BINDING OF CALCIUM IONS ON ACID POLYSACCHARIDES OF PEACH GUM AND DISTRIBUTION PATTERN OF CARBOXYL GROUPS IN THE MACROMOLECULE

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The acid polysaccharide of peach gum and its macromolecular products of a gradual degradation by acid hydrolysis were characterized by molecular weight ($\bar{M}_w$) and molar ratios of both uronic acids and neutral saccharide units. The single-ion activity coefficient of calcium ions ($\gamma_{Ca^{2+}}$) bound to these acid polysaccharides and to the corresponding aldobiouronic acids was measured and CD spectra of potassium and calcium salts of these compounds were recorded. It was ascertained that Ca^{2+} ions are bound to carboxyl groups of polysaccharides under study by an electrostatic bond in molecular disperse solutions. The mean distance of the neighbouring carboxyl groups $b = 0{\cdot}83$ to $1{\cdot}00$ nm, calculated from the measured values $\gamma_{Ca^{2+}}$ indicates a very close arrangement of uronic acid units, which does not undergo alteration with the stepwise degradation of the macromolecule. The obtained results let us conclude that the main chain of the polysaccharide is substituted in longer segments by D-glucuronic acid units and its 4-O-methyl derivative (monomeric side chains), at least at each second D-galactose unit.

Polysaccharide of the peach gum is a branched acid polysaccharide. Its main chain consists of D-galactopyranose units with glycosidic β(1→6) and β(1→3) bonds[1] and of D-mannopyranose units. The character of glycosidic bonds of D-mannose is subjected to a further study. Uronic acids (D-glucuronic acid and its 4-O-methyl derivative) exist mainly as monomeric side chains bound to D-galactose units by a β(1→6) bond and to D-mannose units by a β(1→2) bond[2]; it is assumed that uronic acid units occur also as a constituent of the main chain similarly, as with the acid polysaccharide of the apricot gum[3]. A relatively low ratio of uronic acid units to neutral saccharide units was found in the native polysaccharide. Uronic acid units are here present mainly in form of salts; they bind a very wide spectrum of cations. Thus, *e.g.* of bivalent cations bound to carboxyl groups of acid polysaccharide of the related apricot gum Ba^{2+} ions were found to prevail; Fe, Pb, Sn, Ca, Cu, Mg *etc.* are present to a lower extent[4]. The content of uronic acid units increases with a stepwise degradation of the polysaccharide. So far, data concerning the distribution pattern of carboxyl groups in the native polysaccharide are missing.

The activity coefficient of counterions (γ_i) bound by an electrostatic bond to polyelectrolytes is a function of the linear charge density of the macromolecule. As we

already showed, when studying the effect of pectinesterase on pectin[5] or the esterification of pectic acid with methanol[6], the $\gamma_{Ca^{2+}}$ values measured in such systems are, to some extent, a criterion of the distribution pattern of the ionized groups in the macromolecule. This paper deals, therefore, with the binding of Ca^{2+} ions to carboxyl groups of native and partially degraded polysaccharides of peach gum aiming to contribute to the elucidation of distribution pattern of carboxyl groups in the molecule of the polysaccharide.

EXPERIMENTAL

Material and chemicals: Preparation of the native peach gum polysaccharide and polysaccharides partially degraded by acid hydrolysis was already described[7]. Preparation and characteristic data of aldobiouronic acids (6-O-β-D-glucuronopyranosyl-D-galactopyranose and 2-O-β-D--glucuronopyranosyl-D-mannopyranose) were published earlier[2]. The molar ratios of acid and neutral saccharide components of polysaccharides (samples 1—4) were determined by the same methods as reported in our paper[8]. Molecular weight of polysaccharides ($\overline{M}_w$) was estimated by ultracentrifugation with extrapolation to zero concentration (ultracentifuge MOM G 110). Used were 0·05M-KOH and a saturated calcium hydroxide solutions (*c.* 0·21M). Other chemicals were of analytical grade. Specific conductance of redistilled water was less than $2 \cdot 10^{-4}$ Sm^{-1}.

