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ON THE SPECIFICITY AND MODE OF ACTION OF A XYLANASE FROM *Trametes hirsuta* (WULF.) PILÁT

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ABSTRACT

The mode of action of the extracellular endo-(1→4)-β-D-xylanase produced by *Trametes hirsuta* on a (4-*O*-methyl-D-glucurono)-D-xylan and a modified, essentially neutral D-xylan from white willow (*Salix alba* L.) has been studied. Xylotetraose and xylohexaose, together with aldotetraouronic and aldohexaouronic acids, were the main products. The acidic oligosaccharides had a 4-*O*-methyl-D-glucopyranosyluronic acid group attached to the non-reducing D-xylosyl end-group. The action pattern of the xylanase corresponds to that of a typical endo-enzyme that acts more readily in the middle of chain, and the specific region of its action appears to involve five D-xylosyl residues. The products of the enzymic treatment of the D-xylan have revealed a regular distribution of the 4-*O*-methyl-D-glucopyranosyluronic acid groups attached to the D-xylan backbone.

INTRODUCTION

Chemical procedures for the structural analysis of biopolymers are being supplemented to an ever-increasing extent by enzymic methods. Highly purified enzymes may provide extremely quick and accurate information about the fine structure of investigated polysaccharides, if their specificity and mode of action are known.

Thus, for the investigation of plant cell-wall xylans, xylanases may be useful and, therefore, considerable attention has been devoted to their study. The effect of endo-xylanases from various sources or commercial enzyme-preparations on various xylans has been investigated[1]. Some of the xylanases were highly purified and sufficiently characterized[2–5], but the structures of the oligosaccharides formed by enzymic hydrolysis have been investigated in only a few cases[6–9].

We now report on the specificity and action pattern of a purified endo-(1→4)-β-D-xylanase [(1→4)-D-xylan xylanohydrolase EC 3.2.1.8] isolated from the wood-destroying fungus *Trametes hirsuta*.

EXPERIMENTAL

Enzyme. — The endo-(1→4)-β-D-xylanase was isolated from *Trametes hirsuta*

(Wulf.) Pilát, str. No. 121. The fungus was obtained from the Laboratory of Anatomy and Physiology of Plants, Faculty of Sciences, J. E. Purkyně University, Brno.

Substrates. — (4-*O*-Methyl-D-glucurono)-D-xylan from the willow *Salix alba* L.[10] had d.p. 121 and a molar ratio of D-xylose and uronic acid of 11 : 1. (4-*O*-Methyl-D-glucurono)-D-xylan, d.p. 40, uronic acid content 2.0%, was obtained from deacetylated *O*-acetyl-(4-*O*-methyl-D-glucurono)-D-xylan[11] after uronic acid elimination. (1→3)-β-D-Xylan from the green seaweed *Caulerpa filiformis* was kindly supplied by Dr. E. E. Percival (Royal Holloway College, University of London). Sodium carboxymethylcellulose (2.96 mequiv./g) and laminarin (ex. *Laminaria hyperborea*) were commercial samples (Koch–Light Laboratories). D-Mannan was obtained from *Saccharomyces cerevisiae*[12], D-gluco-D-mannan from willow[13], L-arabino-D-galactan from *Larix sibiricana*[14], and D-galactan and L-arabinan from willow bark[15,16]. β-D-(1→4)-Xylo-oligosaccharides of d.p. 2–6 were isolated from acid hydrolysates of (4-*O*-methyl-D-glucurono)-D-xylan from willow, and gave reduced xylo-oligosaccharides of d.p. 3–6.

General methods. — Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter for aqueous solutions at 20°. The enzymic degradation of the oligosaccharides was followed by determination of the change in optical rotation at 436 nm (10-cm microcell at 40°).

T.l.c. was performed on Kieselguhr G with solvent *A*, 65% 2-propanol–ethyl acetate (1 : 1), and detection with anisaldehyde–sulphuric acid[17].

P.c. was performed on Whatman No. 1 and 3MM papers with *B*, ethyl acetate–acetic acid–water (18 : 7 : 8); and *C*, ethyl acetate–acetic acid–formic acid–water (18 : 3 : 1 : 4). The compounds were detected with aniline hydrogen phthalate[18] and silver nitrate–ammonium hydroxide[19]. The mobilities (R_{Xyl}) and (R_{MGA}) are expressed relative to those of D-xylose and 4-*O*-methyl-D-glucuronic acid.

