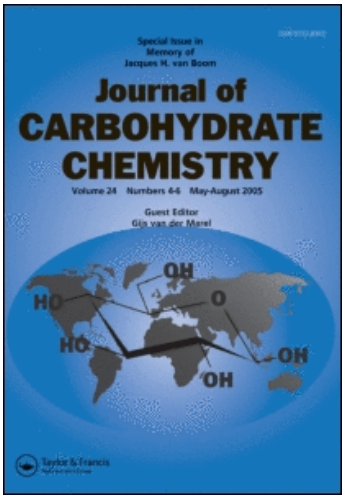

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CHARACTERIZATION OF PECTINS ISOLATED FROM MONGOLIAN PLANTS

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ABSTRACT

Pectic polysaccharides (HP, RP) were extracted from Mongolia hawthorn berries and from rhubarb stalks by hot aqueous oxalic acid treatment. Both polysaccharides contained approximately 78% of galacturonate. HP displayed a low degree of esterification (DE) and arabinose/galactose molar ratio 1.7:1. RP was higher in DE and contained these sugars in a molar ratio 0.5:1. Rhamnose, fucose, xylose, glucose, mannose, 2-*O*-methylxylose, and 2-*O*-methylfucose were found in lower amounts. Results from ^{13}C NMR spectroscopy and methylation analysis indicated the presence of branched arabinans, (1->4)- and (1->3,6)-linked galactans and hemicelluloses of the xylan and glucan types. The pectins differed in their molecular properties. HP exhibited a low molecular weight ($\bar{M}_w$ = 45,000), whereas RP had a broad M_w-distribution with $\bar{M}_w$ = 360,000. Independently of these differences, the binding capacity of both pectins towards divalent cations was influenced by the DE only and increased in the order: $Ca^{2+} < Cd^{2+} < Pb^{2+} < Cu^{2+}$.

INTRODUCTION

Pectins are widely used as gelling and thickening agents in the food and pharmaceutical industry.[2,3] They are important dietary fibers which have remarkable physiological effects. It is also known that pectins can serve as prophylactic agents against poisoning of living organisms with heavy metal cations.[4,5] As a part of a polysaccharide research program, Mongolia hawthorn berries (*Crataegus sanguinea* L.) and stalks of the wild growing rhubarb (*Rheum palmatum* L.) have been investigated as potential domestic sources for the industrial production of pectin. These pectins were found to be effective in treatment of burns.[6] The aim of the present paper was to elucidate the chemical, structural and molecular characteristics of the pectins and to compare their binding properties towards some divalent cations with those of commercial pectins.

RESULTS AND DISCUSSION

Crude pectins were isolated from hawthorn berries and rhubarb stalks by treatment with hot, aqueous oxalic acid, and then with acidified 60% ethanol to remove salts, low molecular weight organic substances and soluble carbohydrates. The purified pectins HP and RP were obtained in the yields of 2.2% and 3.0%, respectively, based on dry plant weight. Both HP and RP contained approximately 78% of galacturonate, but differed in their DE and molecular properties (Table 1). HP displayed a low DE. Its intrinsic viscosity [η] was also low, in accordance with data reported for other low methoxy pectins.[3,7] RP was higher in DE and its [η] was similar to those of commercial high methoxy pectins.[3,7] High performance gel permeation chromatography (HPGPC) of the pectins on Pullulan-calibrated Separon HEMA BIO-S columns gave single but broad peaks with average-molecular weight $\bar{M}_w$ = 45,000 for HP, and $\bar{M}_w$ = 360,000 for RP.

Table 1. Analytical data from the hawthorn and rhubarb pectins before and after one-year storage.

