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Biochemical and catalytic properties of an endoxylanase purified from the culture filtrate of *Thermomyces lanuginosus* ATCC 46882

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Abstract

An endoxylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) from the culture filtrates of *T. lanuginosus* ATCC 46882 was purified to homogeneity by DEAE–Sepharose and Bio-Gel P-30 column chromatographies. The purified endoxylanase had a specific activity of 888.8 μmol min^{-1} mg^{-1} protein and accounted for approximately 30% of the total protein secreted by this fungus. The molecular mass of native (non-denatured) and denatured endoxylanase were 26.3 and 25.7 kD as, respectively. Endoxylanase had a pI of 3.7 and was optimally active between pH 6.0–6.5 and at 75 °C. The enzyme showed > 50% of its original activity between pH 5.5–9.0 and at 85 °C. The pH and temperature stability studies revealed that this endoxylanase was almost completely stable between pH 5.0–9.0 and up to 60 °C for 5 h and at pH 10.0 up to 55 °C for 5 h. Thin-layer chromatography (TLC) analysis showed that endoxylanase released mainly xylose (Xyl) and xylobiose (Xyl_2) from beechwood 4-*O*-methyl-D-glucuronoxylan, *O*-acetyl-4-*O*-methyl-D-glucuronoxylan and rhodymenan (a β-(1→3)–β(1→4)-xylan). Also, the enzyme released an acidic xylo-oligosaccharide from 4-*O*-methyl-D-glucuronoxylan, and an isomeric xylotetraose and an isomeric xylopentaose from rhodymenan. The enzyme hydrolysed [1-^{3}H]-xylo-oligosaccharides in an endofashion, but the hydrolysis of [1-^{3}H]-xylotriose appeared to proceed via transglycosylation, since the xylobiose was the predominant product. Endoxylanase was not active on pNPX

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and pNPC at 40 and 100 mM for up to 6 h, but showed some activity towards pNPX at 100 mM after 20–24 h. The results suggested that the endoxylanase from *T. lanuginosus* belongs to family 11.

Keywords: *T. lanuginosus*; Endoxylanase; Family 11

1. Introduction

Microbial endoxylanases have attracted considerable research interest in recent years mainly due to their potential application in food, animal feed, paper and pulp industries [1–3]. However, such applications prefer thermostable and cellulase-free xylanase with broad pH and temperature optima. The thermophilic fungus, *T. lanuginosus* has been shown to produce high levels of thermostable and cellulase-free xylanase with broad pH and temperature optima, when grown on cheap carbon source such as corn cob [4,5]. Further research on the xylanase system of this fungus is expected to facilitate the full realisation of its commercial potential.

Grouping of microbial endoxylanases, according to physico-chemical properties led to the discovery of two groups of endoxylanases, which can be differentiated on the basis of their molecular mass and pI values [6]. This grouping correlated well with the classification of endoxylanase into families 10 (formerly family F) and 11 (formerly family G) based on hydrophobic cluster analysis and amino acid sequence homologies [7,8]. Previous studies with endoxylanases of *Streptomyces lividans* (families 10 and 11) [9], *Trichoderma reesei* (two family 11 xylanases) [10] and an endoxylanase of *Cryptococcus albidus* (family 10) [11] suggested that high molecular mass/low pI endoxylanases (family 10) exhibit greater catalytic versatility than low molecular mass/high pI endoxylanases (family 11), which hydrolyse the naturally occurring polysaccharides to a greater extent. In addition, it was reported that endoxylanases of family 10 liberated aldotetrauronic acid as the shortest acidic oligosaccharide from glucuronoxylans, while endoxylanases of family 11 released aldopentauronic acid as the shortest acidic oligosaccharide [11]. Similarly, family 10 endoxylanases released an isomeric xylotriose (Xyl_3) as the shortest product containing one β-(1→3)-linkage from rhodymenan, a seaweed xylan with β-(1→3)-β-(1→4)-linkage, while endoxylanase of the family 11 released an isomeric tetrasaccharide [11]. Also, family 10 xylanases hydrolysed acetyl xylan to a higher degree and released short acetylated oligosaccharides, whereas the degree of hydrolysis of acetyl xylan by family 11 xylanases was rather low, and either acetylated xylobiose (Xyl_2) or xylotriose (Xyl_3) was hardly found among the products of hydrolysis [9,11].

The main purpose of the present study was to investigate the biochemical and catalytic properties of purified endoxylanase from a thermophilic fungus, *T. lanuginosus* ATCC 46882 and to establish its relationship with the recognised endoxylanase families. According to the molecular mass, the enzyme is expected to have catalytic properties of the family 11 xylanase.

2. Experimental

Materials and methods.—T. lanuginosus ATCC 46882 was purchased from American type culture collection and maintained on potato dextrose-agar (PDA) medium. DEAE–Sepharose (fast flow), Bio-Gel P-6DG and Bio-Gel P-30 were from Pharmacia and Bio-Rad, respectively. Beechwood 4-*O*-methyl-D-glucuronoxylan was isolated from sawdust as described [12]. *O*-Acetyl-4-*O*-methyl-D-glucuronoxylan was obtained by the extraction of beechwood holocelluose with dimethylsulphoxide [13]. Rhodymenan, a β-(1→3)-β-(1→4)-xylan from *Rhodymenia stenogona* was a gift from Dr. A.I. Usov (Academy of Sciences, Moscow, Russia). Xylo-oligosaccharides (Xyl_2 up to Xyl_5) were purchased from Megazyme (Australia). 1-^{3}H-Reducing-end labelled xylo-oligosaccharides with specific radioactivity of approximately 10 MBq/μmol, were obtained by catalytic tritiation of unlabelled compounds by the method of Evans et al. [14] at the Institute for Research, Production and Application of Radioisotopes in Prague (Czech Republic). Crude products obtained from the

Nomenclature: DNS: dinitrosalicylic acid; MeGlcUA: 4-*O*-methylglucuronic acid; pNPC: 4-nitrophenyl β-cellobioside; pNPX: 4-nitrophenyl β-xyloside; Xyl: xylose; Xyl_2: xylobiose; Xyl_3: xylotriose; Xyl_4: xyloteraose; Xyl_5: xylopentaose.

