Purification and characterization of an endoxylanase from *Trichoderma koningii* G-39

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Trichoderma koningii G-39 produced xylanases in submerged culture using oat spelt xylan or crystalline cellulose, Avicel, as the sole carbon source. A low- M_r xylanase was purified from the culture filtrate by ion-exchange chromatography on SP-Trisacryl-M and gel filtration on Fractogel TSK HW-50F. It was homogeneous on SDS/PAGE and isoelectric focusing. A typical procedure provided about 11-fold purification with 4.5 % protein yield and 50 % activity recovery. The purified enzyme has an M_r value of about 21 500 and a pI of 8.9. Its specific activity was 6100 units/mg of protein, with optimal activity towards 0.5 % xylan at about pH 5.5 and 60 °C. The purified enzyme had no activity against CM-cellulose with a degree of substitution of 0.63. It also showed no β -xylosidase activity. The K_m and V_{max} values, as determined with the soluble fraction of oat spelt xylan as substrate, were 0.70 mg/ml and $1.85 \times 10^6 \mu\text{mol/min per mg}$ of enzyme respectively. Hg^{2+} (1 mM) and SDS (10 mM) completely inhibited xylanase activity, whereas Ca^{2+} showed no significant effect on the enzyme activity at 1 mM, but gave 80 % inhibition at 10 mM. The enzyme contained about 4.4 % carbohydrate and showed an immunological relationship to a cellobiohydrolase from the same fungal strain.

INTRODUCTION

Natural xylan is a heterogeneous polysaccharide consisting of a backbone chain of β -1,4-linked D-xylopyranosyl residues and side chains of different substituents, including O-acetyl, arabinosyl and glucuronyl residues [1]. The backbone is hydrolysed by endoxylanase (1,4- β -D-xylan xylanohydrolase; EC 3.2.1.8) and the xylo-oligosaccharides formed are hydrolysed to xylose by β -xylosidase (1,4- β -D-xylan xylohydrolase; EC 3.2.1.37). A complete xylanolytic system also requires activities that hydrolyse the substituted non-xylose components as well [2].

Xylanases production by many micro-organisms, including fungi, yeasts and bacteria, has been reported. It is commonly observed that micro-organisms secrete into the medium several kinds of xylanases with different properties. Examples include *Aspergillus*, *Bacillus*, *Clostridium*, *Streptomyces* and *Trichoderma* species, taxa in which xylanase multiplicity has been most extensively examined [3]. The potential of various fungal and bacterial xylanolytic enzymes in the hydrolysis of different lignocellulosic substrates has also been evaluated [4].

As is the case with fungal cellulolytic enzymes, xylanolytic enzymes of *Trichoderma* spp. are also inducible ones [5]. A study of the variation in the pattern of extracellular enzymes produced by an efficient lignocellulose-degrading fungal strain, *T. koningii* G-39, in response to various inducer compounds, showed that microcrystalline cellulose, Avicel, strongly induced synthesis of a low- M_r xylanase in addition to cellulolytic enzymes [6]. However, phosphoric acid-swollen Avicel, and soluble inducers such as sophorose and cello-oligosaccharides, were not efficient inducers of this xylanase. We now describe some of the properties of a cellulase-free low- M_r endoxylanase and a simple procedure for the purification of this enzyme from *T. koningii* G-39. In addition, we report immunological relationships between this endoxylanase and other lignocellulolytic components of the same fungal strain.

