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CARBOHYDRATES OF *Symphytum asperum*

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It has been shown that the carbohydrates of *Symphytum asperum* L. — a promising fodder plant — include water-soluble polysaccharides, pectin substances, protopectin, hemicelluloses, a glucomannan, and cellulose. The quantitative amounts of the polysaccharide fractions in the raw material have been established and their characteristics are given. The protopectins of the leaves and stems of *Symphytum asperum* L. have been characterized. Their macromolecules are based on a fragment constructed of D-galacturonic acid residues linked by α-glycosidic bonds.

Symphytum asperum L. (prickly comfrey) is a perennial plant of the family Boraginaceae which is distinguished by valuable biological characteristics and a high crop yield and is promising as a fodder and foodstuff.

The Boraginaceae contain alkaloids, vitamins, polyphenols, and other physiologically active compounds and have long been used in folk medicine [1, 2]. We have shown previously [3] that the comfrey contains a considerable amount of protein and ash substances, free sugars, and polysaccharides. The free sugars are represented by sucrose, glucose, and fructose, and accumulate mainly in the stems.

TABLE 1. Amounts of Polysaccharides in the Leaves and Stems of *Symphytum asperum* L. (% on the absolutely dry weight of the substance) and Their Monosaccharide Compositions

Extractant	Object of investigation	Amt. of polysaccharide	Monosaccharide (molar ratios)								
			UA	Gal	Glc	Man	Ara	Xyl	Rha	Rib	unidentified sugar
Water, 20°C	Leaves	4.01	6.0	1.0	0,9	—	2,1	0,5	1.1	1,0	Tr.
	Stems	2,47	6.9	3.9	1.1	—	4.3	1,9	0.9	1,2	Tr.
Water, 90°C	Leaves	0,33	3,6	5,2	1,8	—	4,3	1,6	5.6	2,0	1.9
	Stems	0,81	1.5	7,8	1,0	—	7,3	1,4	3,3	0,4	Tr.
0.5% ammonium oxalate	Leaves	1,27	10,0	1.1	0,7	—	0,9	Tr.	1.7	0,8	0,3
	Stems	3,69	11,2	1,0	0,5	—	1,1	0,5	1,9	0,9	0 5
10% KOH	Leaves HMC A	5,39	—	6,0	24,0	—	13,0	15,0	Tr.	Tr.	—
	Leaves HMC B	3,63	1.4	1,1	1,6	—	1,2	14,0	Tr.	Tr.	—
	Stems HMC B	7,68	4,2	0,6	1 8	—	0.5	7,5	Tr.	Tr	—
18% NaOH + 4% H_3BO_3	Leaves	1 06	—	—	0.3	28,3	—	12 5	—	—	Tr.
	Stems	1,23	—	—	1,3	15,2	—	5.6	—	—	Tr.
Residue	Leaves	5,09	—	—	19,5	4.7	—	2,0	—	—	—
	Stems	12,45	—	—	10,0	—	—	1,8	—	—	—

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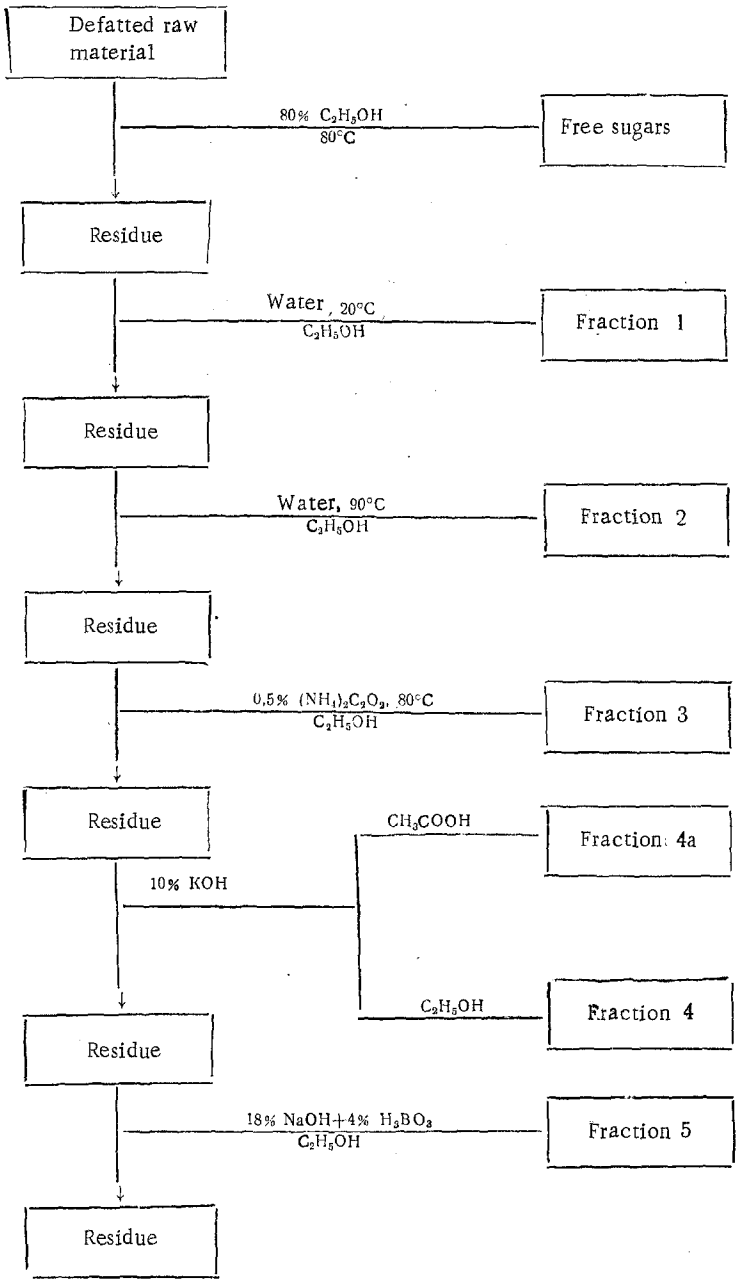

Scheme of fractionation of Symphytum asperum L.

