DISTRIBUTION OF 4-O-METHYL-D-GLUCURONIC ACID UNITS IN XYLAN OF THE BARK OF WHITE WILLOW (*Salix alba* L.)

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Homogeneous, water soluble 4-O-methyl-D-glucurono-D-xylan was isolated from the bark of white willow (*Salix alba* L.). The equivalent weight of the polymer was found to be 990, *i.e.* each sixth unit of D-xylose on average was substituted by 4-O-methyl-D-glucuronic acid. The single-ion activity coefficient of calcium counterions $\gamma_{Ca^{2+}}$ was estimated in a molecular disperse solution of calcium 4-O-methyl-D-glucurono-D-xylan. The mean distance of adjacent carboxyl groups, $b = 1{\cdot}1$ nm, was obtained from the experimentally determined value $\gamma_{Ca^{2+}} = 0{\cdot}39$ using the relation $\gamma_{Ca^{2+}} = f(b)$. Comparison of the value $b = 1{\cdot}1$ nm with the D-xylose unit length (0·50 nm) and the calculated value of the equivalent weight indicated the presence of blocks with a close arrangement of uronic acids in the D-xylan chain. In this case, as it follows from the conformation of the macromolecule, at least each second D-xylose on average bears the side monomeric 4-O-methyl-α-D-glucuronic acid unit at $C_{(2)}$. Blocks with a high concentration of uronic acid alternate with approximately three times greater blocks of unsubstituted, or very little substituted D-xylose units.

Our previous study[1] showed that 4-O-methyl-D-glucurono-D-xylan of the white willow bark has a β(1→4) linked xylopyranosyl backbone with the single unit side chains of 4-O-methyl-α-D-glucopyranosyluronic acid bound to $C_{(2)}$ of D-xylose. At that time, however, we did not dispose of a reliable method for determination of a sequential arrangement of 4-O-methyl-D-glucuronic acids in the D-xylan macromolecule. Routine methods, *i.e.* the partial acid or enzymic hydrolysis, do not offer sufficient information on the problem at hand, since in both cases only a mixture of aldouronic acids and neutral xylooligosaccharides was formed according to investigations so far published[2-4]. This phenomenon is explained by a different stability, and/or accessibility of glycosidic bonds of D-xylan towards both acid[2] and enzymic[2,4] hydrolysis. Nevertheless, it has generally been accepted[2,3,5,6] that monomeric side units of uronic acid are randomly distributed along the D-xylan chain. Shimizu and coworkers[7] isolated, however, from the hydrolysate of hemicellulose present in the spruce neutral-sulfite liquor also a dimer of D-xylose bearing two vicinal 4-O-methyl-D-glucuronic acid units in $C_{(2)}$ position of the corresponding D-xylose. The same author with coworkers[8] isolated oligosaccharides containing two vicinal 4-O-methyl-D-glucuronic acid units as single unit side chains in a con-

siderable amount also from the hydrolysate of D-xylan of larch wood. The used procedure, *i.e.* partial acid hydrolysis followed by separation of fragments on columns packed with ion exchangers did not make it possible to draw conclusions on distribution of these vicinal uronic acids along the D-xylan backbone.

Our preceding paper[9] showed that determination of the activity of calcium counterions bound to carboxyl groups of linear acid polysaccharides constitutes a novel methodic approach in examination of uronic acid distribution in their macromolecules. This paper utilizes this technique for examination of distribution of 4-O--methyl-D-glucuronic acid in D-xylan of the bark of white willow.