Preparation of potassium and calcium salts of acid polysaccharides and aldobiouronic acids: Concentration of solutions, prepared from the investigated substances from which other low-molecular electrolytes were perfectly removed, was approximately 4 mmol [COOH]/l. The solutions were further percolated over Dowex 50WX2 (H^+ form) column in order to remove the trace amount of metal cations bound to carboxyl groups; subsequently, they were neutralized by potentiometric titration with 0·05M-KOH, or 0·021M-$Ca(OH)_2$ solutions to the point of equivalence (pH 7·2—7·5). This way obtained solutions of potassium and calcium salts were adjusted to 2 and 3 mmol [COOM]/l concentration (M = K or $Ca_{0.5}$).

Methods: Potassium and calcium salts of the above-mentioned concentrations were subjected to circular dichroism measurement on a Roussel-Jouan Dichrograph, model 185/II. The single--ion activity coefficient $\gamma_{Ca^{2+}}$ in solutions of calcium salts of acid polysaccharides and aldobiouronic acids was determined by the metallochromic indicator method (tetramethylmurexide)[9,10]. Measured were 3·00 mmol [$COOCa_{0.5}$]/l solutions without addition of any further electrolyte. The viscosity of the partially degraded polysaccharide (sample 3) was determined with an automatic viscometer FICA in 0·155M-NaCl-0·005M sodium oxalate at pH 7·2 (K^+ form) and pH 2·0 (H^+ form). Solutions were adjusted to pH 2 by addition of HCl. Janetzki VAC 601 preparative ultracentifuge was used for preparative purposes. Calcium in solutions of calcium salt of the acid polysaccharide was chelatometrically determined using 0·01M-Complexon IV under spectrophotometric indication of the point of equivalence (interference filter Zeiss, Jena, IF 600 nm).

RESULTS AND DISCUSSION

Structural characterization of peach gum polysaccharides is given in Table I in terms of molar ratios of the individual saccharide components. Molar ratios of neutral saccharides relate to one uronic acid unit. Sample 1 describes the structure of the

native polysaccharide, samples 2 to 4 that of polysaccharides stepwise degraded by partial acid hydrolysis. Table II lists the sum of all neutral saccharide units corresponding to one uronic acid unit (NS/UA) in the respective samples. These data show that the partial degradation of polysaccharide results first of all in cleavage of the side chains consisting of D-xylose, L-arabinose, L-rhamnose and partially also of D-galactose units.

Binding of Ca^{2+} *Ions to Carboxyl Groups of Polysaccharides and to Corresponding Aldobiouronic Acids*

Binding of Ca^{2+} ions to carboxyl groups of polysaccharide is characterized by the single-ion activity coefficient $\gamma_{Ca^{2+}}$, determined in solutions of calcium salts of substances under investigation at 3·00 mmol $[COOCa_{0.5}]/l$ concentration (Table II). The activity coefficients $\gamma_{Ca^{2+}}$ of the first three samples are very close to each other in the interval of values $\gamma_{Ca^{2+}}$ 0·32–0·34; a value slightly higher ($\gamma_{Ca^{2+}} = 0{\cdot}37$) was found with sample 4 having the lowest molecular weight ($\bar{M}_w$ 25000). The results show that the electric charge density of the macromolecule remains virtually unaltered during the stepwise degradation of the polysaccharide macromolecule (the same distribution pattern of carboxyl groups) although the degradation considerably altered both the molar ratios of individual saccharide units in polysaccharides and their molecular weight.

Prior to interpretation of the measured $\gamma_{Ca^{2+}}$ values it is necessary to elucidate the kind of Ca^{2+} ions binding to carboxyl groups of the polysaccharide, whether electrostatic or chelate bonds are involved. In addition to carboxyl groups, oxygen atoms of saccharide units in the chelate binding would participate similarly as encountered with aggregates of calcium D-galacturonan and L-guluronan[11–13].

TABLE I

Structural Characterization of Native Peach Gum Polysaccharide and Polysaccharides Partially Degraded (molar ratios of saccharide units)

Sample No	Molecular weight $\bar{M}_w$	GlcUA	4-O-Methyl--GlcUA	Gal	Man	Xyl	Ara	Rha
1	460 000	0·60	0·40	3·78	0·28	1·67	5·92	0·27
2	213 000	0·63	0·37	3·31	0·22	1·81	4·88	0·24
3	53 000	0·73	0·27	4·04	0·36	0·30	1·05	0·08
4	25 000	0·90	0·10	2·17	0·69	[a]	[a]	[a]

[a] Traces.

