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Purification and properties of an extra cellular xylanase enzyme of *Clostridium* strain SAIV

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Abstract

An extracellular xylanase enzyme fraction A from a mesophilic *Clostridium* strain SAIV was purified by ammonium sulfate precipitation, Sephadex G-50 gel filtration and DEAE-Sephadex A-50 ion exchange. The xylanase exhibited a molecular weight of ~ 30,000 and it was stable upto 55° C with an optimum temperature of 50° C. It was most stable between pH 5–7, with an optimum pH of around 6. The Km value was 7.0 mg·xylan ml^{-1} and V_{max} was 36 μmol·xylose liberated mg^{-1} min^{-1}. Carboxymethyl cellulose, filter paper cellulose and 4-p-nitrophenyl β-D-xylopyranoside were not hydrolysed. The specific activity of xylanase fraction A (9.8 U mg^{-1}) is 2–10 fold higher than the specific activity of xylanase in other mesophilic, xylanolytic, obligate anaerobic bacteria. A minor fraction of xylanase activity designated as xylanase B was also obtained supporting the view that the multiplicity of xylanases is common in microorganisms.

Introduction

Xylanases are produced by many organisms viz. fungi, bacteria, animals and plants (Dekker and Richards 1976). The important characteristics and biotechnological applications of xylanase has been reviewed (Dekker and Richards 1976; Reilly 1981; Woodward 1984; Biely 1985; Wong et al. 1988). Among anaerobic bacteria xylanase has been purified and well characterized only from mesophilic *Clostridium acetobutylicum* (Lee et al. 1987) and two thermophilic species *C. stercorarium* (Berenger et al. 1985) and *C. thermolacticum* (Debeire et al. 1990). This is despite the fact that clostridia are now recognized to have much potential for lignocellulosic biomass conversion to fuels and chemicals.

Against this background we undertook screening of a number of anaerobic xylanolytic bacteria from various habitats which could be promising for biomass conversion (Murty Ph.D. thesis 1989). One of the isolates that we obtained from decaying carpentry scrap is a *Clostridium* strain SAIV and its characteristics were reported (Murty and Chandra 1989, 1991). The features of note in the present context are that it forms 5–7 by 1–3 μm motile rods with subterminal spores. It utilizes several substrates including xylan, xylose, filter paper cellulose, cellobiose, maltose, glucose, mannose etc. producing ethanol, acetate, formate, CO_2 and H_2 as major fermentation products. It is mesophilic with optimum growth temperature of 37° C at pH 7.0. During growth on xylan or xylose it excretes extra-cellular xylanase with activity of 520 mU ml^{-1} which is significantly higher than that recorded in other anaerobic mesophilic, xylanolytic *Clostridium, Ruminococcus flavefaciens, Butyrivibrio fibrisolvens* or *Bacteroides ovatus* (Murty and Chandra

1991). Hence, the present investigation on the purification of this xylanase and a comparison of its properties with xylanases purified from *C. acetobutylicum* and *C. stercorarium* was taken up.

Materials and methods

Chemicals

Sephadex G-50 and DEAE Sephadex A-50 were procured from Pharmacia (Sweden). Acrylamide, N,N'-methylene-bis acrylamide, N,N,N',N'-tetramethyl ethylene diamine (TEMED), larchwood xylan, ammonium persulfate and p-nitrophenyl β-D-xylopyranoside (4PNPX) were obtained from Sigma (St. Louis, MO, U.S.A.). Rainbow TM protein molecular weight markers were obtained from Amersham (U.S.A.).

Microorganism and culture methods

Clostridium strain SAIV isolated and characterized in our laboratory (Murty & Chandra 1989, 1991) was grown anaerobically without agitation in mineral base medium of Murty and Chandra (1989) with 1.5% (W/V) larchwood xylan and 0.2% yeast extract. Cells were separated from the culture broth after 70–72 h of growth by centrifugation at 12,000 × g for 2 h at 4° C when the maximum amount of the extra-cellular xylanase was released. The supernatant was used as a crude preparation of xylanase enzyme. Dithiothreitol (10 mM) was added to keep the enzyme reduced thereafter.