EXPERIMENTAL

Optical rotations were measured in aqueous solutions with a Perkin–Elmer, model 141, polarimeter at 20°C. Free-boundary electrophoresis of D-xylan solutions (10 mg/ml) was performed in 0·2M sodium tetraborate buffer (pH 7·2) with a Zeiss 35 apparatus at 10 V/cm and 6 mA for 30 min. Hewlett-Packard, model 5711A chromatograph was used for gas chromatography on a 200 × 0·3 cm column packed with 3% OV-225 over Chromosorb W AW DMCS (80—100 mesh) at a preset temperature range 120°C (4 min) to 170°C at a 2°C/min rate. Sugars were analyzed as alditol trifluoroacetates[10]. For paper chromatography on paper Whatman No 1 following solvent systems were used: ethyl acetate–pyridine–water 8 : 2 : 1 (S_1), ethyl acetate–pyridine–acetic acid–water 5 : 5 : 1 : 3 (S_2, v/v) using the descending technique. Reducing sugars were detected with anilinium hydrogen phthalate[11]. The uronic acids content was determined alkalimetrically by a potentiometric titration with 0·05M-KOH. Polysaccharides were hydrolyzed with 2M trifluoroacetic acid at 100°C for 2 h.

Molecular weight (M_w) of D-xylan was estimated by ultracentrifugation (260 000*g*) and extrapolation to zero concentration (ultracentrifuge MOM G 110). Six photographs taken after 30 min at 6 min intervals showed that the polysaccharide sedimented as a single, symmetrical peak. The partial specific volume was determined pycnometrically for a 1% solution of polysaccharide.

The 0·05M-KOH and 0·021M-$Ca(OH)_2$ solutions were carbonate-free. Tetramethylmurexide was of the same origin as in preceding papers[12,13]. Redistilled water free of carbon dioxide with specific conductance less than $2 \cdot 10^{-4}$ Sm^{-1} was used.

Isolation of D-Xylan

Holocellulose of the bark of young twigs of white willow (*S. alba* L.) was stepwise extracted with various solvents[14] (Table I). All extractions were made at room temperature for 3 h with exception of that with hot water (80°C) and were twice repeated. Combined extracts of aqueous 24% KOH were neutralized with acetic acid, dialyzed towards distilled water for 4 days and finally lyophilized. The obtained fraction G (Table I) had $[\alpha]_D$ +44·7°C (*c* 0·8), the methoxyl group and uronic acid content 2·0 and 14·1%, respectively.

To the cooled and stirred mixture of polysaccharides G (15 g) dissolved in water (1 000 ml) at 70°C a 5% aqueous solution of cetyltrimethylammonium bromide (900 ml) was dropwise added. The precipitate was centrifuged, washed with water and dissolved in 5% acetic acid (250 ml); this solution was poured into ethanol (1 500 ml). The precipitated mixture of polysaccharides (5·2 g) was separated, suspended in water, dialyzed and lyophilized. D-Xylose was identified after hydrolysis together with small amounts of D-galactose, L-arabinose and trace amounts

of L-rhamnose (solvent system S_1). From uronic acids the 4-O-methyl-D-glucuronic and trace amount of D-galacturonic acids were present (solvent system S_2). Solubility of D-xylan fraction in water was enhanced after neutralization with 0·05M-KOH. The insoluble residue was separated by a 30 min centrifugation at 20000*g* and the supernatant (500 ml) was poured into ethanol (5000 ml) acidified with concentrated hydrochloric acid (250 ml). The precipitated polysaccharide was gradually washed with acidified and neutral ethanol, ether and finally it was dried overnight at 40°C (yield 3·5 g). Potentiometric titration with 0·01M-$AgNO_3$ did not indicate the presence of Cl^- ions in the obtained material.

The polysaccharide (1 g) was dissolved again in water by neutralization with 0·05M-KOH to a final concentration 5—7 mmol (COOK)/l. This colloidic solution was centrifuged at 190000*g* with a preparative centrifuge Janetzki, model VAC 601, for 3 h. Concentration and a subsequent lyophilization of the combined supernatants gave a water-soluble 4-O-methyl-D-glucurono--D-xylan (0·7 g, K^+ form) homogeneous on free-boundary electrophoresis and sedimentation analysis. Its physicochemical constants are given in Table II. Acid hydrolysis of the polymer afforded only D-xylose and 4-O-methyl-D-glucuronic acid (as detected in solvent systems S_1 and S_2).

