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Purification and characterisation of a β-D-xylosidase from the anaerobic rumen fungus *Neocallimastix frontalis*

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ABSTRACT

A β-D-xylosidase from the anaerobic rumen fungus *Neocallimastix frontalis* was purified by anion-exchange and gel filtration chromatography. The enzyme was isoelectrically homogeneous and had an isoelectric point of pH 4.6. The apparent molecular mass calculated by gel filtration was 150000 Da. Under denaturing conditions, the enzyme appeared as a dimer composed of two polypeptides with molecular masses of 83000 and 53000 Da. The pH and temperature optimum were 6.4 and 37°C, respectively: the activity was very sensitive to temperature. The enzyme was inhibited by copper, silver and zinc ions, EDTA and SDS, and was stimulated by calcium and magnesium ions. It was competitively inhibited by D-xylose with an apparent K_i of 3.98 mM. The β-D-xylosidase exhibited hydrolytic activity on xylobiose and xylo-oligosaccharides of dp up to 7: the specific activities and maximum velocities decreased as the chain length increased. Analysis of the products of hydrolysis by HPLC indicated a typical exo-action. A mixture of β-D-xylosidase and a xylanase acted synergistically in producing high reducing sugar values, using a xylan from oat spelts.

INTRODUCTION

D-Xylans (arabinoglucuronoxylans) are major constituents of woods and agricultural residues. To realise the industrial potential of these xylans, they must be broken down to monosaccharides, which can be used as chemical feedstocks or as growth substrates for the production of alcohol or other chemicals. In both the agricultural and chemical industries, it is important to understand the mechanism by which the xylans are degraded by microbial enzymes in order to initiate other studies that will lead to improvements in the efficiency of the conversion.

Much of the research carried out so far on xylan-degrading enzymes has involved enzymes from aerobic fungi and bacteria. The recent discovery that anaerobic fungi proliferate in the rumen and that these microorganisms produce

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highly active extracellular cellulases and hemicellulases has resulted in considerable research activity[1].

Xylans are generally highly branched heteropolymers. The common substituents, which are attached to the backbone of β-(1 → 4)-linked D-xylopyranosyl residues, are acetyl, arabinosyl and glucosyluronic acid residues. A feature of some xylans is the presence of phenolic substituents which are covalently linked via the arabinosyl residues. Present knowledge clearly suggests that the enzymatic hydrolysis of these complex xylans requires the combined action of endo-(1 → 4)-β-D-xylanases (EC 3.2.1.8), β-D-xylosidases (EC 3.2.1.37), and enzymes responsible for cleavage of side groups[2,3]. In order to understand the role and the efficiency of the various components of the hydrolytic systems, fractionation and purification of individual enzymes is a necessary pre-requisite. We now report the purification and characterisation of a β-D-xylosidase from the anaerobic rumen fungi *Neocallimastix frontalis*. This fungus has already been reported to produce a highly active extracellular cellulase and xylanase[4,5].

A number of reports on the isolation and properties of β-D-xylosidases from aerobic fungi and bacteria are available[6–13]. However, to our knowledge, this is the first communication on the purification and characterisation of a β-D-xylosidase from an anaerobic fungus.

EXPERIMENTAL

Growth conditions.—*N. frontalis* RK 21 and the methanogen *Methanobrevibacter smithii* were co-cultured at 39°C in a liquid medium composed of 150 mL/L mineral salt solution I (3.0 g/L K_2HPO_4), 150 mL/L mineral salt solution II (3.0 g/L KH_2PO_4, 6.0 g/L $(NH_4)_2SO_4$, 6.0 g/L NaCl, 1.23 g/L $MgSO_4 \cdot 7H_2O$, and 0.69 g/L $CaCl_2 \cdot H_2O$), 4 g/L $NaHCO_3$, 1 g/L cysteine hydrochloride, 1.25 mL/L resazurine solution (0.1%), and 20% sterilised cow rumen fluid. The xylan-containing substrate used was non-delignified barley straw at 0.5% concentration. Culture fluids were recovered after 7 days of growth.

Enzyme recovery.—Culture broth (19 L) was centrifuged at 25000 *g* for 30 min and 4°C and the clear supernatant solution concentrated by ultrafiltration in a hollow fibre cartridge (Amicon H1P10-20) with a molecular weight cut-off of 10000. The concentrate was precipitated at 4°C with ammonium sulphate (85% saturation), resuspended in 0.1 M 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.0) and stored at −18°C. Filtrate obtained from this step was concentrated in a hollow fibre cartridge (Amicon H1P3-20) with a molecular weight cut-off of 3000. The concentrate was then precipitated at 4°C with ammonium sulphate (85% saturation), resuspended in 0.1 M MES buffer (pH 6.0), and fractionated as described below.

HiLoad anion-exchange chromatography.—A HiLoad 16/10 Q Sepharose HR column (Pharmacia) equilibrated with 0.01 M bis-Tris buffer (pH 6.0) containing 0.01 M NaCl was used. Elution was effected with a linear NaCl gradient (0.01–0.5

M) in the same buffer at a flow rate of 5 mL/min. Fractions (5 mL) were collected and screened for β-D-xylosidase activity.

Gel filtration.—This chromatography technique was performed on a Superose 6 HR 10/30 column (Pharmacia) equilibrated with 0.1 M ammonium acetate buffer (pH 6.0) and eluted with the same buffer at a flow rate of 0.3 mL/min. Aliquots (200 μL) were applied to the column. Fractions (0.9 mL) were collected and screened for enzyme activity. The following proteins were used for the calibration of the column: thyroglobulin (669 000 Da), ferritin (440 000 Da), aldolase (158 000 Da), and BSA (66 000 Da).

