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Note

A rapid procedure for the purification of an endo-(1→4)-β-D-xylanase from *Polyporus tulipiferae (Irpex lacteus)*

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Enzymes, in association with chemical techiques[1–3], are powerful tools for the elucidation of polysaccharide structures. They have been used[4,5] as gold–enzyme complexes for the localisation of polysaccharides in plant cell-walls. This specific technique is a new approach in the microscopy of sub-structures (walls, starch granules) of plant cells in relation to plant physiology, food technology, nutrition, *etc.*

Only pure enzymes can provide unambiguous results and, in contrast to amylolytic enzymes, cell-wall depolymerases of high purity (xylanases, cellulases, pectinases) are not commercially available. Hence, there is a need for simplified purification procedures starting from commercially available, crude enzyme preparations. Rapid purification schemes[6,7] have been proposed for commercial β-D-glucan hydrolases, but the criteria of homogeneity (analytical electrofocusing) are sometimes missing[7] and the specificity of the enzymes has been tested only on a limited number of substrates.

We have purified[8] an alkaline endo-(1→4)-β-D-xylanase from a commercial, crude enzyme-mixture, Driselase, from the white-rot fungus *Polyporus tulipiferae (Irpex lacteus)*, but the procedure was complex. A rapid and facile procedure is now reported for the purification of another endo-(1→4)-β-D-xylanase from this source.

Ammonium sulfate precipitation (50% saturation) effected a 2.3-fold purification (Table I) of the xylanase activity (~62% yield) by removing ~73% of the initial proteins and almost all of the coloured material.

Application of a zymogram technique (using Xylan–Azure[9]) to both analytical isoelectric focusing and the titration curve of crude Driselase showed that it contained four major xylanases[10] with pI values of 3.7, 6.3, 7.4, and 8.2. In an attempt to purify the xylanase of pI 6.3, chromatofocusing was applied to the above fraction from the ammonium sulfate precipitation with the eluent pH at 8.1. However, only the strongly acidic enzyme (pI 3.7) was bound to PBE 94. Since

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TABLE I

PURIFICATION OF XYLANASE II FROM *P. tulipiferae*

Step	*Volume (mL)*	*Activity (nkat.mL^{-1})*	*Protein (mg.mL^{-1})*	*Specific activity (nkat.mg^{-1})*	*Yield (%)*	*Purification (fold)*
Crude enzyme	190	305	5.83	52.3	100	—
Ammonium sulfate precipitation (50% saturation)	6	6007	50.4	119.2	62.2	2.3
1st chromatography PBE 94 (pH 8.1)	20	1159	1.00	1159	40.0	22.2
2nd chromatography PBE 94 (pH 9.4)	30	180	0.076	2371	9.3	45.3

most of the inert proteins from Driselase had acidic pI values, the unbound xylanase activities were purified 22-fold (Table I) (~40% of the initial activity and 1.8% of the proteins). The behaviour of the xylanase of pI 6.3 was unusual and, on increasing the pH of the eluent to 9.4 (Fig. 1), a major fraction (I), which contained both alkaline xylanases, passed through the column but a minor peak (II) was delayed and corresponded to the xylanase of pI 6.3. The enzyme in peak II was of high purity, as shown by analytical isoelectric focusing (Fig. 1), and is now readily available by this procedure.

Xylanases from *P. tulipiferae* have a low density of ionisable groups over a

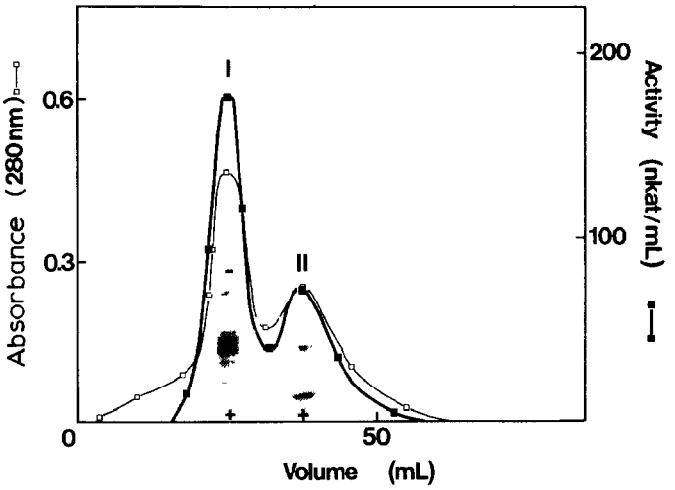

Fig. 1. Elution behaviour of the partially purified (see Experimental) xylanases from *Polyporus tulipiferae* on Polybuffer Exchanger 94 in 25mM ethanolamine–HCl buffer (pH 9.4). Fractions (1.4 mL) were assayed for proteins at 280 nm (—□—) and for xylanase activity (—■—). Isoelectric focusing electrophoregrams (protein staining) of both xylanase fractions (I, II) are inserted under the corresponding peaks.

wide pH range. An alkaline xylanase[8] from this fungus did not bind to PBE 118 even at pH 11.0. Furthermore, titration curves of these enzymes were rather flat and did not diverge much from the central trough between pH 4.0 and 10.0, making their pI values difficult to determine accurately. This general trend explains why xylanase II did not bind but was simply delayed on the exchanger at a pH higher by 3.1 units than its pI (6.3).

