*Biochimica et Biophysica Acta*, 612 (1980) 143—152


BBA 68921

# PURIFICATION AND PROPERTIES OF XYLAN HYDROLASE FROM MUSHROOM *TERMITOMYCES CLYPEATUS*

A.K. GHOSH, P.C. BANERJEE and S. SENGUPTA

*Indian Institute of Experimental Medicine, 4 Raja S.C. Mullick Road, Calcutta 700 032 (India)*

(Received June 6th, 1979)

*Key words: Xylan hydrolase; Polysaccharidase; (T. clypeatus)*

## Summary

The endoxylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) from the culture filtrate of a mushroom, *Termitomyces clypeatus*, has been purified 93-fold by ammonium sulphate precipitation, ion-exchange chromatography (DEAE-Sephadex) and gel permeation chromatography (Bio-Gel P-200). The enzyme preparation gave a single protein band on disc gel electrophoresis at pH 9.5, and has a molecular weight of approx. 90 000. It also acts on amylopectin, arabinoxylan and arabinogalactan.

The enzyme shows maximum activity on xylan (1,4-β-linked D-xylopyranose units) at pH 5.5 and at 55°C and is fairly stable between pH 3 and 10 and temperatures up to 60°C. The $K_m$ is 4 mg of xylan/ml. $Hg^{2+}$ is the most potent inhibitor, whereas $Fe^{2+}$, $Ag^{+}$, iodoacetate and phosphate moderately inhibit the enzyme activity.

## Introduction

Xylanase is an important hydrolytic enzyme splitting 1,4-β-linked glycosidic bonds of D-xylopyranoside units of xylan. This enzyme has been purified from various fungal and bacterial sources and studied in different laboratories [1] but little is known about the same from mushroom fermentation.

The present paper describes the purification and properties of an extracellular endoxylanase obtained during submerged fermentation of the mushroom *Termitomyces clypeatus*.

## Materials and Methods

The synthetic medium for the growth of *T. clypeatus* (Heim) has been described earlier [2]. For the production of enzyme submerged fermentation

was continued at 30 ± 1°C for 7 days in a medium containing (%, w/v) dextrin, 4; $NH_4H_2PO_4$, 2.463; $CaCl_2 \cdot 2\,H_2O$, 0.037; $KH_2PO_4$, 0.087; $MgSO_4 \cdot 7\,H_2O$, 0.05; $H_3BO_3$, 0.057; $FeSO_4 \cdot 7\,H_2O$, 0.025; $MnCl_2 \cdot 4\,H_2O$, 0.0036; $NaMoO_4 \cdot 4\,H_2O$, 0.0032; $ZnSO_4 \cdot 7\,H_2O$, 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-*O*-methyl-α-D-glucopyranoside, arabinogalactan and carboxymethyl cellulose (low viscosity) were purchased from Sigma Chemical Co., U.S.A. Arabinoxylan (arabinose, 34.1%, and xylose, 65.9%) was a gift from Dr. G.B. Fincher, Brewing Industry Research Foundation, England. Biol-Gel P-200 (75—150 μm) was the product of Bio-Rad Laboratories, U.S.A. Iodoacetic acid, dextrin white ($[\alpha]_D > 200°$) and EDTA were purchased from E. Merck, Darmstadt, F.R.G. Horse myoglobin ($M_r$ 17 800), chymotrypsinogen A (25 000), ovalbumin (45 000) and bovine serum albumin (67 000), were the products of Serva Fine biochemicals), Heidelberg, F.R.G. Other chemicals used were of chemically pure quality.

*Assay of xylanase activity*

This was carried out by measuring the amount of liberated reducing sugar according to the method of Nelson [3] as modified by Somogyi [4]. The assay mixture contained 0.1 ml of culture filtrate or enzyme solution, 1.0 ml xylan suspension (10mg/ml in 0.1 M citrate/phosphate/acetate buffer, pH 5.0) and 0.9 ml of the same buffer. Incubation was carried out for 30 min at 40°C, and stopped by adding 2 ml alkaline copper reagent. The mixture was kept for 10 min in a boiling water bath and cooled, followed by the addition of 1.0 ml arsenomolybdate reagent. After 15 min the mixture was centrifuged. The supernatant was diluted 5 times with water and the absorbance measured at 500 nm. Readings were expressed in terms of xylose equivalents. One unit of enzyme activity was expressed as the amount of enzyme protein which produces one μmol of D-xylose per min under the assay conditions.