Institute were extensively purified to radiochemical purity by repeated thin-layer chromatography combined with autoradiography. All the remaining chemicals used were analytical grade and purchased either from Sigma, BDH or other companies.

Production of xylanase.—The mineral medium used for the production of xylanolytic enzymes contained per litre: yeast extract, 14.3 g; $(NH_4)_2SO_4$, 2.1 g; $MgSO_4$, 3.0 g; $CaCl_2$. $2H_2O$, 0.3 g; $FeSO_4$. $7H_2O$, 0.5 g, and KH_2SO_4, 10 g. A 5 mL mycelia and spore suspension of *T. lanuginosus* ATCC 46882 from a 6-day old culture, grown on PDA slope at 45–48 °C, was inoculated to a 250 mL Erlenmeyer flask containing 100 mL of the above mineral medium supplemented with corn cob (20 g/L) as carbon source. The flasks were incubated at 50 °C in an orbital shaker operating at 200 rpm for 2 days. A 10 mL preculture was used to inoculate 90 mL mineral medium having above composition and the same amount of corn cob. The initial pH of both preculture and enzyme production media was adjusted to 6.0. The fungus was cultured under identical conditions as the pre-culture for 6 days and harvested by filtration using a glass fibre filter disc. The clear supernatant was assayed for xylanase as described below and concentrated by spray drying.

Assay of xylanase.—Activity was determined by measuring the reducing sugars released from birchwood glucuronoxylan (Roth 7500) using the DNS method [15]. A 1.5 mL reaction mixture containing 0.9 mL of 1% (w/v) birchwood glucuronoxylan in 50 mM citrate-phosphate buffer, pH 6.0 and 0.5 mL distilled water was pre-incubated at 50 °C for 10 min. A 0.1 mL of appropriately diluted enzyme was added, and the incubation continued for further 10 min before adding 3 mL DNS reagent. The reaction was terminated by placing the tubes in polyethylene glycol 400 boiling bath for 5 min. The colour developed was read at 540 nm against the reagent blank and converted into reducing sugar values using the xylose standard curve prepared under identical conditions. One unit (U) of xylanase activity was expressed as μmol of reducing sugar (xylose equivalent) released in 1 min.

Protein determination.—This was determined by the bicinchoninic acid method according to Pierce instruction manual No. 23235X using bovine serum albumin as a standard. The absorbance at 280 nm was used to monitor the protein in column eluents.

Purification of endoxylanase.—*(a) Desalting*. The spray dried powder (3 g) containing 1334 mg protein and 2.96×10^5 units of xylanase was dissolved in 50 mL distilled water and subjected to 60% $(NH_4)_2SO_4$ saturation. The precipitated protein was collected by centrifugation (20,000×g), dissolved in minimal amount of distilled water and desalted on Bio- Gel P-6DG column (70×2.5 cm) using 20 mM sodium acetate buffer, pH 5.0 at a flow rate of 24 mL/h. The active xylanase fractions were pooled and concentrated using Omega 3 membrane (Flowgen) fitted into a 50 mL Amicon cell.

(b) Ion-exchange chromatography. A 5 mL concentrated sample from the above step containing 379 mg protein and 2.56×10^5 units of xylanase was diluted with 10 mL of 50 mM potassium phosphate buffer, pH 6.0 and applied to a DEAE-Sepharose (fast flow; 28×2.6 cm) column connected to a FPLC system and equilibrated with the same buffer. The bound xylanase was eluted using 0–0.4 M NaCI gradient and the active fractions (46–58) were pooled and concentrated.

(c) Gel filtration. A 4.5 mL sample from the previous step containing 168 mg protein and 1.61×10^5 units of xylanase was applied to a column of Bio-Gel P-30 (100×1.6 cm) connected to a FPLC system and equilibrated with 20 mM potassium phosphate buffer, pH 6.0. The xylanase was eluted at a flow rate of 15 mL/h. The 4 mL fractions collected were assayed for xylanase as described above. The active fractions (19–22) were pooled and concentrated.

Biochemical characterisation of purified endoxylanase.—*(a) Determination of purity and molecular mass*. These were determined by SDS–PAGE as described [16] using a Bio-Rad mini electrophoresis system. Purified endoxylanase (10 μg each) was mixed with SDS sample treatment buffer at a ratio of 1:1 (v/v) and subjected to SDS–PAGE either directly (non-denatured) or after treating at 100 °C for 5 min (denatured).

(b) Determination of isoelectric point (pI). Novex pre-cast polyacrylamide isoelectric focusing (IEF) gel (5%) containing 2% ampholyte, covering a pH range of 3.0–7.0 was used. IEF was performed using Novex Xcell II mini-cell according to the instructions of Novex '96 electrophoresis catalogue. At the end of IEF, the gel was stained using Coomassie blue prepared in propanol:acetic acid:water (60:30:10), destained with propanol: acetic acid:water (60:30:10) and photographed. The isoelectric point of purified endoxylanase was

determined from the plot of pI values of standard marker proteins versus their relative mobility in IEF-gel.

