J. Biosci., Vol. 11, Numbers 1–4, March 1987. pp. 275-285. © Printed in India.

Multisubstrate specifics amylase from mushroom *Termitomyces clypeatus*

ANIL K. GHOSH and SUBHABRATA SENGUPTA
Indian Institute of Chemical Biology, 4 Raja S. C. Mullick Road, Jadavpur, Calcutta 700 032, India

Abstract. An amylase was purified from the culture filtrate of *Termitomyces clypeatus* by ammonium sulphate precipitation, DEAE-Sephadex chromatography and gel filtration on Bio-Gel P-200 column. The electrophoretically homogeneous preparation also exhibited hydrolytic activity (in a decreasing order) on amylose, xylan, amylopectin, glycogen, arabinogalactan and arabinoxylan. The enzyme had characteristically endo-hydrolytic activity on all the substrates tested and no xylose, glucose, arabinose or glucuronic acid could be detected even after prolonged enzymatic digestion of the polysaccharides.

Interestingly the enzyme had similar pH optima (5·5), temperature optima (55°C), pH stability (pH 3-10) and thermal denaturation kinetics when acted on both starch and xylan (larch wood). K_m values were found to be 2·63 mg/ml for amylase and 6·25 mg/ml for xylanase activity. Hill's plot also indicated that the enzyme contained a single active site for both activities. Hg^{2+} was found to be most potent inhibitor. Ca^{2+}, a common activator for amylase activity, appeared to be an inhibitor for this enzyme. Thus it appeared that the enzyme had multisubstrate specificity acting as α-amylase on starch and also acting as xylanase on side chain oligosaccharides of xylan containing α-linked sugars.

Keywords. Amylase; xylanase; polysaccharidase; *Termitomyces clypeatus.*

Introduction

Endo-glycosidases, particularly those hydrolysing polysaccharides of biomass into fermentable sugars, have much use in commerce. Different amylases, xylanases (hemicellulases) and cellulases from various microbial sources have largely been screened and studied for their commercial utility. We have reported earlier that mushroom-mycelia under submerged growth elaborates all of these enzymes in good titre (Ghosh and Sengupta, 1982). An interesting endo-β (l→4) xylanase, constitutively produced by mushroom *Termitomyces clypeatus* in dextrin medium, was purified to homogeneity (Ghosh *et al.,* 1980). The enzyme was found to be of high molecular weight (M_r) (~90,000) compared to other reported xylanases (Dekker and Richards, 1976) and also contained hydrolytic activity for amylopectin, arabinoxylan and arabino galactan. It is is to be mentioned that xylan is a complex molecule containing both β-linked xylosidic backbone and α-glycosidically linked glucose, arabinose and glucuronic acid in branch chains. Perhaps for this reason purified xylanases from various sources has been shown to contain carboxymethyl cellulase, amylase (Comtat *et al.,* 1975) and carboxymethyl cellulase, cellulase and amylase activities (Takahashi and Katsumi, 1979). However it was never identified whether origin of all these activities was because of contamination or lied on the same enzyme molecule.

The present paper describes purification of an extra cellular amylase from the

Abbreviations used: M_r, Molecular weight; BSA, bovine serum albumin; PAGE, Polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; TLC, thin-layer chromatography.

culture filtrate of mushroom *T. clypeatus* and to trace xylanase activity during purification steps and finally in the purified amylase molecule.