Determination of Ca^{2+} Ions Activity in Solution of Calcium D-Xylan

Solution of the isolated D-xylan (5—7 mmol (COOK)/l was once more centrifuged at 190000*g*, percolated through a Dowex 50WX2 (H^+) column and neutralized to pH 7·2 with a saturated (0·021M) calcium hydroxide solution. The activity of calcium ions was determined in calcium 4-O-methyl-D-glucurono-D-xylan solution (2·5—3·5 mmol ($COOCa_{0.5}$)/l) using tetramethylmurexide as a metallochromic indicator[12,13]. The total content of calcium was determined by a chelatometric titration with 0·01M Complexon IV with a spectrophotometric indication of the point of equivalence (murexide, interference filter Zeiss, Jena, IF 600 nm). The single-ion

TABLE I

Extraction of Polysaccharides from Holocellulose of the Bark of White Willow

Fraction	Extraction reagent	Yield %	Molar ratios of saccharides							
			Gal	Glc	Man	Ara	Xyl	Rha	Fuc	uronic acids
A	cold water	11·2	1·0	—	—	0·8	traces	traces	—	5·4
B	hot water	1·5	1·0	0·9	0·2	2·8	0·3	0·4	0·1	2·6
C	0·5% $(CO_2NH_4)_2$	3·1	1·0	0·1	—	0·7	0·1	0·1	—	2·7
D	2·5% NH_4OH	2·5	1·0	0·7	0·3	4·3	0·6	0·1	0·1	4·1
E	saturated solution of $Ba(OH)_2$	0·3	1·0	0·5	0·1	0·6	8·8	0·1	0·1	2·4
F	6% KOH	13·1	1·0	1·4	0·1	0·7	6·2	0·2	traces	1·2
G	24% KOH	10·4	1·0	0·5	0·2	0·5	9·4	0·3	—	2·0

activity coefficient $\gamma_{Ca^{2+}}$ was calculated from the activity of calcium ions and the total concentration of calcium in solution. The activity of calcium ions in the solution of calcium 2-O-(4-O-methyl-α-D-glucopyranosyluronate)-D-xylose was determined similarly. The aldobiouronic acid was prepared and characterized by Dr P. Kováč of this Institute.

Determination of 4-O-Methyl-D-glucuronic Acid Units Distribution in D-Xylan

Function $\gamma_{Ca^{2+}} = f(b)$, where b stands for the mean distance of adjacent carboxyl groups in a perpendicular projection to the main macromolecule axis, was employed for calculation. This function was introduced in our previous paper[9] using the value $\gamma_{Ca^{2+}}$ determined in model solutions of pectins[15] of various esterification degree (E >43%). In this range of esterification degree only electrostatic binding of calcium ions to carboxylic acids takes place, this being an unevitable condition of the presented procedure.

RESULTS AND DISCUSSION

Polysaccharides obtained by extraction with cold (A) and hot (B) water (Table I), containing distinct fractions of pectic substances, were already investigated in detail[14,16-18]. Fraction G, having $[\alpha]_D$ +44·7° and the highest content of D-xylose (~68%), was mainly constituted of 4-O-methyl-D-glucurono-D-xylan, the structure of which has been described[1]. This study deals with the distribution of 4-O-methyl-D-glucuronic acid in the D-xylan macromolecule.

Fractional precipitation with Cetavlon afforded a D-xylan fraction containing in addition to D-xylose also a small amount of D-galactose, L-arabinose and traces of L-rhamnose. Analysis of uronic acids indicated the presence of 4-O-methyl-D-glucuronic acid and a trace amount of D-galacturonic acid. Potassium salt of the contaminated polymer was centrifuged at 20000*g* and the supernatant was precipitated with ethanol. Since the deionized polysaccharide was still heterogeneous, it was further centrifuged as potassium salt at 190000*g* for 3 h. This final stage furnished 4-O-methyl-D-glucurono-D-xylan (K^+ form) completely water-soluble, homogeneous by free-boundary electrophoresis and sedimentation analysis. Its physicochemical constants are listed in Table II.