(c) Effect of pH and temperature on the activity and stability of endoxylanase. This was studied in the pH range 4.0–10.0 (50 mM citrate/Na_2HPO_4 buffer, pH 4.0–7.5; 50 mM Tris-HCl buffer, pH 7.5–9.0 and 50 mM borate buffer, pH 9.0–10.0) and temperatures between 50–85 °C. The pH and temperature stabilities of endoxylanase were studied at different pH (4.0–10.0) using the above buffers and at temperatures between 40–80 °C.

(d) Hydrolysis of polysaccharides and arylglycosides. The 2.0% (w/v) polysaccharide solutions in 0.05 M pyridine-acetic acid buffer, pH 6.0 were incubated with appropriately diluted enzyme at 50 °C. The aliquots were analysed at different time intervals, for products of hydrolysis, by TLC on microcrystalline cellulose (Lucefol, Kavalier, Czechoslovakia) in the solvent system ethylacetate-acetic acid-water (3:2:1). Reducing sugars were detected using the aniline-hydrogen phthalate reagent. Radioactive compounds were localised according to the position of standards detected on guide strips. 4-Nitrophenol and 4-nitrophenyl glycosides were detected under UV light.

(e) Bond cleavage frequencies and kinetic parameters of [1-^{3}H]-xylo-oligosaccharides. The initial bond-cleavage frequencies of reducing-end-labelled xylo-oligosaccharides, identical with relative rates of the cleavage of individual glycosidic linkages, were determined using the method of Allen and Thoma [17]. A 0.25 mM of either Xyl_3, Xyl_4 or Xyl_5 containing appropriate addition of respective radioactive xylo-oligosaccharide in 0.05 M pyridine-acetic acid buffer (pH 6.0) was incubated with appropriately diluted enzyme at 50 °C. The 2 μL aliquots were removed at different time intervals and subjected to chromatographic analysis. After the resolution of the enzyme-substrate mixtures by TLC, the radioactivity in the products and substrate was measured in a liquid scintillation counter. The ratio of radioactivity in the substrate and products was determined and used for graphic determination of the initial bond cleavage frequencies, i.e. frequencies corresponding to the zero reaction time.

Initial rates of hydrolysis were obtained by plotting the decrease in radioactivity in the substrate vs. time of incubation, and determining the slope of the function corresponding to the zero time of the reaction. The values were normalised to equal enzyme concentration (U/mL), since different enzyme concentrations were used with shorter and longer oligosaccharides. V/K_m parameters for [1-^{3}H]-xylo-oligosaccharides, expressed in molar terms of endoxylanase (k_0/K_m), were obtained by constructing the plots ln([1-^{3}H] Xyl_i/[1-^{3}H]Xyl_n) versus time, using the method of Suganuma et al. [18].

3. Results

Production of xylanase.—*T. lanuginosus* ATCC 46882, when grown on corn cob at 50 °C for six days, produced 2840 U of xylanase per ml of the culture filtrate. The level of xylanase produced by *T. lanuginosus* ATCC 46882 on corn cob was approximately two-fold higher than that produced on birchwood xylan (1613 U/mL). Also, among the 18 strains of *T. lanuginosus* tested in our laboratory, ATCC 46882 produced the highest xylanase activity on corn cob and birchwood xylan, and none of the culture filtrates showed any cellulase activity.

Purification of endoxylanase.—The endoxylanase from *T. lanuginosus* ATCC 46882 was purified using a simple procedure which involved desalting, ion-exchange and gel-filtration chromatographies. The summary of the purification is presented in Table 1. Desalting on Bio- Gel P-6 DG column separated considerable amount of brown colour (phenolics) from the xylanase sample and increased its specific activity from 222.4 to 677.0 μmol/min/mg protein (Table 1). Fractionation on a DEAE–Sepharose column removed a number of contaminating proteins (Fig. 1(A)) and increased the specific activity and degree of endoxylanase purification to 955.52 μmol/min/mg protein and 4.29, respectively (Table 1). Although further purification on Bio-Gel P-30 column removed a contaminating protein peak from the endoxylanase sample (Fig. 1(B)), this step marginally reduced the specific activity (888.84 μmol/min/mg protein) and degree of purification (4.0) of endoxylanase (Table 1).

General properties.—Purified endoxylanase was homogeneous when tested by SDS–PAGE under non-denaturing and denaturing conditions (Fig. 2(A)) as well as by IEF (Fig. 2(B)). Under non-denaturing conditions, the endoxylanase had a molecular mass of 26.3 kDa while the molecular mass was reduced to 25.7 kDa upon heating with

Table 1
Summary of purification of endoxylanase from *T. lanuginosus* ATCC 46882

Purification step	Total activity (μmol/min)	Total protein (mg)	Specific activity (μmol/min/mg protein)	Fold purification	Recovery (%)
1. Culture filtrate	296,726	1334.2	222.4	—	100
2. Desalting on Bio-Gel P-6-DG	256,923	379.5	677.0	3.04	86.6
3. DEAE–Sepharose chromatography	160,729	168.2	955.6	4.3	54.2
4. Bio-Gel P-30 chromatography	113,007	127.1	888.8	4.0	38.1

SDS-treatment buffer at 100 °C for 5 min (Fig. 2(A)). The endoxylanase had a pI value of 3.7 (Fig. 2(B)) and was optimally active between pH 6.0–6.5 and at 75 °C (Fig. 3(F)). The enzyme showed > 50% of its original activity over a wide range of pH (5.5–9.0) even at 85 °C (Fig. 3(F)). Also, the enzyme showed relatively high activity (~25–30%) between pH 9.0–9.5 at temperatures up to 85 °C (Fig. 3(F)). However, the activity was considerably higher when measured using Tris-HCI buffer, pH 7.5 compared to that obtained with citrate/Na_2HPO_4 buffer at the same pH value (Fig. 3(F)).