Materials and methods

Synthetic medium for the growth of *T. clypeatus* (Heim) has been described earlier (Ghosh *et al.,* 1980). For the production of enzyme, submerged fermentation was continued at 30 ± l°C for 7 days in a medium containing (% w/v) dextrin, 4; $NH_4H_2PO_4$, 2·463; $CaCl_2$–$2H_2O$, 0·037; KH_2PO_4, 0·087; $MgSO_4$–$7H_2O$, 0·05; H_3BO_3, 0·057; $FeSO_4$–$7H_2O$, 0·025; $MnCl_2$–$4H_2O$, 0·0036; $NaMoO_4$–$4H_2O$, 0·0032; $ZnSO_4$–$7H_2O$, 0·03; at pH 3·0. Xylan (1, 4-*β*-linked) from larch wood, yeast mannan, DEAE-Sephadex (A-50), methyl-α-D-xylopyranoside, methyl-*β*-D-xylopyranoside, amylopectin azure, α-methyl-D-mannopyranoside, 1-0-methyl·-α-D-glucopyranoside, 1-0-methyl-*β*-D-glucopyranoside, arabinogalactan, carboxymethyl cellulose (low viscosity), cellulose, dextran *(M_r* 66,900), amylose type III polygalacturonic acid grade III, glycogen type II and sucrose were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Arabinoxylan (arabinose, 34·1% and xylose, 65·9%) was a gift from Dr. G. B. Fincher, Brewing Industry Research Foundation, England. Bio-Gel P-200 (75–150 *μ*m) was the product of Bio-Rad Laboratories, Richmond, California, USA.

Other chemicals used were of chemically pure quality or better.

Assay of enzyme activity

This was carried out by measuring the amount of liberated reducing sugar according to the method of Nelson (1944) as modified by Somogyi (1952) and also described earlier (Ghosh *et al.,* 1980). The assay mixture contained 0·02 ml of culture filtrate or enzyme solution, 0·2 ml of xylan or starch (soluble) suspension or solution (10 mg/ml in 0·1 M acetate bluffer, pH 5·0) and 0·18 ml of the same buffer. Incubation was carried out for 30 min at 40°C and stopped by adding 0·4 ml of alkaline copper reagent. Readings were expressed in terms of glucose or xylose equivalents. One unit of enzyme activity was expressed as the amount of enzyme protein which produced one *μ*mol of D-glucose or D-xylose per min under the assay condition.

Protein determination

Protein was estimated according to Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard or by measuring absorbance at 280 nm during colum elution.

Polyacrylamide gel electrophoresis (*PAGE*)

It was carried out in glycine-Tris bluffer at pH 9·5 using 7·5% acrylamide (Gabriel, 1971). A constant current of 2·5 mA per gel (7·0 cm) was applied for 3 h at 25°C. Gels were stained with Coomassie brilliant blue for 12 h and destained with methanol/acetic acid/water.

Sodium dodecyl sulphate (SDS)-PAGE using Polyacrylamide gel (7·5%) was made

according to Weber *et al.* (1972). Protein standards (Bio-Rad): lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), BSA (66,200) and phosphorylase B (92,500) or enzyme were dissolved in 1·25% SDS buffer, heated on a boiling water bath and electrophoresed. A constant current of 8mA per gel (8.0cm) was applied for 6h at 25°C.

Purification of the enzyme

This was carried out at 4°C unless otherwise specified using the following steps.

Ammonium sulphate precipitation: The filtered broth (1660 ml) was brought to pH 6·5 with slow additions of K_2 H P O 4 , kept overnight and centrifuged at 50,000 g for 30 min. The supernatant was then brought to 80% saturation with $(NH_4)_2SO_4$, kept overnight and centrifuged at 105,000 *g* for 60 min. The precipitate was dissolved in 16ml of 0·01 M acetate buffer (pH 5·0), dialysed against the same buffer, and applied onto a DEAE-sephadex column.

Chromatography on DEAE-Sephadex (*A-50*): The column (4·6×27·8 cm) was equilibrated with 0·01 M acetate buffer (pH 5·0). The enzyme solution (15·5ml) was applied to the column and eluted with the same buffer at a flow rate of 40 ml/h. After passing 4 bed volumes of the buffer (1300 ml), a 0–0·8 M linear NaCl gradient was used.

