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Studies on the Xylan from the Red Seaweed *Rhodymenia palmata*

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Two fractions of the xylan from *Rhodymenia palmata* (L.) Grev. have been investigated by methylation analysis. In agreement with previous studies both polysaccharides were found to have a linear structure, the chain containing β-(1→3)- and β-(1→4)-linkages. The ratio between these linkages of approximately 1:2 was slightly different in the two samples. One of the xylan fractions was hydrolysed by a specific enzyme and a mixture of a hexasaccharide, two pentasaccharides and two tetrasaccharides, all of which contained one β-(1→3)-linkage, was isolated and the components were characterised. The significance of these findings, with regard to the structure of the polysaccharides and the specificity of the enzyme, are discussed.

The xylan from the red seaweed *Rhodymenia palmata* (L.) Grev., first isolated by Barry and Dillon [1] is composed of β-(1→3)- and β-(1→4)-linked xylose residues as indicated by periodate oxidation and methylation studies.[2,3] Evidence obtained from the Barry [4] and Smith [5] degradations has indicated that the polysaccharide is homogeneous and not a mixture of a β-(1→3)- and β-(1→4)-linked xylan. These findings are substantiated by the result of a partial hydrolysis experiment,[6] using a bacterial suspension, when among other products, a trisaccharide containing both linkages was obtained.

Jensen and coworkers [7] have recently observed that stepwise extraction of the dried seaweed, first with water and then with 0.2 M hydrochloric acid, yielded two fractions, of which the former gave considerably more viscous solutions than the latter. They suggested that the two fractions are chemically similar but differ in molecular weight. In the present communication methylation studies on two fractions of the *Rhodymenia palmata* xylan are reported as well as the enzymatic hydrolysis of one of the fractions, using a highly purified cellulase.

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METHYLATION STUDIES

Two fractions of the xylan, one obtained by extraction with water (xylan I-6) and the other by subsequent extraction of the residue with 0.1 M sulphuric acid (xylan II-2) were investigated by periodate oxidation. Xylan I-6, according to periodate consumption, contained 29 % β-(1→3)- and 71 % β-(1→4)-linkages. Its degree of polymerisation, determined osmometrically on the nitrate, was 64. On periodate oxidation one mole of formic acid per 26 pentose residues was formed. After sodium borohydride reduction and periodate oxidation one mole of formic acid per 46 pentose residues was formed. Assuming a linear structure and a statistical distribution of the linkages, these values correspond to a $\bar{P}_n$ of 71 and 78, respectively. A soluble nitrate of xylan II-2 could not be prepared. According to periodate consumption it contained 38 % β-(1→3)- and 62 % β-(1→4)-linkages. From the liberation of formic acid, before and after sodium borohydride reduction, the $\bar{P}_n$ was estimated to be 100 and 76, respectively. The $\bar{P}_n$ values determined by this method are not very accurate but give some idea as to the order of magnitude and also indicate that the polysaccharide is essentially unbranched.

Two xylan fractions (I-4 and II-1) were exhaustively methylated, hydrolysed, and the methyl ethers were separated and characterised. 2,3,4-Tri-*O*-methyl-D-xylose, 2,3-di-*O*-methyl-D-xylose and 2,4-di-*O*-methyl-D-xylose were obtained. Mono-*O*-methyl-D-xyloses were obtained in trace amounts only. Small portions of the methylated polysaccharides were subjected to methanolysis and the relative proportions of 2,3,4-tri-, 2,3-di- and 2,4-di-*O*-methyl-D-xylose were determined to be 2:72:26 for xylan I-4 and 3:57:40 for xylan II-1 by gas liquid chromatography.

The ratio between β-(1→4)- and β-(1→3)-linkages in the two polysaccharides, as determined by the methylation analysis, agrees fairly well with the values estimated by periodate oxidation. The amount of mono-*O*-methyl-D-xyloses was low. These ethers probably have no structural significance but result from undermethylation and demethylation during the hydrolysis. The methylation analysis therefore indicates that both xylans are unbranched but have slightly different proportions of β-(1→4)- and β-(1→3)-linkages.