Stability.—This was studied at temperatures between 40–80 °C and pH values 4.0–10.0 (Fig. 3(A)–(E)). At pH 4.0 and up to 45 °C, the enzyme retained 70–80% of its original activity up to 5 h, whereas at and > 50 °C and at pH 4.0, the enzyme was less stable (Fig. 3(A)). At pH 5.0, the endoxylanase retained approximately 80% of its original activity up to 55 °C for 5 h, but gradually lost its activity above 60 °C (Fig. 3(B)). At pH 6.0, the enzyme was most stable and retained ~80% of its original activity up to 5 h at 60 °C (Fig. 3(C)). Also, the enzyme was stable at pH 9.0 and 10.0, up to 5 h at 60 and 55 °C, respectively (Fig. 3(D),(E)).

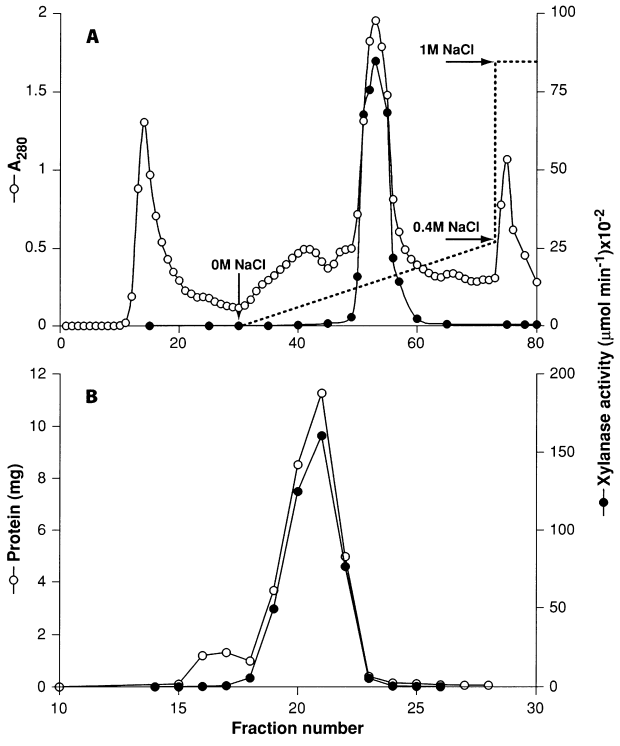


Fig. 1. Purification of endoxylanase from the culture filtrate of *T. lanuginosus* ATCC 46882. (A) Fractionation of desalted endoxylanase sample by DEAE–Sepharose column (fast flow; 28×2.6 cm) chromatography. (B) Purification of endoglucanase sample obtained from DEAE–Sepharose column by Bio-Gel P-30 column (100×1.6 cm) chromatography.

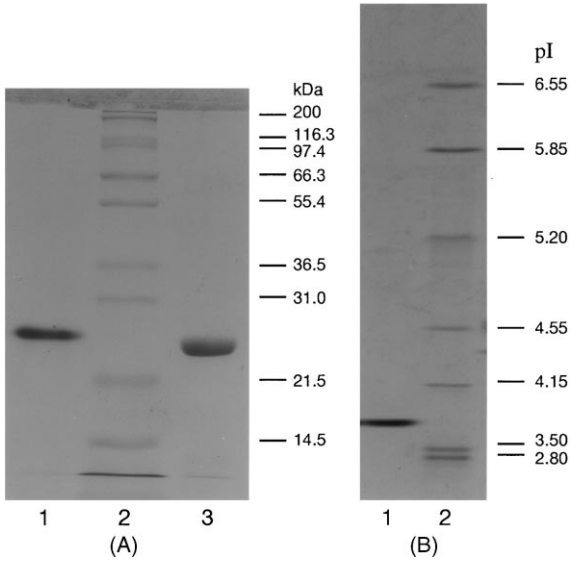
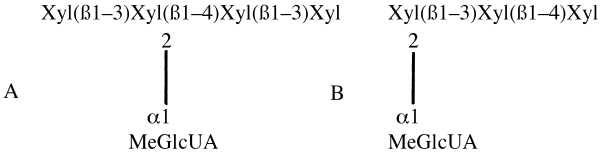


Fig. 2. (A) SDS–PAGE of purified endoxylanase from *T. lanuginosus* ATCC 46882 under non- denaturing and denaturing conditions. Lane 1, endoxylanase, non-denatured; lane 2, standard protein markers in the order of increasing molecular mass: lysozyme (14.5 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31.0 kDa), lactate dehydrogenase (36.5 kDa), glutamic dehydrogenase (55.4 kDa), bovine serum albumin (66.3 kDa), phosphorylase; (B) (97.4 kDa), β-galactosidase (116.3 kDa) and myosin (200 kDa); lane 3, endoxylanase, denatured; (B) IEF–PAGE of purified endoxylanase from *T. lanuginosus* ATCC 46882. Lane 1, endoxylanase; lane 2, standard protein markers in the order of increasing pI: pepsinogen (pI, 2.80); amyloglucosidase (pI, 3.50); glucose oxidase (pI, 4.15); soybean trypsin inhibitor (pI, 4.55); β lactoglobulin (pI, 5.20); bovine carbonic anhydrase (pI, 5.85) and human carbonic anhydrase (pI, 6.55).

Above 65 °C, the enzyme was less stable after 2 h at all pH values tested.