Polyacrylamide gel electrophoresis.—Polyacrylamide gels were prepared from the monomers (National Diagnostics, Aylesbury, Bucks., UK). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a discontinuous buffer system: stacking gel (4% T, 2.5% C) at pH 6.8, and resolving gel (11.2% T, 1.8% C) at pH 8.8. Samples were treated at 100°C for 5 min in the presence of "sample treatment buffer" (2% SDS, 10% glycerol, 0.02% Bromophenol Blue, and 5% mercaptoethanol in 0.0625 M Tris-HCl, pH 6.8) and run at 20 mA for 1 h. The running buffer was Tris-glycine (0.025 M Tris, 0.19 M glycine, and 0.1% SDS) at pH 8.3. Gels were stained either with Coomassie Brilliant Blue R-250 or with silver stain (Biorad). Low and high molecular weight calibration mixtures (Sigma) were used as standard.

Isoelectrofocusing (IEF).—IEF was carried out in polyacrylamide gels (4.1% T, 0.12% C) containing 3.4% Ampholine (Pharmacia, UK), pH range 3.0–5.5, and 1.68% Ampholine, pH range 5.5–10.0. The resultant gradient pH in the gels was 3.0–5.8. A voltage of 1 000 V was applied for 2 h. Gels were stained either with Coomassie Brilliant Blue or with silver stain.

Zymogram.—Enzyme activity was detected in situ by overlaying IEF gels with 0.025 M methylumbelliferyl β-D-xylopyranoside (MUX) in 0.1 M MES buffer (pH 6.4) containing agar (1.0%). Incubations were done at 37°C. MUX hydrolysis was revealed in UV light.

Enzyme assays.—*(a) β-D-Xylosidase activity*. This was determined by measuring the release of *p*-nitrophenol (*p*NP) from *p*-nitrophenyl β-D-xylopyranoside (*p*NPX). Assay mixtures (0.1 mL) containing 0.5 mg/mL pNPX in 0.1 M MES buffer at pH 6.4 were incubated for 30 min at 37°C in microtitre plates. Reactions were stopped by the addition of one volume of 0.4 M glycine–NaOH buffer (pH 10.8). The absorbance of the liberated *p*NP was measured at 405 nm, using a Titertek plate reader (Flow Laboratories Ltd, Rickmansworth, Herts, UK). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*NP per min at pH 6.4 and 37°C.

(b) α-L-Arabinofuranosidase, β-D-glucosidase, β-D-galactosidase, and acetyl esterase activities. These were measured by following the liberation of *p*NP from *p*NP-α-L-arabinofuranoside, *p*NP-β-D-glucopyranoside, *p*NP-β-D-galactopyranoside, and *p*NP-acetate (all from Sigma), respectively. Conditions were the same as described above.

(c) Xylanase activity. This was determined by assaying the reducing sugars released from a suspension of oat-spelt arabinoxylan (10 g of xylan were dissolved in 250 mL of 1 M NaOH, brought to pH 6.0 by adding 1 M acetic acid; 10 mL of 2% NaN_3 were added and the solution was made up to 1 L). Assay mixtures (1 mL) containing 0.5% of the xylan suspension were incubated for 30 min at 37°C in 0.1 M MES buffer at pH 6.4. Reducing sugars were determined colorimetrically at 520 nm, using the Nelson–Somogyi method[14], and expressed as xylose equivalents. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugars per min at pH 6.0 and 50°C.

(d) Cellobiase activity. This was measured by assaying the D-glucose liberated from cellobiose. Assay mixtures (1 mL) containing 0.4 mg/mL of cellobiose in 0.1 M MES buffer at pH 6.4 were incubated for 30 min at 37°C. D-Glucose was determined colorimetrically at 520 nm by the D-glucose oxidase method[15]. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of D-glucose per min at pH 6.4 and 37°C.

(e) Carboxymethylcellulase (CM-cellulase) activity. This was determined by assaying the reducing sugars released from carboxymethylcellulose (CM-cellulose). Assay mixtures (1 mL) containing 1% of CM-cellulose were incubated for 30 min at 37°C in 0.1 M MES buffer (pH 6.4). Reducing sugars were determined by the Nelson–Somogyi method, as outlined above for the measurement of xylanase activity.

(f) Activity on xylo-oligosaccharides ranging from xylobiose to xyloheptaose[3]. This was determined as follows: 0.4-mL mixtures containing xylo-oligosaccharide (X_2–X_7) (1 mg/mL) in 1 mM phosphate buffer (pH 6.4) and 0.4 mIU of purified enzyme solution were incubated at 37°C. Samples were removed at intervals (0, 5, 10, 15, 20, and 30 min in the cases of X_2–X_4 and at 30 min in the cases of X_5–X_7), the reaction was terminated by immersing the tubes in a boling water bath for 5 min, and the products were analysed by HPLC as described below.

Concentrations of xylo-oligosaccharides ranging from 0 to 1.2 mg/mL were used when analysing the effect of substrate concentration. In these analyses, the incubation time was 30 min.

Separation of xylo-oligomers by HPLC.—A 0.3-mL sample from the 0.4-mL mixtures was passed through two columns: one of cation-exchange resin IR-45 (HCO_3^- form) and one of anion-exchange resin IR-120 (H^+ form). The columns were eluted five time with 0.4 mL of deionised water. The eluents were freeze-dried and dissolved in 0.1 mL of deionised water. The samples were centrifuged and finally analysed by HPLC, using a HICHROM Spherisorb S5ODS2 column (25 × 0.46 cm), at a flow rate of 1.5 mL/min. The mobile phase was acetonitrile–water–amine modifier 1 at 65:35:0.01 (v/v) when analysing xylose and short xylo-oligomers (X_2–X_4), and 58:42:0.01 when analysing longer xylo-oligomers (X_5–X_7). The xylo-oligosaccharides were detected by a refractive-index detector.