ENZYMATIC HYDROLYSIS

A purified enzyme fraction from *Stereum sangvinolentum* was used. (The isolation and purification of this enzyme will be described elsewhere). This enzyme had strong cellulase activity and also hydrolysed β-(1→4)-linked xylans. It is not yet established whether the enzyme preparation contains a single component, capable of hydrolysing cellulose and xylan or is a mixture of a cellulase and a xylanase. Starch, laminaran, and pustulan (β-(1→6)-glucan) were not attacked. The effect of the enzyme upon the cellodextrins and the β-(1→4)-linked xylodextrins (X_2, X_3 *etc.*) was also investigated. The di- and trisaccharides were not hydrolysed. The higher oligosaccharides were hydrolysed to give mixtures of the di- and trisaccharides. Transglycosidation was, however, also observed and higher oligosaccharides were formed, *e.g.* from X_4 and X_5. Starting from cellopentaose and cellohexaose, higher oligo-

saccharides of this series, which have low solubilities, precipitated from the solution. By using low concentrations of substrate (0.01 %) these transglycosidation reactions were negligible.

A 0.01 % solution of the polysaccharide (xylan I-6) was treated with the enzyme, at pH 5 and 37°, until practically constant optical rotation (28 days)

Table 1. Fractionation of enzymatic hydrolysate from xylan I-6 (2.4 g).

Fractions	Weight (mg)	Composition
1	1140	Higher oligosaccharides
2	710	Oligosaccharides of DP 7—10
3	53	» » » 5—7
4	104	» » » 5—7
5	74	» » » 4—6
6	38	» » » 3—5
7	21	Xylotriose
8	11	Xylotriose + xylobiose
9	5	Xylobiose

was observed. The hydrolysate was then concentrated and fractionated by chromatography on a Sephadex G-25 column. The results are given in Table 1 and Fig. 1.

Solutions (1 %) of fractions 1 and 2 were hydrolysed at a low rate by the enzyme. Further fractionations of fractions 3, 4, 5, and 6 by chromatography on a Sephadex G-25 column yielded three fractions, which, according to their relative mobilities on the column, should consist of hexa-, penta-, and tetrasaccharides, respectively.

A crystalline component, A (125 mg), m.p. 169—173°, $[\alpha]_{436}$ —154°, and another, B (55 mg), m.p. 160—165°, $[\alpha]_{436}$ —144°, were obtained from the hexa- and pentasaccharide fractions, respectively. The tetrasaccharide fraction gave two spots on paper chromatography, that with the lower R_F-value corresponding to X_4. The component, C, giving the other spot, was isolated by paper chromatography. The degrees of polymerisation of A and B, determined essentially as described by Timell [8] were in agreement with the expected values.

On paper electrophoresis in germanate buffer [9] a 3-*O*-substituted xylose has a considerably higher mobility than a 4-*O*-substituted xylose. As A, B, and C had the same mobilities as X_6, X_5, and X_4, it was concluded that the reducing end-groups of all three are not linked in the 3-position.

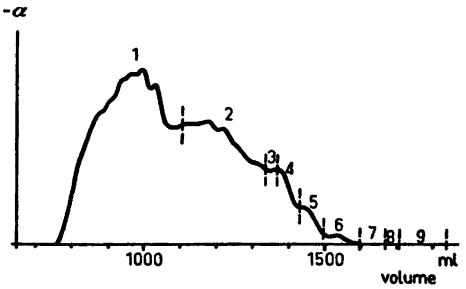

Fig. 1. Fractionation of enzymatic hydrolysate from xylan I-6 (2.4 g) on a Sephadex G-25 column.

Table 2. Optical rotations in water and in cuprammonium of xylo-, di-, and oligosaccharides.

Substance	$\bar{P}_n$	$[M]_{436}^{20}$ in cuprammonium	$[M]_{436}^{20}$ water	$\Delta[M_n]$	$\Delta[M_{n+1}]-\Delta[M_n]$	
X_2		− 490	− 90	− 400	−1500	
X_3		− 2280	− 380	−1900	−2000	
X_4		− 4550	− 650	−3900	−2000	−1700
X_5		− 6810	− 910	−5900	−1800	
X_6		− 8840	−1140	−7700	−1400	
X_7		−10510	−1410	−9100		
$B(X_5^*)$	5	− 5400	−1000	−4400		
$A(X_6^*)$	6	− 7250	−1250	−6000		