*Protein determination*

Protein was estimated according to Lowry et al. [5] using bovine serum albumin as standard.

*Polyacrylamide gel electrophoresis*

Gel electrophoresis was carried out in glycine-Tris buffer of pH 9.5 using 7.5% acrylamide [6]. 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) gel electrophoresis of the β-mercaptoethanol-treated enzyme on polyacrylamide gel (7.5%) was performed according to Weber et al. [7]. A constant current of 8 mA per gel (8.0 cm) was applied for 6 h at 25°C.

*Purification of xylanase*

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

*(i) Ammonium sulphate precipitation.* The filtered broth (2 l) was brought to pH 6.5 by slow addition of $K_2HPO_4$, which caused a heavy precipitate. The mixture was kept overnight and centrifuged. The supernatant was then brought to 80% saturation with $(NH_4)_2SO_4$ and kept overnight. The precipitate was dissolved in 20 ml 0.005 M citrate/phosphate buffer (pH 5.0), dialysed against the same buffer and applied onto a DEAE-Sephadex column.

*(ii) Chromatography on DEAE-Sephadex* (A-50). The column (2.8 × 39.0 cm) was equilibrated with 0.005 M citrate/phosphate buffer (pH 5.0). The enzyme solution (20 ml) was applied to the column and eluted with the same buffer at a flow rate of 12 ml/h. After passing 4 bed volumes of the buffer (650 ml), a 0—0.8 M linear NaCl gradient was used.

*(iii) Chromatography on Bio-Gel P-200.* The column (2.2 × 51 cm) was equilibrated with 0.1 M citrate/phosphate buffer (pH 5.0). Xylanase fraction (60—80) eluted with NaCl gradient from the DEAE-Sephadex column was lyophilized to approx. 4 ml, dialysed against the same buffer and applied to the column. The enzyme was eluted (5 ml/tube) with the same buffer at a rate of 4.5 ml/h. The fraction (22—33) showing activity was used as enzyme source for further studies.

*Studies on the properties of the purified enzyme*

*(a) Optimum temperature.* Xyalanase activity was measured at 10—80°C to determine the optimum temperature.

*(b) Thermal stability.* Enzyme solution (10 μg protein per ml) was kept at pH 5.0 for 1 h at different temperatures (30—80°C) and for several hours (40°C) to days (30°C) and residual enzyme activity was measured. The same enzyme solution was lyophilized, frozen and thawed, and enzyme activity was measured.

*(c) Effect of pH on activity.* Universal buffers (citrate/phosphate/borate/barbiturate) of pH 3.0—10.0 were used in the incubation to determine the optimum pH for enzyme activity.

*(d) Stability at different pH values.* The stock enzyme solution was brought to different pH values (pH 3—10) by dilution (10 times) with universal buffers and kept for 1 h at 37°C. Activity of these preincubated samples was measured.

*(e) Rate of xylan hydrolysis.* This was done by determining the reducing groups liberated at different time intervals (10—150 min) in an incubation mixture containing $1.1 \cdot 10^{-2}$ unit of enzyme and 10 mg xylan.

*(f) Effect of some metal ions and inhibitors.* Enzyme activity was measured in presence of the compounds using acetate buffer (0.1 M, pH 5.0).

*(g) Effect of substrate concentration on enzyme activity.* Variable amounts (0.25—40 mg) of xylan in acetate buffer were incubated with the same amount of enzyme ($3.91 \cdot 10^{-2}$ unit).

*(h) Activity towards different substrates.* In the assay mixtures different carbohydrates including xylan were incubated for 2 h at the concentration of 5 mg/ml in 0.1 M acetate buffer with the same amount of enzyme. Activities were expressed in terms of liberated xylose equivalents.