Action on polysaccharides.—The hydrolysis products released from polysaccharides by endoxylanase were analyzed by TLC. From beechwood 4-*O*-methyl-D-glucuronoxylan, the endoxylanase liberated mainly xylose, xylobiose and an acidic oligosaccharide which had the value of R_{xyl} 0.36 (Fig. 4). This was the shortest acidic oligosaccharide liberated by this endoxylanase, with chromatographic mobility compatible with an aldopenta uronic acid, A. The endoxylanase did not liberate an aldotetrauronic acid B, having a value of R_{xyl} 0.53–0.55 and the shortest acidic product released from 4-*O*-methyl-D-glucuronoxylan by the family 10 xylanases [11].



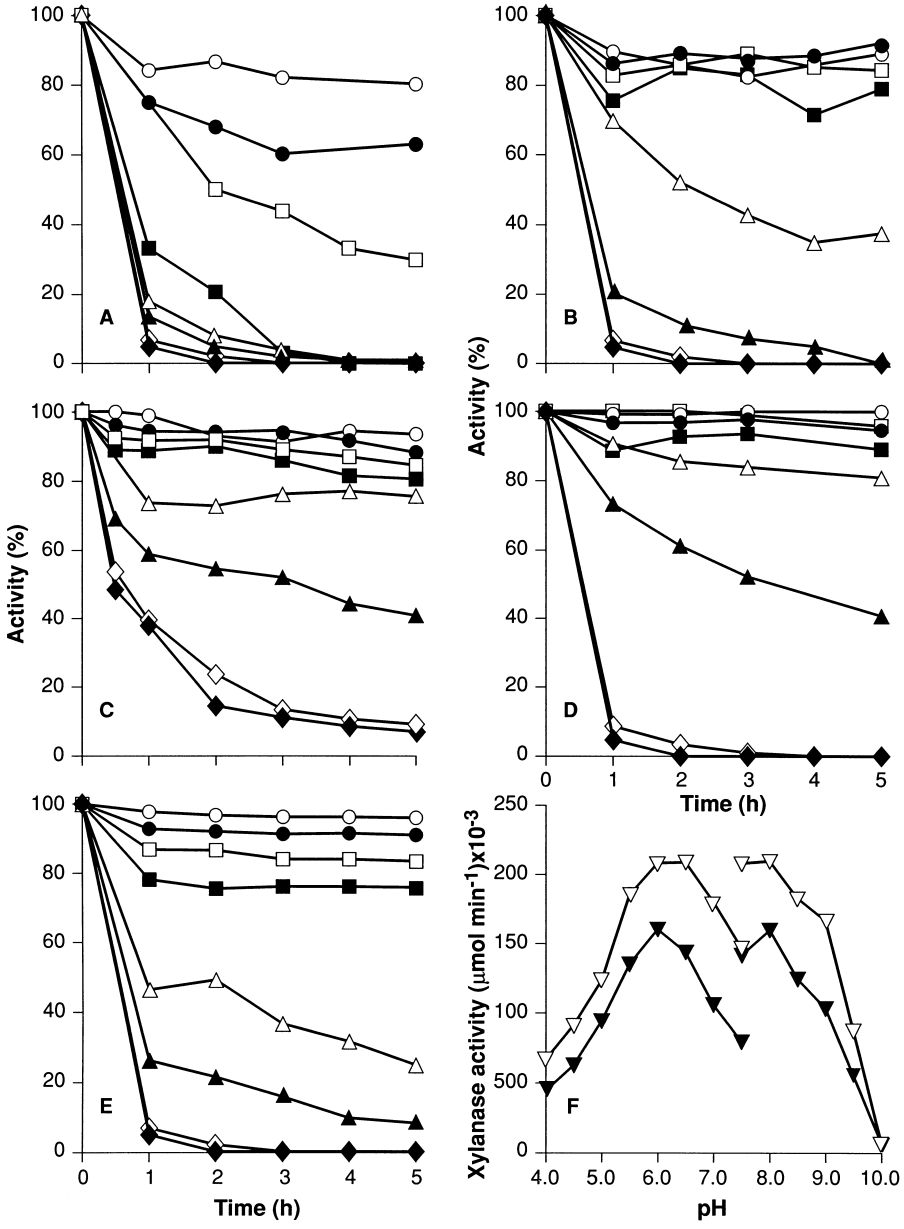


Fig. 3. Stability of endoxylanase from *T. lanuginosus* ATCC 46882 at different pH and temperature as a function of time: (A) pH 4.0; (B) pH 5.0; (C) pH 6.0; (D) pH 9.0 and (E) pH 10.0. Symbols ○, ●, □, ■, △, ▲, ◇ and ◆ correspond to 40, 45, 50, 55, 60, 65, 70 and 80 °C, respectively; (F) activity of endoxylanase from *T. lanuginosus* ATCC 46882 as a function of pH at 75 (▽) and 85 °C (▼).

The time course of the liberation of products from rhodymenan is depicted in Fig. 5. The main hydrolysis products were Xyl, Xyl_2 and an isomeric oligosaccharide with (β1–3)-linkage, which had a chromatographic mobility of an isomeric Xyl_4 (R_{xyl} 0.43). Isomeric xylotriose liberated by xylanases of family 10 [11] had the structure Xyl(β1–3)Xyl (β1–4)Xyl and RXyl 0.62, which did not appear in the rhodymenan hydrolysate of *T. lanuginosus* endoxylanase.

When endoxylanase was tested on beechwood *O*-acetyl-4-*O*-methylglucuronoxylan, the acetylated xylo-oligosaccharides which showed chromatographic mobility higher than Xyl and found in the hydrolysates by xylanases of family 10 [9], were not observed among the hydrolysis products (Fig. 6). Also, the endoxylanase from *T. lanuginosus* was capable of releasing only small amounts of non-acetylated products Xyl and Xyl_2 from *O*-acetyl- 4-*O*-methylglucuronoxylan (Fig. 6).

