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Measurement of xylanase activity with insoluble xylan substrate

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Insoluble xylan was prepared from ground birch (*Betula pubescens*) pulp by alkali extraction and precipitation with ethanol. The only sugar detected after acid hydrolysis of the preparation was xylose. The insoluble xylan was used as substrate in a nephelometric assay to determine the xylanase (EC 3.2.1.8, 1,4-β-D-xylan xylanohydrolase and EC 3.2.1.37, 1,4-β-D-xylan xylohydrolase) activities of *Aspergillus* and *Trichoderma* enzymes. The nephelometric method is reliable in evaluating xylanase hydrolysis of insoluble xylan.

The neutral carbohydrate fraction of hardwood species, e.g. the birch *Betula pubescens*, contains 55% (w/w) glucose and 39% xylose (Timell, 1964). In an exhaustive enzymic hydrolysis of wood, the enzymes acting on xylopolysaccharide should thus be considered as important as those acting on glucopolysaccharide. In order to analyse the hydrolytic properties of xylanolytic enzymes, it is necessary to have a substrate as analogous as possible to natural xylan in order to obtain reliable estimates of wood hydrolysis. The ligno-hemicellulose fraction of native wood is insoluble in biological solvents. Less than 0.5% can be extracted with water (Ebringerova *et al.*, 1967).

The aim of the present work was to prepare an insoluble homogeneous xylan substrate and to use it for measuring xylanolytic activities. In our previous studies a nephelometric method was used for the assay of enzymes hydrolysing amorphous cellulose (Nummi *et al.*, 1981). This method is now adapted for the prediction of the hydrolysis rates of insoluble xylan (hemicellulose) in wood.

Materials and methods

Preparation of insoluble xylan

Xylan was prepared by modifying the alkali-extraction method of Adams (1965). Birch (*Betula pubescens*) wood was pulped by using a laboratory grinding machine equipped with a polishing stone (diameter 0.3 m, breadth 0.05 m) rotating at 25 m/s. Pulp consisting of 40% of particles smaller than 200 mesh was used to prepare insoluble xylan. A 100 g portion of pulp was washed with 2.5 litres of 0.5 M-sodium citrate buffer, pH 5.0, containing 0.1 M-EDTA (Merck, Darmstadt, Germany), at room temperature for 20 h. The washed pulp was extracted with 1.7 litres of 1 M-NaOH for 20 h. After centrifugation the extract was filtered through a Whatman (Maidstone, Kent, U.K.) GF/C 11 filter. The pH of the filtrate was adjusted to pH 4.5 by adding 10 M-acetic acid. The suspension was stirred for 2 h, after which the liquid was decanted and the precipitate obtained was suspended in 0.1 M-sodium acetate buffer, pH 4.0. After 20 h the suspension was centrifuged and the pellet was dissolved in 400 ml of 0.6 M-NaOH. After 20 h the insoluble material was eliminated by centrifugation and filtration of the supernatant through a Whatman GF/C 11 filter. The clear filtrate was precipitated with 800 ml of ethanol. The precipitate was washed with 0.1 M-sodium acetate buffer, pH 4.0, and then resuspended in the same buffer. The stability of the substrate was checked during long-term storage.

Analysis of insoluble xylan

Xylan (20 mg) was hydrolysed with 1 ml of 0.5 M-H_2SO_4 for 10 h at 100°C (Nurmesniemi & Pulkkinen, 1981). The insoluble and soluble fractions were separated. For dry-weight determination, the insoluble fraction was washed with deionized water. The soluble fraction was fractionated into neutral and acidic fractions (Nurmesniemi & Pulkkinen, 1981). The soluble fraction after acid hydrolysis was neutralized with $Ba(OH)_2$. The possible lactones and the uronic acids were split under N_2 with NaOH at pH 8 at room temperature for 20 h. After filtration the solution was diluted to 1.5 ml with water and the cations were adsorbed on a Dowex 50W (Cl^- form) resin (Fluka, Buchs, Switzerland; column vol. 5 ml). Neutral and acidic components were eluted with 10 ml of water. The solution was neutralized with NaOH and passed

through an anion-exchanger, Dowex 1 (acetate form) (Fluka; column vol. 5ml).

The neutral fraction was eluted with 10ml of water and the acidic fraction with 10ml of 2M-acetic acid. Fractions were concentrated by evaporation and analysed on Whatman 3MM paper. The solvent was ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.). The spray reagent was 0.1M-*p*-anisidine phthalate in 95% (v/v) ethanol (Nurmesniemi & Pulkkinen, 1981). For quantitative determination of uronic acids, a portion of the acidic fraction was run on paper under the same conditions and left unstained. The areas expected to be aldobiouronic and 4-*O*-methylglucuronic acids according to their R_F values of 0.72 and 1.3 respectively (Nurmesniemi & Pulkkinen, 1981) were eluted from the paper with water. The water extracts were analysed by the cysteine/carbazole methods for uronic acids (Ashwell, 1957). The neutral fraction was quantitatively analysed by liquid chromatography. The equipment used was an Optilab (Vallinsby, Sweden) 931 HSRI with multiref 902C. The column contained Aminex HPX-87C in water. Analyses were performed at 65°C.