Uronic acid units are bound to neutral saccharides in the main chain. Therefore, we also determined the $\gamma_{Ca^{2+}}$ values in solutions of calcium salts of the corresponding aldobiouronic acids (Table II, samples 5 and 6). The determined $\gamma_{Ca^{2+}}$ activity coefficients 0·761 and 0·754 are virtually identical with the value $\gamma_{Ca^{2+}} = 0{\cdot}759$ calculated according to Debye and Hückel for solution of a strong electrolyte ($CaCl_2$) of the same ionic strength. The accordance of data evidences that no chelate binding of calcium occurred at the binding site of uronic acid units to the main chain.

Solutions of calcium salts of polysaccharide at 3·00 mmol $[COOCa_{0.5}]$/l concentration are perfectly clear; neither the ultracentrifugation in a preparative ultracentrifuge at 190000*g* (30 min) proved the presence of micro-gel particles. The content of calcium bound to carboxyl groups was determined in the starting solution of the Ca-salt of the polysaccharide (sample 3) and in the supernatant after ultracentrifugation; the supernatant contained 98·4% of the starting concentration of the polysaccharide.

As we proved earlier, the CD-spectra of solutions of salts of polyuronic acids[14] and their derivatives[15] are not influenced by the kind of counterions (Ca^{2+}, Mg^{2+}, K^+), as long as cations are bound by pure electrostatic bonds to carboxyl groups. In contrast, when an aggregation of macromolecules associated with a remarkable change of the molecular environment of the chromophore took place also a considerable change of the CD-spectrum occurred[11,14]. We investigated, therefore, circular dichroism of potassium and calcium salts of 6-O-β-D-glucuronopyranosyl--D-galactopyranose (sample 5) and of the partially degraded polysaccharide (sample 3; Fig. 1). The solutions of potassium and calcium salts of the examined substances revealed virtually identical spectra in both cases with deviations close to experimental

TABLE II

Single-Ion Activity Coefficient $\gamma_{Ca^{2+}}$ in Solutions of Calcium Salts of Polysaccharides of Peach Gum and the Corresponding Aldobiouronic Acids

Sample No	Saccharide	*NS/UA*	$\gamma_{Ca^{2+}}$	*b* nm
1	acid polysaccharide	11·9	0·326 ± 0·003	0·86
2		10·5	0·339 ± 0·001	0·90
3		5·8	0·317 ± 0·008	0·83
4		2·9	0·367 ± 0·007	1·00
5	GlcpUA β(1→6)-Galp	1·0	0·761 ± 0·006	—
6	GlcpUA-β(1→2)-Manp	1·0	0·754 ± 0·001	—

$[COOCa_{0.5}]$ = 3·00 mmol/l.

errors. The CD-spectrum of salts of aldobiouronic acid (curve *1*) is very close to that of sodium methyl β-D-glucuronopyranoside[16]. Although the spectrum of salts of the acid polysaccharide (curve *2*) traces the maximum and minimum of the spectrum of the corresponding aldobiouronic acid, a shift of the band position toward shorter wavelengths and to the higher negative values of ellipticity $[\Theta]$ occurred. The identity of spectra of both potassium and calcium salts of the acid polysaccharide together with the preceding results evidence that the solutions of these substances are molecular disperse with an electrostatic binding of Ca^{2+} ions to carboxyl groups.