From the difference in molecular rotation at 436 nm of the β-(1→4)-linked xylodextrins, in water and in cuprammonium solution [10] (Table 2), the contribution to the shift of one (1→4)-linked xylose residue can be estimated to be about −1700°. A β-(1−3)-linked xylose residue should not give a copper complex, and from the values of the shifts for A and B, the presence of one such residue in each of these oligosaccharides is indicated. The symbol X_n^* is used below for a linear oligosaccharide composed of β-D-xylose residues, one of which is linked 1→3 and the others 1→4. The symbol includes all possible isomers which seem to have similar R_F-values on paper chromatography. On paper chromatograms, the members of this series have higher R_F-values than the β-(1→4)-linked xylodextrins, and are easily distinguishable from the latter as has already been observed by Howard.[6] Partial acid hydrolysis studies on the oligosaccharides and the derived alditols gave further information concerning their structures. A and reduced A both yielded X_3 as the largest exclusively β-(1−4)-linked oligosaccharide, indicating the structure X 1→4 X 1→4 X 1→3 X 1→4 X 1→4 X. B and reduced B also yielded X_3 as the highest member of this series, indicating the structure X 1→4 X 1→4 X 1→3 X 1→4 X. C, but not reduced C, also yielded X_3, indicating the structure X 1→3 X 1→4 X 1→4 X. The results, however, only indicate the presence of these compounds but not if the oligosaccharides are pure or mixtures of isomers.

More conclusive results were obtained with the aid of an enzyme fraction, obtained from guar beans (*Cyamopsis tetragonoloba*). This enzyme fraction acts as a β-D-xylosidase and degrades the xylodextrins from the non-reducing end. Thus, when reduced X_6 was treated with the enzyme, xylose was the only reducing sugar formed and all the xylosidic linkages were successively hydrolysed. When A was treated with the enzyme, the sugars formed were X_5^*, X_4^*, X_3, X_2, and X, showing that the oligosaccharide has the structure X 1→4 X 1→4 X 1→3 X 1→4 X 1→4 X, in agreement with the result from the partial acid hydrolysis. B, however, yielded X_4^*, X_3^*, X_3, X_2, and X, showing it to be a mixture of two pentasaccharides B_1, X 1→4 X 1→4 X 1→3 X 1→4 X and B_2, X 1→4 X 1→3 X 1→4 X 1→4 X. Similarly C yielded X_3^*, X_3, X_2, and X, showing it to be a mixture of two tetrasaccharides C_1, X 1→4 X 1→3 X 1→4 X and C_2, X 1→3 X 1→4 X 1→4 X.

From these results it appears that the enzyme used for the partial hydrolysis of the xylan preferentially attacks β-(1→4)-linkages, when these are flanked by other β-(1→4)-linkages, as in the structure —4 X 1→4 X 1→4 X 1→4 X 1→4 X 1—. If this were a rigorous requirement, only one hexasaccharide (A), two pentasaccharides (B_1 and B_2) and one tetrasaccharide (C_1) containing one β-(1→3)-linkage, should be formed. The yields of these, decreasing from the hexa- to the tetra-saccharide, indicate that a β-(1→3)-linkage not only inhibits the cleavage of an adjacent linkage, but also reduces the rate of cleavage of the linkage two removed. This effect must be pronounced, as structural elements giving rise to the hexasaccharide (A) should be less frequent than those giving rise to the pentasaccharides (B_1 and B_2) or the tetrasaccharide (C_1), assuming a statistical distribution of the β-(1→4)- and β-(1→3)-linkages.

The formation of the other tetrasaccharide (C_2) is not in agreement with the assumed requirements of the enzyme. One possibility is that it is formed from the non-reducing end of some polymers of appropriate structures. Another possibility is that the restrictions assumed for the action of the enzyme are not rigorous. It was also found that traces of X_2 and an X_4* oligosaccharide were formed when A was subjected to prolonged treatment with the enzyme.

The polysaccharide contains β-(1→4)- and β-(1→3)-linkages in the approximate proportions 2:1. The results of the enzymatic hydrolysis show that these linkages are not regularly arranged. A purely random arrangement of the residues seems to be the more probable. If this is so, then there should be the probability of finding, in the hydrolysate, oligosaccharides containing two β-(1→3)-linkages, *e.g.* the hexasaccharide X 1→4 X 1→3 X 1→4 X 1→3 X 1→4 X and the pentasaccharide X 1→4 X 1→3 X 1→3 X 1→4 X. Both for statistical reasons and because of the requirements of the enzyme, discussed above, these oligosaccharides should be formed in low yields only and may easily have been overlooked. The high yield of higher oligosaccharides (DP ≥ 7) resistant or very slowly attacked by the enzyme, is also in agreement with the assumption of a random distribution of the linkages.