Pectin	HP	HP1	RP	RP1
Galacturonan, %[a]	77.1	80.6	77.8	78.5
Degree of esterification, DE, %	12.4	11.9	51.9	64.0
Protein, %[b]	nd	1.10	nd	1.41
$[\eta]$, mL/g[c]	119	92	530	472
$\bar{M}_w$[d]	45,000	47,000	360,000	230,000
$\bar{M}_n$[d]	19,000	26,000	180,000	89,000
$\bar{M}_w/\bar{M}_n$[d]	2.37	1.81	2.00	2.58

a. Estimated and expressed as the K^+ salt of galacturonic acid (M_o = 214.1).
b. % Nitrogen x 6.25.
c. In 0.155M NaCl at 25 °C.
d. Average-molecular weight values estimated by HPGPC on Separon HEMA BIO (100 and 1000) columns.
nd, is not determined.

However, these values are not absolute because they could be influenced by differences in the hydrodynamic properties of the compared pectins[8] as well as of the applied calibration standards.

As seen from Table 2, HP and RP contained the same neutral sugars, *i.e.*, arabinose, galactose, and minor amounts of rhamnose, xylose, glucose, fucose, and mannose. 2-*O*-Methylxylose and 2-*O*-methylfucose, the diagnostic components of rhamnogalacturonan RG-II,[9] detected as alditol trifluoroacetates,[10] were also present in trace amounts in both pectins. HP and RP differed significantly in the molar ratios of arabinose and galactose showing 1.7:1 in HP and 0.5:1 in RP. Pectins rich in galacturonate and arabinose were isolated from leaves and roots of radish,[10] apple,[11] apricot[12] and sugar beet pulp.[13] Because the same experimental conditions were applied for the isolation of the pectins studied, hy-

Table 2. Carbohydrate composition of HP, RP, and the Cetavlon-precipitated fractions HPC and RPC.

Sugar	HP	HPC	RP	RPC
Galacturonic acid[a]	66.2	70.0 (8.1)[b,c]	64.5	68.5 (7.0)[b,c]
Neutral sugars[d]				
Galactose	30.0	22.6.(56.7)	47.3	66.9 (87.0)
Arabinose	51.9	47.1 (34.0)	23.0	8.3 (5.6)
Rhamnose	4.2	13.8 (5.7)	9.5	7.5 (2.9)
Fucose	0.7	3.3 (0.4)	1.6	1.0 (0.3)
Xylose	2.1	6.2 (0.8)	6.8	3.8 (0.7)
Glucose	9.7	5.0 (3.4)	9.8	11.7 (3.2)
Mannose	1.3	2.0 (0.4)	2.0	0.8 (0.3)
2-*O*-Methylxylose	*	nd	*	nd
2-*O*-Methylfucose	*	nd	*	nd

a. In %, as anhydro unit of galacturonic acid (M_o = 176).
b. Values in parentheses are data for the carboxyl-reduced polymers HPC-R and RPC-R.
c. Carbazol assay.
d. In mol.%, estimated as alditoltrifluoroacetates on OV-225.
* Amounts <0.3%.
nd, is not determined.

drolytic cleavage could not be the reason for the lower proportion of arabinose in RP. This fact coinciding with the low DE and [η] of HP implies a different stage of ripeness of both plants[3,14] which were harvested at the same time.

HP and RP moved as single although broad bands at free-boundary electrophoresis. However, the pectins yielded precipitable fractions HPC and RPC by treatment with cetyltrimethylammonium hydroxide (Cetavlon) in the yields of 89% and and 64.5%, respectively, with changed sugar composition (Table 2). Whereas the galacturonic acid content increased slightly in both pectins, more galactose than arabinose was lost from HP, and mainly arabinose from RP. The appearance of new signals at δ 163.4, 141.3, and 112.5 in the ^{13}C NMR

spectra of HPC and RPC correponding[15] to unsaturated structures indicates ß-eliminative degradation[16] of the galacturonan chains under the alkaline precipitation conditions. Based on the recent structural conception of pectic polysaccharides,[17] we assume that a part of arabinose- and galactose-containing side-chains was released during the β-eliminative degradation of the pectins. The higher recovery of HPC is consisent with the higher alkali-stability of low methoxy pectins.[3] However, the presence of co-extracted neutral polysaccharides[18] cannot be ruled out.