Protein determination.—The protein content of enzyme preparations was determined by the bicinchoninic acid (BCA) method as described by Redinbaugh and Turley[16].

RESULTS

Enzyme purification.—Initial fractionation of the enzyme concentrate was effected by anion-exchange chromatography on a HiLoad 16/10 Q Sepharose HP column under the conditions described in the Experimental. As Fig. 1 shows, two peaks of β-D-xylosidase activity (I and II) were detected. Peak I, which eluted at 0.1 M NaCl and contained only ~ 12% of the total activity, was rejected. Peak II, which eluted at 0.15 M NaCl, was purified further.

The fractions representing peak II (52–65) (Fig. 1) were pooled, concentrated, desalted on a Pharmacia PD-10 column equilibrated with 0.01 M ammonium acetate buffer (pH 6.0), and further purified by gel filtration on a column of Superose 6 HR 10/30. A typical elution profile obtained, under the conditions described in Experimental, is shown in Fig. 2. β-D-Xylosidase activity appeared as a major peak and a very minor peak. The elution volume of the major peak indicated an apparent molecular mass of 150000 Da.

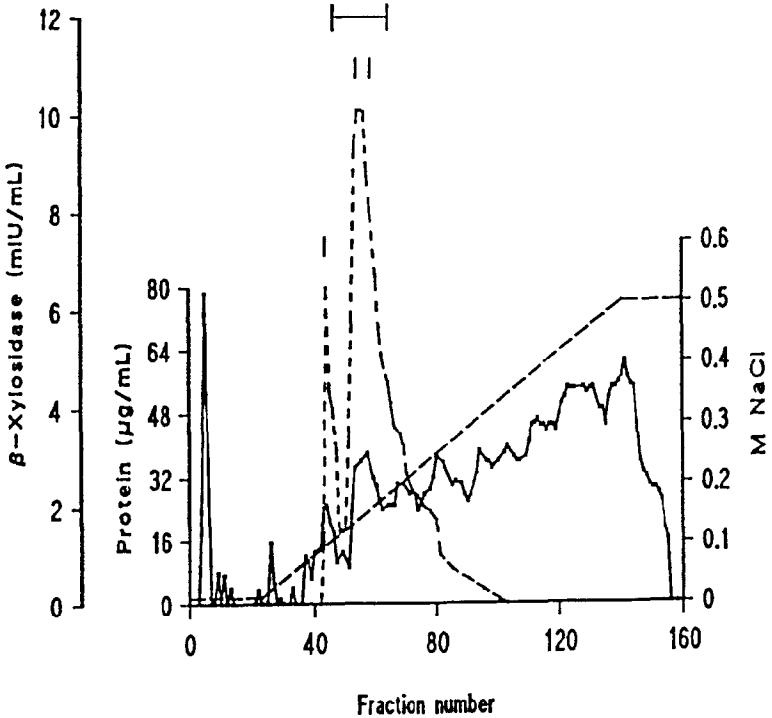

Fig. 1. Anion-exchange chromatography of enzyme concentrate on HiLoad 16/10 Q Sepharose HR High Performance column. Chromatography conditions were as follows: flow rate, 5 mL/min; fraction size, 5 mL; equilibrium buffer, 0.01 M bis-Tris (pH 6.0) containing 0.01 M NaCl; elution, NaCl linear gradient (0.01–0.5 M) in equilibration buffer. Fractions 52–65 ———, designed peak II, were pooled; ———, protein; ---, β-D-xylosidase; – – –, NaCl molarity.

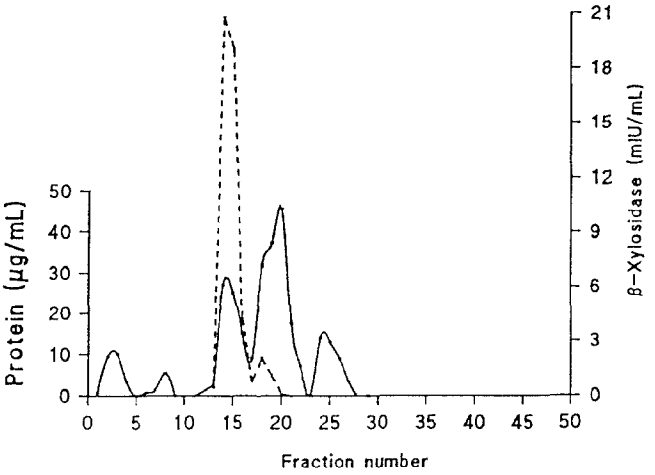

Fig. 2. Gel filtration chromatography of the peak II fraction from Fig. 1. Chromatography conditions were as follows: flow rate, 0.3 mL/min; fraction size, 0.9 mL; equilibration and elution buffer, 0.1 M ammonium acetate (pH 6.0); ———, protein; — — —, β-D-xylosidase activity.

At this stage of the fractionation, the enzyme had been purified 40.82-fold to a specific activity of 0.898 IU/mg of protein. A summary of the purification procedure is given in Table I.

Fractions containing β-D-xylosidase activity were examined for homogeneity by IEF-PAGE (Fig. 3) and SDS-PAGE (Fig. 4) at all stages of fractionation. IEF-PAGE analysis of the β-D-xylosidase component isolated by gel filtration showed a single protein band at an isoelectric of pH 4.5 after staining with silver (Fig. 3, Lane 2). The enzyme exhibited activity on MUX when assayed by the overlay technique (Fig. 3, Lane 4): no contaminating proteins were observed.