G.l.c. was performed on a Hewlett–Packard Model 5711 A gas chromatograph with *D*, a column (200 × 0.2 cm) of 3% of OV-225 on Chromosorb W AW DMCS (80–100 mesh) at 140° with nitrogen as carrier gas at 28 ml/min; *E*, a column (200 × 0.2 cm) of 3% of SP 2340 on Chromosorb W AW DMCS (80–100 mesh) at 120→170° (2°/min) with nitrogen as carrier gas (26 ml/min); and *F*, a column (200 × 0.3 cm) of 3% of ECNSS-M on acid-washed Chromaton N-DMCS (70–80 mesh), at 164→180° (2°/min).

Reducing end-groups were determined by the spectrophotometric method of Somogyi[20]. The appearance of sugars in column eluates was monitored by the phenol–sulphuric acid method[21]. Uronic acid content was determined by the carbazole method[22].

Preparation of xylo-oligosaccharides. — (4-*O*-Methyl-D-glucurono)-D-xylan (10 g) in 98% trifluoroacetic acid (200 ml) was kept at room temperature for 12 days[23]. The hydrolysate was evaporated to a small volume at 35°, and traces of acid together with the acidic portion of the hydrolysate were retained on a column of Dowex-1 X8 ($CH_3CO_2^-$) resin. The neutral and acidic sugars, after elution of the Dowex column, were separated on a charcoal–Celite column by using a linear gradient

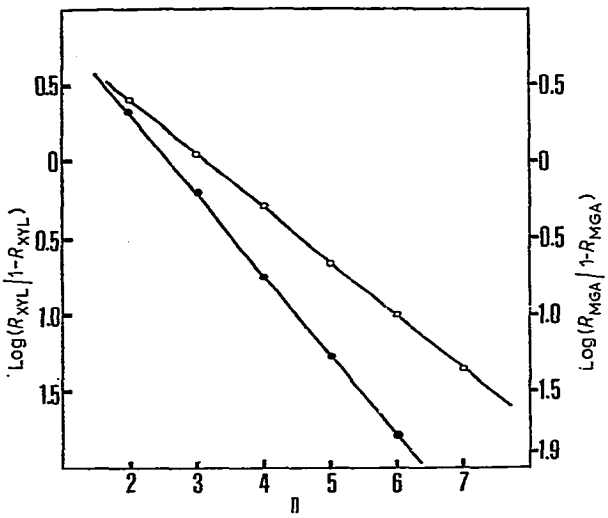

Fig. 1. Plot of log $R_{XYL}/(1 - R_{XYL})$ for neutral (—○—) and log $R_{MGA}/(1 - R_{MGA})$ for acidic (—●—) oligosaccharides versus n: R_{MGA} refers to 4-*O*-methyl-D-glucuronic acid; Whatman No. 1 paper; solvent *B* for neutral and *C* for acidic oligosaccharides.

of aqueous ethanol (0→50% for the neutral and 5→60% for the acidic oligosaccharides). Further separation of the neutral oligosaccharides (d.p. ≤4) was performed by t.l.c. on Kieselguhr G plates (20 × 20 cm) buffered with 0.02M sodium acetate (solvent *A*)[24]. The oligosaccharides with d.p. ≥4 were separated on Whatman No. 3MM paper (solvent *B*). Thus, a homologous series of β-(1→4)-linked D-xylose oligomers[10] having d.p. 2–7 was obtained. The homologous, acidic oligosaccharides (d.p. 2–6) containing D-xylose and 4-*O*-methyl-D-glucuronic acid were isolated by preparative p.c. (solvent *C*) (see Fig. 1).

To assay the d.p., each neutral oligosaccharide was treated with a solution of sodium borohydride (15 mg) in water (2 ml) at room temperature for 24 h. The deionized product was hydrolysed with 90% formic acid at 100° for 2 h, followed by hydrolysis for 1 h after dilution of the acid to 5%. After trifluoroacetylation[25], the molar ratio of xylitol to D-xylose was determined by g.l.c. (column *D*). Acidic oligosaccharides were reduced by the procedure of Taylor and Conrad[26] applied three times. After hydrolysis and reduction with sodium borohydride, the resulting *O*-methylglucitol and xylitol were determined by g.l.c. (column *E*) as their trifluoroacetyl derivatives.