Exhaustive enzymic hydrolyses

Hydrolyses were performed by using 100mg of insoluble xylan and 5mg of *Trichoderma* or *Aspergillus* enzymes. The hydrolysis time was 5 days. The hydrolysis products were analysed by liquid chromatography and also fractionated by ion-exchange chromatography and analysed for neutral and acidic sugars (Nurmesniemi & Pulkkinen, 1981; Ashwell, 1957).

Preparation of enzymes

Aspergillus awamori VTT-D-79103 was fermented in 3.0% (w/v) wheat-bran medium, and *Trichoderma reesei* VTT-D-80133 was cultivated on sulphite cellulose and distiller's spent grain (Linko *et al.*, 1977).

The protein contents of the clarified fermentation broths were determined by the method of Lowry *et al.* (1951), bovine serum albumin (Sigma) being used as the standard. The liquids were used as enzyme preparations to hydrolyse the insoluble xylan. A purified cellobiohydrolase (Nummi *et al.*, 1983) was used as a control cellulolytic enzyme.

Measurement of xylanase activities

The hydrolysis mixture for nephelometric measurements contained 1 mg of xylan and 5–500 μg of enzyme protein in 3.2ml of 0.05M-sodium acetate buffer, pH 4.0. A nephelometric Perkin–Elmer (Oak Brook, IL, U.S.A.) amylase/lipase analyser (model 91) was used to monitor the hydrolysis (Nummi *et al.*, 1981). The temperature of the incubation chamber was 40°C. The decreases in nephelometric values were measured and plotted as a function of the amount of enzyme protein used. The hydrolysis mixtures were rapidly frozen to halt the enzymic reaction. The thawed solutions were applied to a mixed-bed ion-exchanger (Amberlite MB 3; BDH, Poole, Dorset, U.K.), to eliminate buffer ions, and were quantitatively analysed by liquid chromatography.

Results and discussion

Table 1 summarizes the analysis of the prepared xylan. The only sugar detected by both paper and

Table 1. *Composition of insoluble xylan*

For full experimental details, see the text. The insoluble fraction was quantified by dry-weight estimation, the soluble neutral fraction by liquid chromatography, and the acidic fraction by the method of Ashwell (1957).

	Hydrolysis (% dry wt. of insoluble xylan)		
Treatment ...	Acid	Exhaustive enzymic	
		Trichoderma	*Aspergillus*
Insoluble fraction	4	13	8
Soluble fraction			
Neutral			
Xylose	104	86	49
Xylo-oligomers	0	7	29
Glucose	0	0	0
Mannose	0	0	0
Galactose	0	0	0
Acidic			
Uronic acids	0.5	0	0
Total	109	106	86

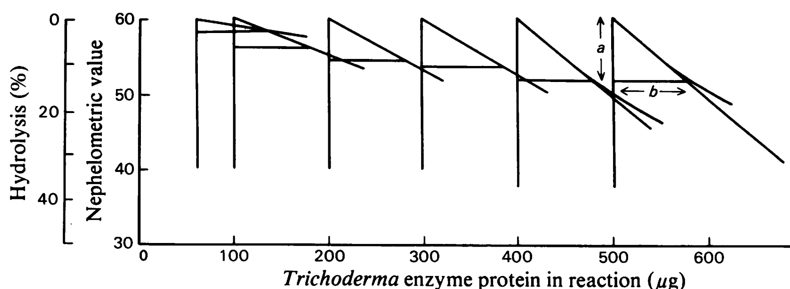

Fig. 1. *Nephelometric plots of the hydrolysis of insoluble xylan using Trichoderma reesei culture filtrate*
The decrease in nephelometric value (*a*) in unit time (*b*) corresponds to the extent of substrate hydrolysis.

liquid chromatography after acid hydrolysis was xylose. The yield, measured by both dry-weight determination and liquid chromatography, was calculated taking into consideration the destruction of pure xylose under the hydrolysis conditions. The insoluble product of acid hydrolysis, 4% of the starting material, was most probably lignin. It was soluble in 1M-NaOH and gave a u.v. spectrum similar to those of aromatic carboxylic acids (Chang *et al.*, 1980). The methoxy content of the insoluble xylan was 1.85% (H. Malissa & G. Reuter, Analytical Laboratory, 5270 Gummersbach 1, Elbach, Germany). Calculated from this value, the amount of lignin would be 6.5% of the original xylan preparation (Ebringerova *et al.*, 1967). According to these results, the insoluble xylan preparation represents the pure polyxylose of birch. Table 1 also reveals differences between the hydrolytic properties of the *Trichoderma* and *Aspergillus* enzymes.

