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PURIFICATION AND PROPERTIES OF XYLANASE FROM *PICHIA STIPITIS*

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ABSTRACT

A xylanase was purified from the culture medium of Pichia stipitis by chromatography on Sephacyl S-300. The purified enzyme showed a pH optimum of 5 and was stable at pH 3-6. A temperature optimum for the activity was 40 °C and was stable up to 40 °C for 30 min. The apparent molecular mass was 43 kDa from SDS-PAGE. The enzyme had a Km of 1.43 mg/ml and V_{max} value of 1.54 μmol/min. Activity was inhibited by 10 mM Cu^{++}, EDTA and 1% SDS. The hydrolysis pattern on xylan demonstrated that the enzyme is an exo-xylanase which produces xylobiose as a final product.

INTRODUCTION

After cellulose, xylan is the next most abundant renewable polysaccharide in nature[1]. Appreciable quantities of xylan are present in material released in the pulp processing and food processing industries[2-5]. It is presently regarded as waste and often deposited in streams and rivers, where it is ecologically harmful. 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. The enzymatic hydrolysis of xylan, which is a heteropolymer of β-1,4-linked D-xylose, α-1,3-linked L-arabinose and α-1,2-linked D-glucopyranose, is accomplished by the action of endo-1,4-β-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37). These two enzymes are widely distributed among fungi and bacteria and properties of the enzymes produced have been studied (review by Coughlan and Hazlewood[6]). Reports on their occurrence in yeast and yeast-like microorganisms are very rare[7-9]. Among the yeasts, the information on xylanase is largely derived from *Cryptococcus albidus*[8]. 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[10]. In 1986, Lee *et al.*[11] described the ability of *P. stipitis* to convert xylan into ethanol. Investigation of the enzymes responsible for its degradation and gene cloning are important for the development in construction of recombinant microorganisms which are able to produce ethanol from renewable resources.

In this report, we describe the purification and some properties of the xylanase from *P. stipitis*.

MATERIALS AND METHODS

Microorganism and cultivation

The yeast *Pichia stipitis* CBS 5773 was obtained from Professor Dr. C.P. Hollenberg, Institut fur Mikrobiologie, Herich Heine Universität Düsseldorf, Germany. For the production

of xylanase, yeast cell was grown on a reciprocal shaker at 30 °C in an inducing medium containing 0.67% yeast nitrogen base and 0.5% soluble β-1,4-xylan isolated from oat spelts (Sigma). Following cell growth the absorbance of the cell suspension was measured at 420 nm 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 72 h culture were removed by centrifugation and the supernatant (crude xylanase preparation) used for xylanase purification.

Enzyme Assays

Activity of endo-1,4-β-xylanase was measured by following the release of reducing sugars from oat spelts xylan. The assay mixture of total volume of 0.4 ml containing enzyme and 1 mg substrate in 50 mM citrate buffer pH 5.1 was incubated for appropriate time at 40 °C. Released reducing sugars were determined as xylose after termination of the reaction with dinitrosalicylic acid reagent and boiling for 10 min[12]. Blanks to correct 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 sodium tetraborate (pH 9.4). Released *p*-nitrophenol was determined spectrophotometrically at 410 nm.

One unit of β-xylanase is defined as the amount of enzyme which is capable of liberating from xylan 1 μmol equivalents of xylose in 1 min. One unit of β-xylosidase is defined as the amount of enzyme which hydrolyzes 1 μmol *p*-nitrophenyl-β-D-xylopyranoside in 1 min.

Purification of xylanase

Unless otherwise indicated, all operations were performed at 4 °C. A 72 h culture of *P. stipitis* on xylan medium was centrifuged to remove the cells. The supernatant was lyophilized. Dry crude enzyme was redissolved in 7 ml of 50 mM sodium acetate buffer pH 5.1. The solution was dialyzed against a solution of the same buffer.

The dialyzed sample was loaded on a column (2.2 cm x 30 cm) of Sephacyl S-300. The column was eluted with the same buffer at a rate of 0.5 ml/min and fraction of 2.0 ml was collected.

