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Note

Mode of action of xylanase II from *Polyporus tulipiferae* on oat-spelt arabinoglucuronoxylan and larchwood 4-*O*-methylglucuronoxylan

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Xylans are the major hemicelluloses in most land plants[1], are typical constituents of secondary cell walls in close association with lignin, occur in primary cell walls of monocots, and, after cellulose, are the major constituents of non-endospermic tissues from *gramineae*[2] and hardwoods. Xylans are built of (1→4)-linked β-D-xylopyranosyl residues carrying, variously, single units of α-L-arabinofuranose at O-3 and/or 4-*O*-methyl-α-D-glucuronic acid at O-2, or short side-chains including arabinose, xylose, galactose, and glucuronic acid residues[3–5].

Enzymes, especially endo-xylanases (EC 3.2.1.8), have been used for the elucidation of their structures in association with chemical techniques[5–7]. Xylanases are able to split the (1→4)-β-D-xylopyranosidic linkages occurring in unsubstituted regions of the xylan backbone, whereas α-L-arabinofuranosidases and β-D-xylosidases act on the terminal non-reducing units of side-chains.

We have purified an endo-(1→4)-β-D-xylanase II produced by *Polyporus tulipiferae*[8] and now describe its mode of action on commercial, lightly substituted, oat-spelt arabinoglucuronoxylan and larchwood 4-*O*-methylglucuronoxylan.

When suspensions (0.5%) of crude xylans were incubated for 24 h with the xylanase II, the liberated reducing sugars increased progressively with concomitant disappearance of the insoluble material and reached a quasi-plateau after 24 h. The soluble total (neutral + acidic) sugars increased from ~35% of the carbohydrate content of oat and larch xylans to 91.4% and 98.0%, respectively, at the end of the reaction (24 h). The maximum degrees of hydrolysis were 35% and 37% for oat and larch xylan, respectively (xylose basis), giving an identical figure of 41% when related to the unsubstituted xylose residues (2,3-di-*O*-methylxylose basis) occurring in the xylans, which must be related to the similar degree of branching of both xylans, namely 16.2% and 13.6%, respectively. The average d.p. of the (neutral + acidic) oligosaccharides produced within 24 h was ~3 for each xylan.

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T.l.c. (Fig. 1) of the products released from larch and oat xylans and h.p.l.c. of the borohydride-reduced products revealed a homologous series of xylo-oligosaccharides, namely, xylose, xylobiose (R_{Xyl} 0.77), xylotriose (R_{Xyl} 0.55), up to xylo octaose. The pattern of release (Fig. 2) is typical of an endo-mechanism of enzyme action. Thus, xylobiose was produced increasingly and in major amounts, and resisted further hydrolysis, whereas xylotriose increased up to 2 h and then was slowly cleaved into xylobiose and xylose. The higher oligosaccharides produced at the beginning of the reaction rapidly disappeared. Similar profiles were obtained from both xylans, which reflects similar degrees of branching. In addition to the xylo-oligosaccharides, larchwood hydrolysates (Fig. 1) contained slowly migrating components (I–III), two of which (I major, II minor) accumulated and migrated at rates which were the same as those of (4-*O*-MeGlcA)Xyl_3 and (4-*O*-MeGlcA)Xyl_4. According to the structure of larch xylan (4-*O*-methylglucuronic acid side-chains attached to O-2 of xylosyl residues) and since they resisted β-D-xylosidase, these compounds are believed to be the aldotetrao- and aldopentao-uronic acids, the acidic substituent being attached to terminal non-reducing xylosyl groups. Similar

