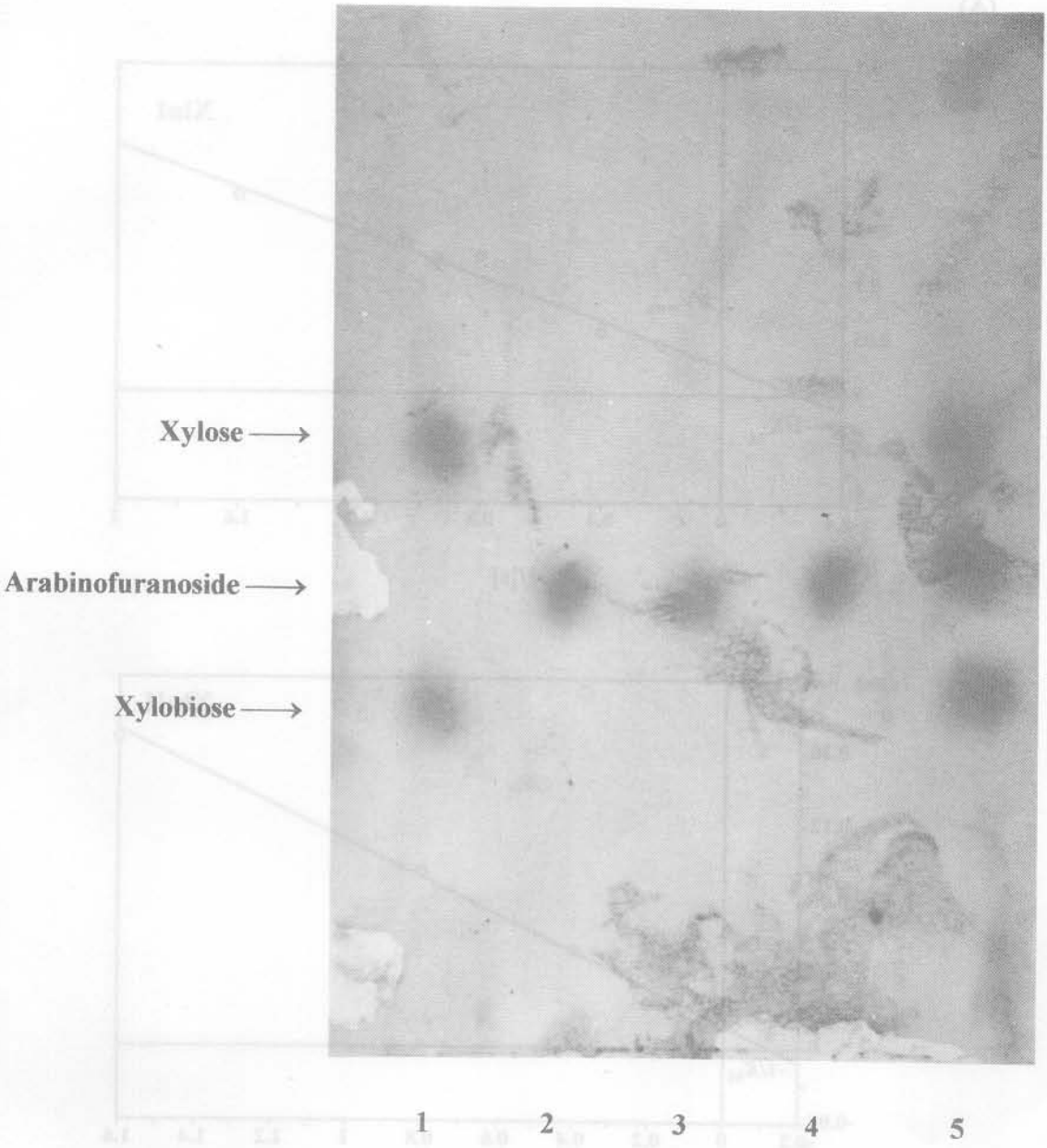

Fig. 6 Paper Chromatogram of Hydrolysis Products of Xln I and Xln II on Oat Spelts Xylan.

1, Standard; 2-4, Hydrolysis products of Xln I at 20 min. 3,

0.25 units of enzyme were incubated at 50°C in solution containing 1% xylan and 50 mM phosphate buffer pH 4.0. At given time intervals, 25 ml aliquots were spotted on the Whatman paper no. 1. 40 mg xylose (X1) and xylobiose (X2) were used as standards.