SDS-PAGE of the same component showed the presence of two protein bands with molecular masses of 83 000 and 53 000 Da (Fig. 4, Lane 2). The sum of these molecular masses (136 000 Da) approximated the molecular mass obtained by gel filtration (150 000 Da). β-D-Xylosidase activity was not observed by the overlay technique with MUX as substrate, presumably because the enzyme had been

TABLE I

Summary of the purification of the β-D-xylosidase from *Neocallimastix frontalis*

Purification stage	Volume (mL)	Protein (mg)	β-D-Xylosidase activity		
			IU	Specific activity (IU/mg of protein)	Purification
Cell-free culture filtrate	18.8 L	13720	301	0.022	1.00
$(NH_4)_2SO_4$ precipitation	240	5912	168	0.028	1.27
Q HiLoad [a]	108	6.19	0.74	0.120	5.45
Superose 6 [a]	10	0.21	0.19	0.898	40.8

[a] See Experimental.

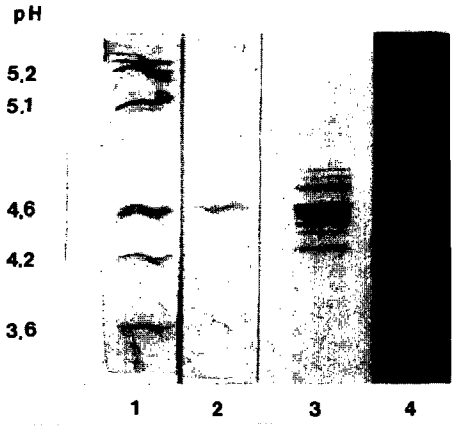

Fig. 3. IEF-PAGE of the β-D-xylosidase recovered after several fractionation steps. Proteins were visualised by silver stain. Lane 1, pI standards; lane 2, purified enzyme from Fig. 2; lane 3, pooled fractions recovered from anion-exchange chromatography (Fig. 1); lane 4, as in lane 2 but enzyme activity detected by the zymogram technique (see Experimental).

denaturated after SDS-PAGE. Equally unsuccessful was the in situ activity assay for β-D-xylosidase performed on the SDS gels which had been washed with a 2.5% solution of Triton X-100 after electrophoresis.

Characterization of the β-D-xylosidase. Effect of temperature on the enzyme activity.—The β-D-xylosidase activity was measured after 30-min incubation of the enzyme in 0.1 M MES buffer (pH 6.4) at temperatures ranging from 25 to 60°C. Maximum activity was observed at 37°C (Fig. 5). Temperatures higher than the maximum caused a rapid decrease of the activity; however, at 60°C, the enzyme still exhibited about 15% of the initial activity.

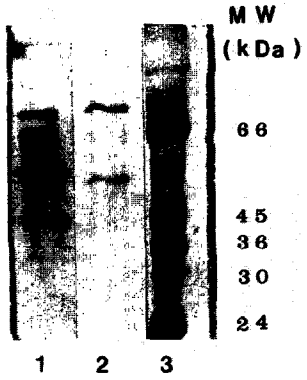

Fig. 4. SDS-PAGE of the β-D-xylosidase fractions recovered at different fractionation steps. Proteins were visualised by silver stain. Lane 1, Fraction II obtained from a HiLoad Q Sepharose column (see Fig. 1); lane 2, β-D-xylosidase isolated by gel filtration on a column of Superose 6 (Fig. 2); lane 3, molecular weight standards.

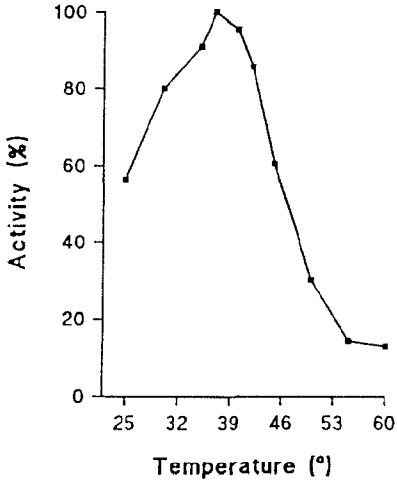

Fig. 5. Effect of temperature on the purified β-D-xylosidase from *N. frontalis*. Incubations were performed at pH 6.4 for 30 min. The control activity was 7.6 mIU/mL.

Thermostability.—The effect of temperature on the enzymatic activity was investigated by incubating the enzyme in 0.1 M MES buffer (pH 6.4) in the absence of substrate at various temperatures ranging from 30 to 50°C (Fig. 6). The residual β-D-xylosidase activity was measured at intervals under standard assay conditions.

The half-life of the enzyme at 30 and 40°C was, respectively, 80 and 45 min. Temperatures of 50°C and above caused a dramatic loss in activity: only 27% of the initial activity was retained after 5 min at 50°C, and no activity was observed

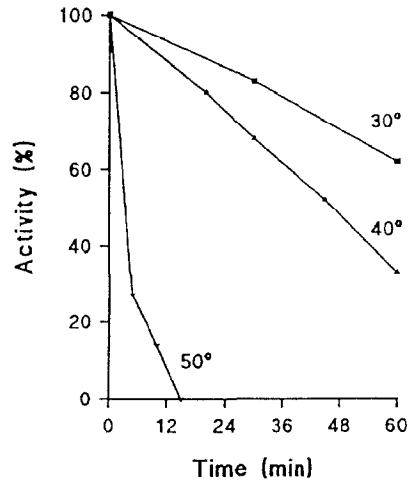

Fig. 6. Thermal stability of the purified β-D-xylosidase. The enzyme was preincubated in the absence of substrate at various temperatures. The residual activity was measured at intervals under the standard assay conditions. The control activity was 6.5 mIU/mL.