Action on xylo-oligosaccharides.—The action of endoxylanase on [1-^{3}H]-xylo-oligosaccharides at 0.25 mM concentration was examined. Since the activity of this endoxylanase on different xylo-oligosaccharides was significantly different, various concentrations of the enzyme had to be used with shorter and longer xylo-oligosaccharides. The reaction course was followed by measuring the distribution of radioactivity in the substrates and products resolved by TLC. Product ratios were calculated and used for the determination of initial bond cleavage frequencies (Fig. 7), initial rates of hydrolysis and k_0/K_m parameters (Table 2). The initial rates of hydrolysis and k_0/K_m parameters refer to the same concentration of the enzyme. Xyl_2 was not attacked by the enzyme at 0.25 mM,

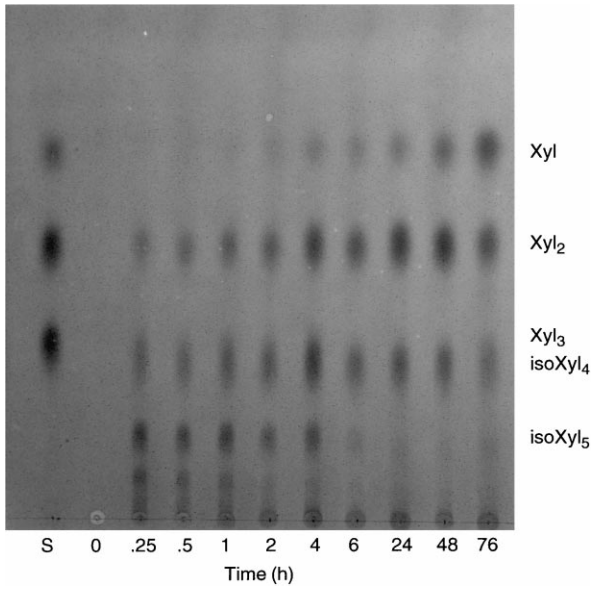


Fig. 5. TLC analysis of the hydrolysis products released from rhodymenan β-(1–4)-β-(1–3)-xylan) by endoxylanase from *T. lanuginosus* ATCC 46882. Position of standards (S) and time of incubation in h are indicated. $IsoXyl_4$ and $isoXyl_5$ correspond to isomeric tetrasaccharide and pentasaccharide containing at least one β-(1–3)-linkage.

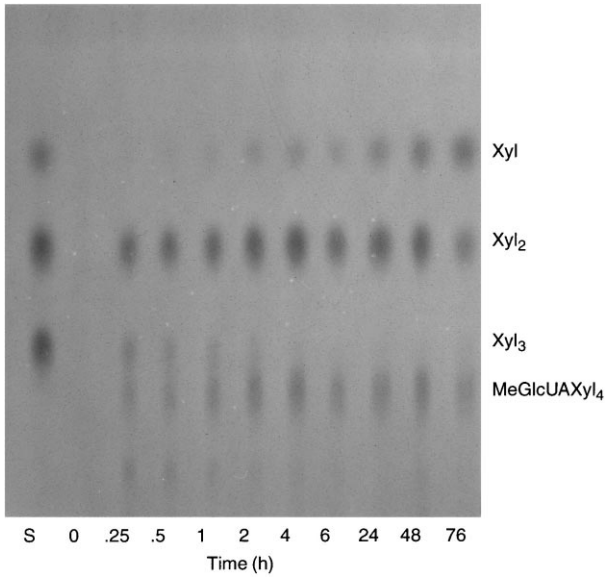


Fig. 4. TLC analysis of the hydrolysis products released from 4-*O*-methyl-D-glucuronoxylan by endoxylanase from *T. lanuginosus* ATCC 46882. Position of standards (S) and time of incubation (h) are indicated.

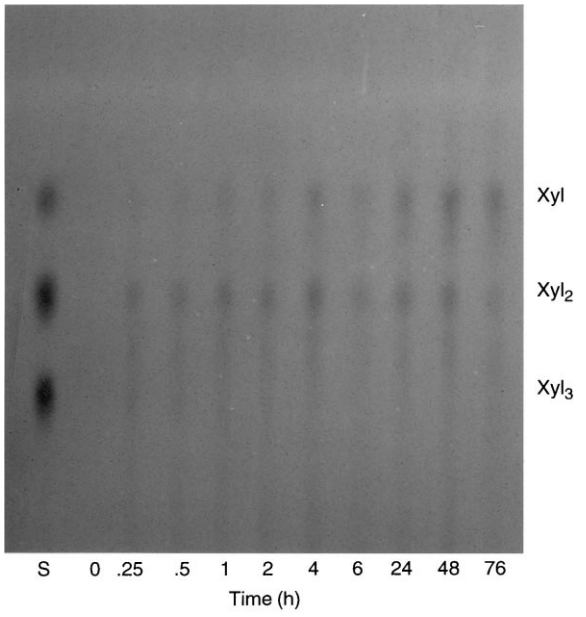


Fig. 6. TLC analysis of the hydrolysis products released from *O*-acetyl-4-*O*-methyl-D-glucuronoxylan by endoxylanase from *T. lanuginosus* ATCC 46882. Position of standards (S) and time of incubation (h) are indicated.