(Though standard L-arabinofuranoside is not available in this experiment, it was shown in the chromatogram from Kubata et al.[28] that the R_f value of L-arabinofuranoside is in between those of xylose and xylobiose)

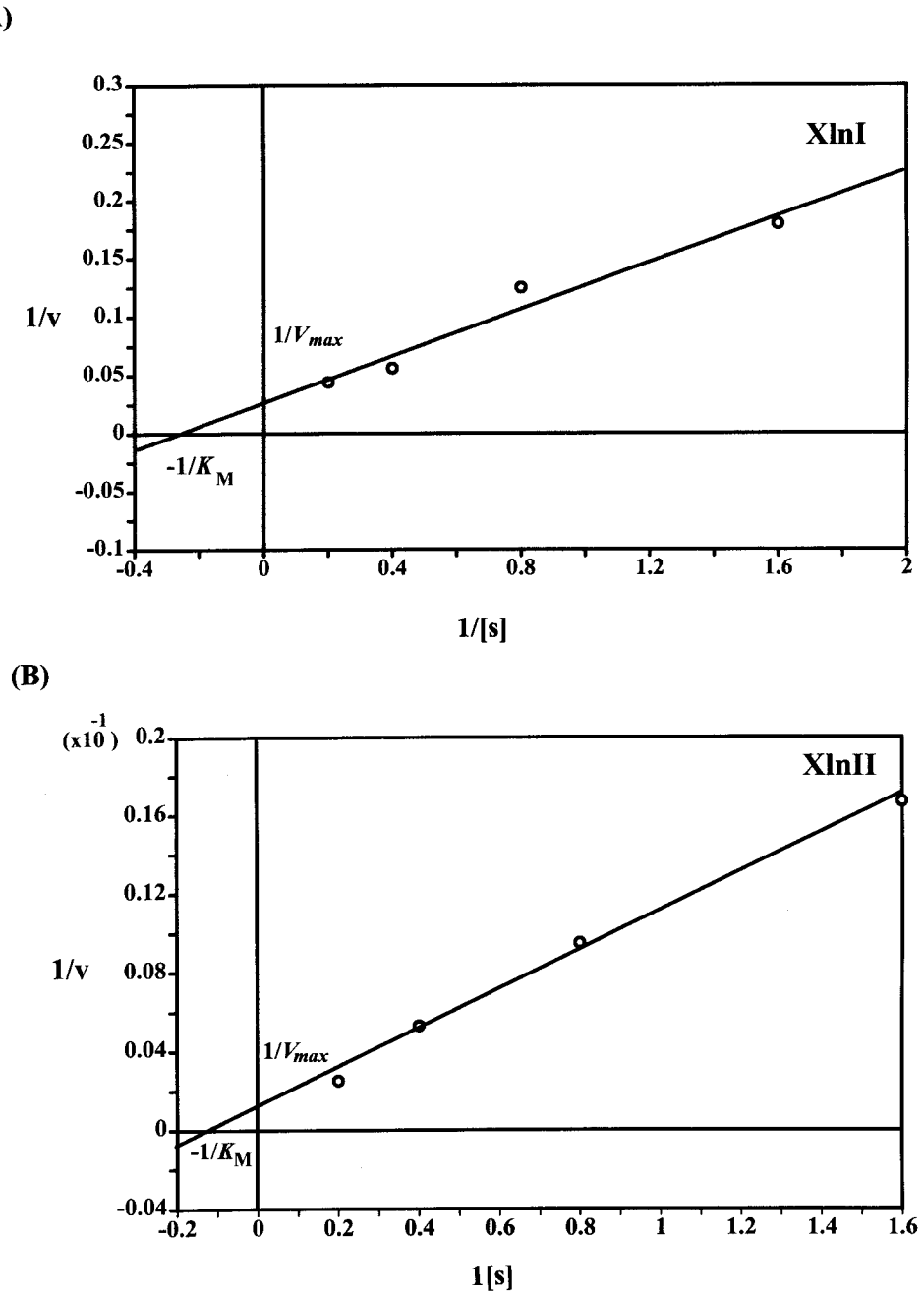

Fig. 7 Lineweaver-Burk plots of XlnI (A) and XlnII (B) activity on oat spelt xylan.