The reduced, neutral xylo-oligosaccharides (d.p. 3–6) were obtained after sodium borohydride reduction and their purity was verified by p.c. (solvent *B*).

Substrate studies of the endo-(1→4)-β-D-*xylanase.* — The substrate specificity of the enzyme was studied by incubating 0.5% solutions (1 ml) of (1→3)-β-D-xylan from green seaweed, and the various non-xylan polysaccharides listed above, in 0.1M sodium acetate buffer (pH 5) with xylanase (0.1 mg of protein, *i.e.*, 0.9 unit) at 40° for 48 h, followed by an assay of reducing power[20] and p.c. (solvents *B* and *C*).

TABLE I

NEUTRAL PRODUCTS OF DEGRADATION OF (4-*O*-METHYL-D-GLUCURONO)-D-XYLAN BY THE ENDO-(1→4)-β-D-XYLANASE

Compound	R_{XYL}[a]	$[\alpha]_D$ *(degrees)*	*D.p.*
Xylobiose	0.72	−24.9	2.1
Xylotetraose	0.33	−59.4	3.9
Xylohexaose	0.09	−77.0	6.0

[a]Solvent *B*.

TABLE II

ACIDIC PRODUCTS OF DEGRADATION OF (4-*O*-METHYL-D-GLUCURONO)-D-XYLAN BY THE ENDO-(1→4)-β-D-XYLANASE

Compound	R_{MGA}[a]	$[\alpha]_D$ *(degrees)*	*D.p.*	*Methylated sugars*[b]		
				2,3-Me₂-Xyl	*3,4-Me₂-Xyl*	*2,3,4-Me₃-Glc*
Aldotetraouronic acid	0.15	+25.0	4	2	1	1
Aldopentaouronic acid	0.05	+ 0.6	5	3	1	1
Aldohexaouronic acid	0.02	−12.0	6	4	1	1

[a]Mobility relative to 4-*O*-methyl-D-glucuronic acid; solvent *C*. [b]From carboxyl-reduced oligosaccharide. Values in mol/mol of oligosaccharide.

Enzymic hydrolysis of willow (4-O-methyl-D-glucurono)-D-xylan. — The polysaccharide (1 g) in 0.1M sodium acetate buffer (pH 5, 50 ml), containing sodium azide (5mM) as preservative, was incubated with xylanase (2 mg of protein, *i.e.*, 18 units) in dialysis tubing at 40° for 7 days. The digest was dialysed against distilled water which was changed every 24 h. Hydrolysis was monitored by the determination of reducing end-groups[20]. The combined dialysis solutions were concentrated and deionized on a column (1.5 × 90 cm) of Sephadex G-10 (40–120 μm). After inactivation of the enzyme (100°, 10 min), the dialysed material was fractionated on a column (1.5 × 95 cm) of Sephadex G-75 (40–120 μm). The products of hydrolysis were separated on a column of Dowex-1 X8 ($CH_3CO_2^-$) resin into neutral and acidic portions, which were successively purified by p.c. (solvents *B* and *C*).

The neutral and acidic oligosaccharides were identified by comparison of their mobilities (R_{Xyl}, R_{MGA}) with those of the authentic compounds in p.c. (solvents *B* and *C*), and by the optical rotation and d.p. values. Aldouronic acids (20 mg) were methylated by using methyl iodide in the presence of sodium methylsulphinylmethanide[27]. The permethylated samples were reduced with lithium aluminum hydride in tetrahydrofuran, hydrolysed (0.25M H_2SO_4, 4 h), transformed into alditol acetates, and analysed by g.l.c. (column *F*). The products detected are listed in Tables I and II.

The molar ratio of neutral (xylotetraose and xylohexaose) and acidic (aldotetrao- and aldohexao-uronic acids) oligosaccharides was determined densitometrically on the basis of calibration curves after p.c. (solvents *B* and *C*).