Enzyme purification

Step 1
The total culture filtrate was concentrated by precipitation with ammonium sulfate (60% W/V). The precipitate separated by centrifugation was dissolved in 0.05 M potassium phosphate buffer (pH 6.0), dialyzed against the same fresh buffer and concentrated by lyophilization.

Step 2
The concentrated sample (65 mg of protein) from step 1 was applied to a Sephadex G-50 column (2 × 80 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 6.0). The fractions exhibiting xylanase activity between 125 and 190 ml of the elution volume were pooled, dialyzed against 0.02 M sodium phosphate buffer (pH 6.0) and concentrated by lyophilization.

Step 3
The concentrated sample (22 mg of protein in 25 ml buffer) of step 2 was applied to a DEAE Sephadex A-50 column (2.5 × 30 cm), equilibrated with 0.02 M sodium phosphate buffer (pH 6.0). First 150 ml of 0.02 M sodium phosphate buffer was passed. The proteins were then eluted from the column by applying a NaCl gradient of 0.0 to 0.5 M in 0.02 M sodium phosphate buffer (pH 6.0). This was followed by a second higher NaCl gradient of 0.5 to 1.0 M using the same buffer. The protein peaks were monitored by absorption at 280 nm using a Shimadzu UV VIS 160 spectrophotometer.

The fractions collected between an elution volume from 430–470 ml and from 525–555 ml contained xylanase activity which were designated as xylanase A and B fractions, respectively. They were pooled separately, dialyzed against 0.02 M sodium phosphate buffer, pH 6.0, concentrated by lyophilization to dry powder, and stored at − 20° C. The enzyme was reconstituted by suspending 1–2 mg in 0.02 M sodium phosphate buffer pH 6.0.

Enzyme assays

Xylanase was assayed according to Berenger et al. (1985) with some modifications using larchwood xylan (1% W/V) in 25 mM citrate-phosphate buffer, pH 6.0 and incubated at 50° C for 30 min. The amount of reducing sugars released was measured with the dinitrosalicylic acid method (Miller 1959). One unit of xylanase activity was defined as the amount of enzyme releasing reducing sugar equivalent to 1 μmol of D-xylose per min.

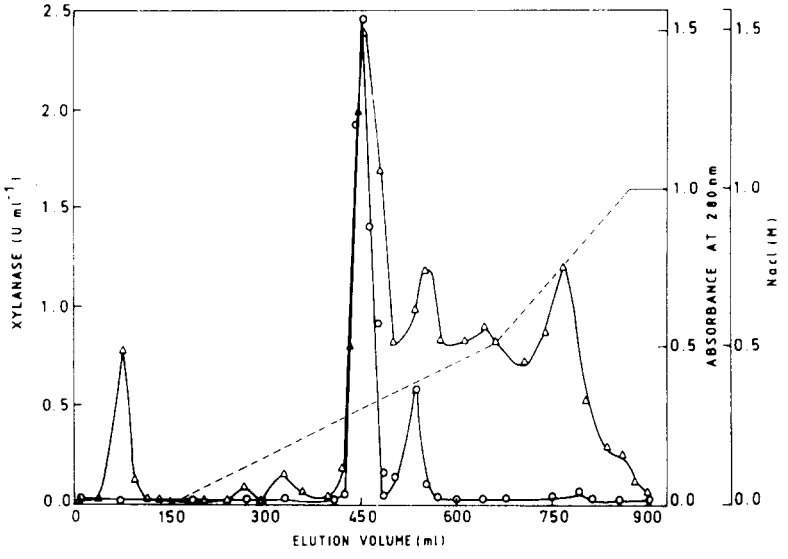

Fig. 1. Elution pattern on DEAE-Sephadex A-50 (○) Xylanase; (△) Absorbance at 280 nm; (----) NaCl gradient.

β-Xylosidase was assayed according to Tanaka et al. (1986) and carboxymethylcellulase (CMCase) and filter paper activity (FPase) according to Saddler and Chan (1982).