*Thin-layer chromatography of enzyme hydrolysed products*

Aliquots of the incubation mixture at various time intervals were chromatog-

raphed on buffered Kieselguhr plates for detection of liberated xylose and other carbohydrates according to Brown and Anderson [8]. The incubation mixture contained 2 ml 1% (w/v) xylan in 0.1 M acetate buffer (pH 5.0) and 0.3 ml enzyme (10 μg protein/ml) and the solvent system used was isopropanol/ethyl acetate/water (13 : 20 : 7). Spots were detected on the chromatogram with anisaldehyde reagent. Xylose was chromatographed as reference sugar.

## Results

*Purification of xylanase*

The enzyme was purified from the culture filtrate of *T. clypeatus* in a state of homogeneity following different steps (Table I).

*(i) $(NH_4)_2SO_4$ precipitation.* It has been observed that direct addition of ammonium sulphate in the culture filtrate caused about 80—90% loss in enzyme activity. This loss could be checked by prior buffering of the culture filtrate to pH 6.5 by adding solid $K_2HPO_4$. This treatment caused precipitation which is free from any enzyme activity but the supernatant has only 40% activity. However, the total activity could be recovered by subsequent ammonium sulphate precipitation. It has also been found that subsequent dialysis of ammonium sulphate fraction resulted in an increase in total enzyme activity from 38.4 to 42.3%. All these steps caused a 6-fold purification of the enzyme.

*(ii) Chromatography on DEAE-Sephadex (A-50).* The elution pattern of proteins including xylanase activities from the column charged with ammonium sulphate precipitate is shown in Fig. 1.

It was observed that during initial buffer elution a small fraction of total xylanase activity, applied on the column, came out in the fractions 12—38. These fractions were curiously assayed for CM-cellulase activity and found to have a high titre of the same. This portion having high cellulase and low xylanase activity was not included in the subsequent purification steps. Elution with a NaCl gradient liberated the major xylanase from the column without any contaminated cellulase activity (fractions 60—82, 116 ml). From Table I, it

TABLE I

PURIFICATION OF XYLANASE FROM THE CULTURE FILTRATE OF *T. CLYPEATUS*

| Enzyme sample | Protein (mg) | Total activity (Units × $10^{-2}$) | Specific activity (Units per mg of protein) | Recovery yield (%) | Purification (-fold) |
|---|---|---|---|---|---|
| Culture filtrate (2000 ml) | 1306.0 | 7.87 | 0.597 | 100 | 1 |
| Buffered culture filtrate (2000 ml) | 366.0 | 3.02 | 0.825 | 38.37 | 1.38 |
| Ammonium sulphate (0—80%) saturation (20 ml) | 95.0 | 3.33 | 3.50 | 42.31 | 5.86 |
| DEAE-Sephadex (A-50) fractions 60—82 (116 ml) | 9.3 | 2.32 | 25.0 | 29.48 | 41.88 |
| Bio Gel P-200 fractions 22—33 (55 ml) | 2.2 | 1.22 | 55.5 | 15.50 | 92.96 |

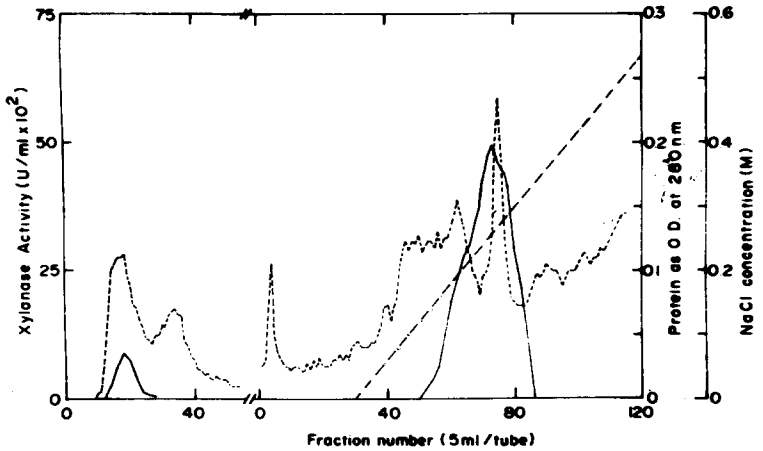


Fig. 1. DEAE-Sephadex A-50 column chromatography (Step 4). Procedures used are described in Methods. ———, xylanase activity; - - - - -, protein as determined from $A_{280}$; · — · — ·, NaCl concentration.

is evident that 7-fold purification of this enzyme takes place at this step. Thus, up to this step 30% enzyme activity was recovered with 42-fold purification.