Distribution Pattern of Carboxyl Groups in the Molecule of Acid Polysaccharides

The activity coefficient $\gamma_{Ca^{2+}}$ can be employed as a criterion of distribution pattern of carboxyl groups in the macromolecule involving a pure electrostatic binding of Ca^{2+} ions to carboxyl groups of polysaccharide. In such a case, the mean distance of neighbouring carboxyl groups can be calculated from the measured $\gamma_{Ca^{2+}}$ values. As we have already shown, the Ca^{2+} ions are bound to pectin of an esterification

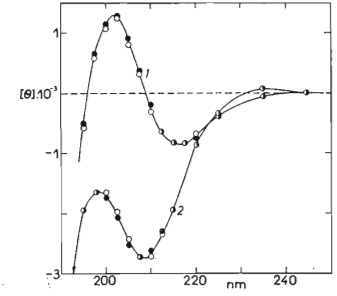

FIG. 1

Circular Dichroism of Solutions of Potassium and Calcium Salts of Acid Polysaccharide of Peach Gum and the Corresponding Aldobiouronic Acid

1 6-O-β-D-Glucuronopyranosyl-D-galactopyranose, *2* partially degraded acid polysaccharide (sample 3); ○ potassium salt, ● calcium salt; $[\Theta]$ (degree cm^2 $dmol^{-1}$).

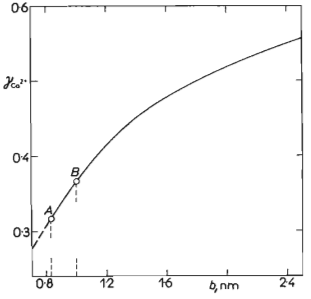

FIG. 2

Relationship Between the Single-Ion Activity Coefficient $\gamma_{Ca^{2+}}$ in Solutions of Calcium Polyuronates and the Mean Distance of Free Carboxyl Groups (b) ($[COOCa_{0.5}] = 3{\cdot}00$ mmol/l)

degree (E) of carboxyl groups (esterified with methanol) greater than 40% by a pure electrostatic bond[17]. Under these circumstances, the activity coefficient γ_i of counterions does not depend on the kind of uronic acid units but it does on the linear charge density of the macromolecule. Hence the $\gamma_{Ca^{2+}}$ values measured in solutions of calcium pectinates[17] can be utilized for interpretation of $\gamma_{Ca^{2+}}$ values determined under the same experimental conditions in solutions of calcium salts of the investigated acid polysaccharides.

In solutions of polyanions free of a further electrolyte it is assumed that, due to the high linear charge density of macromolecules, their chains are more or less stretched. This holds good either for highly flexible macromolecules (polyacrylates, polymethacrylates), or for relatively rigid macromolecules (D-galacturonan, L-guluronan, pectin of a high content of uronic acid units *etc.*). The mean distance of two adjacent anionic groups is described by their perpendicular projection on the main axis of the macromolecule (b).

The distance between two neighbouring carboxyl groups in the molecule of pectic acid ($E = 0\%$), its sodium[18] as well as calcium[19] salts is, according to X-ray diffraction analysis, $b_0 = 0{\cdot}435$ nm. On the basis of this value we calculated the distance b for pectin molecules of various esterification degree (E) and then we calculated the relationship of $\gamma_{Ca^{2+}}$ on the mean distance of adjacent carboxyl groups of polyuronates ($\gamma_{Ca^{2+}} = f(b)$) using the corresponding data $\gamma_{Ca^{2+}}$ determined earlier[17], (Fig. 2). This function and $\gamma_{Ca^{2+}}$ values determined in solutions of calcium salts of acid polysaccharides served for calculation of values b (Table II, Fig. 2, the section on the curve limited by points A and B). The investigated polysaccharides behave, when binding Ca^{2+} ions, as a linear polyuronate with a mean distance of neighbouring carboxyl groups $b = 0{\cdot}83$ to $1{\cdot}00$ nm. These values demonstrate a very close grouping of uronic acid units, which does not depend on the degree of degradation of macromolecules.

The exact interpretation of results is so far impossible, since the data concerning the conformation of the main chain are still missing; moreover, uronic acid units occur here as monomeric side chains what causes a considerable difficulty in evaluation of experimental data. The close grouping of carboxyl groups can correspond to two various structures of macromolecules: *1*) to a linear chain of neutral saccharides with a high substitution degree by uronic acid units; *2*) to a polysaccharide with a lower substitution degree by uronic acid units, having the main chain twisted so as the carboxyl groups are close to each other. Respecting the relatively high linear charge density of the macromolecule and also with respect to the bulky substituents (uronic acid units) we suggest the main chain to be stretched and the short distance between carboxyl groups is given by the really high degree of substitution of the chain by uronic acid units. This conception is backed also by the low flexibility of the macromolecule what is indicated by viscometric measurements. The specific viscosity η_{sp} of solutions of the polysaccharide (sample 3) in an undissociated form (pH 2·0)

was found to be only slightly lower (by *c.* 10%) than that of the solution of the ionized polysaccharide (K^+form, pH 7·2). (Polyelectrolytes having a flexible macromolecule, as *e.g.* polymethacrylates, show a many times lower viscosity of the polyelectrolyte in an undissociated form than in a dissociated one[20]).