The purified xylanase (~9% of the initial activity) was purified 45-fold, with a specific activity of 2371 nkat.mg^{-1}, and gave a sharp band in SDS–polyacrylamide gel electrophoresis and analytical isoelectric focusing (pI 6.3). It had no activity against glycosides and non-xylan polysaccharides, but, as exhibited by the previously purified xylanase[8], it had a low (~0.5%) activity against *o*-nitrophenyl β-D-xylopyranoside. Activity of endo-xylanases towards aryl β-D-xylosides has been attributed[11] to a transglycosylation mechanism.

The purification procedure for xylanase II is simple, highly reproducible, and can be completed in ~2 days. The yield of enzyme was 2.3 mg/5 g of Driselase.

EXPERIMENTAL

Enzyme and substrates. — The enzyme was a commercial, crude powder (~20% soluble proteins) from the basidiomycetes *P. tulipiferae*, marketed as Driselase (Kyowa Hakko Kogyo Co., Tokyo) and generally used for the preparation of protoplasts.

Commercial oat-spelts xylan (Sigma, X-0376; Xyl/Ara 1:0.12) was used, without further purification, for measuring the endo-(1→4)-β-D-xylanase activity. Other polymeric substrates were larchwood xylan (Sigma, X-0375), larchwood arabinogalactan (Sigma, A-2012), carboxymethylcellulose sodium salt (Sigma, C-4888, medium viscosity), gum arabic (Sigma, G-9752), avicel (Merck), Cellulose Azure (Calbiochem 219481), arabinan (Koch–Light 52867), dextran (Sigma, D-4751), polygalacturonic acid (ICN 102771), lichenan from *Cetraria islandica* (Sigma, L-7378), laminarin (Fluka 61400), mixed-linked barley β-D-glucan from barley (gift of Dr. B. Stone), *Phoenix canariensis* mannan (gift from Dr. F. Percheron), waxy-maize beta-limit dextrin[8], and white-lupin acidic galactan (prepared in this Department).

Purification of the xylanase. — Driselase (5 g) was suspended by stirring with 10mM phosphate buffer (200 mL, pH 7.0). After homogenisation, the suspension was immediately centrifuged at 20,000*g* for 10 min, ammonium sulfate (59.4 g) was added to the dark-brown supernatant (190 mL), and the resulting precipitate was allowed to settle for 1 h in the cold. After centrifugation (20,000*g*, 10 min), the pellet was washed once with 10mM phosphate buffer (pH 7.0) containing 50% of ammonium sulfate and redissolved in 25mM Tris–HCl buffer (6 mL, pH 8.1), and the solution was dialysed against the same buffer for 4 h. The pH of the retentate was adjusted to 8.1, if necessary, with 0.5M Tris, and the solution was injected at 60 mL/h on to a column (1.0 × 40 cm) of Polybuffer Exchanger 94 (PBE 94,

Pharmacia) previously equilibrated with 25mM Tris–HCl buffer (pH 8.1). Eluate containing unbound proteins (elution volume, 15→35 mL) was collected, concentrated to 2 mL, and dialysed for 2 h against 25mM ethanolamine–HCl buffer (pH 9.4). The pH of the retentate was adjusted to 9.4, if necessary, with 0.5M ethanolamine, and the solution was injected at 24 mL/h on to a column (1.0 × 40 cm) of PBE 94 equilibrated with the ethanolamine–HCl buffer. The u.v. absorption at 280 nm of the eluate revealed two peaks (I and II; Fig. 1); the minor (II) (eluting at 20→50 mL) was collected.

Xylanase activity was measured by mixing enzyme solution (100 μL, <4 nkat) with 1 mL of a 2% dispersion of xylan in 0.2M acetate buffer (pH 4.5). The volume was made up to 2 mL with distilled water. After 20-min incubation at 40°, 900 μL were withdrawn and analysed for reducing sugars by the Somogyi–Nelson[12] procedure, using D-xylose as the standard; 1 nkat of enzyme liberated 1 nmol of xylose equivalent per s. The specific activity of the Driselase was 52 nkat.mg^{-1} of soluble proteins.

Possible contaminating glycosidases and glycanases were determined by using *p*-nitrophenyl glycosides or polysaccharides as previously described[8]. Other analytical methods have been described[8]. Analytical isoelectric focusing was performed on Agarose IEF (Pharmacia, Sweden) in the pH range 3–10 according to the supplier's recommendations. Calibration of pI was effected with a broad pI calibration kit. Bands were detected by staining with Coomassie Brilliant Blue G.

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