Protein measurement

Protein concentration was measured by the method of Bradford[13] with bovine serum albumin as standard.

Gel electrophoresis

This was performed on 8% polyacrylamide gels at pH 8.9 according to Laemli[14]. Proteins were stained with Coomassie brilliant blue and silver nitrates. The carbohydrate part of the protein was visualized by staining with Periodic acid Schiffís reagent. 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 the molecular weight of the enzyme.

Paper Chromatography

Products of hydrolysis of xylan formed upon the action of β-xylanase were separated by chromatography on Whatman paper No.1 with a solvent system of acetate/acetic acid/water

(18:7:8. v/v/v). Reducing sugars were detected with the silver nitrate reagent. Xylose (X1) and xylobiose (X2) were included as standards.

Determination of kinetic constants

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

RESULTS

Production of xylanolytic enzymes from *P. stipitis*

A medium containing 0.5% oat spelt xylan was inoculated with an overnight culture of *P. stipitis* and shaken at 30 °C for 72 h. The activities of the enzymes xylanase and xylosidase were assayed from cell free cultured fluid and fluid from disintegrated cells. It was found that *P. stipitis* secretes xylanase into the culture medium whereas the activity of xylosidase was detected mainly in the cell homogenate.

The time course of production of extracellular activities of xylanase during growth of *P. stipitis* on medium using 0.5% of oat spelt xylan, 0.5% glucose, and 0.5% xylose as carbon source was examined (Fig 1). Synthesis of xylanase in *P. stipitis* cells was induced strongly by xylan, while xylose and glucose had very low inducing activity. When a low inoculum was used, xylanase activity appeared in the growth medium after a lag of 24-30 h. and showed the highest activity of 32.35 unit/mg at 72 h.

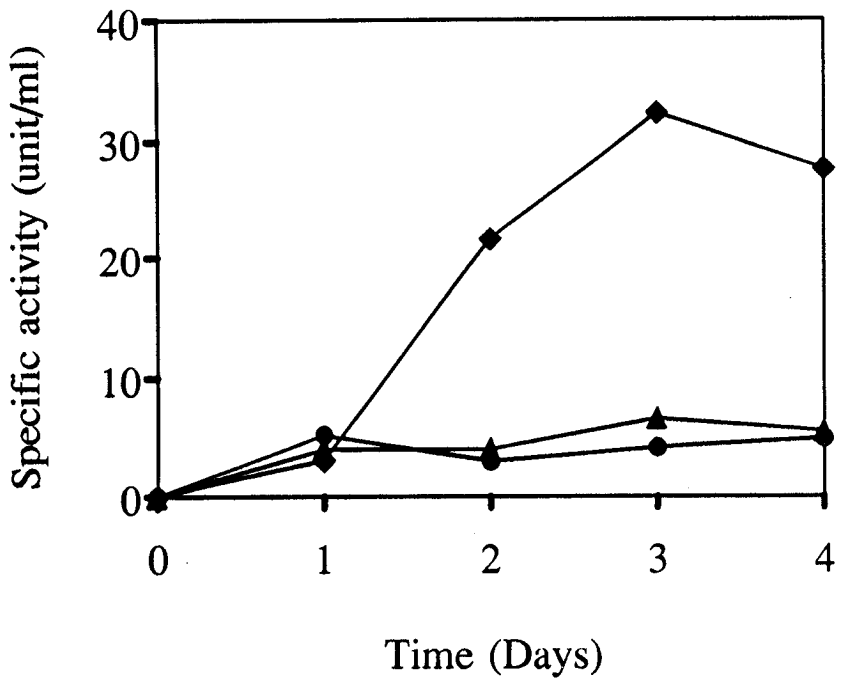

Fig. 1. Effect of carbon sources on enzyme production.