Chromatography on Bio-Gel P-200: The column (3·0× 30cm) was equilibrated with 0·1 M acetate buffer (pH5·0). The enzyme fraction (116–141) eluted with NaCl gradient from the DEAE-*Sephadex* column was lyophilized to approximately 2·16 ml, dialysed against the same buffer and applied to the column. The enzyme was eluted (3·1 ml/tube) with the same buffer at a flow rate of 5 ml/h. The fraction (30-40) showing activity was used as enzyme source for further studies.

Studies on the properties of the purified enzyme

Optimum temperature: Both of the enzyme activities were tested at temperatures between 10—80°C for the determination of optimum enzyme activity.

Thermal stability: Enzyme solution (2·6 μg of protein for xylanase assay and 0·52 μg of protein for amylase assay) was kept at pH 5·0 for 0–240 min at 60°C and 0–30 min at 65°C following measurement of residual enzyme activities lefts.

Effect of pH on activity: Universal buffers (citrate/phosphate/borate/barbiturate) of pH between 2·5-10·0 were used in the incubation mixture to determine optimum enzyme activities using either starch or xylan.

Stability at different pH values: The stock enzyme solution was brought to difference

pH values (pH 2·5-10·0) by dilution (10 times) with universal buffers and kept for 1 h at 37°C. Residual activities of these preincubated samples were measured separately using either starch or xylan as substrates.

Effect of some metal ions and inhibitors: Enzyme activities were measured in the presence or absence of the test compounds using acetate buffer (0·1 M, pH 5·0).

Activity towards different substrates: Assay mixtures containing different carbohydrates (5 mg/ml) were incubated separately for 30 min in 0·1 M acetate buffer, pH 5·0, with 1·0 μg of enzyme.

Effect of substrate concentration on enzyme activity: Variable amounts of xylan (1·0–45 mg) and starch soluble (0·2-5·0 mg) in acetate buffer (0·1 M, pH 5·0) were incubated with 2 μg of enzyme during assay of xylanase activity and 0·52 μg of enzyme during assay of amylase activity.

Mode of action on different xylodextrins

Several xylodextrins were prepared according to Brown and Anderson (1971, 1972). The xylodextrins (X_2–X_8) thus prepared were then further purified by passing through a column (1·8 × 33·5 cm) of Sephadex G-25 using water as the eluent and then compared with authentic samples obtained from Proof. G. O. Aspinall, Depart ment of Chemistry, York University, Ontario, Canada. Enzymatic action on xylan, starch, arabinoxylan, arabinogalactan, glucose, xylose and xylodextrins (X_2-X_8) were studied separately. Incubation mixtures containing different saccharides were made in 2·0 ml of 0·1 M, pH 5·0 acetate buffer and 52 μg of enzyme and incubated at 40°C for 17 h and 41 h, respectively. The reactions were stopped with the addition of 2 ml of absoluted alcohol, shaken vigorously and centrifuged. The supernatants were then deionised and taken out for analysis by thin-layer chromatography (TLC). Plates were made using Kieselguhr buffered with 0·02 M acetate buffer, pH 5·0. The mobile phase used were ethyl acetate: isopropanol: water (75:49:26) for lower dextrins dp =1–3and (63:54:33) for higher dextrins dp = 4–8. Sugars of varied chain lengths were detected on the TLC plate using anisaldehyde reagent (Brown and Anderson, 1971). Dextrins (dp > 3) were compared with authentic xylodextrins only. Side by side blank experiments using boiled enzyme solution was also made. Both quantitative and qualitative measurement were made visually with the intensity of the colour produced on the chromatogram. Higher dextrins (dp > 8) were not available for further studies.