Preparation and enzymic hydrolysis of the modified (4-O-methyl-D-glucurono)-D-xylan. — *O*-Acetyl-(4-*O*-methyl-D-glucurono)-D-xylan from willow[11] was deacetylated with sodium methoxide in methanol[28]. After being dried (100°, 3 h), the deacetylated polysaccharide (160 mg) and toluene-*p*-sulphonic acid (15 mg) were dissolved in anhydrous *N*,*N*-dimethylformamide (10 ml) and then the solution was cooled to 10–15°. Methyl vinyl ether (5 ml) condensed at −20° was added, and the solution was kept at 12–15° for 3 h and then concentrated to dryness. The residue was treated[29] with 0.7M sodium methylsulphinylmethanide in methyl sulphoxide (3.5 ml) at room temperature for 20 h. After neutralisation of the mixture and extraction with chloroform, the clear extract was applied to a column (3.2 × 30 cm) of Sephadex LH-20, which was then eluted with dry chloroform. After concentration of the eluate, the residue was treated with 20% aqueous acetic acid (30 min, 100°). The modified D-xylan, $[\alpha]_D$ −55.9° (*c* 0.76, water), recovered by precipitation with ethanol, contained 97.9% of D-xylose and 2.1% of 4-*O*-methyl-D-glucuronic acid.

The modified polysaccharide (100 mg) was incubated with xylanase in 0.1M acetate buffer (pH 5, 50 ml) in dialysis tubing for 3 days. The degradation products were isolated and identified by the procedures described above.

The action of endo-(1→4)-β-D-xylanase on xylo-oligosaccharides. — The series of β-(1→4)-linked D-xylose oligosaccharides (d.p. 2–6) and reduced oligosaccharides (d.p. 3–6) were tested. The enzyme (0.1 mg of protein, *i.e.*, 0.9 unit) in 0.1M sodium acetate buffer (pH 5, 2 ml) containing oligosaccharide (25 mg) was incubated at 40° for 48 h. The resulting products were fractionated by preparative t.l.c. (solvent *A*) and p.c. (solvent *B*), and the individual compounds were identified by comparison of their chromatographic mobilities with those of the authentic compounds and by determination of d.p. values. The Michaelis parameters K_m and V for the enzyme were determined from Lineweaver–Burk plots[30] for oligosaccharides of d.p. 3–6. The initial velocities for the enzymic hydrolysis of these compounds were obtained by measuring the change in optical rotation with time, using the polarimetric method described by Pettersson[31]. The velocities were derived for different concentrations of the oligosaccharide from the slope of the part of the curves immediately after the lag-phase.

RESULTS AND DISCUSSION

We have described[32–34] the isolation, purification, and some properties of the extracellular endo-(1→4)-β-D-xylanase produced by the fungus *Trametes hirsuta* using wood meal as the carbon source. This enzyme has now been found to be highly specific for the (1→4)-β-D-xylopyranosyl linkages of (4-*O*-methyl-D-glucurono)-D-xylan. (1→3)-β-D-Xylan was not attacked by the enzyme, and β-(1→4), α-(1→4), β-(1→3), and β-(1→6) glycosidic bonds of various non-xylan polysaccharides were

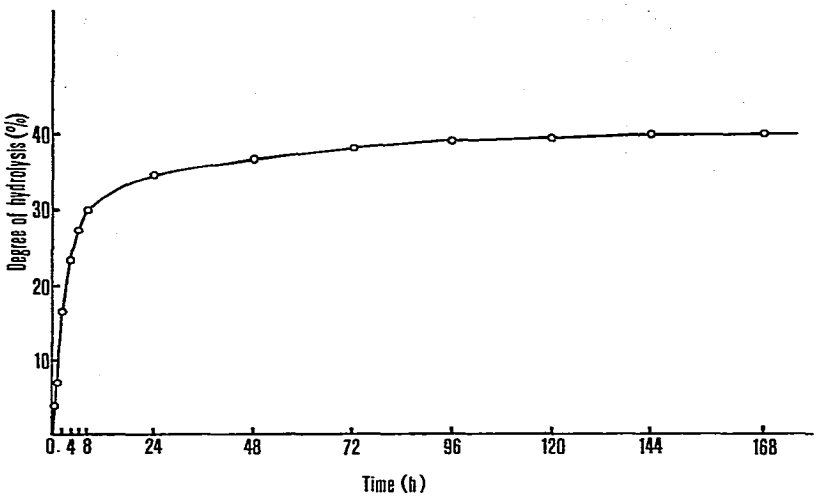

Fig. 2. Hydrolysis of willow (4-*O*-methyl-D-glucurono)-D-xylan by endo-(1→4)-β-D-xylanase, expressed as the percent of cleaved β-D-(1→4)-xylosidic bonds calculated from the μequiv. of reducing sugars formed.