Yeast cell was grown in medium containing different carbon sources: ●, 0.5% glucose; ▲, 0.5% xylose; and ◆, 0.5% xylan. The xylanase activity was followed for four days. The amount of cell counts on each day are $2x10^7$, $7x10^8$, $5x10^9$ and 10^9 cells per ml.

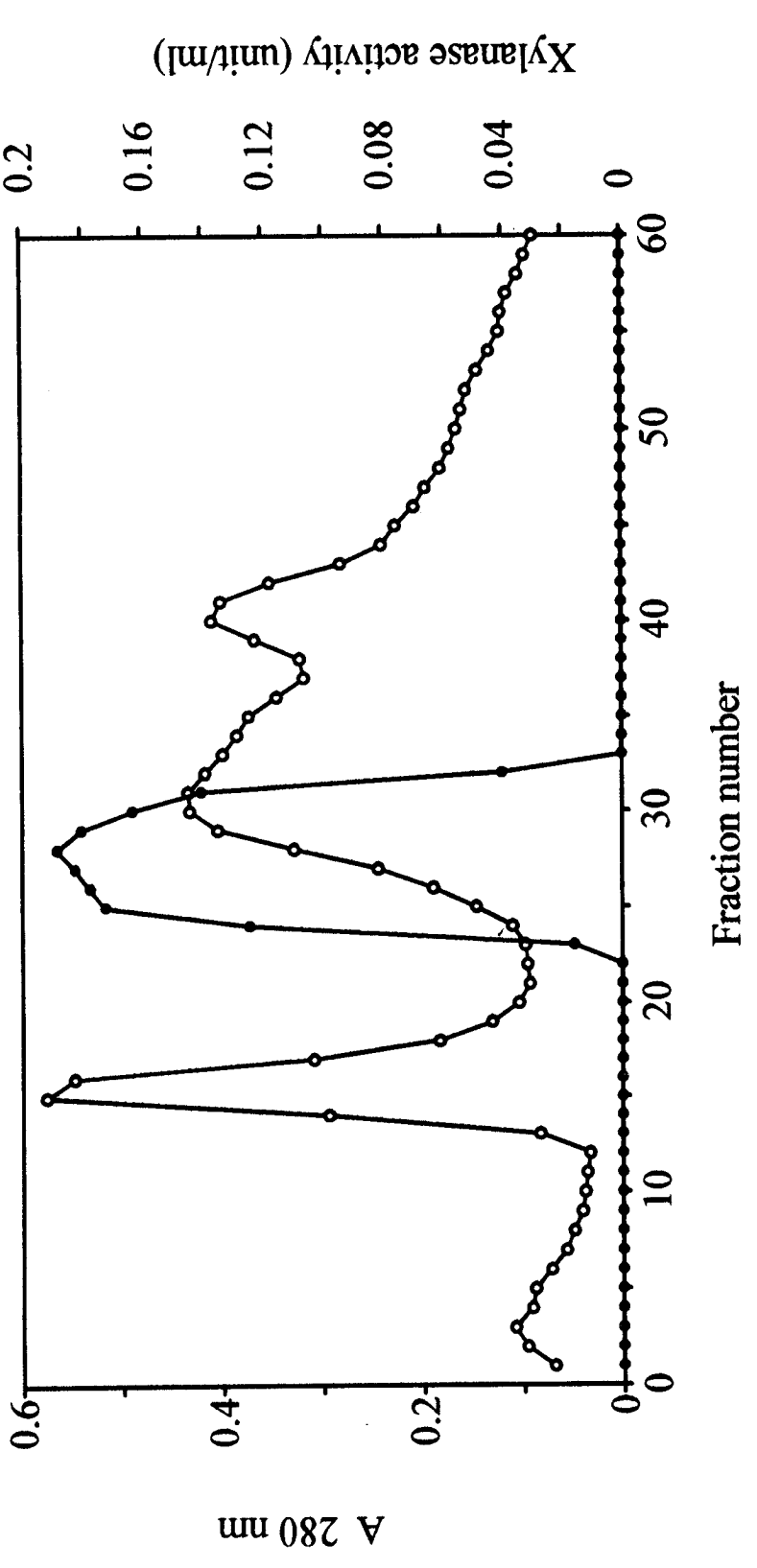

Fig. 2. Gel filtration of crude enzyme from lyophilization.