Results

Hydrolytic activity towards soluble starch and larch wood xylan was measured as a function of growth periods (figure 1). Activity towards hydrolysing both xylan (xylanase) and starch (amylase) remained almost same during early periods of growth. From 5th day onwards, amylase activity increased significantly while xylanase activity was not found to be increasing similarly. Though from 10th day

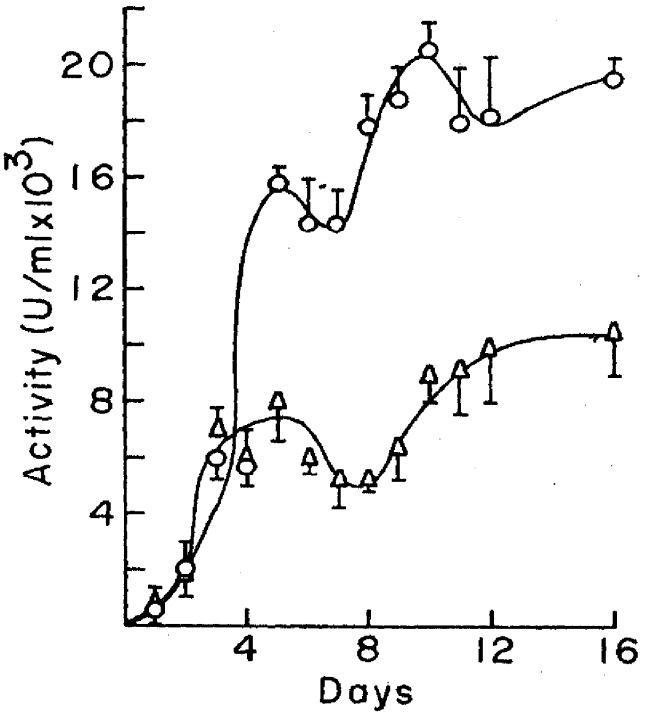

Figure 1. Production of extracellular xylanase and amylase during submerged growth of mushroom *T. clypeatus*.

Activities (O, amylase activity and Δ, xylanase activity) measured represent mean ± standard error of mean were determined as detailed in 'materials and methods'.

onwards about 1·5-fold increase in xylanase activity was detected but amylase activity was almost double or more than double than xylanase activity from 5th day onwards.

The enzyme was purified from the culture filtrate following different steps (table 1).

During purification of the enzyme it was observed that direct addition of ammonium sulphate to the culture filtrate caused significant loss in enzyme activity. This loss could be avoided by prior adjustment of pH of the culture filtrate to 6·5 with the addition of solid K_2 HPO_4 . This treatment caused a precipitation with no such loss in enzyme activity present in the supernatant (~90% of total activity). Unfortunately after $(NH_4)_2SO_4$ precipitation of the buffered culture filtrate about 10–30% of the total activity could only be obtained with 2-6 fold purification.

The $(NH_4)_2SO_4$ precipitated fraction was chromatographed on a DEAE-Sephadex (A-50) column. During washing with buffer a small fraction of enzyme activity was eluted in between fractions 51-54. Later, elution with NaCl gradient, majority of the enzyme activity (fractions 116-141, 156 ml) containing both xylanase and amylase were eluted in the same peak. At this point the enzyme was further purified 3-5-fold with practically no loss in recovery. Next the enzyme was purified more by gel filtration using Bio-Gel P-200. Fractions collected between 27-39 (~40ml), were again found to contain both the activities. After this step of purification, about 9-fold increase in xylanase activity was obtained with only 4%

Table 1. Purification of enzyme from the culture filtrate of *T. clypeatus*.

Enzyme sample	Protein (mg)	Total activity ($U \times 10^{-3}$)		Specific activity (U/mg of protein)		Recovery yield (%)		Purification (-fold)	
		Xylanase	Amylase	Xylanase	Amylase	Xylanase	Amylase	Xylanase	Amylase
Culture filtrate (1660 ml)	1344·6	5·902	4·55	4·39	3·38	100	100	1	1
Buffered culture filtrate (1650 ml)	808·5	5·42	4·28	6·71	5·29	91·95	94·07	1·53	1·57
Ammonium sulphate (0–80%) saturation (16 ml)	65·92	0·626	1·42	9·5	21·54	10·61	31·21	2·16	6·37
DEAE-Sephadex (A–50) fractions 116–141 lyophilised to 12·6 ml	20·03	0·56	2·33	27·96	116·3	9·49	51·21	6·37	34·41
Bio-Gel P-200 fractions 27–39 lyophilised to 10 ml	6·8	0·276	1·77	40·6	260·3	4·68	38·90	9·25	77·01