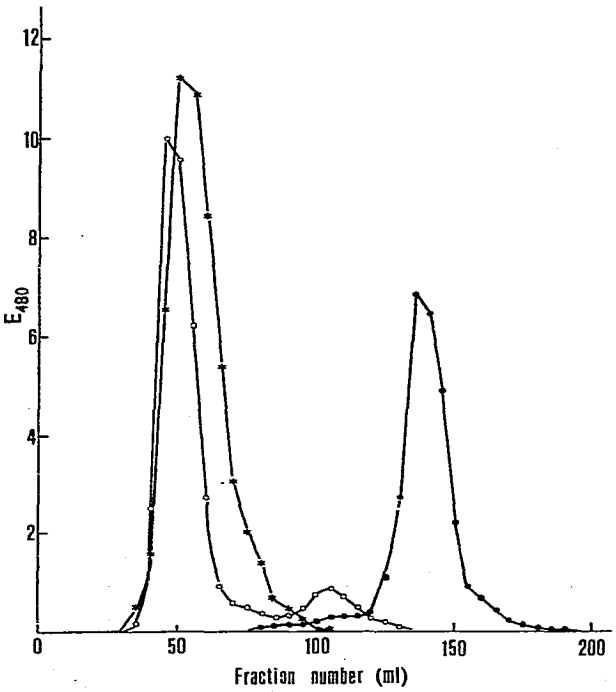

Fig. 3. Elution patterns from Sephadex G-75 of willow (4-*O*-methyl-D-glucurono)-D-xylan before and after enzymic hydrolysis: original xylan (○—○), control (*—*), and degraded xylan (●—●).

also resistant. Similarly, xylobiose and *p*-nitrophenyl β-D-xylopyranoside were not hydrolysed.

To study the action pattern of this enzyme on substrates of known structure, a water-soluble (4-*O*-methyl-D-glucurono)-D-xylan[10] and a modified *O*-acetyl-(4-*O*-methyl-D-glucurono)-D-xylan[11] from willow were used. In the former polymer (d.p. 121), every eleventh β-(1→4)-linked D-xylosyl residue, on the average, carries a single, terminal 4-*O*-methyl-D-glucopyranosyluronic acid group. The latter polysaccharide (d.p. 40), obtained by the selective removal of 4-*O*-methyl-D-glucuronic acid residues from the deacetylated D-xylan[11] by a modification of the method of Aspinall and Rosell[29], contained only 2.0% of uronic acids. For the action of the xylanase, the dialysis system described by Timell[35] was used, which protected the degradation products from further hydrolysis.

The initial phase of the enzyme action upon (4-*O*-methyl-D-glucurono)-D-xylan, characterized by a rapid increase in reducing power, was followed by a longer period during which hydrolysis was much slower. As shown in Fig. 2, 40% degradation of the β-(1→4)-xylosidic linkages of the D-xylan occurred during 7 days. Within this time, the polysaccharide was completely split into oligosaccharides, as determined by gel chromatography on Sephadex G-75 (Fig. 3). Xylose and 4-*O*-methyl-D-glucuronic acid were not obtained. After fractionation of this mixture of saccharides, the resulting neutral and acidic fractions of xylo-oligosaccharides were separated by preparative t.l.c. and p.c.

The main neutral oligosaccharides were xylotetraose and xylohexaose (molar ratio of 2:1), together with a small proportion of higher xylo-oligosaccharides and traces of xylobiose. The optical rotations and d.p. values, which were in agreement with the known β-D-(1→4)-xylosidic linkages of the willow (4-*O*-methyl-D-glucurono)-D-xylan[10], showed that the main products were 4-*O*-β-D-xylopyranosyl-D-xylotriose and 4-*O*-β-D-xylopyranosyl-D-xylopentaose (Table I).