Open circle denote absorbance at 280 nm, closed circles denote xylanase activity.

Purification of xylanase

Details of enzyme purification are summarized in Table 1. Xylanase was purified 4.5-fold over the specific activity detected in concentrated culture supernatant with a recovery yield of 4.46%. Figure 2 shows the final step of xylanase purification on Sephacyl S-300 column chromatography. Proteins were separated into four peaks. Xylanase activity peak was eluted with the third protein peak. The fractions No. 25 through 30 had high activity. The purified enzyme was shown as a discrete protein band on SDS polyacrylamide gel electrophoresis, indicating that it was electrophoretically homogeneous (Fig 3). In addition, this protein band stained strongly with Schiffís reagent, suggesting that it was a glycoprotein.

Molecular weight determination

Electrophoretic mobilities of purified xylanase and of reference proteins on SDS polyacrylamide gel electrophoresis were plotted versus their molecular weight. The mobility of the purified xylanase corresponded to a molecular weight of 43 kDa

Enzyme properties

Figure 4 shows the effects of temperature on the xylanase activity. The xylanase had an optimum temperature at 40° C. The enzyme retained 70% of the activity on standing at 40 °C for 30 min at pH 5.0 and lost 95.2% of activity at 50 °C in 30 min. The optimum pH was 5 and the enzyme was stable at pHs from 3-6. The effect of various ions and chemicals on the activity of enzymes are summarized in Table 2. The activity was slightly increased upon addition of Zn^{++}, Ca^{++}, Mg^{++}, K^{+} and was inhibited by Cu^{++}, EDTA and 1% SDS.

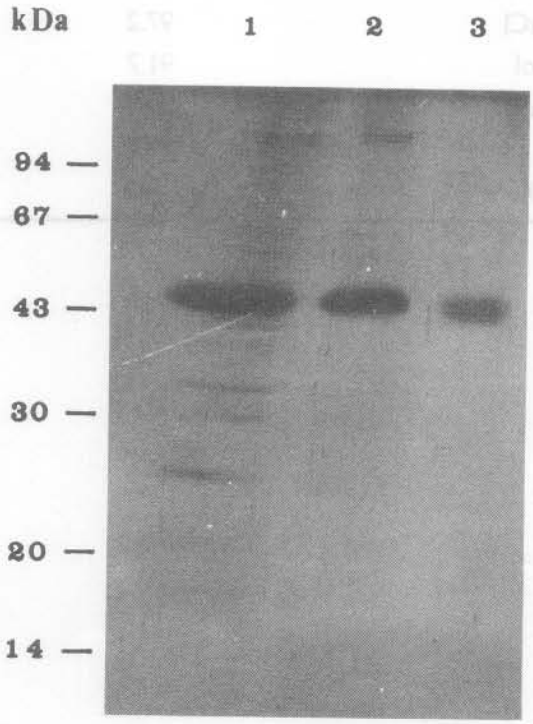

Fig. 3. SDS-PAGE of various stages of protein purification.
12.5 μg of each samples were load on gel containing 8% polyacrylamide. After the electrophoresis the gel was stained with silver nitrates. Lane 1, crude enzyme; Lane 2, lyophilized protein; Lane 3; a pool from gel filtration.

TABLE 1. Summary of xylanase purification step.

Step	Total protein (mg)	Volume (ml)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Crude enzyme	7.650	450	207.01	27.06	100
Lyophilized	0.623	7	11.83	18.99	5.71
Sephacyl S-300	0.11	12	9.24	86.52	4.46

TABLE 2. Effect of some ions and chemicals on xylanase.