recovery. Surprisingly, the same fraction contained 77 fold increase in amylase activity with 39% recovery. The fraction collected from the Bio-Gel P-200 column was found to be homogeneous upon PAGE and on SDS-PAGE. No mobility of the enzyme protein was observed at lower pH values. M_r of the enzyme protein was estimated to be 85,000 upon SDS-PAGE and found to contain no subunit or similar subunits.

Enzymatic activities were then measured as a function of temperature. Both xylanase and amylase activities were found to reach optimum at 55°C. Activation energy (E) for xylanase and amylase activity were found to be 12·8 KCal and 11·8 KCal, respectively. Preincubation of the enzyme solution for 1 h at 35°C or below did not cause any loss in activity but similar treatment at 60°C cause 40-50% decrease in enzyme activity.

Preincubation of the enzyme solution at 60°C for 0-240 min (figure 2a) or at 64°C for 0–30 min (figure 2b) was made following measurement of residual enzyme activity left. The rate of loss in both amylase and xylanase activities were biphasic either at 60°C or at 64°C. At 60°C about 65 % of amylase and 43 % of xylanase activities were lost by initial 72 min of preincubation while after 240 min of preincubation about 88% of amylase and 66% of xylanase activities were lost. Similarly at 64°C about 40% of amylase and 18% of xylanase activities were lost by only initial 9 min period of preincubation while after 30 min of preincubation 82% of amylase and 43% of

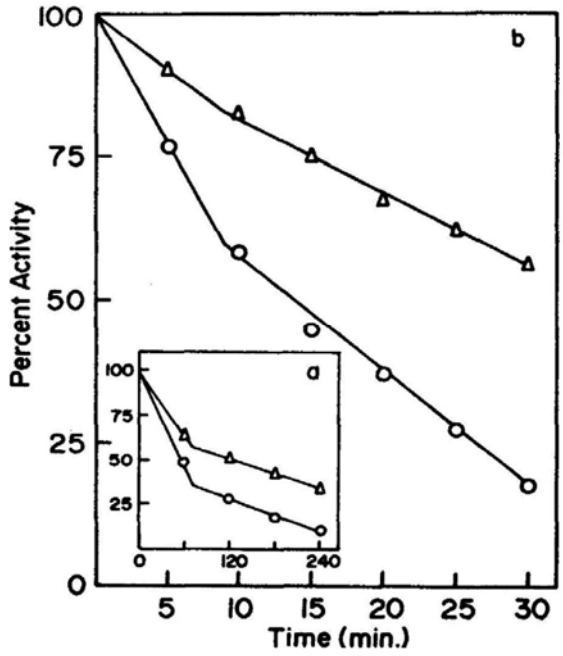

Figure 2. Thermal stability kinetics of the enzyme.

Enzyme solution containing both amylase (O) and xylanase (Δ) activities were pre-incubated either at 60°C (a) or at 64°C (b) for different time periods and the residual activity left were measured as detailed in 'materials and methods'.

xylanase activities were lost. About 40% loss in both xylanase and amylase activities were noticed when the enzyme solution was kept at 30°C, pH 5·0 for several days but about 65% loss in both of the activities were found to be gone by 4 h only after keeping at 40°C (data not shown). The enzyme was sensitive towards freezing and lyophilisation with 15% loss in activity per freezing and thawing cycle.

Optimum pH for both of the activities were found to be 5·5. About 50% decrease in optimum enzyme activity was noticed either at pH 3·0 or at pH 6·5. No activity was left when measured at pH more than 7·0. Stability of the enzyme for both of the activities at different pH values were measured at 40°C. It was noticed that the enzyme was relatively stable over a wide range of pH values (3·0-10·0) at least for 1 h.