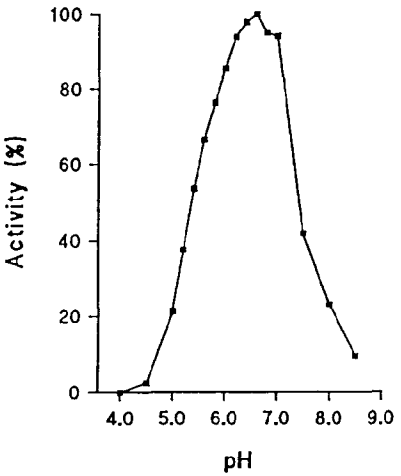

Fig. 7. Effect of pH on the purified β-D-xylosidase. Incubations were performed at 37°C for 30 min. The control activity was 19.0 mIU/mL.

after the same time interval at 60°C (not shown in Fig. 6). However, it was interesting to note that at 60°C (Fig. 5) some hydrolysis of the substrate had been effected: this indicated that, in the presence of substrate, the enzyme partially retained its activity.

Effect of pH on enzyme activity.—The β-D-xylosidase activity was measured at several pH values ranging from 4.0 to 8.5. Incubations were performed at 37°C for 30 min. Fig. 7 shows a typical pH–activity profile. Maximum activity was observed at pH 6.4. The enzyme was not active below pH 4.0 and less than 10% of the initial activity was detected at pH 8.5.

Effect of various metallic ions and reagents on enzyme activity.—The effects of various metallic ions on the activity of the purified enzyme was investigated. As shown in Table II, activity in the presence of 1 mM salts containing Zn^{2+}, Cu^{2+}, or Ag^{+} was significantly less than the control. These ions, respectively, caused 40, 83, and 90% reduction of the maximal activity.

EDTA and SDS also inhibited the enzyme. Approximately 30% of the activity was lost in the presence of EDTA: similar values were obtained between 0.5 and 5.0 mM concentration, showing that the inhibitory effect was not related to the concentration. However, the loss in enzyme activity was clearly dependent on the concentration of SDS. Thus, 23% of the initial activity was lost with SDS at 0.05%, but 78% was lost when the concentration was 1.0%.

The enzyme was slightly stimulated by Ca^{2+} and Mg^{2+}.

End-product inhibition.—As Fig. 8 shows, concentrations of xylose up to 1.4 mM did not significantly affect the activity, using *p*NP-X substrate. However, higher concentrations were markedly inhibitory. Thus, in the presence of 6.6-mM xylose, the initial activity was reduced by ~ 85%. The inhibition was of a competitive type and the apparent kinetic constant (K_i) was 3.98 mM.

TABLE II

Effect of metallic ions and other additives on the β-D-xylosidase purified from *N. frontalis*

Reagent	Reagent concentration [a]	Relative activity (%)
Control		100
$CaCl_2$	1	127
$MgSO_4$	1	117
$ZnSO_4$	1	59
$CuSO_4$	1	17
$AgNO_3$	1	5.2
EDTA	0.5	72
	1.0	73
	2.5	74
	5.0	73
SDS	0.05	67
	0.10	37
	0.50	28
	1.00	22

[a] Concentration of metallic ions and EDTA is given as mM; concentration of SDS is given as percent.

Substrate specificity.—As Table III shows, the purified β-D-xylosidase did not exhibit xylanase, endoglucanase (CM-cellulase), galactosidase, or acetyl esterase activities. Similarly, β-D-glucosidase activity was not observed when cellobiose was used as substrate; however, a very low level of hydrolysis of *p*NP-β-D-glucopyranoside was obtained. In this case, the specific activity observed was 44.5 times lower than that for *p*NP-β-D-xylopyranoside and was not considered significant.

Very low levels of arabinofuranosidase activity were also detected: the specific activity of the enzyme for *p*NP-α-L-arabinofuranoside was 7.4 times lower than that measured for *p*NP-β-D-xylopyranoside.

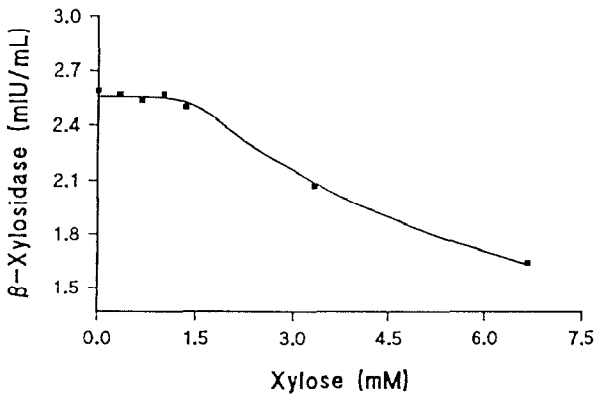

Fig. 8. Effect of different concentrations of D-xylose on the activity of the purified β-D-xylosidase. Incubations were performed under the standard assay conditions.

TABLE III

Substrate specificity of the purified β-D-xylosidase

Substrate	Specific activity
*p*NP-β-D-xylopyranoside	0.89
Xylan	0
Carboxymethylcellulose	0
Cellobiose	0
*p*NP-β-D-glucopyranoside	0.02
*p*NP-α-L-arabinofuranoside	0.12
*p*NP-β-D-galactopyranoside	0
*p*NP-acetate	0

Hydrolysis of xylo-oligosaccharides: kinetic properties.—Xylo-oligosaccharides up to dp 7 were used. The specific activity of the enzyme on each of the substrates and the kinetic constants obtained using Lineweaver–Burk plots are summarised in Table IV.

As Table IV shows, the purified β-D-xylosidase exhibited a higher affinity, higher maximum velocity, and higher specific activity for xylobiose than for *p*NP-X. However, there was no direct relationship between the increase in the affinity (decrease in K_m) and the increase in the maximum velocity and specific activity. Thus, the affinity of the enzyme for xylobiose was slightly higher than the affinity for *p*NP-X, while the maximum velocity (measured by the release of xylose) for xylobiose was much greater than for *p*NP-X.