Effect of temperature and pH on xylanase activity

The effect of temperature was determined by incubating the standard xylanase reaction mixture at pH 6.0 in citrate-phosphate buffer (25 mM) at various temperatures for 30 min before assaying the activity by addition of substrate. Similarly the effect of pH was determined using citrate-phosphate buffer (25 mM) for pH between 3.5 and 7.5 and glycine-NaOH buffer (25 mM) for pH 8.0. For determining pH stability the enzyme was allowed to stand at various pH values for 24 h at 4° C before use in assay.

In all cases the enzyme activity was expressed in terms of percentage of the control.

Effect of various compounds on xylanase activity

The effects of metal ions and chemical agents was tested by pre-incubating the enzyme with the compounds for 15 min at 50° C and then assaying activity by addition of substrate. The activity of untreated enzyme was taken as 100%.

Estimation of molecular weight

Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Laemmli (1970). Molecular weight was calculated as per Weber and Osborn (1969) with reference to standard proteins.

Protein was determined according to Lowry et al. (1951) using bovine serum albumin as standard.

Results

Purification of xylanase

The concentrated protein obtained from the culture filtrate following ammonium sulfate precipitation and dialysis was fractionated on Sephadex G-50 column. A single xylanase peak was observed between elution volume 125 ml to 190 ml. None of the other fractions showed any activity of xylanase, β-xylosidase, CMCase or FPase. This xylanase

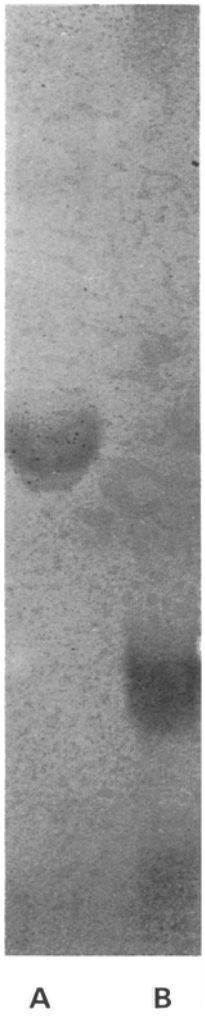

Fig. 2. SDS – Polyacrylamide gel electrophoreses.
Lane A: Xylanase fraction A.
Lane B: Xylanase fraction B of strain SAIV.

fraction was dialyzed, concentrated and loaded on a DEAE Sephadex A-50 column (Fig. 1). Gradient elution with NaCl (0.0–0.5 M) revealed two peaks of xylanase activity. The main peak of xylanase activity eluted from 430–470 ml and was referred to as xylanase fraction A. The second smaller peak of xylanase activity eluted between 525–555 ml and was referred to as xylanase fraction B. Other fractions exhibited neither xylanase, β-xylosidase, FPase nor CMCase activities. A summary of the separation and purification steps is presented in Table 1. An increase of approximately 16-fold of the specific activity of fraction A and 2.5 fold of the specific activity of fraction B was achieved during purification (Table 1).

Properties of the xylanase fractions

The xylanase A fraction on SDS-PAGE (12 μg protein on 9% polyacrylamide) showed a single major prominent protein band (Fig. 2). However, the band was not very sharp and a sharper band could not be achieved even after several repetitions of electrophoresis using varying concentrations of polyacrylamide (8 to 10%) or protein (5 to 25 μg). Although xylanase B fraction was eluted as a single peak on DEAE Sephadex A-50, it did not appear to be homogeneous on electrophoresis (Fig. 2). In addition the bands were more diffused. The purity of xylanase A and B need further study.

The molecular weight of xylanase in fraction A was estimated to be around 30,000 and that of xylanase in fraction B as around 20,000 by SDS-PAGE.