EXPERIMENTAL

Materials

Potato dextrose agar (PDA), Noble agar and adjuvant were purchased from Difco. Carboxymethylcellulose sodium salt [CMC; medium viscosity; degree of substitution (DS) 0.63 ± 0.01] was obtained from Wako. Oat spelt xylan, *p*-nitrophenyl β -D-xylopyranoside (pNXP) and BSA were from Sigma. Avicel, 2-hydroxy-3,5-dinitrobenzoic acid (DNS), Coomassie Brilliant Blue R-250 and Fractogel TSK HW-50F were from Merck. Serva Blue W and Servalyt Precotes were from Serva Co. Bio-Gel P-6DG was obtained from Bio-Rad Laboratories, and SP-Trisacryl-M was from IBF Biotechnics. Protein M_r markers were from Pharmacia. ^{25}I -Protein A and Hybond-C nitrocellulose were purchased from Amersham. All buffer salts and other reagents were of analytical grade.

Micro-organism

T. koningii G-39 was used for the study. The strain is a mutant derived from the wild strain W-10 [7,8] by u.v. mutagenesis. It was isolated as an enhanced cellulase producer in the presence of 5 % (v/v) glycerol by an agar-plate-screening technique [9]. Stock cultures were stored on 3.9 % (w/v) PDA slants.

Enzyme production

The basal medium used for growth and enzyme induction was the same as that described by Mandels & Sternberg [10]. Spores for inoculation were obtained by culturing at 28 °C in Petri dishes, each containing 15 ml of 3.9 % PDA. After 2 weeks of incubation, the spores from two PDA plates were suspended in the basal liquid medium and inoculated into a 1-litre culture flask containing 500 ml of medium. The flask was shaken at 180 rev./min for 24 h at 28 °C. The seed culture was then filtered through G3 fritted-glass filter and mycelia washed twice with 2 vol. of water. Fermentation was carried out in a 2.5-litre fermenter containing 2 litres of induction medium at 28 °C and

Abbreviations used: PDA, potato dextrose agar; CMC, carboxymethylcellulose; DS, degree of substitution; pNXP, *p*-nitrophenyl β -D-xylopyranoside; DNS, 2-hydroxy-3,5-dinitrobenzoic acid; CMCase, carboxymethylcellulase; i.e.f., isoelectric focusing; BBS, borate-buffered saline.

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at 300 rev./min. Inoculum was mycelia from 1.5 litres of seed culture.

Enzyme purification

Crude enzyme preparation. Culture filtrate was prepared for column chromatography by using a three-phase partitioning method [11]. The protein phase was then separated, redissolved in 20 mM-ammonium acetate buffer, pH 5.0, and loaded on to a Bio-Gel P-6DG desalting column (2.6 cm $\times$ 100 cm), equilibrated with the same buffer, and eluted at a flow rate of 250 ml/h. Major protein fractions were pooled and freeze-dried. The freeze-dried crude enzymes were kept at 4 °C.

Column chromatography. Crude enzymes induced by an insoluble carbon source, Avicel or xylan, were applied on to a cation-exchanger (SP-Trisacryl-M) column for separating the xylanases from the cellulases. The adsorbed xylanases were eluted by a linear salt gradient (0–0.2 M-NaCl). Major active fractions were pooled, concentrated by freeze-drying, and subjected to gel filtration on Fractogel TSK HW-50F for further purification. The detailed conditions used are given in the legends to the Figures.

Enzyme activity assay

Xylanase activity was determined by incubating 10 μ l of enzyme solution with 1 ml of 0.5 % xylan in 0.1 M-sodium acetate buffer, pH 5.0, for 10 min at 50 °C. The reducing sugar released was determined by the DNS method [12] with D-xylose as standard. One unit of xylanase activity is defined as 1 μ mol of xylose equivalent produced/min under the assay conditions. Carboxymethylcellulase (CMCase) activity was assayed by the methods of Wang *et al.* [6] using CMC [medium viscosity; DS 0.63 $\pm$ 0.01, determined by means of the acid-wash method ASTM(American Standards for Testing Materials)-D1436-83] as the substrate. β -D-Xylosidase activity was assayed using pNXP as the substrate. The assay mixture contained 10 μ l of enzyme solution and 1 ml of 10 mM-pNXP in 20 mM-sodium acetate buffer, pH 5.0. After 10 min incubation at 50 °C, 1 ml of 1 M-Na₂CO₃ was added and the liberated *p*-nitrophenol was estimated from its absorbance at 400 nm.