The degree of potency in terms of inhibitory effect of all the chemicals tested has been presented in a decreasing order in table 2. Very little, 2·5% and 5·5% of total amylase and xylanase activity respectively could be detected when Hg^{2+} was present in the incubation mixture at a level of 2 mM only. Ag^{+} and Fe^{2+} also had some inhibitory effect on both of the enzymatic activities. BSA (100 μg/ml) and dextran (0–0·45%, w/v) had no effect.

Activity towards different carbohydrates were next studied (table 3). The enzyme

Table 2. Efect of some metal ions and inhibitors.

	Residual activity (%)	
Chemicals	Amylase	Xylanase
Hg^{2+} (2 mM)	2·5	5·5
Ag^{+} (2 mM)	52·0	61·5
Fe^{2+} (2 mM)	64·0	67·5
Ca^{2+} (20 mM)	75·0	86·0
Ca^{2+} (100 mM)	10·0	15·5
Na_2HPO_4 (100 mM)	45·0	50·0
CH_2ICOOH (20 mM)	11·5	5·0
EDTA (20 mM)	30·0	17·5

Incubations were made using 2·0 μg of enzyme for measuring xylanase activity and 0·52 μg of enzyme for measuring amylase activity as detailed in 'mate rials and methods'.

Table 3. Enzymatic activity on different substrates.

Substrate (5 mg/ml)	Activity ($U \times 10^{-1} \pm SEM$)
Starch soluble	17·20 ± 1·2
Amylose	16·37 ± 0·9
Amylopectin	1·33 ± 0·05
Glycogen	1·33 ± 0·20
Arabinogalactan	0·52 ± 0·02
Arabinoxylan	0·62 ± 0·02
Xylan	3·20 ± 0·18

Enzymatic action towards different polysaccharides were tested separately and the activities were expressed as glucose or xylose equivalents produced per mg of enzyme protein per min.

had no α- or *β*-xylosidic or glucosidic or α-mannosidic activity. Cellulose, CM-cellulose, sucrose, mannan, dextran and polygalacturonic acid were not at all attacked by the enzyme. Among the carbohydrates tested, starch (soluble) and amylose were primarily hydrolysed by the enzymatic action. Hydrolysing activity towards xylan was less than one fifth of the activity towards starch (soluble).

Mode of action of the enzyme towards xylan or starch or xylodextrins (only dextrins available) were carried out by incubating respective substrates in the presence of the enzyme and the products obtained were analysed by TLC as des cribed earlier. Incubation of the enzyme with arabinoxylan or arabinogalactan could produce very minute quantity of xylose or galactose. Presence of arabinose in the enzyme-hydrolysed incubation mixture was never noticed. Incubation containing xylan or starch with the enzyme, produced dextrins of varied chain length (dp ≥ 3) among which xylotetraose (X_4) was appeared to be the principal product. Xylobiose (X_2) and xylotriose (X_3) remained almost unattacked by the enzyme. Xyloheptaose (X_7) and xylooctaose (X_8) were readily attacked by the enzyme producing mainly xylotetraose (X_4) while xylohexaose (X_6) was attacked by the enzyme to make xylobiose (X_2) and xylotriose (X_3) together with xylotetraose (X_4). Xylopentaose (X_5) also was attacked by the enzyme to liberate mainly xylotetraose (X_4).