*(iii) Gel-fractionation with Bio Gel P-200.* The fractions collected between tubes 22 and 33 (55 ml), were found to have xylanase activity (Fig. 2). This step increased the specific activity of the enzyme by more than 2-fold with about 50% loss in recovery yield.

*(iv) Disc electrophoresis on polyacrylamide gel.* The xylanase eluted from Bio Gel P-200 column upon gel electrophoresis at pH 9.5 gave a single band (Fig. 3, I). No mobility of the enzyme protein was observed at lower pH values. The enzyme also gave a single band on SDS gel electrophoresis (Fig. 3, II).

*General properties of the purified enzyme*

*(a) Optimum temperature.* The enzyme shows optimum activity at 55°C

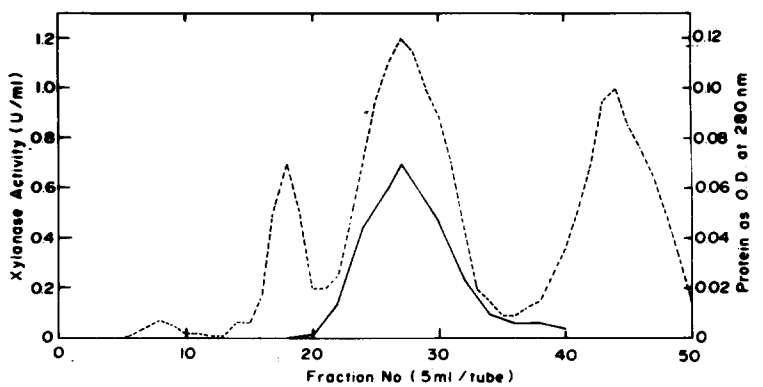


Fig. 2. Bio Gel P-200 gel filtration (Step 5). Procedures used are described in Methods. ———, xylanase activity; - - - - -, protein as determined from $A_{280}$.

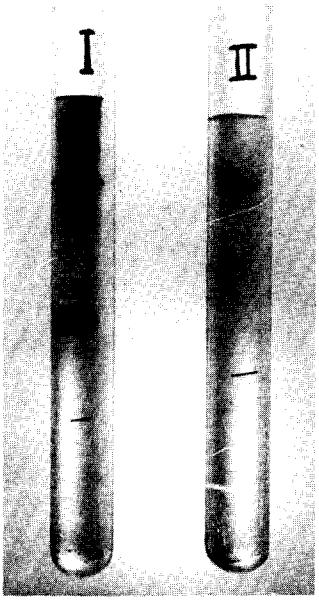


Fig. 3. Polyacrylamide gel electrophoresis of the purified xylanase. (I) Electrophoretogram of the enzyme on 7.5% polyacrylamide gel. (II) Electrophoretogram of the reduced and denatured enzyme on 7.5% polyacrylamide gel with 0.1% SDS.

with xylan as substrate and retains 20% of its maximum activity even at 10 or 75°C.

*(b) Thermal stability.* Preincubation of the enzyme for 1 h at 35°C or below does not affect its activity, but at higher temperatures (up to 60°C) 40—50% loss was noted. A sharp fall from 60°C leading to a total loss in activity at 70°C was observed. Preservation at 30°C even for several days caused only about 42% loss in activity, while incubation at 40°C for only 4 h lowered the enzyme activity to 35% (Table II). The enzyme is stable towards freezing and lyophilization with 15% loss in activity per freezing and thawing cycle.

*(c) Effect of pH.* Optimum pH for xylanase activity was found to be 5.5. About 50% decrease in the optimum activity occurs both at pH 3.0 and 7.0; surprisingly, it was noted that the enzyme has some activity even at pH 10.0.

*(d) Stability at different pH values.* The enzyme is relatively stable over a wide range of pH values (3.0—10.0) at least for 1 h.