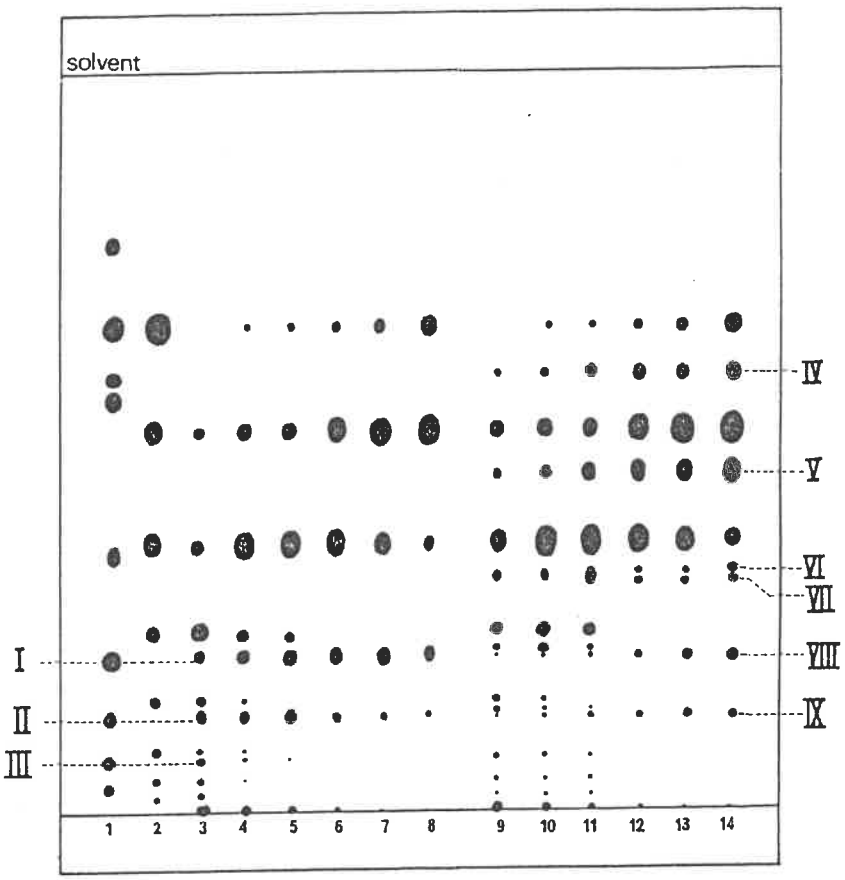

Fig. 1. T.l.c. of the products of hydrolysis of larch and oat xylans by endo-xylanase II from *Polyporus tulipiferae*. Larch xylan (time of hydrolysis), 3 (0.25 h), 4 (0.5 h), 5 (1 h), 6 (4 h), 7 (6 h), 8 (24 h); oat xylan, 9 (0.25 h), 10 (0.5 h), 11 (1 h), 12 (4 h), 13 (6 h), 14 (24 h). Standards: 1, (4-*O*-MeGlcA)Xyl_6→(4-*O*-MeGlcA)Xyl_3, glucuronic acid, glucose, arabinose, xylose, and rhamnose; 2, (xylooctaose→xylose).

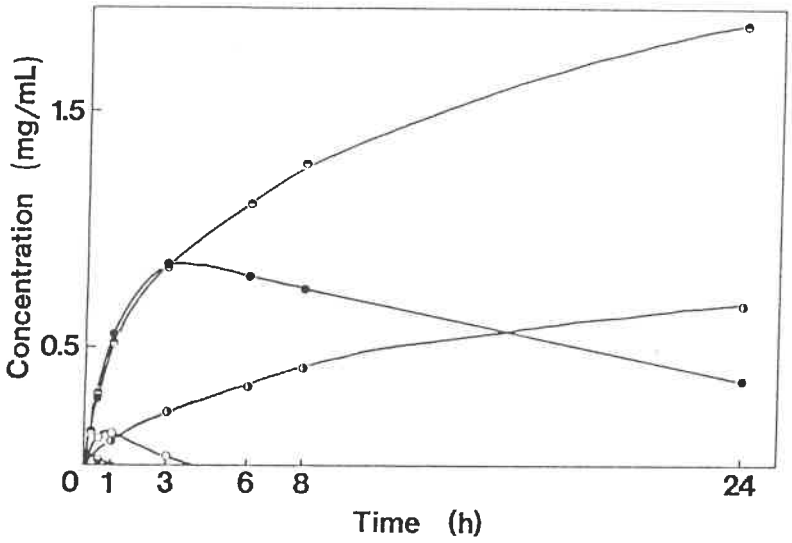

Fig. 2. Concentration profiles of xylose (—◑—), xylobiose (—●—), xylotriose (—●—), and xylotetraose (—○—) produced by the action of endo-xylanase II on oat xylan. Similar profiles were obtained for larch xylan. Oligosaccharides were analysed, after borohydride reduction, by h.p.l.c. on an RSil C18 column (see Experimental).

compounds (VIII and IX) were present in the hydrolysate of oat-spelt xylan. Such acidic oligosaccharides have been detected in xylanase hydrolysates of 4-*O*-methylglucuronoxylans from white birch[9] and redwood[7], the lowest major homologue accumulating being 2^3-(4-*O*-methylglucuronosyl)-xylotriose.