Aldotetrao- and aldohexao-uronic acids (molar ratio of 2.9:1) were the main acidic products together with a small proportion of aldopentaouronic acid (Table II). Aldouronic acids having d.p. >6 were also present in small amount, but no mono-, aldobio-, and aldotrio-uronic acids were detected. The structures of these compounds were established by methylation analysis to be *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-D-xylotriose, *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-D-xylotetraose, and *O*-(4-*O*-methyl-α-D-glucopyranosyluronic acid)-(1→2)-D-xylopentaose. In all cases, 4-*O*-methyl-D-glucuronic acid was linked to C-2 of the terminal D-xylosyl group of β-(1→4)-linked tri-, tetra-, and penta-saccharides (Table II). Aldouronic acids of the same structure were obtained by Timell[35] and Shimizu[9] by enzymic degradation of (4-*O*-methyl-D-glucurono)-D-xylans from white birch and shiraka birch. The high content of aldotetraouronic acid and the lack of the uronic acids of lower d.p. indicate, in agreement with the results of previous work[7,35], that the 4-*O*-methyl-D-glucuronic acid terminal residue sterically hinders the enzymic cleavage of the two (1→4)-β-D-xylosidic linkages in the vicinity of the branch-point.

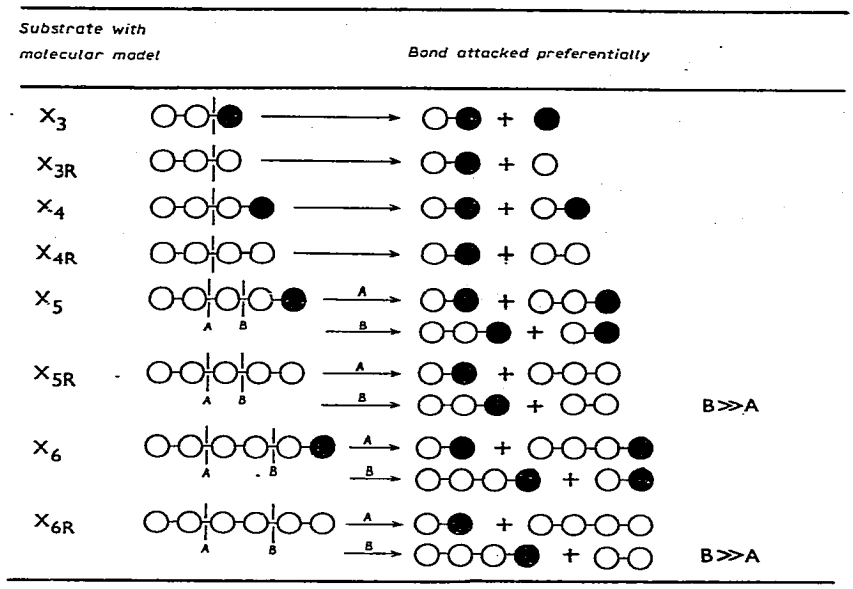

Fig. 4. Action pattern of endo-(1→4)-β-D-xylanase on non-reduced and reduced β-D-(1→4)-xylo-oligosaccharides: ○ and ●, non-reducing and reducing D-xylosyl residues; ——, β-(1→4)-glycosidic bonds; R, reduced xylo-oligosaccharides.

For unknown reasons, the linkage immediately to the left of the branch-point seems to be easily hydrolysed by the enzyme.

The presence of xylotetraose and xylohexaose as the main products, with only minor amounts of xylobiose, and the absence of xylotriose and xylopentaose reveal an interesting regularity in the mechanism of the attack of *T. hirsuta* endo-(1→4)-β-D-xylanase upon the linear part of the (4-*O*-methyl-D-glucurono)-D-xylan backbone. Such action of D-xylanases on polysaccharides of similar structure has not been observed hitherto. Enzymic hydrolysis of (4-*O*-methyl-D-glucurono)-D-xylans from poplar[36,37], beech[38,39], and birch[5,9], using partially or highly purified endo-xylanases, afforded a homologous series of neutral xylo-oligosaccharides.

To understand better the mode of action of endo-(1→4)-β-D-xylanase, without a steric-hindrance effect by the terminal 4-*O*-methyl-D-glucopyranosyluronic acid group, the enzymic degradation of a modified D-xylan and xylo-oligosaccharides was studied. The modified, essentially neutral D-xylan was completely split by the xylanase during 3 days. The products formed in the hydrolysis, xylotetraose and xylohexaose as the main constituents and a small proportion of xylo-oligosaccharides having higher d.p., were identical with those obtained by enzymic hydrolysis of the (4-*O*-methyl-D-glucurono)-D-xylan.