Nephelometric plots of the hydrolysis of insoluble xylan by *Trichoderma* enzymes are shown in Fig. 1. It can be seen that the nephelometric method is suitable for determining the hydrolysis of macromolecular xylan. A clear relationship was obtained between the decrease in nephelometric value and the amount of enzyme protein used (Fig. 2). The smallest amount of enzyme that gave a clear slope in the nephelometric analysis was 5–10μg in the reaction mixture of 3.2ml. When the enzymic activity was analysed by determining the amount of xylose released, it was necessary to use 10-fold amounts of enzyme, 50–100μg in the reaction mixture, to obtain detectable amounts of xylose for liquid-chromatographic estimation. Further, a linear relationship was not obtained between the amount of xylose released and the amount of enzyme, because the enzymic hydrolysis produces variable amounts of soluble xylo-oligodextrins in addition to xylose, depending on the xylanase preparation used (Fig. 3). According to the results of liquid chromatography, the amount of xylose produced was 86% and 49% of the original insoluble xylan in an exhaustive hydrolysis by *Trichoderma* and *Aspergillus* enzymes respectively (Table 1). *Trichoderma* enzymes produced 7%, and *Aspergillus* 29%, soluble xylo-oligomers. The insoluble fraction after enzymic hydrolysis

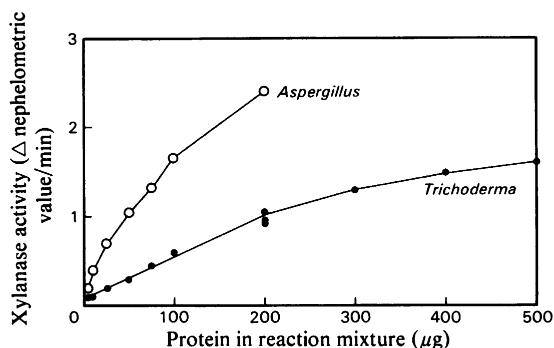

Fig. 2. *Decrease in nephelometric values corresponding to the action of xylanase enzymes from Trichoderma and Aspergillus culture filtrates*

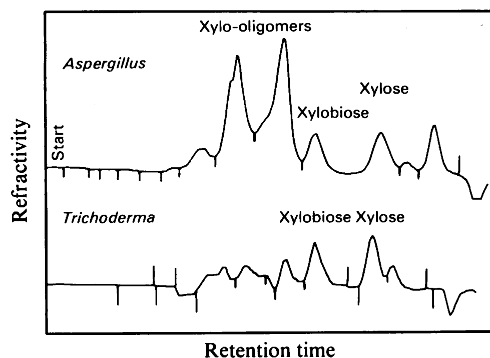

Fig. 3. *Hydrolysis products from insoluble xylan after 10min of hydrolysis by Trichoderma and Aspergillus culture filtrates*
The reaction mixture contained 1mg of insoluble xylan and 500μg of *Trichoderma* or 100μg of *Aspergillus* protein. The deionized samples were analysed by liquid chromatography in an Aminex HPX-87C column eluted with water at 65°C.

seemed to contain lignin and its reaction products with components of the culture filtrates of *Aspergillus* and *Trichoderma*.

Insoluble xylan is a specific substrate for xylanases. Pure cellobiohydrolase from *Trichoderma reesei* (Nummi *et al*., 1983) had no action whatsoever against the insoluble xylan.

It is reasonable that insoluble substrate should be used to estimate the activities of xylanases that are intended for the hydrolysis of insoluble hemicellulose. The activity estimates obtained with artificial soluble substrates do not correspond to practical yields. The material to be hydrolysed by xylanases, the hemicellulose fraction of wood, is insoluble under the conditions of the enzymic hydrolysis before enzyme attack. The insoluble xylan prepared here is suitable for analysis of the degradation of natural xylan and it is a specific substrate for xylanases. If the hydrolysis is monitored by a nephelometric method, a good correlation is obtained between the amount of enzyme protein used and the degradation of the insoluble xylan. The pattern of end products and the purity and composition of the enzyme preparation do not give an erroneous prediction of the hydrolysis, because it is the disappearance of substrate that is monitored and not the appearance of products. The amounts of enzyme needed for analysis are lower than those needed to produce detectable amounts of end products. Thus the nephelometric method using insoluble xylan is suitable for the analysis of the activities of xylanolytic enzymes intended for wood hydrolysis.

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