The electrostatic bond between calcium ions and carboxyl groups of the polymer is a prerequisite for the estimation of uronic acid distribution pattern in a linear acid polysaccharide[9]. In this case, regardless of the kind of uronic acid, the function $\gamma_{Ca^{2+}} = f(b)$, expressing the relationship between the single-ion activity coefficient of calcium counterions $\gamma_{Ca^{2+}}$ and the mean distance b of free adjacent carboxyl groups, has a general validity for linear acid polysaccharides containing uronic acids. In other words, the $\gamma_{Ca^{2+}}$ determined in solutions of calcium salts of acid polysaccharides can be used as a criterion of carboxyl groups distribution in the macromolecule under investigation.

As shown in the preceding paper[19], calcium ions are bound to acid polysaccharides (*e.g.* pectinic acids, alginates) by a pure electrostatic bond only in a molecular-

-disperse system with an intramolecular bond of Ca^{2+} ions. If an intermolecular Ca^{2+} bond is involved under a concurrent formation of aggregates or microgel particles, this is of chelate character[20]. It was further proved, when studying the distribution pattern of D-glucuronic acid in the peach gum polysaccharide[9], that no intramolecular chelate binding of Ca^{2+} ions occurred at the site of uronic acid linkage to D-galactose or D-mannose of the polysaccharide main chain. Similarly, it has been evidenced that in the point of attachment of 4-O-methyl-D-glucuronic acid to D-xylose by a glycosidic $\alpha(1\rightarrow2)$ bond no chelatation of calcium ions takes place. The activity coefficient $\gamma_{Ca^{2+}}$ determined in solution of calcium salt of the respective aldobiouronic acid ($\gamma_{Ca^{2+}} = 0{\cdot}75$; $(COOCa_{0.5}) = 3{\cdot}00$ mmol/l) had the same value as $\gamma_{Ca^{2+}}$ calculated according to Debye and Hückel for a strong electrolyte of the same concentration ($\gamma_{Ca^{2+}} = 0{\cdot}759$).

Ultracentrifugation of solution of calcium 4-O-methyl-D-glucurono-D-xylan at 190000*g* showed that this solution was molecular-disperse and did not contain aggregates of macromolecules. Also $\gamma_{Ca^{2+}} = 0{\cdot}39$ determined in the solution of calcium salt of 4-O-methyl-D-glucurono-D-xylan immediately after its preparation was almost identical with that found in the same solution after ultracentrifugation. The deviation from the original value was less than $\pm 0{\cdot}005$. These results together with the presented facts entitle to propose that calcium ions are bound to carboxyl groups of D-xylan macromolecule exclusively by an electrostatic bond.

When using the relation $\gamma_{Ca^{2+}} = f(b)$ (Fig. 1), the determined value $\gamma_{Ca^{2+}} = 0{\cdot}39$ corresponds to $b = 1{\cdot}1$ nm, which represents the mean distance of adjacent monomeric side units of 4-O-methyl-D-glucuronic acid. As it follows from the study of a fiber diagram of 4-O-methyl-D-glucurono-D-xylan of birch wood[21] the D-xylan backbone has a threefold screw symmetry with a rotation of 120° for each D-xylose unit and a repeating length of 1·5 nm. Conformation of the 4-O-methyl-D-glucuronic

TABLE II
Physicochemical Constants of 4-O-Methyl-D-glucurono-D-xylan

Methoxyls, %	3·1
4-O-Methyl-D-glucuronic acid, %	21·1
Equivalent weight	990
Xylose : uronic acid ratio	6
Diffusion coefficient D_{20}	$4{\cdot}03 \cdot 10^{-11}$ m^2 s^{-1}
Sedimentation constant S_{20}	$4{\cdot}33 \cdot 10^{-13}$ s^{-1}
Partial specific volume p_{20}	$0{\cdot}683 \cdot 10^{-3}$ m^3 kg^{-1}
Molecular weight $\bar{M}_w$	82 300
$[\alpha]_D$	−43·7°

acid relative to the main D-xylan chain[21] was established, too. It could be presumed that the D-xylan under study has most likely an extended chain in solution similar to those of other synthetic polyelectrolytes, due to a relatively high linear charge density of the macromolecule and bulky monomeric side units of 4-O-methyl-D-glucuronic acid.