In the ^{13}C NMR spectra of the deesterified HP and RP (K^+ form, D_2O) already assigned[10,13] signals of (1->4)-α-D-galacturonosyl moieties are dominating. For HP these signals were observed at δ 100.4-101.0 (C-1), 69.4 (C-2), 69.9 (C-3), 79.2 (C-4) 72.3 (C-5), and 174-176 (C-6). Corresponding signals in the spectra of HPC and RPC, which gave gels in DMSO-d_6, were broader and shifted upfield, *e.g.*, at δ 98.6, 68.0, 68.1, 76.7, 69.6, and 170.2. Their complex of at least five signals at δ 108.6-107.3 was related to C1 of α-L-arabinofuranosyl residues engaged[10,19,20] in various linkages. In spite of the high abundance of arabinose in HP and HPC, only resonances of C1 to C5 of terminal (δ 108.42, 82.56, 77.80, 85.40, and 62,34) and 5-linked (δ 108.62, 82.01, 78.01, 83.4, and 68.50) α-L-arabinofuranosyl residues could be well resolved in HP. This indicates that these units are located in mobile chains. The signal at high field (107.3 ppm) is typical for C1 of arabinose linked in position 2.[21-23] In the anomeric region, where the resonance of β-D-galactopyranosyl residues is known,[23,24] several unresolved signals at δ 104.5-105.8 were present in all pectins studied. Similarly, the complexity of C-5 and C-6 resonances at δ 61.0-70.0 reflects a variety of linkage types of the galactose component. In both pectins the presence of rhamnose and fucose is documented by small resonances at δ 17.5- 18.2.[21]

The complex nature of both arabinose and galactose components in HP and RP derived from their ^{13}C NMR spectra was confirmed by the methylation analysis (Table 3). Due to

Table 3. Methylation analysis of HP, RP, and the carboxyl-reduced, Cetavlon-precipitated fractions HPC-R and RPC-R

Sugar residue	Linkages	Glycosidic linkage composition, mol.%[a]			
		HP	HPC-R	RP	RPC-R
Rhap	Terminal[b]	1.7	1.8	3.9	1.8
	1-2	tr	2.4	0	tr
	1-2,3	tr	0	tr	1.1
	1-3,4	1.3	2.0	2.7	0
	1-2,3,4	2.3	0.8	2.6	0.8
Araf	Terminal	13.9	10.6	16.5	2.0
	1-2	4.5	1.2	0.8	1.1
	1-3	7.9	3.8	0.3	0
	1-5	19.7	8.4	2.5	1.9
	1-2,5	5.5	5.7	3.1	0
	1-3,5	1.5	3.4	0.5	0.4
Galp	Terminal	7.6	8.6	9.8	8.9
	1-3	2.6	2.2	4.3	1.1
	1-6	0.5	0	1.1	4.9
	1-3,6	4.8	7.0	17.9	3.1
	1-2,3,4,6	0.7	0	2.1	4.0
	1-4	10.9	34.5[c]	12.1	62.7[c]
	1-3,4	1.7	3.6[c]	1.2	2.7[c]
	1-2,3,4	1.5	0	1.0	0.9
Glcp	Terminal	0	0.4[c]	0	1.9[c]
	1-4	5.9	2.4	6.1	2.7
	1-4,6	2.3	1.2	4.7	tr
Xylp	Terminal	0.5	tr	3.0	tr
	1-4	1.5	tr	4.9	tr

a. Partially methylated alditol acetates were determined on SSQ-710 capillary column.
b. Terminal fucopyranosyl units are included.
c. Deuterium-labelled.
tr, traces.