Ion and Chemicals	% Activity
none	100
10mM $ZnSO_4$	126.6
10mM $CaCl_2$	119.3
10mM $MgCl_2$	119.3
10mM KCl	110.1
10mM NaCl	97.2
5% Ethanol	91.7
10mM $CuSO_4$	20.2
1% SDS	13.8
10mM EDTA	8.3

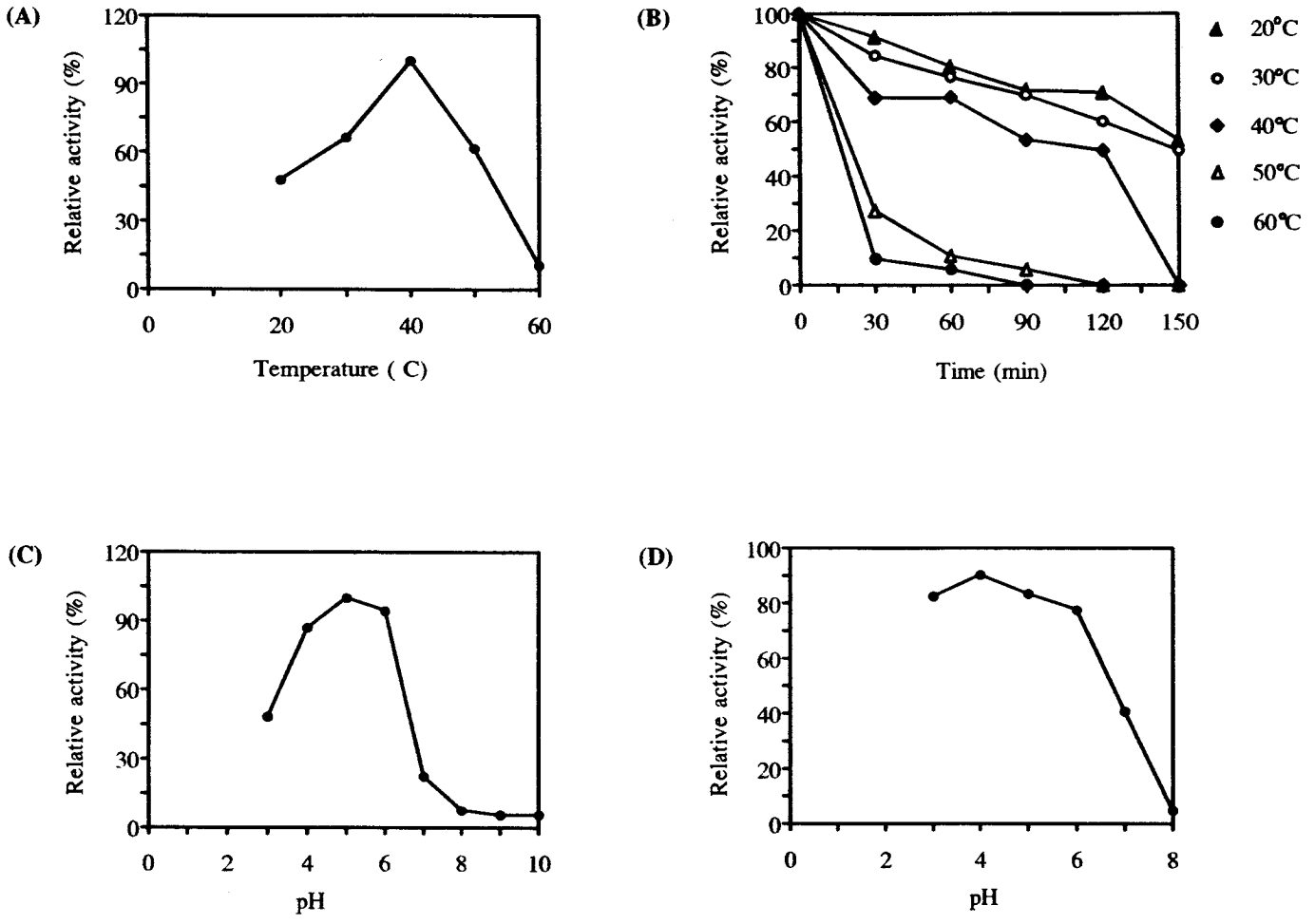

Fig. 4. Enzyme properties of xylanase.