The optimal temperature for purified xylanase A

Table 1. Summary of the purification of xylanase from strain SAIV.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture Supernatant	3500	2980	1855	0.62	100	1
60% $(NH_4)_2SO_4$	200	130	468	3.6	25	5.8
Sephadex G-50	65	65	377	5.8	20	9.3
DEAE Sephadex A-50						
(Xylanase A)	40	14	138	9.8	7	15.8
(Xylanase B)	30	12	20	1.6	1	2.5

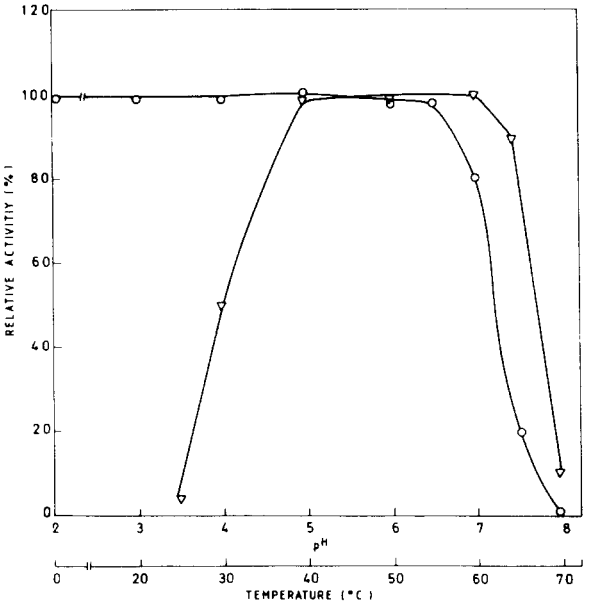

Fig. 3. Thermal and pH stability of xylanase A 100% activity = 9.8 U·mg^{-1} protein (○) Temperature; (▽) pH.

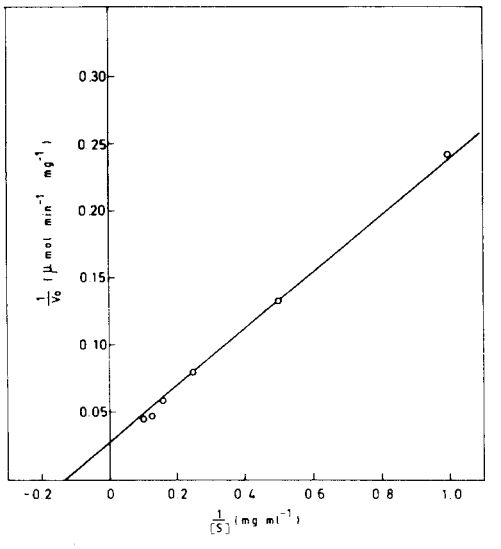

Fig. 4. Lineweaver-Burk plot of enriched xylanase A fraction using xylan as substrate.

activity was 50° C and the optimal pH was 5.5 to 6.5. Xylanase A was stable upto 55° C and between pH 5 to 7 (Fig. 3). Lyophilized enzyme sample reconstituted at pH 6.0 showed a loss of no more than about 15% of the total activity after storage for a period of two months.

The Km and V_{max} of xylanase in fraction A were calculated to be 7.0 mg of xylan ml^{-1} and 36 μmol of xylose liberated min^{-1} mg^{-1} respectively from the Lineweaver-Burk plot (Fig. 4) when the kinetics was studied using larchwood xylan as the substrate. The activity on xylan did not change when assayed under aerobic or anaerobic conditions and hence was insensitive to oxygen.

With regard to the effect of metal ions and inhibitors on xylanase A, 10^{-3} M NaCl or KCl did not inhibit activity; 10^{-3} M LiCl, $ZnCl_2$, $MgCl_2$ and $BaCl_2$ inhibited upto 20%, 10^{-3} M $FeCl_3$, $CuSO_4$, $Pb(CH_3COO)_2$ and $MnCl_2$ upto 35–40%, 10^{-3} M $CaCl_2$ and $HgCl_2$ upto 88%, 10^{-3} M EDTA upto 55%, and p-chloromercuribenzoate upto 95%.