Enzyme (15 mg) was incubated with soluble oat spelt xylan at 50°C and samples were removed and assayed for product formation as described in Materials and Methods. Initial velosity (v) was the mean of the slope of the plot of product formation versus time of duplicate experiments and was expresses as unit of enzyme/ mg protein.

s = concentration of xylan (mg/ml)

brilliant blue, both F1 and F2 contain two polypeptides having molecular weight of 48 kDa and 23 kDa. The reason for separation of the enzyme into two fractions on the used of ion-exchangers may be due to an interaction of the enzyme with a large excess of polysaccharide-containing material[13]. These polypeptides were further purified by subjecting F1 and F2 on separated columns of Sephacryl S-300 column and resulted in separation of proteins into four peaks (Fig. 3, peak 1-4) respectively. Two of them, peak 2-3, showed xylanase activity. The purity of enzymes was examined by SDS-PAGE. Each enzyme recovered from gel filtration was shown to be a discreted protein band (Fig. 4). This result suggested that *Cryptococcus* sp. X-1 released at least two xylanases to the culture medium and the proteins with molecular masses of 48 kDa and 23 kDa were designated Xln I and Xln II respectively.

Substrate specificity

Crude and purified preparations of β-xylanases did not show any ability to liberate reducing saccharides from CM-cellulose, arabinan, arabinogalactan, and *p*-nitrophenol-β-D-xylopyranoside (Table 3)

Properties of the purified enzymes

Effects of temperature and pH on the enzyme activity and stability

Figure 5 shows the effect of temperature and pH on the xylanase activity. The optimum temperatures of Xln I and Xln II are 50° C and 50°-55° C respectively. Xln II shows a very broad temperature range for the activity. The optimum pHs of Xln I and Xln II were 4 and 2 respectively. Such an acid xylanase as Xln II has not been reported in yeast except for the fungal xylanase, Xln C of *Aspergillus kawachii*[20].

Thermal stability of the purified enzymes has investigated by determining the residual activity after incubating the enzymes at different temperatures for 2 h. The enzymes were stable at 40°C, and the activity was lost when incubated at 50°C.

Action of Xln I and Xln II on oat spelts xylan

The hydrolysis of xylan with 0.25 unit of purified Xln I gave final products corresponding to a product with an R_f value correspond to arabinofuranoside. Xln II gave final products corresponding to xylose, xylobiose and arabinofuranoside. These products occurred within a short digestion time (Fig. 6). Under prolonged incubation, there were no changes in the final products (data not shown).

Effect of various chemicals on enzyme activity

From an analysis of the effects of some metal ions and chemicals on the activities of enzymes (Table 4) it was concluded that activities of both enzymes was inhibited by 10 mM Cu^{2+} (80% inhibition), 1%SDS and EDTA (80- 92% inhibition). The acitivities were slightly enhanced in the presence of 10 mM Mg^{2+}

Enzymes kinetics

The K_m and V_{max} values for Xln I (Fig. 7) as calculated by the x and y intercepts in the plot of 1/[s] versus $1/v$ were 4 mg/ml and 28.31 U/mg protein, respectively. Calculated K_m and V_{max} values for Xln II were 7.69 mg/ml and 603.86 U/mg protein. The regression analysis showed R square value of 0.997

Amino acid sequences of Xln II

In order to compare the Xln I and Xln II with other xylanases of known sequences, we attempted to determine the N-terminal sequence of these proteins. Unfortunately, the amount

of Xln I was not enough for the sequencing and N-terminal of yeast proteins are often modified and block the sequencing. Therefore, only the Xln II protein were digested to several peptides which were further sequenced. The partial amino acid sequences of Xln II are concluded as follows:

QTINYVQNYNGNLANFKYNEGAGTYSGGWLAPFDFNI.....

HTEYNQPSIQGTKTFGQYFSVRASKRNSGTVTLGNHFNAWKQYGFANGAANPDF.....

A protein similarity search was done by using SWISS-PROT database. Comparision of amino acid sequences showed that Xln II had about 55-60% similarity with xylanases from *Aspergillus kawachii*[22], *Humicola inolens*[23], *Trichoderma reesei*[24], *Thermonospora fusca*[25] and *Ruminococcus flavefaciens*[26].