T.l.c. of hydrolysates of oat-spelt arabinoglucuronoxylan revealed a more complex pattern (Fig. 1), numerous components (IV–IX) being consistently observed in addition to xylo-oligosaccharides. Arabinose was not detected, which indicates the absence of arabinosidase from xylanase II as previously stated[8]. Also, glucuronic acid was not detected. The α-L-arabinofuranosidase from *Dichomitus squalens* cleaved products IV (R_{Xyl} 0.90) and V (R_{Xyl} 0.69) (Fig. 1), yielding arabinose (R_{Xyl} 0.89), xylobiose and xylotriose. Likewise, h.p.l.c. revealed, in addition to xylo-oligosaccharides, products which were cleaved by arabinofuranosidase but not affected by β-D-xylosidase. Therefore, these products must be α-L-arabinofuranosides devoid of unsubstituted terminal non-reducing xylosyl groups. After the treatment with arabinosidase, the peaks corresponding to the xylo-oligosaccharides were enriched and some higher oligosaccharides (for example, xylohexaose) which were initially absent were detected and must have arisen from xylo-oligosaccharides carrying one or more α-L-arabinose side-chains or arabinose attached to (1→2)-linked arabinose. The two latter possibilities are unlikely according to the known distribution of arabinose substituents in weakly ramified *gramineae* xylans[2,5] and the characterisation of oligosaccharides produced by xylanases working on arabinoxylans[5,10,11]. Therefore, by subtracting the peaks of the xylo-oligosaccharides produced by the action of xylanase II from those obtained after the action of arabinosidase (see Experimental), the variations in the concentration of arabinosyl-xylo-oligosaccharides up to the heptaose (Fig. 3) have been

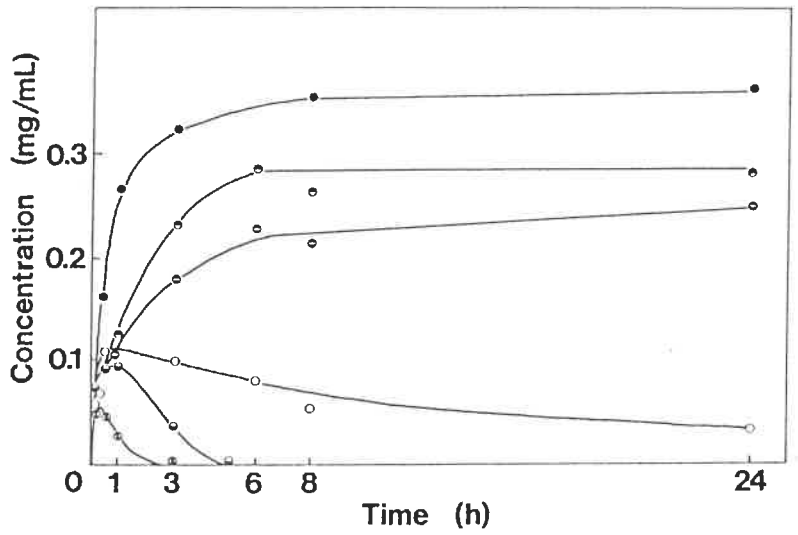

Fig. 3. Curves of hydrolysis of arabinosyl-xylo-oligosaccharides (AX) released by endo-xylanase II from oat-spelt xylan: AX_2 (—◓—), AX_3 (—●—), AX_4 (—○—), AX_5 (—◒—), AX_6 (—⊗—), and unknown α-L-arabinofuranoside AF1 (—⊖—). The concentration of AF1 was obtained by the same method as for arabinosyl-xylo-oligosaccharides (see text).

quantified. The rate of production of arabinosyl-xylo-oligosaccharides reflected also an endo-mechanism of action modified by the presence of arabinose substituents. The major product of hydrolysis was arabinosyl-xylotriose followed by arabinosyl-xylobiose, both of which accumulated as shown by t.l.c. It was not possible to determine if arabinosyl-xylose was produced, since the liberation of arabinose by the arabinosidase yielded a massive peak which partially overlapped that of xylose and thus prevented an accurate determination of the xylose. Arabinosyl-xylotetraose was the lowest oligosaccharide undergoing slow hydrolysis with a pattern similar to that of xylotriose (Fig. 2). Higher arabinosyl-xylo-oligosaccharides were rapidly degraded. In addition, another α-L-arabinofuranoside (AF1), which accumulated in the medium (Fig. 3), yielded an unknown peak U (eluted between xylotriose and xylotetraose) on treatment with arabinosidase.

Thus, the presence of arabinose side-chains on (1→4)-β-D-xylo-oligosaccharides limits the action of xylanase II, in contrast to the findings with hemicellulase II from *Ceratocystis paradoxa*[10]. Also, the d.p. of the lowest oligosaccharide undergoing hydrolysis is increased by 1 when arabinose is present. These observations are consistent with the stereospecificity of the binding site of enzymes. The presence of a projecting α-L-arabinofuranosyl group, in contrast to a β-D-xylopyranosyl group, prevents a good fit between substrate and enzyme binding site. The kinetics of hydrolysis of arabinosyl-xylo-oligosaccharides (Fig. 3) by xylanase II suggests that they are homologues with the arabinosyl group attached to the non-reducing end of the xylo-oligosaccharide as observed in hydrolysates of *gramineae* xylans by hemicellulase from *Ceratocystis paradoxa*[10].