TABLE II

THERMAL STABILITY OF *T. CLYPEATUS* XYLANASE AT 30 AND 40°C

| Temperature (°C) | Time | Loss of activity (%) |
|---|---|---|
| 30 | 24 h | 31.07 |
| 30 | 7 days | 41.75 |
| 40 | 4 h | 66.02 |

TABLE III

EFFECT OF SOME METAL IONS AND INHIBITORS ON XYLANASE ACTIVITY

Activity was assayed by incubating 0.025 units of enzyme in 2 ml (final volume) containing 0.1 M acetate buffer (pH 5.0), 10 mg xylan and the chemicals at 40°C for 30 min, and measuring the xylose liberated therein following the method already described.

| Chemicals | Residual activity (%) | |
|---|---|---|
| | 2 mM | 20 mM |
| $Hg^{2+}$ | 11.5 | 0.0 |
| $Ag^{+}$ | 61.5 | 8.0 |
| $Fe^{2+}$ | 67.5 | 10.0 |
| $Cu^{2+}$ | — | 75.0 |
| $Zn^{2+}$ | — | 75.0 |
| $Mg^{2+}$ | — | 98.0 |
| $Ca^{2+}$ | — | 86.0 |
| $NaN_3$ | — | 98.0 |
| $CH_2ICOOH$ | 94.5 | 5.0 |
| EDTA | 100.0 | 17.0 |

*(e) Rate of xylan hydrolysis.* It has been observed that by the enzymatic action on xylan, reducing groups are exponentially liberated with time for a period up to 90 min and the rate of hydrolysis is uniform right from the beginning. Beyond this period, the rate of hydrolysis decreases gradually with time.

*(f) Effect of some chemical agents.* Among the metal ions tested (Table III), $Hg^{2+}$, $Ag^{+}$ and $Fe^{2+}$ are strong inhibitors while $Cu^{2+}$, $Zn^{2+}$ and $Ca^{2+}$ caused mild inhibition at 20 mM concentration. Iodoacetic acid and EDTA also inhibit activity strongly at 20 mM and very weakly at 2 mM concentration. Among the inhibitor tested $Hg^{2+}$ may be accounted as the most potent one. It was also interestingly noted that about 50% loss of activity occurs in the presence of 100 mM $NaH_2PO_4$. Bovine serum albumin (100 μg/ml) has no effect on enzyme activity.

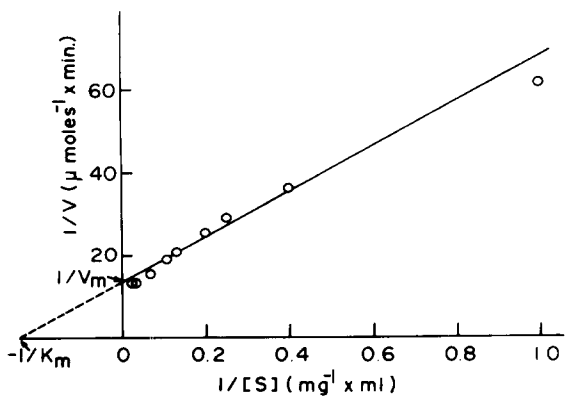


Fig. 4. Lineweaver-Burk plot. Variable amounts (0.25—45 mg per ml in 0.1 M acetate buffer, pH 5.0) of xylan were incubated.

TABLE IV

SPECIFIC ACTIVITIES OF *T. CLYPEATUS* XYLANASE ON DIFFERENT SUBSTRATES

Specific activity is expressed in terms of xylose equivalents produced per mg of enzyme protein per min.

| Substrate (5 mg/ml) | Specific activity ($\mu mol \cdot mg^{-1} \cdot min^{-1}$) |
|---|---|
| Arabinogalactan | 5.3 |
| Arabinoxylan | 5.5 |
| Amylopectin | 14.7 |
| Xylan | 55.5 |

*(g) Effect of substrate concentration.* From the Lineweaver-Burk plot (Fig. 4), $V$ and $K_m$ values are found to be $7.41 \cdot 10^{-2}$ $\mu$mol of xylose/min per $\mu$g of enzyme and 4 mg xylan per ml, respectively.