(A) optimum temperature; (B) thermal stability, the enzyme solution was heated for 30-150 min at each indicated temperature and residual activity was measured; (C) optimum pH; (D) pH stability, the enzyme solution was incubated in buffer at each indicated pH for 30 min and residual activity was measured

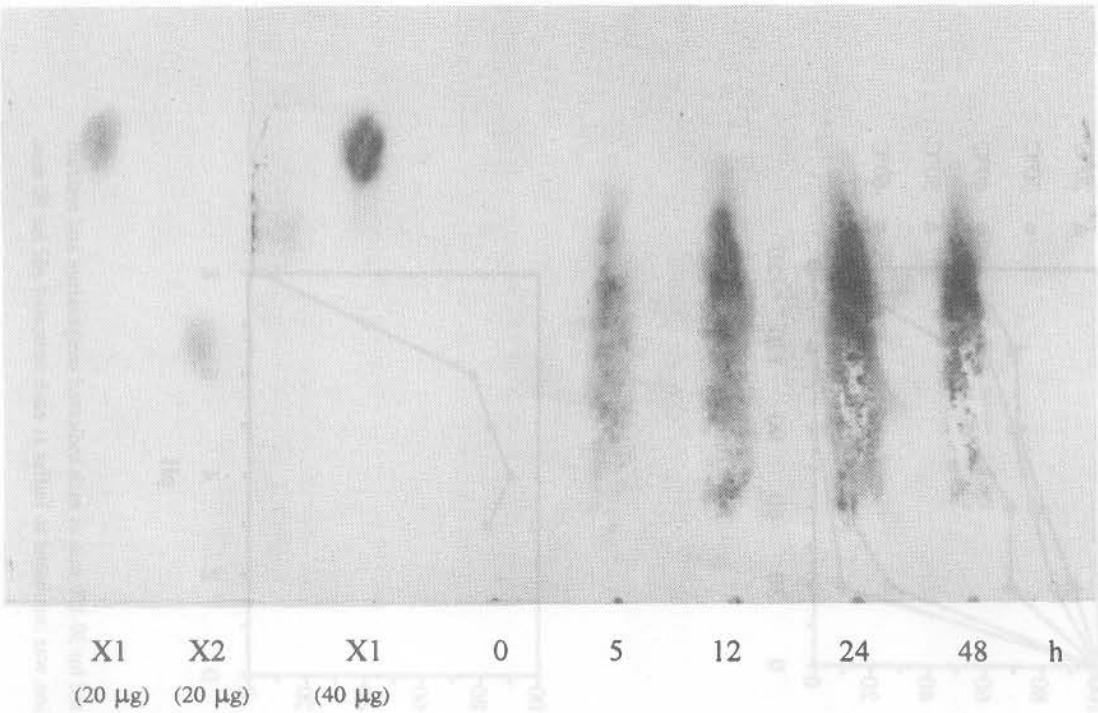

Fig. 5. Paper chromatography of hydrolysis products of purified xylanase on oat spelt xylan. The enzyme was incubated at 40 °C in solution containing 1% xylan and 50 mM phosphate buffer pH 5.0. At given time intervals, 100 µl aliquots were spotted on the Whatman paper no. 1, 20 µg and 40 µg xylose (X1) and 20 µg xylobiose (X2) were used as standards

Determination of kinetic constant of enzymes

Purified enzyme (1.1125 µg/ml) was incubated with various concentrations of soluble xylan (1.75 mg/ml to 3.25 mg/ml) in acetate buffer pH 5.1. The Michaelis constant (K_m), and the maximal rate (V_{max}) of enzyme can be derived from rates of catalysis measured at different substrate concentration. A plot of 1/v versus 1/[S] (Lineweaver-Burk plot) showed the K_m value to be 1.43 mg/ml and the V_{max} 1.54 (mol/min.) The regression analysis showed R square value of 0.997 and standard deviation of 0.68.