EXPERIMENTAL

Optical rotations were measured at room temperature (20—23°) using a Perkin-Elmer "141" photoelectric polarimeter. All melting points are corrected. Evaporations were carried out under reduced pressure at a bath temperature below 45°.

Thin-layer chromatography. Absorbent: Kieselguhr G nach Stahl, E. Merck A. G. Solvent: Butan-1-ol-ethanol-water (5:3:2). Spray reagent: Sulphuric acid (98 %, 5 ml) and α-naphtol (1 g) in butan-1-ol (100 ml).

Paper chromatography. Paper: Whatman No. 1 for analytical, and Whatman No. 3 MM for preparative separations. Solvents: A. Butan-1-ol-ethanol-water (10:3:5). B. Ethyl acetate-pyridine-water (2:1:2). Spray reagent: Anisidine hydrochloride.

Paper electrophoresis. Paper: Whatman No. 3 MM. Buffer: 0.1 M germanate buffer at pH 10.7. Spray reagent: Anisidine hydrochloride.

Isolation of the xylans. The dried powdered algae (2 kg) were extracted first with ethanol in a Soxhlet apparatus to remove most of the colouring matter and then six times with water at room temperature. The residue was extracted four times with 0.1 M aqueous sulphuric acid at room temperature. The polysaccharide material in each extract was precipitated with ethanol. In Table 3 are given the yields of the various fractions, the optical rotations in cuprammonium solution,[11] and the results of periodate oxidation of some of the fractions. The periodate oxidations were performed in unbuffered solutions of 0.1 M sodium metaperiodate and the periodate consumption was determined according

Table 3. Xylan fractions obtained from *Rhodymenia palmata*.

Fractions	Yield (g)	$[\alpha]_{436}^{20}$ in cuprammonium	$\bar{P}_n$	Periodate consumption mole/mole anhydrosugar	Formic acid mole/mole anhydrosugar Before reduction	After reduction
Xylan I-1	5.1	− 800	129	0.66	0.019	0.013
I-2	4.2	− 850				
I-3	5.7	− 910				
I-4	12.9	−1045				
I-5	17.6	−1070				
I-6	18.0	−1075	64	0.71	0.038	0.022
Xylan II-1	2.3	− 790				
II-2	2.6	− 823		0.62	0.026	0.021
II-3	2.0	− 810				
II-4	1.4	− 838				

to the Fleury-Lange method.[12] The formic acid released on the oxidation was determined both before and after reduction with sodium borohydride. The degrees of polymerisation were determined osmometrically on the nitrated products, following the procedure used by Lindberg and Meier.[13]

Methylation studies

Each of the xylans I and II (xylan I−4 and xylan II−1) was methylated with sodium hydroxide and dimethyl sulphate as described by Srivastava *et al.*[14] After two methylations almost fully methylated products were obtained. (Calc. for a xylan with $\bar{P}_n$ 60: OCH_3 39.2 %. Found: xylan I OCH_3 39.3 %, xylan II OCH_3 39.2 %). Yields: xylan I: 66 %, xylan II: 34 %. Each of the methylated polysaccharides was hydrolysed in 90 % aqueous formic acid for 30 min at 100°. The solutions were concentrated to dryness and the residues were treated with 0.125 M aqueous sulphuric acid (100 ml per g material) at 100° overnight. After neutralisation with Dowex 3 (free base), the solutions were concentrated to small volumes and fractionated on a carbon-Celite (1:1) column. A gradient of 6 % to 30 % aqueous ethanol eluted mono-(trace) and dimethylxyloses. Subsequent elution with 50 % aqueous ethanol eluted trimethylxylose. The separation was followed by paper chromatography using solvent A. In the dimethylxylose fraction 2,3-di-*O*-methyl-D-xylose was eluted first and then a mixture of 2,3- and 2,4-di-*O*-methyl-D-xylose. The proportions of the two sugars in the mixture were determined by gas chromatography as described below. A preparative separation of the two dimethylxyloses was made by paper chromatography using solvent A.