As it follows from the molar ratios of uronic acid units, D-mannose and D-galactose in the native polysaccharide (1 : 0·28 : 0·78) and isolated aldobiouronic acids, the prevailing portion of uronic acid (at least 72%) is bound to D-galactose units by a β(1→6) glycosidic bond. The way of binding of D-mannose units in the main chain was not yet ascertained. This fact let us consider only the binding of uronic acids to the segment of the main chain consisting exclusively of D-galactose units. With respect to the above-mentioned glycosidic bond of side chains of uronic acids and their close grouping in the macromolecule, this galactan chain includes mostly β(1→3) bonds; in an extreme case β(1→3) and β(1→6) bonds in a 1 : 1 ratio.

A linear D-galactan with β(1→3) bonds and a stretched chain, under condition that $C_{(6)}$ atoms are alternately located on the reverse side of the chain, reveals the length b_0 corresponding to one saccharide unit approximately 0·40 nm (a perpendicular projection on the main axis of the macromolecule). D-Galactan with alternate β(1→3) and β(1→6) bonds and stretched chain reveals mean value b_0 for one saccharide unit 0·52 to 0·54 nm estimated by means of Dreiding models for the $^4C_1$ conformation of D-galactose units. For comparison purposes we cite the distances of carboxyl groups (b_0) of polyuronates with glycosidic (1→4) bonds as found by X-ray diffraction analysis. The lowest value $b_0 = 0{\cdot}435$ nm display polyuronates with *trans*-diaxial glycosidic bonds (D-galacturonan[18,19], L-guluronan[21]), the greatest distance of carboxyl groups $b_0 = 0{\cdot}515$ nm have polyuronates with *trans*-diequatorial glycosidic bonds (D-mannuronan[21], carboxymethylcellulose of $DS = 1$).

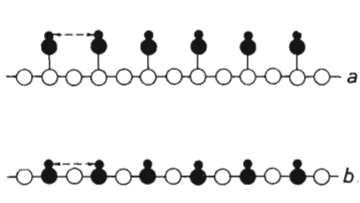

FIG. 3

Schematic Plot of Various Distribution Patterns of Uronic Acid Units in the Molecule of Acid Polysaccharide

○ D-Galactose unit, ●● uronic acid unit, ● COO^-.

If considering the arrangement of side chains exclusively on the same side of the macromolecule (the comb arrangement) then the experimentally determined values $b = 0{\cdot}83$ to $1{\cdot}00$ nm correspond to a substitution of D-galactan chain by uronic acid units roughly at each second saccharide unit (Fig. 3, Scheme *a*). Should the conformation of the macromolecule be close to a helical arrangement with a multifold screw symmetry, then the substitution of each D-galactose unit by uronic acid is also possible. Due to the fact that uronic acid units occur as side chains, a mean linear charge density of the chain corresponding to the measured values can be achieved at a suitable conformation of the macromolecule. Should the uronic acid units be only a constituent of the main chain, an alternation of D-glucuronic acid and D-galactose units in the main chain is involved (Fig. 3, Scheme *b*).

As it follows from the structural analysis (Table I), the main chain of degraded acid polysaccharides is substituted by uronic acid only at each third or fourth saccharide unit on average. On the other hand the $\gamma_{Ca^{2+}}$ values let us conclude that the main chain is substituted by uronic acid units at least at each second saccharide unit. It cannot be determined at the time being whether all, or each second saccharide units are substituted.

The high degree of substitution evidenced in this paper by physico-chemical methods in comparison with results of structural chemical analysis let us conclude that the main chain of the polysaccharide is not homogenously substituted by uronic acid units. It probably consists of segments with a high substitution degree alterning with those consisting only of neutral saccharide units.

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