For determination of K_m and V_{max} , suitably diluted xylanase was incubated with different amounts of xylan under the assay conditions given. K_m and V_{max} were determined from Lineweaver–Burk plots [13].

Electrophoretic analysis

Protein purity and estimation of the M_r value of the purified enzyme was carried out by SDS/PAGE using the discontinuous buffer system of Laemmli [14]. Protein bands were revealed by staining with Coomassie Brilliant Blue R-250.

Isoelectric focusing (i.e.f.) was performed on a Hoefer HE 900 horizontal slab-gel unit. A commercial slab-gel system, Servalyt Precotes, was used as recommended by the supplier. Protein bands were revealed by staining with Serva Blue W.

Antiserum preparation

The purified enzymes were separately used to raise the corresponding antiserum from New Zealand White rabbits. The rabbits were immunized over a period of 50 days with four intradermal injections of antigens emulsified with an equal volume of Freund's Adjuvant. The amounts of antigens used for the first injection were about 0.1 mg with complete Freund's Adjuvant. The next three injections were performed 30 days after the first injection and at 10 days intervals with about 0.2 mg antigen and incomplete Freund's Adjuvant. At 10 days after the fourth injection, the rabbits were bled from the ear artery and antisera prepared from the whole bloods.

Immunodetection

Ouchterlony double diffusion was performed as described by Garvey *et al.* [15], using agar plates containing 1 % Noble agar in borate-buffered saline (BBS) solution (0.1 M-borate buffer containing 75 mM-NaCl, pH 8.4). Each well was loaded with 10 μ l each of antiserum or antigen. The antigen concentration was 2 mg/ml for purified enzyme and 6 mg/ml for crude enzyme. The agar plates were incubated at room temperature in a wet box for 1 day and the precipitation lines were revealed by staining with Coomassie Brilliant Blue R-250.

Western blotting was performed using a Hoefer TE-52 Transphor transfer electrophoresis unit with power lid for electrophoretic transfer of proteins from SDS/PAGE to unmodified nitrocellulose. The apparatus was used as recommended by the manufacturer. Hybond-C nitrocellulose paper was then applied to perform radioactive detection with antibody and ¹²⁵I-Protein A. Finally, the air-dried nitrocellulose papers were exposed to the X-ray film with an intensifying screen at –70 °C for an appropriate time, followed by general developing procedures.

Other analysis

Protein was determined by the Bradford method [16], with BSA as standard. Total carbohydrate was measured by the phenol/H₂SO₄ method described by Dubois *et al.* [17] using glucose as standard. Picomolar-level amino acid analysis was carried out by combination of gas-phase hydrolysis and the dimethylaminoazobenzenesulphonyl chloride/h.p.l.c. procedure [18]. Product analysis was performed by h.p.l.c. as described previously [6].

RESULTS

Enzyme production

T. koningii G-39 could be induced by various carbon sources other than glucose to produce various cellulolytic and xylanolytic enzymes in the culture medium [6]. The induction time for the enzyme production depended on the carbon source used. For a 2-litre fermentation, the induction times were about 30–40 h for 2 % Avicel and 50 h for 1 % xylan as inducer. On the basis of the SDS/PAGE protein pattern of crude enzymes and enzyme activity assay (results not shown), 1 % xylan was selected as the

Fig. 1. Chromatography of 1%-xylan-induced crude enzymes on an SP-Trisacryl-M column (1.6 cm $\times$ 20 cm)

The column was equilibrated with 20 mM-sodium acetate buffer, pH 4.5, and followed by an elution with a linear NaCl gradient (0–0.2 M) in the same buffer at a flow rate of 36 ml/h. The fraction size was 15 ml for buffer-eluted fractions (nos. 1–23) and 5 ml for 0–0.2 M-NaCl (0 M-NaCl in 150 ml mixing chamber and 0.2 M-NaCl in a 150 ml reservoir)-salt-gradient-eluted fractions (nos. 24–40). ●, Protein (A_{280}); ◇, xylanase activity (A_{600}).