*(h) Activity towards different substrates.* The enzyme has no $\alpha$- or $\beta$-xylosidic or $\alpha$-glucosidic or $\alpha$-mannosidic activity. Cellulose, CM-cellulose, sucrose and mannan were not at all attacked by the enzyme, but appreciable reducing sugar formation was noted by its action on two unrelated carbohydrates like amylopectin and arabinogalactan (Table IV). Almost same

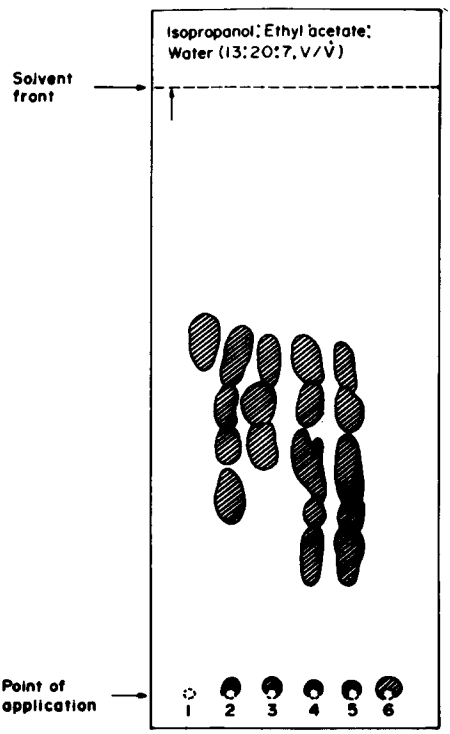


Fig. 5. One-dimensional thin-layer chromatogram of incubation mixtures containing *T. clypeatus* xylanase with xylan as substrate. (1), D-xylose; (2), (3), (4), (5) and (6) are reaction mixtures incubated for 0.5, 1.0, 4.0, 24.0 and 0 h, respectively. Procedures are described in Methods.

enzyme activity was noticed for both arabinogalactan and arabinoxylan.

*(i) Characterisation of xylose in the enzyme hydrolysed products.* The schematic diagram of the developed thin-layer chromatographic plate (Fig. 5) represents that xylose is one of the products in all the incubation mixtures (from 0.5—24 h). But total hydrolysis to xylose was not achieved even in 24 h of incubation.

*(j) Molecular weight and subunit localisation of the xylanase.* Molecular weight of xylanase as determined from the relative mobility of different standard proteins on SDS electrophoresis was approximated to be 90 000. The enzyme also gave a single protein band (Fig. 3, 11) indicating that it contains similar subunits or not subunit.

## Discussion

Xylanases are nowadays receiving much attention due to their huge applications in commerce as well as in various fields of biochemistry. Preliminary reports on xylanases from the mushrooms, *Agaricus bisporus* [9], *Polysporus betulinus* [10] and *Schizophyllum commune* [11] are available, but the extracellular production of the enzyme from mushroom so far has not been reported in detail.

The extracellular production of a xyalanase from a mushroom, *T. clypeatus*, as observed in the laboratory appeared to be interesting as the enzyme is constitutive and produced in high titre. In the process of purification, ammonium sulphate precipitation of the enzyme from the unbuffered culture filtrate caused considerable loss in the enzyme activity and that was only partially prevented by prior buffering of the culture filtrate. This phenomenon was reported by Dekker and Richards [12] during xylanase purification from the culture filtrate of a lower fungus.

Chromatography of the ammonium sulphate precipitate on DEAE-Sephadex gave two well separated xylanase fractions. The first fraction, eluted with the void volume, has high cellulase but low xylanase activity and was not purified further. The second major xylanase fraction having no cellulase activity was eluted by using a linear gradient (0—0.8 M) of NaCl. Two protein bands were detected in the fraction by polyacrylamide gel electrophoresis at pH 9.5. Subsequent purification of the enzyme preparation by gel fractionation on Bio Gel P-200 gave a homogeneous enzyme solution. These procedures caused about 93-fold purification of the enzyme with a recovery yield of about 15% (Table I).

Studies on the physicochemical properties of the mushroom xylanase indicate that the enzyme has a pH optimum at 5.5, temperature optimum at 55°C, temperature stability up to 60°C and pH stability between pH 3 and 10. All these observations are very similar to the properties of xylanases from various sources [1]. But the molecular weight of *T. clypeatus* xylanase is relatively high (about 90 000) compared to that reported from other sources [1].