The degradations of non-reduced and reduced xylo-oligosaccharides of d.p. 3–6 are shown in Fig. 4. Xylotriose was the lowest homologue of the D-xylose oligosaccharides attacked by D-xylanase, yielding xylobiose from the non-reducing end and D-xylose. Xylotetraose was completely cleaved into xylobiose. Xylopentaose was

TABLE III

THE EMPIRICAL CONSTANTS FOR THE HYDROLYSIS OF β-D-(1→4)-XYLO-OLIGOSACCHARIDES BY THE ENDO-(1→4)-β-D-XYLANASE

Substrate	10^4 K_m *(mol.litre^{-1})*	$K_f{}^a = V/K_m$ *(degree.litre.mol^{-1}.min^{-1})*
Xylotriose	700[a]	0.09
Xylotetraose	146[a]	0.82
Xylopentaose	7.5[a]	5.2
Xylohexaose	6[a]	6.8
(4-*O*-Methyl-D-glucurono)-D-xylan	0.57[b]	—

[a]Determined from polarimetric measurements. [b]Determined by the reducing end-group method.

split at one or other of the inner linkages, since no xylotetraose was detected during hydrolysis and xylotriose was the main product. Xylohexaose yielded xylotetraose and xylobiose. The main product, xylotetraose, resulting from enzymic hydrolysis of the reduced hexasaccharide, indicates the preferential cleavage of tetrasaccharide from the non-reducing end. Thus, the products of enzymic hydrolysis of the reduced oligosaccharides enabled the preferred points of cleavage to be specified. It is evident that the endo-(1→4)-β-D-xylanase from *T. hirsuta* most readily cleaves the xylosidic linkages near the middle of the chain.

The rate of enzymic hydrolysis of oligosaccharides was determined by a polarimetric technique used by Pettersson for the β-D-(1→4)-oligoglucosides[31]. In Figs. 5 and 6, the results of the enzymic hydrolysis of xylotetraose and the determination of K_m are reproduced. The kinetic parameters K_m and k_f are summarized in Table III;

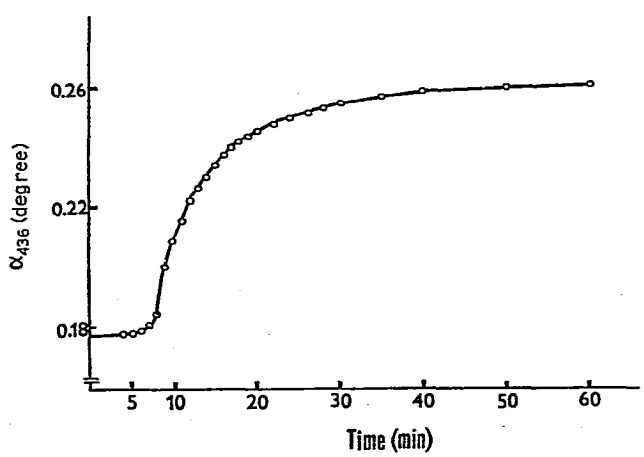

Fig. 5. The change in optical rotation during hydrolysis of xylotetraose with endo-(1→4)-β-D-xylanase: 10mM solution of substrate in acetate buffer (0.1M, pH 5) containing enzyme (0.01 mg of protein); 40°; 10-cm cell.

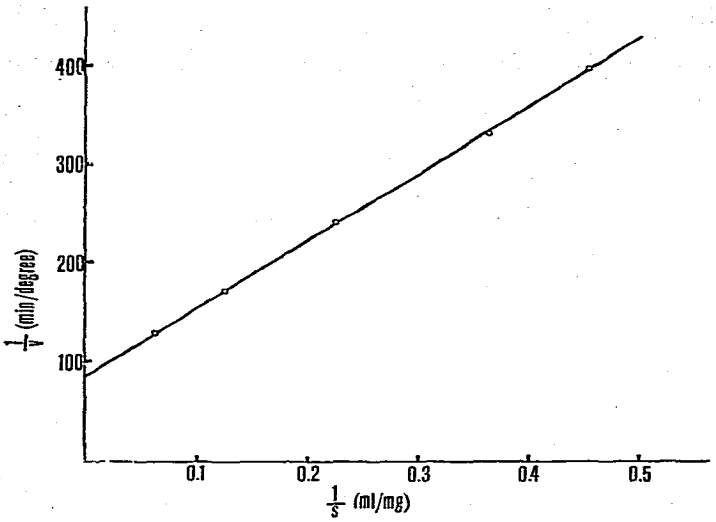

Fig. 6. Double reciprocal plot of rate of hydrolysis of xylotetraose catalysed by the xylanase from *Trametes hirsuta*.