losses of the highly methylated alditol acetates during concentration in vacuo, only qualitative conclusions could be drawn from the methylation analysis data. Arabinofuranose was mainly terminal, (1->5)-, (1->3,5)-, and (1->2,5)-linked, indicating a highly branched arabinan. The presence of 2-

and 3-linked arabinosyl residues is indicative for arabinosyl-protein linkages.[25] Both pectins contained ~1% of proteins rich in Asp, Glu and Ser (See Experimental). Proteins found in purified pectins[26,27] were assumed to be implied in arabinogalactan-proteins linkages. Galactopyranose was mainly terminal, (1->4)- and (1->3,4)-linked in HP. Higher proportions of (1->3)- and (1->3,6)-linked galactopyranosyl residues were detected in RP. This suggests the presence of arabinogalactan type I and II.[28] Arabinogalactan I was reported to build neutral side-chains in the pectin isolated from apple,[1] sugar beet,[13] onion,[23] and apricot.[12] Although type II is more abundant in arabinogalactans of coniferous woods,[28] it was detected also in pectic polysaccharides isolated from sugar beet,[7] grape,[26] and apple.[17,19]

The linkage type of xylose and glucose units as well as the detection of deuterium-labelled glucose in the carboxyl-reduced pectins pointed out the presence of co-extracted hemicelluloses of the glucuronoxylan and glucan types.

The high abundance of 3,4- and 2,3,4-linked rhamnosyl residues implies a high branching of the "hairy" regions in both pectins. After a partial digestion of deesterified HP and RP with EPGase, polymeric fractions with $\bar{M}w$ = 13,000 and $\bar{M}_w$ = 15,200, respectively (Tab. 4), were isolated indicating that both pectins contain large "smooth" regions. HPGPC of the fractions showed very small proportions (<5%) of a high molecular-weight component which was visible as a shoulder in the chromatogram of the original samples and may contain the neutral carbohydrates.[29] In the ^{13}C NMR spectra, sharp signals of the galacturonan chain dominated. Besides the minor signals at δ 97.5, 93.2, 71.2, 72.1 and 73.9 assigned to the resonances of terminal residues[21,22] no signals corresponding to the neutral sugar components were observed.

The presence of free carboxyl groups of the galacturonan chain in the pectin molecule determines its ability to interact with oppositely charged molecules. In this connection it was interesting to study the binding of divalent

Table 4. Molecular weight distribution of the polymeric fractions obtained after digestion of HP and RP with EPGase.

Pectin	$\bar{M}_w$	$\bar{M}_n$	$D = \bar{M}_w/\bar{M}_n$
HP-P	13,100	7,700	1.70
RP-P	15,200	7,900	1.92

cations to carboxyl groups of HP and RP, especially with regard to the toxic cation binding.

Due to possible degradation of pectins during storage, prior to these experiments, both pectins were purified with 60% acidified ethanol (See Experimental). As seen from Table 1, the purification procedure caused changes in the composition and molecular properties of both pectins. Their galacturonate content increased slightly to approximately 80%, accompanied by an increase of DE in RP. HPGPC revealed that in the case of the low molecular-weight HP, a part was degraded and solubilised by the aqueous ethanol, decreasing the DE, $[\eta]$, and the polydispersity, but increasing slightly $\bar{M}_w$. In the case of RP, $[\eta]$ decreased only $\sim$10%, whereas the average-M_w lost 1/3 of its original value. It is evident that $[\eta]$ does not reflect changes of molecular properties of pectic polysaccharides[30] indicated by HPGPC. From the broad molecular-weight distribution curve, two fractions separated. The first one ($\sim$10%) remained at $\bar{M}_w$ = 600,000 and the main peak was shifted to lower value ($\bar{M}_w$ = 150,000). The results confirm that the high methoxy pectins are more susceptible to degradation than pectins with low DE.

Our earlier studies[4,5,31] proved that divalent cations are bound to carboxyl groups of pectin stoichiometrically. The results obtained with divalent cations Ca^{2+}, Cd^{2+}, Pb^{2+}, and Cu^{2+} are presented in Table 5. The results for purified commercial citrus and apple pectins are given for comparison. In the case of Ca^{2+}, the binding is expressed as the degree

Table 5. Binding properties of various pectins.