Table 2
Initial rates of hydrolysis of tritiated xylooligosaccharides (all 0.25 mM) by *T. lanuginosus* xylanase recalculated to a unit concentration of the enzyme and the k_0/K_m parameters (Table 2) referred to the same molar enzyme concentration

Oligosaccharide	[E] (U/ml)	Initial rate of hydrolysis (mM/min/U/ml)	Multiple	k_0/K_m (1/M/min)	Multiple
Xylotriose	6000	4×10^{-7}		56.5	
			275		266
Xylotetraose	30	1.1×10^{-4}		15×120^3	
			13		13
Xylopentaose	3	1.4×10^{-3}		19×10^4	

therefore it was not included in Table 2. Xyl_3 was cleaved almost exclusively at the first glycosidic linkage from the non-reducing end, as only radioactive xylobiose was formed from the [1-^{3}H]-xylotriose. Xyl_4 was hydrolysed mainly in the middle, however, significant cleavage also occurred at the first glycosidic linkage from the non-reducing end (~10%). From [1-^{3}H]-xylopentaose, the enzyme released two products, Xyl_2 and Xyl_3 (Fig. 7), where the [1-^{3}H]-Xyl_3 clearly predominated.

The data presented in Table 2 indicates that the enzyme had the lowest affinity towards Xyl_3, and the Xyl_3 was >250 times less favourable substrate than Xyl_4. The Xyl_4 was around 13 times less preferred substrate than Xyl_5. However, there was almost no Xyl_3 present in the polysaccharide hydrolysates (Figs 4–6). At 40 mM xyltroise and enzyme concentration of 1500 U/mL, the Xyl_3 was converted mainly into Xyl_2 without the formation of corresponding amount of Xyl (Fig. 8). The conversion of Xyl_3 into mainly Xyl_2 presumably involved glycosyl transfer reaction, where one xylosyl unit from one Xyl_3 molecule was transferred to another Xyl_3 with the formation of Xyl_4. The Xyl_4 was however, not released from the enzyme, but rapidly cleaved into two molecules of Xyl_2. The overall reaction can be envisaged as follows:

$$Xyl_3 + E = E(Xyl) + Xyl_2$$

$$E(Xyl) + Xyl_3 = E(Xyl_4)$$

$$E(Xyl_4) = E + 2Xyl_2$$

where E, free endoxylanase; E(Xyl), endoxylanase xylose complex; $E(Xyl_4)$, endoxylanase xylotetraose complex.

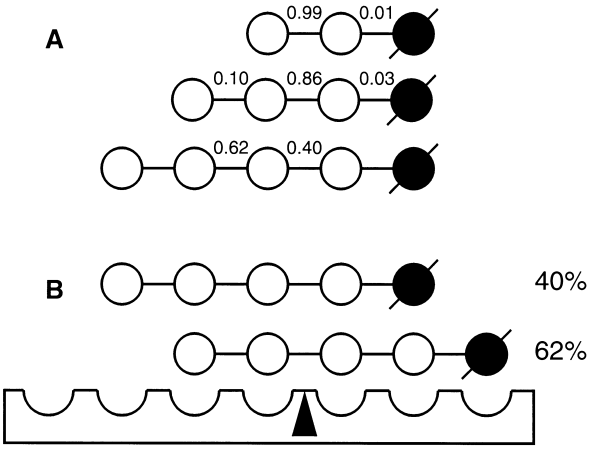


Fig. 7. (A) Initial bond cleavage frequencies (relative rates of hydrolysis of individual glycosidic linkages in oligosaccharides at zero time of the reaction) of [1-^{3}H]-xylooligosaccharides at 0.25 mM substrate concentration with endoxylanase from *T. lanuginosus* ATCC 46882. Enzyme concentration: 6000 U/mL for xylotriose, 30 U/mL for xylotetraose and 3 U/mL for xylopentaose. Symbols ○ and ● correspond to xylopyranosyl and labelled, reducing-end D-xylose residues, respectively. (B) Hypothetical substrate binding site of the enzyme comprising seven subsites (three right and four left of the catalytic groups) and two productive complexes of [1-^{3}H]-xylopentaose. The probability of their formation is indicated as a percentage.

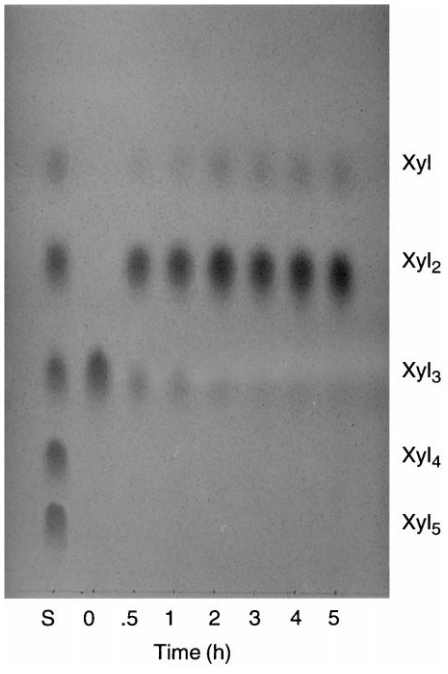


Fig. 8. TLC of products formed from 40 mM xylotriose during incubation with endoxylanase from *T. lanuginosus* ATCC 46882. The position of standards (S) and time of incubation (h) are indicated.

Also, the experiment with Xyl_2 at 40 mM and xylanase concentration of 1500 U/mL was repeated. The enzyme was found to catalyze extremely slow conversion of Xyl_2 after several hours of a long lag-phase. After 6 h of incubation, Xyl and Xyl_3 were observed in the reaction mixture which could be due to glycosyl transfer reaction of endoxylanase.