For substrates (xylotriose and xylotetraose) of longer chain length, the affinity of the enzyme increased slightly as the chain length increased, but the maximum velocity and the specific activity decreased.

Mode of action on xylo-oligosaccharides.—With xylobiose as substrate (Fig. 9a), the enzyme effected approximately 20% degradation after incubation for 30 min and acted at a relatively constant rate. The enzyme hydrolysed xylotriose at a similar rate to xylobiose in the first 15 min of incubation (Fig. 9b), but an increase in the rate was observed after that. After 30-min incubation, 35% of the substrate

TABLE IV

Specific activities and kinetic constants of β-D-xylosidase for different xylose-containing substrates

	Specific activity	K_m (mM)	V_{max} [a]
*p*NP-X	0.89	2.98	0.27×10^{-3}
Xylobiose	18	2.42	24.8×10^{-3}
Xylotriose	15	1.24	18.5×10^{-3}
Xylotetraose	7.5	1.18	8.6×10^{-3}
Xylopentaose	2.17	nd	nd
Xylohexaose	2.17	nd	nd
Xyloheptaose	2.04	nd	nd

[a] V_{max} expressed as μmol/min/μg. Abbreviations: *p*NP-X, *p*-nitrophenyl β-D-xylopyranoside; nd, not determined.

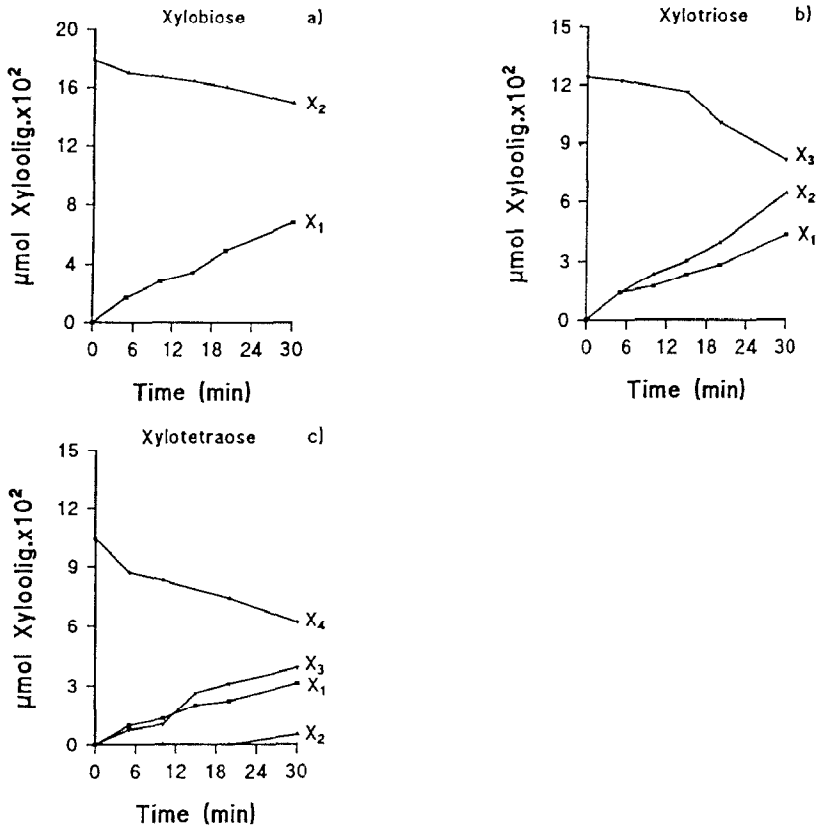

Fig. 9. Time course of hydrolysis of xylobiose and xylo-oligosaccharides up to degree of polymerization 4 by β-D-xylosidase purified from *N. frontalis*. Abbreviations: Xyloolig., xylo-oligosaccharides; X_1, xylose; X_2, xylobiose; X_3, xylotriose; X_4, xylotetraose.

had been degraded. The reaction products, analysed by HPLC as described in Experimental, consisted only of xylobiose and xylose: no evidence for higher xylo-oligosaccharides resulting from transglycosylation was obtained by this technique.

Xylotetraose was degraded at a similar rate and extent to xylotriose (Fig. 9c) in 30 min. Equal amounts of xylose and xylotriose were observed during the early stages of the hydrolysis. Xylobiose was not detected until the reaction mixture had been incubated for 30 min, at which time only trace amounts of it were observed.

The products of hydrolysis of xylo-oligomers longer than xylotetraose were analysed only after 30-min incubation (Table V). Xylose, xylotriose, and xylotetraose were released from xylopentaose, but no xylobiose. In the case of xylohexaose, equimolar amounts of xylose and xylopentaose were the major products, while the enzyme released mainly xylose and xylohexaose from xyloheptaose.

In no case were xylo-oligosaccharides with a dp higher than the substrates detected.

TABLE V

Sugars released from xylo-oligosaccharides [a]

Substrate	Hydrolysis products (μmol $\times 10^2$)					
	X_1	X_2	X_3	X_4	X_5	X_6
Xylopentaose	3.90	0	5.07	8.94		
Xylohexaose	3.90	0	0.69	2.36	3.91	
Xyloheptaose	3.67	0	0	0.76	0.75	3.18

[a] Time of incubation was 30 min. Reaction mixtures contained 0.4 mIU of β-D-xylosidase and 1 mg/mL of substrate (see Experimental for further details). Abbreviations used: X_1, xylose; X_2, xylobiose; X_3, xylotriose, X_4, xylotetraose; X_5, xylopentaose; X_6, xylohexaose.