Fig. 2. Chromatography of pooled xylanase fractions from SP-Trisacryl-M on a TSK HW-50F column (1.6 cm $\times$ 100 cm)

The column was eluted with 20 mM-sodium acetate buffer, pH 5.0, containing 0.1 M-NaCl at a flow rate of 40 ml/h. Fractions (5.5 ml) were assayed for protein [A_{280} (●)] and xylanase activity [A_{600} (◇)].

Fig. 3. SDS/PAGE analysis on a 10% separation gel

The content of sample loading was 3 μ g for purified enzymes and 25 μ g for crude enzymes: 1, phosphoric acid-swollen-Avicel-induced crude enzyme; 2, Avicel-induced crude enzyme; 3, non-binding fractions from SP-Trisacryl-M step; 4, xylan-induced crude enzyme; 5, purified β -xylosidase (X1); 6, purified endoxylanase (X2); M, M_r markers. Protein bands were revealed by staining with Coomassie Brilliant Blue R-250.

carbon source for xylanase production. The yield of prepared crude enzymes was about 90–100 mg/litre of culture filtrate.

Enzyme purification

Crude enzyme in 20 mM-sodium acetate buffer, pH 4.5, was applied to a SP-Trisacryl-M column (1.6 cm $\times$ 20 cm) pre-equilibrated with the same buffer at a flow rate of 36 ml/h, and the column was subjected to linear gradient elution with 300 ml of the same buffer containing 0–0.2 M-NaCl (Fig. 1). The fractions

Fig. 4. Slab i.e.f. analysis

Lane 1, pI marker (protein mixture 9; Serva); lane 2, purified β -xylosidase (X1); lane 3, purified endoxylanase (X2); lane 4, xylan-induced crude enzyme. Protein bands were revealed by staining with Serva Blue W.

(nos. 31–36) with xylanase activity were pooled and concentrated by freeze-drying. Further purification was by gel filtration on a TSK HW-50F column (1.6 cm $\times$ 100 cm), equilibrated with 20 mM-sodium acetate buffer, pH 5.0, containing 0.1 M-NaCl, and eluted with the same buffer at a flow rate of 40 ml/h. Xylanase-active fractions appearing in the single protein peak (Fig. 2) were then pooled and freeze-dried after dialysis against 5 mM-ammonium acetate buffer, pH 5.0.

The purification procedure, summarized in Table 1, results in about 11-fold overall purification with about 50% recovery of activity. The purified enzyme has a specific activity of 6100 units/mg with 0.5% oat spelt xylan as substrate at pH 5.0 and 50 $^{\circ}$ C. The enzyme migrated as a single sharp band (M_r 21 500) on SDS/PAGE (Fig. 3) and on i.e.f. (pI 8.9, Fig. 4), indicating the homogeneity of the preparation.

Amino acid composition

The amino acid composition of the purified xylanase reported here (Table 2) is relatively close to that of the 20000- M_r xylanase (pI 9.4) from *T. harzianum* E58, but distinct from that of the 22000- M_r xylanase (pI 8.5) from the same source [19,20]. The 21 500- M_r xylanase of *T. koningii* G-39 had lower tyrosine, isoleucine and valine contents and contained 1.6 half-cystine residues, whereas the 20000- M_r xylanase of *T. harzianum* E58 had a lower content of glutamic acid + glutamine and contained no half-cystine. The xylanase of *T. koningii* G-39 contained 4.4% carbohydrate and, as shown by amino-acid-sequencing analysis, was blocked at the *N*-terminus.