The inhibition of enzyme activity by $Hg^{2+}$, $Ag^{+}$, $Cu^{2+}$, $Fe^{2+}$ and also by iodoacetate was similarly observed for a xylanase from *B. subtilis* [13]. The $Hg^{2+}$ was found to be the most potent inhibitor, and its effect on enzymic activity suggests reaction with thiol groups. But the inhibition by $PO_4^{3-}$ of this enzyme

activity as observed here has not been reported so far. During purification, the loss of xylanase activity by buffering with $PO_4^{3-}$ and also the subsequent slight regain in activity upon dialysis may be accounted for by the same reason. So in the subsequent studies, citrate-phosphate buffer was replaced by acetate buffer.

The rate of hydrolysis of xylan decreased in the higher range of substrate concentration, presumably because of substrate inhibition.

The substrate specificity of this enzyme is interesting as it liberates appreciable reducing groups from amylopectin and arabinogalactan. Compared to xylan, arabinoxylan was found to be a poor substrate. Activities towards amylopectin and arabinogalactan as observed here, are not peculiar for xylanases. The purified xylanase from commercial cellulase preparation is also capable of hydrolysing both cellulose and amylose [14,15]. Takenishi et al. [16] reported that a highly purified xylanase is also capable of hydrolysing (1 → 3)-L-arabinofuranosyl linkages at the branch points of arabinoxylan. A similar observation was also reported by Toda et al. [17].

Characterisation of the enzyme-hydrolysed products (Fig. 5) indicates that the enzyme is an endoxylanase.

## Acknowledgement

The authors are grateful to Professor B.K. Bachhawat, director of the institute for his kind interest and valuable suggestions during the progress of the work. A fellowship grant to A.K.G. by the Council of Scientific and Industrial Research is also gratefully acknowledged. Thanks are also due to Dr. G.B. Fincher, Brewing Industry Research Foundation, Nutfield, Redhill, Surrey, U.K. for the gift of arabinoxylan.

## References

1 Dekker, R.F.H. and Richards, G.N. (1976) in Advances in Carbohydrate Chemistry and Biochemistry (Tipson, R.S. and Horton, D., eds.), Vol. 32, pp. 227—352, Academic Press, New York
2 Ghosh, A.K. and Sengupta, S. (1978) J. Food Sci. Technol. 15, 237—242
3 Nelson, N. (1944) J. Biol. Chem. 153, 375—380
4 Somogyi, M. (1952) J. Biol. Chem. 195, 19—23
5 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
6 Gabriel, O. (1971) in Methods in Enzymology (Jakoby, W.B., ed.), Vol. 22, pp. 565—578, Academic Press, New York
7 Weber, K., Pringle, J.R. and Osborn, M. (1972) in Methods in Enzymology (Hirs, C.H.W. and Timasheff, S.N., eds.), Vol. 26, pp. 3—27, Academic Press, New York
8 Brown, W. and Anderson, O. (1971) J. Chromatogr. 57, 255—263
9 Hashimoto, K. and Takahashi, Z. (1970) Toyo Shokuhin Kogyo Tauki Daigaku, Toyo Shokuhin Kenkyusho Knekyu Hokokusho 9, 320—326
10 Lyr., H. (1960) Arch. Mikrobiol. 35, 258—278
11 Varadi, J., Necesany, V. and Kovacs, P. (1971) Drev. Vysak. 16 (3), 147—159
12 Dekker, R.F.H. and Richards, G.N. (1975) Carbohydr. Res. 39, 97—114
13 Takahashi, M. and Hashimoto, Y. (1963) Hakko Kogaku Zasshi 41, 116—119
14 Comtat, J., Ruel, K., Joseleau, J. and Barnoud, F. (1975) Symposium on Enzymatic Hydrolysis of Cellulose, Aulanko, Finland, 12—14 March, 1975, p. 351.
15 Hrazdina, G. and Neukom, H. (1966) Biochim. Biophys. Acta. 128, 402—403
16 Takenishi, S. and Tsujisaka, Y. (1973) Agric. Biol. Chem. 37, 1385—1391
17 Toda, S., Suzuki, H. and Nisizawa, K. (1971) Hakko Kogaku Zasshi 49, 499—521