Analysis of the hydrolysis product

Figure 5 shows a paper chromatogram of the hydrolyzate of oat spelt xylan with the xylanase. The end-products after 24-hour digestion were xylobiose (X2). The enzyme did not show xylosidase activity examined with *p*-nitrophenyl-β-xyloside.

DISCUSSION

During growth on oat spelts xylan, the yeast *P. stipitis*, CBS 5773, elaborates two enzymes related to xylan degradation. One enzyme, which is secreted into culture fluid, was identified as an β-xylanase. The other enzyme, which is found in cell fraction, was β-xylosidase. The xylanase is an inducible enzyme but xylan itself cannot function as a direct inducer as shown by the long lag period, i.e., 24 hour, before the enzyme appear in the medium. Xylan in the culture medium was first degraded by the xylanase which was produced constitutively from

the yeast cell. The small oligosaccharides which obtained from the degradation entered the yeast cell and induced the production of β-xylanase. The similar mode of xylan utilization was also described in other yeast, e.g. *Cryptococcus albidus* which suggested xylobiose and higher xylose oligosacchrides as the natural inducer of β-xylanase[8]. The enzyme was purified to homogeniety by lyophilization of culture fluid and passed the sample through column chromatography. The lyophilized material appeared as a sticky pellet with brown colour, and was difficult to redissolve. This has also been reported in other studies and some have used non-metabolic substrates, e.g. β-methylxyloside, as an inducer in the purification[9]. However, these substrates are not commercially available, therefore in this experiment, we still used xylan as an inducer. In the process of xylan hydrolysis reported in many microorganisms, xylanases were found in cellulosome or xylanosome (a cell bound multi enzymes complex or extracellular bag of enzymes) and in noncellulosomal or xylanosomal fraction. The xylanosome or enzyme itself carried xylan binding domain which is responsible for adherence to and hydrolysis of substrate[15]. The occurrence of xylanosome may be the reason for the low concentration of protein appearing in the culture filtrate. Upon lyophilization the pellet may be composed mostly of enzyme-xylan associated complex and free-floating enzyme, and only the latter may easily be dissolved. This could be the reason why protein and enzyme were lost at this step. To solve this difficulty, the culture filtrate was passed through a DEAE-cellulose column and concentrated by ammonium precipitation, or ethanol precipitation or evaporation instead of lyophilization. But none of these methods could resolve the problem. Purity of the enzyme from gel filtration was demonstrated by a single protein band on SDS polyacrylamide gel electrophoresis detected by silver stain and suggesting a single subunit of the enzyme. The purified xylanase from this study shares some physical properties with other yeast and fungal xylanases[16,17] that it is a glycoprotein with molecular masses of 43 kd. The K_m values measured is 1.43 mg/ml and V_{max} value is 1.54 (mol/min.) They display activity with optimal temperature at 40 °C and the optimal pH is 5. The enzyme was stable at 40 °C and at pH range of 3-6. The activity of xylanase was unaffected by most of the ion tested, except for Cu^{++} which inhibited the enzyme. The enzyme was also inhibited by EDTA and 1%SDS.

Purified xylanase catalyzed the hydrolysis of oat spelt xylans producing exclusively a single compound with *Rf* value corresponding to xylobiose on paper chromatography. Transient formation of larger products at the early stage within one hour of hydrolysis was not observed. These results indicated that xylanase of *P. stipitis* was an exo-xylanase producing xylobiose as a smallest product. A similar mode of action has been examined by Xylanase V of *Aeromonas caviae* ME-1[18].

The xylanase was active only on xylan substrate and was inactive on other carbohydrate, such as cellulose and starch. The result suggest that it is a true xylanase. In addition, it did not hydrolyze *p*-nitrophenyl-β-D-xyloside, indicating that it does not possess β-xylosidase activity. As *P. stipitis* does not produce cellulolytic enzymes, create a good opportunity to use the enzyme in many applications which specifically removal of hemicellulose are required[5]. Cloning of related genes of xylanolytic enzymes from this yeast strain are of our interest because it is possible to enhance the ethanol production from hemicellulose by using recombinant DNA technology.