Table 1. Purification of endoxylanase from *T. koningii* G39

Step	Total volume (ml)	Total protein (mg)	Total activity (units)	Activity recovery (%)	Specific activity (units/mg)	Purification (fold)
1. Crude enzyme	50	45.1	24 767	100.0	549.2	1
2. SP-Trisacryl-M	52	3.78	17 029	68.8	4504.9	8.2
3. TSK HW-50F	21	2.04	12 395	50	6089.2	11.1

Table 2. Amino acid composition of endoxylanase

Amino acid	Residues/molecule*	Amino acid	Residues/molecule
Asx	19.3	Tyr	7.5
Glx	12.4	Ala	9
Lys	3.5	Val	9.5
Arg	7.6	Leu	7.9
His	2.8	Ile	5.3
Gly	26.5	Pro	8.6
Ser	19.0	Met	1.0
Thr	15.4	Phe	6.2
$\frac{1}{2}$ -Cys	1.6		

* The calculated number of residues/molecule is based upon an M_r of 215000.

Fig. 5. H.p.l.c. chromatograms of the hydrolysis products after incubating oat spelt xylan with the purified xylanase

The conditions of the enzymic reaction are as described in the Materials and methods section. (a) Xylose (40 μ g); (b) after 0 h incubation; (c) after 5 min incubation; (d) after 10 min incubation; (e) after 20 min incubation; (f) after 50 min incubation. A Bio-Rad column (HPX-42A) was used with water as the eluent at 0.6 ml/min. The post-column reactor temperature was 130 $^{\circ}$ C, with 0.5% boric acid and 0.5% monethanolamine as reagents, also at 0.6 ml/min. The fluorescence detector was set to 320 nm (excitation and wavelength) and 450 nm (emission wavelength).

Properties

Several physicochemical properties of the purified enzyme were determined. The K_m and V_{max} values with oat spelt xylan as substrate were 0.70 mg/ml and $1.85 \times 10^6 \mu$ mol/min per mg respectively, as determined from the Lineweaver-Burk plot. Activity was optimal at 60 $^{\circ}$ C and at pH 5.0–5.5. The xylanase is devoid of CMCase or β -D-xylosidase activity. It was completely

Fig. 6. Western-blotting autoradiogram

Proteins were firstly separated by SDS/PAGE, followed by electroblotting. Antiserum used was that against X2. Protein loaded in each lane were: 1, M_r markers; 2, phosphoric acid-swollen-Avicel-induced crude enzyme (SA); 3, a purified cellobiohydrolase (C1); 4, Avicel-induced crude enzyme (A); 5, a purified endoglucanase (Cx1); 6, a purified endoglucanase (Cx2); 7, a purified low- M_r endoglucanase (Cx3); 8, xylan-induced crude enzyme (X); 9, purified β -xylosidase (X1); 10, purified endoxylanase (X2).

Fig. 7. Double-immunodiffusion test

Antiserum against X2 was placed in the centre wells of both (a) and (b). Outer wells containing antigens in (a) (abbreviation as in Fig. 6): (1) C1; (2) Cx1; (3) Cx3; (4) X1; (5) X2; (6) control (phosphate buffer). Outer wells containing antigens in (b): (1) Blank; (2) A; (3) SA; (4) X; (5) X2; (6) X1.

inhibited by 1 mM- Hg^{2+} , whereas Mg^{2+} , Ca^{2+} , EDTA, urea, SDS or guanidinium chloride had no significant effects on the enzyme activity at the same concentration. On the other hand, the enzyme was almost completely inhibited by a high concentration (10 mM) of SDS or Ca^{2+} .

H.p.l.c. analysis of the hydrolysis products of oat spelt xylan (Fig. 5) showed no xylose or xylobiose in the initial phase of reaction, indicating that the purified xylanase is an endo-acting enzyme. On further incubation, the baseline between xylan and xylose peaks increased, indicating the xylan was degraded to various xylo-oligomers (not identified). After 50 min incubation, increased accumulation of xylose was observed.