Effect of the addition of β-D-xylosidase on the hydrolysis of xylan by xylanase.— The effect of the simultaneous or the sequential addition of xylanase and β-D-xylosidase on the hydrolysis of xylan was tested. For this study, different concentrations of β-D-xylosidase purified from *N. frontalis* were added to a reaction mixture containing 12.7 mIU of partially purified xylanase from the same source.

Fig. 10 shows that the amount of reducing sugars released by the xylanase acting alone (0.29 μmol xylose equivalent) was increased by 16% by the sequential action of xylanase (inactivated by boiling 5 min) and 0.13 mIU of β-D-xylosidase: the addition of 0.26 mIU of β-D-xylosidase resulted in a 36% increase. When the xylanase and β-D-xylosidase were acting in a concert, a similar enhancement in the release of reducing sugars was observed for all the concentrations of β-D-xylosi-

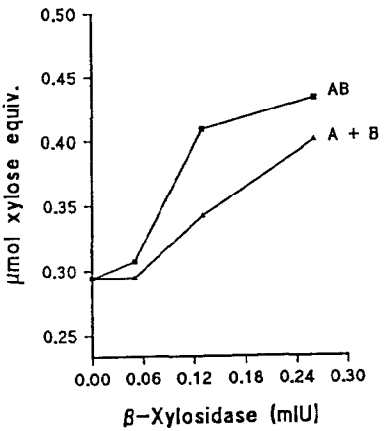

Fig. 10. Effect of the combination of different concentrations of β-D-xylosidase with xylanase on the release of product from xylan. AB, simultaneous action of xylanase and β-D-xylosidase at 37°C for 30 min; A + B, sequential action of xylanase and β-D-xylosidase (xylanase was incubated for 30 min at 37°C with xylan and inactivated by heat (100°C; 5 min) prior to incubation (30 min) with β-D-xylosidase. Controls: initial β-D-xylosidase activity on xylan was nil, and initial xylanase activity on xylan was 0.291 μmol xylose equiv/12.7 mIU of partially purified enzyme.

dase tested, but the release was higher than that observed when xylanase and β-D-xylosidase acted sequentially.

DISCUSSION

A β-D-xylosidase component was purified to homogeneity by anion-exchange and gel filtration chromatography. The β-D-xylosidase had an apparent molecular mass of 150000 Da when determined by gel filtration, but on denaturing conditions of SDS-PAGE it separated into two protein bands with molecular masses of 83000 and 53000 Da. As the sum of these molecular masses is approximately the molecular mass calculated by gel filtration chromatography, this suggested that the purified enzyme could be a dimeric protein composed of two subunits. β-D-Xylosidases composed of two different subunits have been reported in the xylan-degrading enzyme systems produced by the aerobic fungi *Trichoderma lignorum*[17] and *Chaetomium trilaterale* strain B[18]. The β-D-xylosidase from the actinomycete *Termomonospora fusca*[19] consisted of three subunits and exhibited a molecular mass of 168000 Da. In the case of the anaerobic bacteria *Clostridium acetobutylicum*[20], the enzyme consisted of two subunits of 85000 Da and one of 63000 Da.

It is not clear if the β-D-xylosidase activity resides in one of the subunits produced by *N. frontalis* or if both components are necessary for the catalytic activity. We have shown that the β-D-xylosidase activity was inhibited by low concentrations of SDS. This high sensitivity to SDS made it impossible to detect β-D-xylosidase activity, after SDS-PAGE, by the zymogram technique using 4-methylumbelliferyl β-D-xylopyranoside as substrate. However, the fact that removal of the SDS, prior to the application of the overlay did not result in the recovery of activity could indicate that both subunits may be required for catalytic activity. It is, however, still possible that the enzyme denaturated by SDS does not regain its activity after removal of the reagents.

The isoelectric point of the purified β-D-xylosidase was estimated by IEF-PAGE to be 4.5, indicating that the enzyme is an acidic protein. Similar pI values have been observed for β-D-xylosidases purified from aerobic fungi[9–11] and aerobic bacteria[12]. A more basic pI (5.85) has been reported for the same enzyme isolated from the anaerobic bacterium *C. acetobutylicum*[20].

The optimal conditions for the enzymatic activity of the purified enzyme from *Neocallimastix frontalis* were 37°C and pH 6.4. These results are consistent with the values obtained for the crude enzyme. Hébraud and Fèvre[21] have reported similar pH and temperature optima for the β-D-xylosidase found in crude culture filtrates of the rumen fungi *Sphaeromonas communis* and *Piromonas communis*.

It is not surprising that the optimum activity for the enzymes of different rumen fungi are similar and that these conditions are clearly related to the physiological conditions observed in the rumen. As expected, fungi from other sources than the rumen exhibited optimum conditions for β-D-xylosidase, which are clearly different from those observed for *N. frontalis*. Thus, higher temperature optima and more

acidic pH optima were reported for *T. lignorum*[17], *T. viride*[10], *Emicerella nidulans*[10], and *Sclerotium rolfsii*[8] β-D-xylosidases.

The study of the thermostability of the purified β-D-xylosidase from *N. frontalis* indicated that the enzyme was highly sensitive to temperature, losing part of the activity even after 1 h at 30°C, and being totally inactivated after 5 min at 60°C. As expected, the thermostability of the enzyme appeared to be significantly enhanced in the presence of the substrate *p*-nitrophenyl β-D-xylopyranoside, probably as a result of the stabilising effect of the enzyme–substrate complex.

The heat stability of the β-D-xylosidase from *N. frontalis* was remarkably low in comparison with the stability of the β-D-xylosidase from *Trichoderma lignorum*[17], which retained 70% of its activity on incubation at 70°C for 1 h: a β-D-xylosidase from *Aspergillus niger*[22] had a half-life of 75 h at 60°C.