DISCUSSION

Xylan is a complex polymer which is made up of a backbone containing β-1,4-linked xylosyl residues and side chains. Complete hydrolysis of xylan requires multi-enzymes with different modes of action acting in cooperation. Different microorganisms produce different kinds of xylanolytic enzymes. Here, we report the isolation of a strain of yeast, *Cryptococcus* sp. X-1 which elaborates two xylanolytic enzymes during growth on oat spelts xylan. One enzyme, which is secreted into culture fluid, was identified as an β-xylanase. The other enzyme, which is found in the cell fraction, was β-xylosidase. Both extracellular xylanase and cell-bound β-xylosidase were induced by growth on xylan but not glucose and xylose. In the production of xylanase by *Cryptococcus* sp. X-1 using oat spelts xylan as inducer, the culture filtrate obtained from the inducing culture was clear, not viscous and gave high enzyme activity. The enzyme was purified by ammonium precipitation and column chromatography. After ammonium precipitation, solution of the redissolved pellet was very viscous due to coprecipitation of a large amount of polysaccharide. The specific activity in concentrated solution was lower than expected, probably because of improper removal of ammonium salt from the solution (Table 2). Chromatography on ion-exchange was necessary to remove other proteins because the xylanase does not bind to the column. The high level of secretion of xylanase was evident from the results of gel filtration, SDS-PAGE of the crude enzyme and from the observed homogeneity after only a threefold purification. The high concentration and specific activity of Xln II suggest that it is the major extracellular xylanase produced by *Cryptococcus* sp. X-1. Xln I and Xln II shares many physical properties with fungal and other yeast xylanases[5, 14], however, the enzymatic properties of the purified enzyme were somewhat different from those of xylanase from *C. albidus*[13, 27]. Our major enzyme (Xln II) has a molecular weight of 23 kDa and the optimum conditions for enzyme activity are different. Result from amino acid sequences of Xln II also confirm that Xln II is a true xylanase. Apart from the homology of amino acid sequences, the enzyme shows some similar properties to Xly C of *A. kawachii,* i.e., small molecular size and low optimum pHs (2.0).

Characterization of the enzyme hydrolysis products from oat spelts xylan indicates that the Xln I and Xln II attacks the polymer at branch points and were classified as debranching type endo-xylanases[21]. This type of mode of action of the enzyme has not been reported in other yeast. In general, endoxylanases are often prevented from cleaving the xylan backbone by the presence of substituents[10] Therefore, these must be removed before extensive degradation of the backbone can occur. This reason made Xln I and Xln II as good choice enzymes for the complete degradation of xylan in industrial uses. In addition, the fact that the xylanase from *Cryptococcus* sp. X-1 does not show cellulolytic activity, create a good opportunity to use the

enzyme in many applications which removal of hemicellulose in specifically are required. More detailed studies of mode of hydrolysis of the enzymes are in progress. Cloning of related genes of xylanolytic enzymes from this yeast strain are also of our interest.

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บทคัดย่อ

การทำบริสุทธิ์เอนไซม์ cyclodextrin glycosytransferase (CGTase) ที่ ผลิตจาก *Bacillus* sp. PS304 โดยการตกตะกอนโปรตีนด้วยแอมโมเนียมซัลเฟตและ DEAE-cellulose คอลัมน์โครมาโทกราฟี ตามลำดับ พบว่า สามารถทำให้เอนไซม์ CGTase มีความบริสุทธิ์เพิ่มขึ้น 29 เท่า เอนไซม์ที่เตรียมได้มีความเสถียรที่พีเอช 4 ถึง 12 แต่มีกิจกรรมสูงสุดที่พี เอช 5 และ 8.5 และมีความเสถียรที่อุณหภูมิสูงสุด 45 °ซ ในขณะที่มีกิจกรรมสูงสุดที่อุณหภูมิ 55 °ซ นอกจากนี้ เอนไซม์ยังมีค่า isoelectric point (pI) เท่ากับ 6.5 เอนไซม์ CGTase นี้มีน้ำหนักโมเลกุล 76 กิโลดาลตัน เมื่อตรวจโดย SDS-PAGE กิจกรรมของเอนไซม์ดังกล่าวถูกยับยั้งได้บางส่วนโดย 10 mM คอปเปอร์ไอออน (Cu^{2+}) และถูกยับยั้งได้ทั้งหมดโดย 3,4-dichloroisocoumarin ที่ความเข้มข้น 1 mM.