Each of the methylated polysaccharides (2−5 mg) was treated with methanol containing 3 % hydrogen chloride (0.2 ml) at 100° for 16 h. The resulting solutions (2 µl) were injected onto a Perkin-Elmer "OS 138" (polyphenyl ether) column mounted in a Perkin-Elmer Model 801 gas chromatograph at a column temperature of 140°. The flow rate of nitrogen carrier gas was 21 ml/min. Authentic samples of the monomethylxyloses were readily detected, but only traces of these substances were found in the methanolysis mixtures. The relative proportions of the various components were determined from the previously determined response of the flame ionisation detector to reference samples of the various materials in known proportions.

*Characterisation of the components. 2,3-Di-*O*-methyl-*D*-xylose.* The N-phenylglycosylamine had m.p. 134−136° undepressed on admixture with an authentic sample.

*2,4-Di-*O*-methyl-*D*-xylose.* The crystalline sugar had m.p. 105−107° in good agreement with previously recorded values.

*2,3,4-Tri-*O*-methyl-*D*-xylose.* The crystalline sugar had m.p. 84−86° undepressed on admixture with an authentic sample.

Characterisation of the enzymes. The homologous series of β-(1→4)-linked xylose oligosaccharides was prepared according to Whistler and Tu.[15]

The cellulase-xylanase was isolated from *Stereum sangvinolentum*. When the enzyme solution (0.1 ml) was added to a solution of carboxymethyl cellulose of D.S. 0.7 (50 mg) in 0.1 M sodium acetate buffer at pH 5.0 (10 ml) and the mixture kept at 37° for 15 min, the time of passage of the solution through a viscosimeter at 25° dropped from 100 sec to 20 sec. The buffer alone passed in 7.5 sec. For the enzymatic hydrolyses of the oligosaccharides 0.1 ml of this enzyme solution was used per ml buffer solution containing oligosaccharide, and for enzyme hydrolysis of the xylan one thenth of this enzyme concentration was used.

In the investigations of the action of the enzyme upon the various oligosaccharides a temperature of 37° was used. Samples were withdrawn at time intervals, the enzyme was deactivated by heating at 100° for 10 min, and the solution was examined by paper chromatography in solvent B.

A β-D-xylosidase was isolated from *Cyamopsis tetragonoloba*.[16] The beans were allowed to germinate for a few days, shelled and washed with water. After drying and grinding, the powder was extracted with water. The aqueous extract was precipitated with increasing concentrations of ammonium sulphate. The precipitates from 20 to 30 % ammonium sulphate were desalted by dialysis against running water and, after concentrations, the solution was fractionated on a Sephadex G-75 column. The xylosidase used in this work was one of the first (high molecular weight) fractions. The hydrolyses with this enzyme were performed in 0.1 M ammonium acetate buffer at pH 5.0 at 37°, the substrate concentration being 0.1 %.

Enzymatic hydrolysis of xylan I. Xylan I-6 (2.4 g) was dissolved in toluene-saturated sodium acetate buffer at pH 5.0 (24 l) and incubated with the enzyme solution (24 ml) at 37°. The reaction was followed by paper chromatography and by polarimetry ($[\alpha]_{578}$ initial −121°, $[\alpha]_{578}$ final −81°). The reaction was halted after 28 days, when no further change in optical rotation was observed, by heating at 85° for 15 min. The solution was concentrated to 2 l, deionised with Dowex 50 W-X8 (H^+ form) and concentrated to a small volume. The concentrate was added to the top of a Sephadex G-25 column (4.5 × 100 cm). The column was irrigated with toluene-saturated water. Fractions of 10 ml were collected. The separation was followed by thin layer chromatography and by optical rotation. The results are given in Table 1 and in Fig. 1. After several refractionations on the same column the oligosaccharides A, B, and C were obtained. The degree of polymerisation was determined as described by Timell,[8] using orcinol instead of phenol. The method was checked against the β-(1→4)-linked xylodextrins. The number of β-(1→3)-linkages was determined by measuring the optical rotations of the oligosaccharides in cuprammonium solution.

Partial acid hydrolysis. The oligosaccharide (2—3 mg) was dissolved in 0.125 M aqueous sulphuric acid (1 ml) and kept at 100° for 30 min. The solution was deionised with Dowex 3 (free base), concentrated, and the products were examined by paper chromatography in solvent B. The oligosaccharides were also examined by using the β-D-xylosidase as described above.

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