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REFERENCES

1. Bieley, P. (1985) *Trends Biochem.* **11**, 286-290
2. Senior, D.J., Mayers, P.R. and Saddler, J.N. (1991) *Biotechnol. Bioengineer.* **37**, 274-279
3. Buchert, J., Ranua, M., Kantelinen, A. and Viikari, L. (1992) *Appl. Microbiol. Biotechnol.* **37**, 825-829
4. Bernier Jr, R.L., Gray, A.L. Moser, G.P., Hamilton, J. Roberge, M. and Senior, D.J. (1993) *Bioresource Tech.* **50**, 79-83
5. Jeffries, T.W. (1988) Fermentation of xylose and cellobiose. In: Yeast: Biotechnology and Biocatalysis (Verachert, H. and Demot, R., eds). Marcel Dekker, New York, 376
6. Coughlan, M.P. and Hazlewood, G.P. (1993) *Biotechnol. Appl. Biochem.* **17**, 259-289
7. Stevens, B.J.H. and Payne, J. (1977) *J. Gen. Microbiol.* **100**, 381-383
8. Biely, P., Vrsanska, M., and Kratry, Z. (1980) *Eur. J. Biochem.* **108**, 313-321
9. Nakanishi, K., Araki, H., and Yasui, T. (1984) *J. Ferment. Technol.* **62**, 361-369
10. Boucher, F., Morosoli, R., and Durand, S. (1988) *Nucl. Acids. Res.* **16**,9874
11. Lee, H., Biely, P., Latta, R.K., Barbosa, M.F.S., and Schneider, H. (1986) *Appl. Environ. Microbiol.* **52**, 320-324
12. Miller, G.L. (1959) *Anal. Chem.* **31**, 426-428
13. Bradford, M.M. (1970) *Anal Biochem.* **72**, 248-254
14. Laemmli, U.K. (1970) *Nature (Lond.)* **277**, 680-685
15. Thomson, J.A. (1993) *FEMS Microbiol. Rev.* **104**, 65-82
16. Molosoli, R., Roy, C. and Yaguchi, M. (1986) *Biochemica et Biophysica Acta* **870**, 473-478
17. Bastawde, K.B. (1992) *World J. Microbiol. Biotechnol.* **8**, 353-368
18. Kubuta, B.K., Suzuki, T., Horitsu, H., Kawai, K. and Takamizawa, K. (1994) *Appl. Environ. Microb.* **60**, 531-535

บทคัดย่อ

ได้เตรียมเอนไซม์ไซลาเนสจากยีสต์สายพันธุ์ *Pichia stipitis* ให้บริสุทธิ์โดยการผ่านคอลัมน์ Sephacyl S-300 และศึกษาสมบัติของเอนไซม์ พบว่าเอนไซม์ทำงานได้ดีที่สุดที่ pH 5 มีความเสถียรที่ pH 3-6 มีค่าแอคติวิตี้สูงสุดและเสถียรเมื่อบ่มที่อุณหภูมิ 40 °C นาน 30 นาที เมื่อทำอิเลคโตรโฟรีซีสแบบ SDS-PAGE ปรากฏเป็นแถบโปรตีนเดียวซึ่งมีน้ำหนักโมเลกุล 43 kDa ค่าทางจลนศาสตร์ของเอนไซม์คือ K_m เท่ากับ 1.43 มก./มล. และ V_{max} เท่ากับ 1.54 ไมโครโมล/นาที การทำงานของเอนไซม์ถูกยับยั้งโดย 10 mM Cu^{++}, EDTA และ 1% SDS ผลผลิตสุดท้ายที่ได้จากการย่อยไซแลนคือไซโลไบโอส ซึ่งแสดงว่าเอนไซม์ทำงานแบบ exo-xylanase