Pectin	GalU %	DE %	$\gamma_{Ca^{2+}}$	Association degree, β			
				Ca^{2+}	Cd^{2+}	Cu^{2+}	Pb^{2+}
HP	80.6	11.9	0.099	0.911	0.913	0.981	0.969
RP	78.5	64.0	0.436	0.463	0.700	0.906	0.848
CP	90.6	61.4	0.395	0.518	0.701	0.944	0.903
AP	76.0	61.7	0.405	0.503	0.687	0.919	0.888

CP - citrus pectin; AP - apple pectin.

of association β and as the activity coefficient γ_{Ca2+} as well. Both values are considerably dependent on DE similarly as in the case of Cd^{2+} binding. The association degree for Ca^{2+} and Cd^{2+} decreases with increasing DE of pectin. On the other hand, DE does not influence Pb^{2+} and Cu^{2+} binding appreciably; both cations are almost quantitatively bound to carboxyl groups of pectin even at DE ≈ 60%. However, the binding of Me^{2+} studied is not dependent on the uronic acid content provided that this content is relatively high, as it is in our case. Therefore, binding is practically independent of the content of neutral sugar components as well as of the pectin origin. It was also confirmed that the molecular weight of pectin is the factor not influencing the divalent cation binding. The activity coefficient of Ca^{2+} ions bound to carboxyl groups of oligomeric pectic acid fragments with degree of polymerization DP ≥ 20 exhibits almost the same value as that found for polymeric calcium pectate.[31]

It can be seen that the binding capacity of HP and RP is similar to that of commercial pectins; it increased in the order $Ca^{2+} < Cd^{2+} < Pb^{2+} < Cu^{2+}$. The conclusion about divalent cation binding presented in this paper is in good agreement with our previous works[4,5,31] where the influence of the linear charge density of citrus pectin on the binding of various divalent cations was studied in detail.

EXPERIMENTAL

Materials and general procedures. Hawthorn berries and the stalks of wild grown rhubarb were collected near Ulan-Bator, Mongolia, in August 1986. The endo-polygalacturonase (EPGase) from tomato[32] was kindly supplied by Dr. O. Markovič (Institute of Chemistry, Bratislava). Two commercial pectins were used; citrus pectin (Genu Pectin, Medium Rapid Set, Type A, Pektinfabrik, Copenhagen, Denmark) and apple pectin (East Bohemian Canning Factories and Distilleries, Smiřice, Czechoslovakia). Pullulan standards were from Shodex Standart P-82, Macherey-Nagel GmBH & Co KG, Germany.

The procedures for total hydrolysis, quantitative analysis of sugars, determination of proteins, amino acid analysis, free-boundary electrophoresis, optical rotation, and IR and ^{13}C NMR spectra were already described.[33,34] The amino acid composition[12] (mol. %) of HP was as follows: Asp (18.4), Glu (16.0), Thr (2.8), Ser (8.1), Pro (6.0), Gly (3.3), Ala (4.8), Ile (6.4), Leu (6.1), Tyr (5.3), Phe (5.6), His (5.6), Lys (6.1), and Arg (2.9); that of RP was: Asp (15.7), Glu (10.7), Thr (2.9), Ser (4.9), Pro (5.6), Gly (6.8), Ala (4.5), Val (1.6), Ile (4.3), Leu (7.2), Tyr (5.7), Phe (7.9), His (5.5), Lys (7.5), and Arg (7.2). The content of uronic acid (expressed as K^+ salt of galacturonic acid) and DE were determined potentiometrically as well as by the method of precipitation of copper pectate and pectinate.[35,36] HPGPC was performed on Tessek Separon HEMA BIO-100 and HEMA BIO-1000 columns calibrated with the Pullulan standards P-5, P-10, P-20, P-50, P-100, P-200, P-600, and P-800. For molecular-weight distribution and $\bar{M}_w$, $\bar{M}_n$ determination, a computing procedure[37] based on the linear effective calibration curve was applied.