Compared with the length of one D-xylose unit (0·50 nm) the determined value $b = 1{\cdot}1$ nm for the mean distance of adjacent carboxyl groups indicates a close arrangement of uronic acid units where each second D-xylose unit on average bears a terminal $\alpha(1\rightarrow2)$ linked 4-O-methyl-D-glucuronic acid. Since the uronic acid residues occur as monomeric side units, an exact interpretation of results is not simple. At a certain conformation of the macromolecule, however, the possibility of substitution of every D-xylose unit cannot be excluded considering the spatial arrangement of charges along the macromolecule.

Analysis of uronic acids showed the D-xylose to 4-O-methyl-D-glucuronic acid ratio to be 6 : 1. On the other hand $\gamma_{Ca^{2+}} = 0{\cdot}39$ indicates a much closer arrangement of uronic acids in the xylan under investigation. For this reason we presume that D-xylan chain is irregularly substituted by 4-O-methyl-D-glucuronic acid. It consists, with the greatest probability, of blocks with a high concentration of uronic acid altering with approximately three times greater blocks of unsubstituted or only very little substituted D-xylose units.

Binding of calcium ions to oligo- and polymannuronates having an identical conformation of macromolecule with that of the D-xylan under study was investigated in our previous experiments[22]. The determined relationship of $\gamma_{Ca^{2+}}$ on the polymerization degree (DP) revealed that the activity of calcium ions electrostatically bound to carboxyl groups of D-mannuronan is totally independent on the size of the macromolecule from DP > 30. The course of the function $\gamma_{Ca^{2+}} = f(DP)$

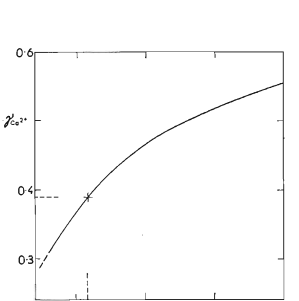

FIG. 1

Dependence of the Single-Ion Activity Coefficient $\gamma_{Ca^{2+}}$ upon the Mean Distance b of Adjacent Carboxyl Groups in Solutions of Calcium Salts of Linear Acid Polysaccharide

Uronic acids concentration 3·00 mmol $(COOCa_{0.5})$/l.

let us further consider that the relatively low value $\gamma_{Ca^{2+}} = 0{\cdot}39$ in solution of calcium 4-O-methyl-D-glucurono-D-xylan indicates the occurrence of greater blocks with a high concentration of 4-O-methyl-D-glucuronic acid in the D-xylan chain. The size of these blocks cannot be unequivocally determined, as the uronic acid residues appear as side monomeric units.

If only the appearance of vicinal couples of 4-O-methyl-D-glucuronic acid units[8], distributed randomly in the D-xylan chain is considered, such a low value of $\gamma_{Ca^{2+}}$ could not be obtained. In this case, both vicinal uronic acid units would be remote from the other pair by 10 D-xylose units on average and the strength of the binding of calcium ions to these "isolated" dimeric groups should be close to the binding of calcium ions to the appropriate dimer. This consideration is based upon findings concerning the binding of Ca^{2+} ions to pectinates of various linear charge density of macromolecule[15]. Values of $\gamma_{Ca^{2+}}$ determined in solutions of calcium digalacturonate[23], diguluronate and dimannuronate[22] were found to be 0·65, 0·64 and 0·68, respectively. Considering these data it follows that even in such a case, where only vicinal pairs of 4-O-methyl-D-glucuronic acid units are present in the D-xylan macromolecule, they cannot be randomly distributed.

One can conclude that D-xylan from the bark of white willow has 4-O-methyl-D-glucuronic acid units concentrated in greater blocks, where at least each second D-xylose unit on average is substituted with a monomeric side unit of 4-O-methyl-D-glucuronic acid by an $\alpha(1\rightarrow2)$ bond. These blocks with a high concentration of uronic acid alter with approximately three times greater blocks of unsubstituted or only very little substituted D-xylose units.

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