Effect of various concentrations of starch and xylan on the enzymatic activities were studied and the corresponding activities measured were analysed on a Lineweaver-Burk plot (figure 3). V_m values were found to be 64·52 μmol glucose

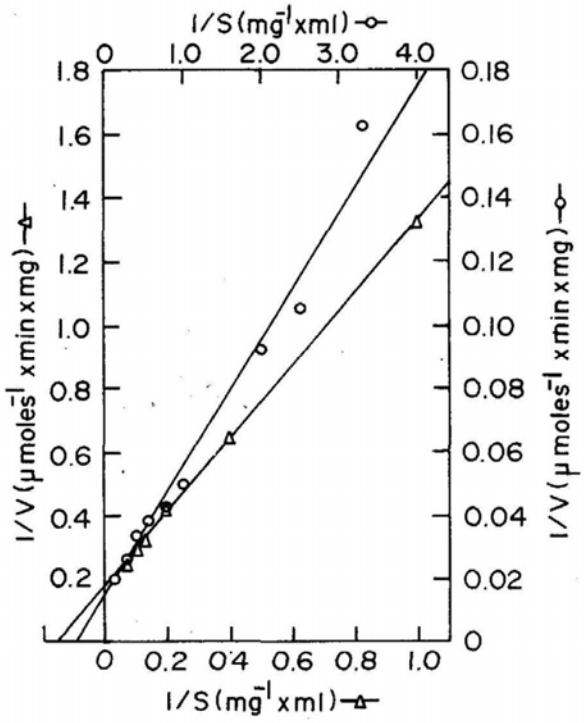

Figure 3. Linewcaver-Burk plot.

Enzymatic activities were measured as a function of either starch (O) or xylan (Δ) concentration using the methods described in 'materials and methods'.

equivalent/min/mg of protein for amylase and 5·56 μmol of xylose equivalent/min/mg of protein for xylanase activity. K_m values were 2·63 mg of starch/ml for amylase and 6·35 mg of xylan/ml for xylanase activity, respectively. Both amylase and xylanase activities were further analysed by the linear plot of Hill equation. Both of the slopes (n = 0·94≈1 using starch and n =1·16≈1 using xylan) had a value of unity. $S_{\frac{1}{2}}$ values were 2·63 mg of starch/ml and 6·17 mg of xylan/ml.

Discussion

Increase in amylase activity with respect to increase in xylanase activity during later periods of growth was not clearly understood (figure 1). This may be related to the increase in some inhibitors from 5th day onwards. Increase in amylase activity was similarly noticed during purification of the enzyme (table 1) which might be due to the removal of some inhibitors. Characterisation of this natural inhibitors had not been ruled out by some additional experiments since we had been principally inte rested in isolating an enzyme with multisubstrate specificity. Loss in enzyme activity during ammonium sulphate precipitation of the unbuffered culture filtrate was very similar to the observation, we have reported earlier (Ghosh *et al.,* 1980). Unfortun ately significant quantity (~80%) of xylanase activity was lost in comparison to loss in amylase activity (~60%) by ammonium sulphate precipitation. Elution pattern of the enzyme having both amylase and xylanase activity either from the ion-exchange column or from the gel filtration column were interestingly similar. Physicochemical properties and stability towards pH and temperature for both of the activities were also same and resemble in various respects to non-arabinose liberating endo-β-D xylanases (Dekker and Richards, 1976). No increase in enzymatic activity in terms of either xylanase or amylase was observed in the presence of Ca^{2+}. Moreover, both the activities were significantly inhibited when measured in the presence of Ca^{2+}. Moreover, both the activities were significantly inhibited when measured in the presence of Ca^{2+} (table 2) which is in contrast to common amylases (Thoma *et al.,* 1971). Inhibition of the enzyme activity in the presence of PO_4^{3-} was quite similar to the observation we reported earlier (Ghosh *et al.,* 1980). So at this point it may be assumed that the present enzyme was a single enzyme capable of hydrolysing both starch and xylan since the enzyme was electrophoretically homogeneous. The degree of starch hydrolysing activity was approximately 6 times more than xylan hydro lysing activity. Xylan from larch wood is an unbranched chain of xylopyranose residues, with every fifth or sixth residue substituted at C_2 with a 4-O-methol-D glucuronic acid unit and with a small number of xylopyranose residues substituted at C-3 with arabinofuranose units (Aspinall and Mckay, 1959). Acid hydrolysis of Sigma xylan has been shown to contain xylose, 48–71%; glucose, 10–13%; arabinose, 10–11%; glucuronic acid 8–29% and some unhydrolysed polymer (Frederick *et al.,* 1981). From the Lineweaver-Burk plot, K_m values for xylanase and amylase were 6·25 mg/ml and 2·63 mg/ml respectively (figure 3) indicating that the enzyme had lesser affinity for attacking xylan. But considering other observations the enzyme may be considered as a xylanase and not a commonly reported amylase (Thoma *et al.,* 1971). The enzyme could cleave α-1,4 glycosyl linkages present either in xylan or in starch with or without additional capacity for breaking β-1,4 and α-l,6 linkages. Similar report is also available for a thermostable xylanase purified from a thermo-