Isolation and purification of pectins. The air- dried rhubarb stalks were ground (particle size, 0.25 mm) and extracted with benzene-ethanol (2:1, v/v) under reflux for 8 h. The isolation of pectin was performed in the pilot plant of the Mongolian Institute. The extractive-free material was

suspended in 1M oxalic acid (pH = 2) and stirred at 70 ^{0}C for 2 h. The acidic extract was poured into four volumes of 96% ethanol. The precipitate formed was treated with HCl-acidified 80% ethanol (pH=3), washed subsequently with 80-96% ethanol and acetone to give the crude pectin. The hawthorn berries were washed with cold water, mixed in distilled water for few minutes and then, the plant residue was removed by filtration. From this material, the crude pectin was subsequently isolated under the above mentioned conditions. The crude pectins were purified by washing with 60% acidified ethanol (5 mL 37% HCl/100 mL), 60% and 96% ethanol. The purified pectins (HP, RP) were dissolved in distilled water by neutralization with 0.05M KOH to the point of equivalence and lyophilized. The same procedure was used for purification of the commercial citrus and apple pectins.

The studies on binding were performed 12 months later. For this purpose, HP and RP were purified and characterized immediately before starting these experiments, using the same procedures as described above. Analysis of these samples (HP1, HP2) is given in Table 1.

HP and RP (2g) dissolved in water (200 mL) were precipitated with Cetavlon (HO^{-}form).[38] The precipitates were separated by centrifugation, washed with water, acidified, and recovered after dialysis (24 h) by lyophilization as HPC and RPC.

Carboxyl- reduction. Carboxyl-reduction of HPC and RPC was effected by the method of Taylor and Sandford[39] using 1--cyclohexyl-3-(2-morpholinomethyl)carbodiimide and sodium borodeuteride. After three treatments, more than 90% of the uronic acids was reduced (carbazol assay). The reduced samples HPC-R and RPC-R were recovered in the yields of 82.7 and 92.2%, respectively.

Methylation analysis. The polysaccharides (100 mg) were methylated according to Ciucanu et Kerek[40] as already described.[34] Methylation was monitored by IR spectroscopy. The methylated products exhibited a negligible absorption of hydroxyl groups. They were converted into alditol acetates

which were analysed and identified by GLC and GC-MS[41] using the molar response factor of Sweet et al.[42]

Enzymatic digestion of pectins. Prior to enzymatic digestion, HP and RP were deesterified with 0.1M NaOH at 10 ^{0}C for 16 h. The EPGase was added (0.5 mL, 50 units/g pectin) to the solutions (0.5%) of deesterified pectins in 0.1M sodium acetate buffer (pH=4.5) containing 0.1M NaCl. The solutions were incubated at 30 ^{0}C and the hydrolysis was checked by the decrease of relative viscosity.[43] After 120 min of incubation time, the viscosity drop levelled off in both pectin solutions. Precipitation with ethanol (1:5, v/v) from the non-dialysable portion afforded fractions HP-P and RP-P, which after centrifugation and subsequent dissolution, were lyophilised. The dialysable and ethanol-soluble parts were collected and freeze-dried, but they are not included in the present study.

The activity determination. The activity of Ca^{2+} ions was determined by the metal-indicator method using tetramethylmurexide as metallochromic indicator.[44,45] The measurements were carried out in calcium pectinate solutions of concentration 3.00 mmol ($-COOCa_{0.5}$)/L without further electrolyte addition. The activities of divalent cations (Cd^{2+}, Cu^{2+}, Pb^{2+}) bound to free carboxyl groups of pectins studied were estimated[46] by ion specific electrodes at 25 ^{0}C. The suspensions contained potassium pectinates in concentration of c_{COOK}=3.0 mmol/L and the corresponding divalent salts [$Cd(NO_3)_2$, $Cu(NO_3)_2$, $Pb(NO_3)_2$] in the concentration of 1.5 mmol/L. The ionic strength of the starting solution was adjusted to 0.01mol/L (KNO_3). The concentration of free divalent cations was measured by ionspecific electrodes and the binding was evaluated according to the degree of association ß which is defined as : $\beta=(c_{Me2+})_{bound}/(c_{Me2+})_{added}$.

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