β-D-Xylosidase from *N. frontalis* was inhibited in the presence of salt solutions containing Zn^{2+}, Cu^{2+}, or Ag^{+} and by EDTA and SDS. It was slightly stimulated by salt solutions containing Ca^{2+} or Mg^{2+}. John and Schmidt[17] observed that Cu^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, or EDTA did not affect the β-D-xylosidase from *T. lignorum*, while Fe^{2+} and Mn^{2+} stimulated activity. Similarly, Hg^{2+}, Ag^{+}, and Cu^{2+} were found to strongly inhibit *S. rolfsii* β-D-xylosidase, but Ca^{2+} or Fe^{2+} did not show any effect[8]. In the case of the aerobic bacterium *Bacillus pumilus*[12], bivalent cations, including Ca^{2+} and Mg^{2+}, had a strong inhibitory effect on the β-D-xylosidase activity while EDTA, at 1.5 M, did not show any effect.

The β-D-xylosidase of *N. frontalis* was strongly inhibited by D-xylose, which is the principal end-product of the action of the enzyme on xylo-oligosaccharides, the natural substrates of the enzyme. The inhibition was competitive and the apparent K_i was 3.98 mM using the *p*-nitrophenyl β-D-xylopyranoside substrate. This value is similar to that reported by Poutanen and Puls[9] for the β-D-xylosidase purified from *Trichoderma reesei* (K_i 2.3 mM).

The mechanism of action of the β-D-xylosidase was studied by an examination of the capacity of the enzyme to hydrolyse various xylo-oligosaccharides as substrate and by an analysis of the products released. The isolated enzyme proved totally ineffective against polysaccharides, such as xylan or carboxymethylcellulose, but catalysed the hydrolysis of xylobiose and short-chain xylo-oligosaccharides: xylobiose was the best substrate of those tested. The enzyme exhibited a higher affinity for xylo-oligosaccharides as the chain length of the substrate increased, but the maximum velocity decreased greatly as the chain length increased, and so too did the specific activity. Similar results have been obtained by Van Doorsleer et al.[23] for the β-D-xylosidase from *Bacillus pumilus* and by Matsuo and Yasui for *Trichoderma viride* and *Emericela nidulans* β-D-xylosidases[10]. In all these cases, the rate of hydrolysis of xylo-oligosaccharides decreased with increasing chain length, but the K_m values reported were almost the same. A β-D-xylosidase isolated from *Trichoderma lignorum*[17], in contrast, released xylose residues from xylohexaose, xyloheptaose, and xylooctaose more rapidly than from shorter chain oligomers and

xylobiose. Also, β-D-xylosidase purified from *Aspergillus niger*[7] hydrolysed xylotriose more rapidly and to a much greater extent than xylobiose.

The β-D-xylosidase of *N. frontalis* appeared to act exclusively by removing xylose from xylo-oligosaccharides. There was no evidence for transferase activity resulting in the synthesis of higher oligomers. The apparent absence of transferase activity is unusual in a glycosidase: β-D-xylosidases from other microbial sources have been reported to be rich in this activity[8,22], and most β-D-glucosidases studied possess this property[24]. However, β-D-xylosidase from *N. frontalis* would appear to be unusual in another respect: it was very specific for the β-D-xylosidase linkage. Unexpectedly, *p*-nitrophenyl β-D-glucopyranoside was not a good substrate. In the case of the brown rot fungus *Tyromyces palustris*[25] and the fungus *Chaetomium trilaterale*[18], β-D-xylosidase and β-D-glucosidase activities resided in the same protein: a β-D-xylosidase from *Neurospora crassa*[11] also hydrolysed both (1 → 4)-β-D-xylosidic and (1 → 4)-β-D-glucosidic linkages. A purified β-D-xylosidase from *Trichoderma reesei*[9] also exhibits bifunctionality, although the activities reported in this case were β-D-xylosidase and arabinofuranosidase: the α-L-arabinofuranosidase activity was about 70% of that of β-D-xylosidase. β-D-Xylosidases of broad substrate specifities have also been shown to be produced by *Bacteroides ovatus*[26] and *Butyrivibrio fibrisolvens*[27]. In the last two cases mentioned, the cloning and expression of the gene encoding these bifunctional proteins has been reported.

Clearly in the circumstances, the β-D-xylosidase isolated from *N. frontalis* apparently appears as a relatively substrate-specific enzyme.

It has been speculated that the structural similarities between the various cellulosic and hemicellulosic substrates that occur together in lignocellulose may be responsible for the apparent evolution of bifunctionality, and this could be of selective advantage in certain environments[27]. However, such enzymes are not attractive for the industrialist wishing to maximise the effectiveness and selectively of enzymes in various process in the food industry or the paper and pulp industry. Clearly, the β-D-xylosidase of *N. frontalis*, which is unusual in being highly substrate-specific, may have some commercial potential.

The importance of β-D-xylosidase in the saccharification of xylan has been clearly demonstrated[28,29]. In our study, reaction mixtures containing xylanase and β-D-xylosidase from *N. frontalis* produced higher reducing sugar values than the xylanase acting alone. Moreover, the observation that the concerted action of both enzymes was greater than the sum of the individual action showed that the enzymes acted synergistically. The cooperative action among the different activities present in microbial xylanolytic systems has received substantial attention[2], but knowledge of the mechanisms involved is incomplete. Similar problems appear to exist in the interpretation of the mechanism of synergistic action of enzymes synthesised by certain cellulolytic fungi[30]. Work is currently being carried out in this laboratory to characterise the molecular basis of the cooperative action

observed between xylanase and β-D-xylosidase from *N. frontalis* in order to better understand the contribution of the β-D-xylosidase to the xylanolytic system.

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