Immunodetection

Antiserum against the purified xylanase showed specific reaction with the purified enzyme and crude enzymes induced by insoluble carbon source, xylan or Avicel, whereas no reaction was shown with the crude enzyme induced by phosphoric acid-swollen Avicel in both Western-blotting (Fig. 6) and Ouchterlony double-diffusion tests (Fig. 7).

DISCUSSION

Previous studies with washed mycelia of *T. koningii* G-39 indicated that the enzymes induced by crystalline cellulose included highly active xylanases in addition to all components of the cellulase system [6]. The present study showed that the same

fungus growing on xylan produced lower amounts of cellulases and, therefore, a higher ratio of xylanase to cellulase activity in comparison with that induced by Avicel. Thus, for the purposes of the present study, xylan was used as the sole carbon source for enzyme production. Cation-exchange chromatography on a SP-Trisacryl-M column, pH 4.5, was used for the separation of xylanases from cellulases. The protein pattern revealed by SDS/PAGE (Fig. 3) suggested that the xylanases, but not low- M_r CMCase, adsorbed to this column. This was an important factor in the success of separation by subsequent gel filtration. The specific activity of xylanase in the culture filtrate was highest at the culture time of 40–50 h. This shorter culture time for enzymes production was adopted for the present xylanase purification. From a 2-litre batch fermentation, the purification gave 4.5% protein yield and 50% activity recovery (Table 1). The specific activity of 6100 units/mg was high in comparison with the range 0.28–1582 units/mg for specific activities reported for D-xylanases isolated from other *Trichoderma* species [19–27].

Examination of xylanase multiplicity in *Bacillus* spp. suggested that these bacteria produce two categories of xylanase [3]. The enzymes in one group are basic (pI 8.3–10.0), with low M_r values (16 000–22 000) estimated by SDS/PAGE, and the other is acidic (pI 3.6–4.5), with high M_r values (43 000–50 000). This pattern, namely low- M_r /basic and high- M_r /acidic, of microbial xylanases also obtained in *Clostridium*, *Streptomyces*, *Aspergillus* and *Trichoderma* species [3]. The M_r (21 500) and pI (8.9) values of the xylanase characterized here are within the ranges previously reported for microbial xylanases. However, its high specific activity and amino acid composition distinguish it from other D-xylanases with similar M_r and/or pI values [20,23–25,27–32]. Interestingly, the present endoxylanase is different from either of the two low- M_r endoxylanases (29 000, 17 700) purified from *T. koningii* IMI 73022 [22], both of which had lower pI (7.24, 7.3) and higher K_m (1.4 mg/ml, 4.2 mg/ml) values and were devoid of carbohydrate. The xylanase characterized here has a smaller K_m (0.7 mg/ml) and a high V_{max} ($1.85 \times 10^6 \mu\text{mol/min per mg of enzyme}$) and contained about 4.4% carbohydrate.

In order to study the immunological relationships between the cellulolytic and xylanolytic enzymes produced by *T. koningii* G-39, a number of these enzymes have been purified to homogeneity (by the criterion of SDS/PAGE [33]). These include one cellobiohydrolase (C1), three endoglucanases (Cx1, Cx2, Cx3), one β -xylosidase (X1) and the endoxylanase (X2) characterized here. From Ouchterlony double-diffusion tests (Fig. 7), it appeared that antisera against X2 showed strong precipitation lines to crude enzymes induced by xylan or Avicel, but not to those induced by phosphoric acid-swollen Avicel. In addition, anti-X2 serum cross-reacts with C1, but not with the other cellulase components from the same fungal strain. The results suggest that the smaller enzyme X2 and the larger C1 may possess a closely related antigenic structure which gave rise to these cross-reaction patterns [34,35]. The possibility that C1 might be contaminated by X2 is very unlikely, as the purified C1 showed no xylanase activity.

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