Discussion

Clostridium strain SAIV excreted two to ten-fold higher levels of extracellular xylanase activity (520 mU ml^{-1}) in culture broth in comparison with xylanase activity reported in other mesophilic obligate anaerobes (Murty and Chandra 1991; Pettipher and Latham 1979; Lemmel et al. 1986; Tanaka et al. 1986; Hespell et al. 1987; Reddy et al. 1983). This stimulated our interest to enrich and purify this xylanase of strain SAIV. Wong et al. (1988) reviewed most of the clostridial xylanases studied so far. Of these only xylanase from *C. stercorarium* a thermophile (Berenger et al. 1985) and *C. acetobutylicum* an industrially useful mesophilic organism (Lee et al. 1987) has been well characterized. Purification and properties of an endo-1,4-xylanase of a thermophilic *C. thermolacticum* has been recently reported (Debeire et al. 1990). In the present study we have been successful in enriching an extracellular xylanase to 7-fold higher activity by $(NH_4)_2SO_4$ precipitation, gel and ion exchange chromatography. Strain SAIV produced two xylanase enzyme fractions viz. a major peak designated as fraction A and a minor peak fraction B.

The presence of two xylanase fractions A and B supports the fact of multiplicity of xylanases in

Table 2. Comparison of properties of xylanase produced by strain SAIV, *C. acetobutylicum* and *C. stercorarium.*

Culture	Enzyme	Molecular weight	Stability		Optimum		K_m (mg xylan per ml)	V_{max} μmol of xylose/min/ mg of protein)	References
			Thermal (°C)	pH	Temperature (°C)	pH			
Clostridium SAIV (mesophilic)	Xylanase A	30,000	upto 55	5.0–7.0	50	5.5–7.0	7.0	36	Present study
	Xylanase B	20,000	–	–	–	–	–	–	-do-
C. acetobutylicum	Xylanase A	65,000	upto 40	5.5–7.0	50	5.0	6.0	22.4	Lee et al. 1987
	Xylanase B	29,000	upto 50	3.5–7.0	60	5.5–6.0	6.7	22.3	
C. stercorarium (thermophilic)	Xylanase A	44,000	upto 75	7.0–12	75	5.5–7.0	3.2	5500	Berenger et al. (1985)
	Xylanase B	72,000	upto 75	7.0–12	75	5.5–7.0	2.9	3500	
	Xylanase C	62,000	upto 75	7.0–12	75	5.5–7.0	3.7	4000	

some microorganisms. For instance *C. stercorarium* and *C. acetobutylicum* produce 3 and 2 fractions respectively (Table 2). The xylanase fraction A of strain SAIV differs in several properties particularly in molecular weight, pH, thermal stability and kinetic behaviour from xylanase of the other two clostridia (Table 2).

Xylanase of strain SAIV did not hydrolyse substrates other than xylan such as CMC, FP cellulose or 4-PNPX, whereas xylanase fraction A of *C. acetobutylicum* could non-specifically hydrolyse CMC, acid swollen cellulose and lichenan, and xylanase B could hydrolyse xylan and lichenan (Lee et al. 1987).

From a practical point of view the combination of low Km and high V_{max} is important. The Km values of xylanases have been shown to range from 0.27 mg·ml^{-1} (in *Ceratocystis paradoxa II* Woodward 1984) to 19.6 mg·ml^{-1} (in *Cryptococcus albidus* Woodward 1984). The Km of xylanase fraction A of strain SAIV is on the lower side (7.0 mg·xylan ml^{-1}) and the V_{max} (36 μmol xylan liberated/min/ mg protein) is higher than in *C. acetobutylicum.*

The specific activity of xylanases purified till now are reported to be 5.33 U·mg^{-1} in aerobic *Bacillus acidocaldarius* (Uchino and Nakane 1981), 6.59 U·mg^{-1} in xylanase fraction B of *C. acetobutylicum,* and an exceptionally high activity of 3626 U·mg^{-1} in the thermophilic *C. stercorarium.* The specific activity of 9.8 U·mg^{-1} is higher than that of *C. acetobutylicum* (6.59 U·mg^{-1}).

After *C. acetobutylicum, Clostridium* strain SAIV is only the second mesophilic anaerobe from which a xylanase has now been enriched and purified. Xylanase in strain SAIV is induced even during growth on xylose, glucose or filter paper plus xylose (Murty and Chandra 1991). It could be of interest to determine which fraction A or B is predominantly expressed during growth on different substrates. Further investigation on the mechanism of activity of xylanase A and B of strain SAIV could aid in a better understanding of hemicellulose hydrolysis.

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