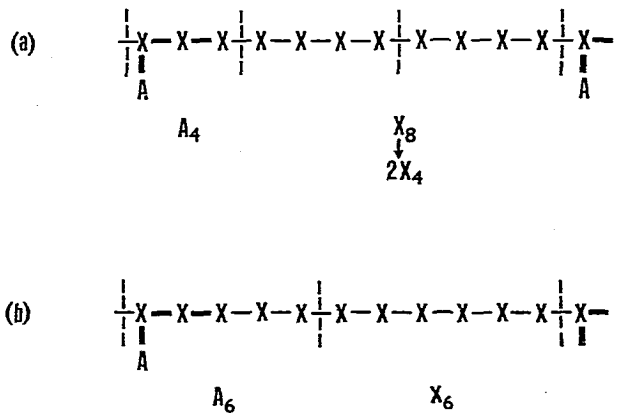

Fig. 7. Possible action of endo-(1→4)-β-D-xylanase on the willow (4-*O*-methyl-D-glucurono)-D-xylan, where X = β-D-(1→4)-linked D-Xyl*p*, and A = 4-*O*-Me-α-D-GlcA*p*: $\frac{|}{|}$, hydrolysed linkages; ——, stable linkages; ——, labile linkages.

k_f is the first-order velocity constant with respect to substrate[40], when $S \ll K_m$. As the method employed has certain limitations (*e.g.*, the wavelength used), the numerical values of these constants have only a limited significance. Nevertheless, the figures are considered good enough to demonstrate a trend in the order of magnitude. Thus, whereas xylotriose was attacked by the enzyme very slowly, the rate of hydrolysis of higher oligosaccharides increased with increase in d.p. This is in accordance with the decrease of K_m and the increase of k_f for the individual oligosaccharides. The decrease of K_m with increasing d.p. may reflect an increasing tendency towards the formation

of enzyme–substrate complexes. As may be judged from the great change of K_m and k_f values from xylotetraose to xylopentaose, the specific region of action of the endo-(1→4)-β-D-xylanase appears to be at least five D-xylose residues.

Based upon the results of enzymic hydrolysis of (4-*O*-methyl-D-glucurono)-D-xylan, a possible mode of action of the xylanase may be proposed (Fig. 7). The double amount of xylotetraose formed, compared with xylohexaose, supports the assumption of preferential cleavage of the D-xylan as shown in Fig. 7a; this is in good agreement with the high content of aldotetraouronic acid in the hydrolysate. The ratio of the K_m values for xylohexaose and xylotetraose is 1:27, suggesting a rapid degradation of xylo-octaose to xylotetraose with only a slight degradation of xylotetraose to xylobiose. The low content of xylobiose in the enzymic hydrolysate confirms this presumption. However, the presence of aldohexaouronic acid and xylohexaose does not exclude the possibility of enzymic attack as shown in Fig. 7b, although to a lower extent.

Enzymic hydrolysis of the essentially neutral and linear D-xylan, obtained after elimination[29] of the uronic acid side-chain, released identical products. It indicates that the binding site of the enzyme is effectively filled by a chain of five D-xylose residues. In addition, a conclusion may be drawn about the fine structure of the willow-xylan macromolecule. The products of xylanase treatment on (4-*O*-methyl-D-glucurono)-D-xylan indicate that the 4-*O*-methyl-D-glucopyranosyluronic acid groups are regularly distributed along the D-xylan backbone, thus excluding blocks having a higher degree of substitution. This contrasts with the situation for birch (*Betula verrucosa*) xylan, where an irregular arrangement was found[41].

The present results do not provide a reliable basis for explaining why the purified endo-xylanase attacks oligosaccharides of d.p. ≥3. The high enzyme-activity towards the longer chains, together with the continuous removal of the hydrolysis products, may provide a possible explanation for the absence of oligosaccharides of lower d.p. in the D-xylan hydrolysates.

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