phillic acidophillic *Bacillus* Sp. (Uchino and Nakane, 1981). This was further confirmed by analysing the enzyme hydrolysed larch wood xylan by TLC. The predominant hydrolysed products from larch wood xylan were xylodextrins of varied chain lengths (dp>3). No xylose, glucuronic acid, arabinose or glucose were detected in the enzyme hydrolysed mixture.

Glycogen and amylo-pectin were poorly attacked by the enzyme in comparison to starch and amylose. Similarly arabinoxylan and arabinogalactan were poorly attacked with respect to xylan. So the enzyme may be considered as an endoenzyme and possibly had a preference in cleaving less branched polysaccharides. On the basis of all these facts the enzyme may be classified as an non arabinose liberating endo xylanase, as mentioned by Dekker and Richards (1976) cleaving principally α, l→4 glycosyl linkages.

Acknowledgement

The authors are grateful to Professor Bimal K. Bachhawat, ex-director of the institute for his kind interest and valuable suggestions during the progress of the work. Uninterrupted support from Dr. S. C. Pakrashi, is highly appreciated. Thanks are also due to Dr. G. B. Fincher, Brewing Industry Research Foundation, Nutfield, Redhill, Surrey, UK for arabinoxylan and Dr. G. O. Aspinall, Trent University, Ontario, Canada for providing xylodextrins (dp 2-8) as generous gift.

References

Aspinall, G. O. and Mckay, J. E. (1959) *J. Chem. Soc.,* **1959,** 1059.
Brown, W. and Anderson, O. (1971) *J. Chromatogr.,* **57,** 255.
Brown, W. and Anderson, O. (1973) *J. Chromatogr.,* **67,** 163.
Comtat, J., Ruel, K., Joseleau, J. and Barnoud, F. (1975) *Symp. Enz. hydrolysis of cellulose,* Aulanko, Finland, 12-14 March, p. 351.
Dekker, R. F. H. and Richards, G. N. (1976) *Adv. Carbohydr. Chem. Biochem.,* **32,** 227.
Frederick, M. M., Frederick, J. R., Fratzke, A. R. and Reilly, P. J. (1981) *Carbohydr. Res.,* **97,** 87.
Gabriel, O. (1971) *Methods* Enzymol., **22,** 565.
Ghosh, A. K. and Sengupta, S. (1982) *Acta Mycol.,* **18,** 113.
Ghosh, A. K., Banerjee, P. C. and Sengupta, S. (1980) *Biochim. Biophys. Acta,* **612,** 143.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.,* **193,** 265.
Nelson, N. (1944) *J. Biol. Chem.,* **153,** 375.
Somogyi, M. (1952) *J. Biol. Chem.,* **195,** 19.
Takahashi, M. and Katsumi, S. (1979) *J. Ferment. Technol.,* **57,** 434.
Thoma, J. A., Spradlin, J. E. and Dygert, S. (1971) *Enzymes,* **5,** 115.
Uchino, F. and Nakane, T. (1981) *Agric. Biol. Chem.,* **45,** 1121.
Weber, K., Pringle, J. R. and Osborn, M. (1972) *Methods Enzymol.,* **26,** 3.