EXPERIMENTAL

Substrates. — Arabinoglucuronoxylan from oat spelts (Xyl/Ara/Gal/U 1:0.10:0.02:0.03) (X-0376) and larchwood 4-*O*-methylglucuronoxylan (Xyl/U 1:0.09) (X-0375) (Sigma) were used without additional purification (identical carbohydrate content of ~97%). 4-*O*-Methylglucuronoxylo-oligosaccharides were generously donated by Dr. P. Debeire (INRA, Villeneuve d'Ascq, France).

Enzymes. — Endo-(1→4)-β-D-xylanase II was purified[8] to homogeneity from a commercial crude enzyme mixture, Driselase, from the white-rot fungus *Polyporus tulipiferae (Irpex lacteus)* (specific activity, 1206 nkat.mg^{-1}). Homogeneous α-L-arabinofuranosidase (426 nkat.mg^{-1}) was obtained from a culture supernatant of the basidiomycetes *Dichomitus squalens* grown on wheat-straw arabinoglucuronoxylan[12]. Partially purified β-D-xylosidase (82 nkat.mg^{-1}; suspension in 3.5M ammonium sulfate) from *Aspergillus niger* (Sigma) and β-D-galactose dehydrogenase from *Pseudomonas fluorescens* (5 U.mg^{-1}; suspension in 3.2M ammonium sulfate) (Boehringer) were commercial products.

General methods. — Total neutral sugars were determined by an automated orcinol method with calibration using xylose[13]. Uronic acids were determined by the *m*-phenylphenol technique[14], using glucuronic acid as the standard. Reducing sugars were analysed by a micro-scale Somogyi–Nelson procedure, using D-xylose as a standard[15]. Mono- and oligo-saccharides were separated by t.l.c. on silica gel F1500 (Schleicher and Schüll), using 1-propanol–ethyl acetate–ethanol–pyridine–acetic acid–water (7:3:3:2:1) and detected with 0.5% thymol in sulfuric acid–ethanol (5:95) at 90° (10 min).

Hydrolysis of xylans by the xylanase II. — To suspensions (1 mL) of xylans [1% in 0.1M acetate buffer (pH 5.0) containing 0.04% sodium azide] were added water (1 mL) and xylanase II (5 μL, 6 nkat), and they were then incubated at 40°. Samples (200 μL) were removed periodically and centrifuged. To each clear supernatant (150 μL) was added M sodium hydroxide (5 μL) in order to stop the reaction before measurement of reducing and total (neutral + acidic) sugars. Mono- and oligo-saccharides produced by the xylanase were analysed by t.l.c. and h.p.l.c.

For h.p.l.c., a Waters Associates Chromatograph equipped with a M510 Waters pump and a column (25 × 0.4 cm) packed with RSil C18 HL (particle size, 5 μm) (Alltech Associates) was used with elution by degassed distilled water at 0.9 mL/min (20°). Detection was effected with an ERMA ERC-7510 differential refractometer (1/4X); the flow-path cuvette was kept at 35°. Prior to analysis, sugars (50 μL) were reduced[16] with 2.5% sodium borohydride in 2M ammonia (200 μL) for 1 h at 60°. After neutralisation with Amberlite IR-120 (H^+) resin and filtration, each solution was concentrated to dryness and methanol was twice evaporated from the residue. The concentration of the major oligosaccharide in the mixture was adjusted with distilled water to 50 μg.mL^{-1} prior to injection (30 μL). The arabinosyl-xylo-oligosaccharides were determined as follows. To a solution (50 μL) of reduced oligosaccharides in water (pH 5.5) were added aqueous 2% sodium

azide (0.5 μL) and α-L-arabinofuranosidase solution (1 μL, 0.02 nkat). After 48 h at 40°, each solution was injected onto the C18 column and the chromatogram was compared with that of the untreated sample. Hydrolysates were also treated under similar conditions with β-D-xylosidase (0.5 μL, 0.02 nkat). The unreduced mixtures of oligosaccharides were treated similarly for detection of the arabinosyl-xylo-oligosaccharides by t.l.c. Xylo-oligosaccharides produced by the action of xylanase II were compared to a standard prepared by hydrolysis of larchwood xylan with dilute acid.

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