Action on artificial substrates.—The useful criterium for differentiating the xylanases of families 10 and 11 is their action on 4-nitrophenyl β-xylopyranoside and 4-nitrophenyl β-cellobioside. Both these substrates were hydrolyzed at the aglyconic linkage by xylanases of family 10. The action of endoxylanase from *T. lanuginosus* was tested on both substrates at low and high substrate concentrations. No evidence has been obtained for the liberation of 4-nitrophenol from any of the two substrates at 40 and 100 mM concentrations within 5 to 6 h, which suggested that the enzyme does not belong to the family 10. However, the enzyme (1500 U/mL) showed signs of positive reaction after 20–24 h, when incubated with 4-nitrophenyl β-D-xylopyranoside (100 mM). The presence of xylose and Xyl_2 in trace amounts was observed in the reaction mixture after 20 h. The formation of Xyl_2 can be envisaged only as a result of glycosyl transfer reactions.

4. Discussion

Production of xylanase by many micro-organisms is generally associated with the presence of cellulase and vice versa [19–21]. However, the availability of cellulase-free xylanase would be of remarkable advantage, especially for biobleaching of paper pulp [3,22]. Recent studies demonstrated that *T. lanuginosus* is a unique fungus which produces a xylanase free of cellulase activity [4,5]. Also, the data presented here revealed that *T. lanuginosus* ATCC 46882 produced the highest xylanase activity per mL of the culture filtrate when grown on corn cob (2840 U/mL) and birchwood xylan (1613 U/mL), and appears to be a considerable commercial and scientific interest.

Endoxylanase from *T. lanuginosus* ATCC 46882 was purified to homogeneity by a simple procedure which included mainly an ion-exchange and gel-filtration chromatographies. Purification of xylanase from two other strains of *T. lanuginosus* has been reported [23,24]. However, the multistage purification procedure reported by other researchers was laborious and resulted in only 5–8% recovery of total xylanase activity compared to the 38% yield in the present investigation. Also, the purification procedure reported here is simple, straight forward and can be easily adopted for the large scale purification of endoxylanase from *T. lanuginosus*.

The molecular mass (25.7 kDa) and the pI value (3.7) of purified endoxylanase from *T. lanuginosus* ATCC 46882 were in the range of that reported for xylanase purified from other strains of *T. lanuginosus* [23,24]. However, the endoxylanase from ATCC 46882 was optimally active at 75 °C compared to 60–70 °C from other strains of *T. lanuginosus* [23,24]. Also, the endoxylanase characterised in the present study showed a broad pH optimum between 6.0–6.5 and appeared to be similar to xylanase from *T. lanuginosus* DSM 5826 [24], but different from other strains of *T. lanuginosus* which showed a pH optimum of 6.0. Like the xylanase characterised from different strains of *T. lanuginosus*, the endoxylanase from *T. lanuginosus* ATCC 46882 was also stable over a wide range of pH and temperature.

Until the present study, the catalytic properties of endoxylanase from *T. lanuginosus* have not been investigated in detail. Also, the family of this enzyme has not been established until the present study. The nature of the fragments liberated from 4-*O*-methylglucuronoxylan, rhodymenan and *O*-acetyl-4-*O*-methylglucuronoxylan indicates that the xylanase of *T. lanuginosus* ATTC 46882 belongs to family 11. The enzyme does not liberate short fragments from the polysaccharides, as the xylanases from family 10. This conclusion is in agreement with its low affinity towards short xylo-oligosaccharides such as Xyl_3, a substrate around 250 times less preferred than Xyl_4. However, a mechanism different from a simple hydrolysis appears to be responsible for the conversion of Xyl_3 at concentrations higher than 0.25 mM. The observation of glycosyl transfer reactions with Xyl_2 and 4-nitrophenyl β-xylopyranoside, after a long-term incubation of the substrates with high xylanase concentration supports the existence of multiple reaction pathways of this endoxylanase.

Based on the molecular mass and catalytic properties, the endoxylanase purified from *T. lanuginosus* ATCC 46882 appears to belong family 11. However, it has been reported that family 10 xylanases have a high molecular mass and a low pI

whereas the xylanases belong to family 11 are of low molecular mass with high pI [9,11]. Endoxylanase from *T. lanuginosus* has a low pI (3.7), but showed identical molecular mass and catalytic properties as family 11 glycosidases. Thus, endoxylanase from *T. lanuginosus* appears to be an exception compared to other family 11 xylanases.

The mode of action of this xylanase on xylotriose justifies further discussion. There are only few xylanases which attack xylotriose predominantly at the first glycosidic linkage from the non-reducing end [9]. Two such enzymes are, endoxylanase II (alkaline glycanase) and endoglucanase I (non-specific glycanase) of *T. reesei* [9]. Like other endoxylanases [9], the ability of xylanase from *T. lanuginosus* to convert Xyl_3 (40 mM) predominantly to Xyl_2 can be considered as an evidence for multiple pathways of substrate degradation, which includes glycosyl transfer reactions. Furthermore, the conversion of Xyl_3 to Xyl_2 was not accompanied by the presence of products larger than xylotriose confirms that the enzyme has high affinity towards xylo-oligosaccharides larger than xylotriose.

The ability of xylanase from *T. lanuginosus* ATCC 46882 to hydrolyze polymeric substrates such as beechwood 4-*O*-methyl-D-glucuronoxylan, *O*-acetyl-4-*O*-methyl-D-glucuronoxylan and rhodymenan mainly to monomer and dimer may be important in view of the application of the enzyme for practical purposes, such as saccharification of xylan-rich materials for subsequent fermentation and preparation of Xyl and Xyl_2. Also, this xylanase could be useful for specific applications where short xylose fragments and high degree of hydrolysis are required.

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