To characterize the carbohydrates of *Symphytum asperum* L., the polysaccharides of the leaves and stems were fractionated separately as shown in the scheme (Table 1). The fractions isolated by cold and hot water made up more than 50% of the dry matter of *Symphytum asperum* L. In the monomeric compositions of their polysaccharides, these fractions were similar to one another. Uronic acids predominated in the hydrolysates of the polysaccharides isolated by cold water, and neutral sugars — arabinose, galactose, and rhamnose — in the fractions isolated by hot water.

In hydrolysates of the polysaccharides isolated by water and 0.5% ammonium oxalate, unidentified sugars were found which were more mobile chromatographically than rhamnose — apparently methyl derivatives of xylose. The presence of glucose is the result of the hydrolysis of the starch present in the leaves, which was confirmed by the amylolysis of the polysaccharides of the aqueous fraction of the leaves.

On a column containing Sephadex G-200, the water-soluble polysaccharides were separated into two fractions, which shows their heterogeneity in relation to molecular mass.

The amount of polysaccharides isolated by ammonium oxalate was greater in the stems than in the leaves. Their hydrolysates consisted mainly of galacturonic acid, neutral sugars being present in only small amount.

TABLE 2. Monosaccharide Compositions of the Fractions of Protopectins Obtained by Chromatography on DEAE-cellulose

Eluent	Yield of the fraction, %	Monosaccharide composition, %							
		Gal·UA	Gal	Gl	Ara	Xyl	Ram	2-O-Me-Xyl	unidentified sugar
0.5M NaH_2PO_4	19.3 16,7	14,89 17,31	36,52 32,08	2,62 0,98	20,87 24,21	8,26 5,99	14,17 17,29	2,67 2,08	— —
0.3 M NaOH	46,7 48,1	70,02 74,42	4,98 3,72	Tr. —	5,01 6,01	8,68 6,78	6,76 5,41	2,55 2,03	Tr. Tr.
0,5 M NaOH	30,4 29,9	78,89 77,96	4,21 3,99	Tr. —	3,97 4,47	2,77 2,98	2,98 1,47	Tr. Tr.	— —

Note. The numerators give the amounts of monosaccharides in the fractions of the protopectins from the leaves, and the denominators those of the protopectin in the stems.

The IR spectra of the polysaccharides contained the following absorption bands: 1745 cm^{-1} (stretching vibrations of the carbonyls of carboxy groups), 1600-1400 cm^{-1} (vibrations of an ionized carboxyl), 1000-1150 cm^{-1} (stretching vibrations of a pyranose ring), 760-960 cm^{-1} (symmetrical and asymmetrical vibrations of a pyranose ring).

The large amount of galacturonic acid (more than 70%), the positive specific rotation of the polysaccharides of the leaves $[\alpha]_D^{20}$ +203° and of the stems $[\alpha]_D^{20}$ +198°, and the results of IR spectroscopy permitted these polysaccharides to be assigned to the class of pectin substances.

The fractions isolated by 10% KOH contained considerable amounts of carbohydrates. From an alkaline extract of the leaves, after neutralization with acetic acid, we isolated HMC fraction A, and from the supernatant ethanol precipitated HMC fraction B. From the stems it is possible to isolate only HMC fraction B.

HMC A contained 60% of the total amount of alkali-soluble polysaccharides of the leaves, but the amount of reducing substances (RSs) in a hydrolysate was low — 8.98%. This fraction was more than twice as rich in nitrogen and mineral substances as HMC B. Chromatography of a hydrolysate of HMC A showed the presence only of neutral sugars.

The HMC B of the leaves and that of the stems were identical in their monosaccharide compositions. Among the monosaccharides, xylose, uronic acids, and glucose predominated.

The negative specific rotations and the presence of an absorption band at 890 cm^{-1} in the IR spectra of the polysaccharides permitted the assumption of the presence of a β-glycosidic bond between the sugars in the HMC B of the leaves and stems.

Gel chromatography of the polysaccharides on a column of G-200 showed their polydispersity. The amounts of carbohydrates in the fractions isolated by 18% NaOH in the presence of boric acid from the leaves and stems were approximately the same. The hydrolysates contained mainly mannose and glucose. Impurities of noncarbohydrate nature were present in very small amount. Solutions of the polysaccharides had a negative rotation: For the leaves, $[\alpha]_D^{20} = -40.1°$, and for the stems $[\alpha]_D^{20} = -35.2°$, which shows the β configuration of the glycosidic bonds between the monose residues.

The polysaccharides forming the residues after extraction consisted mainly of glucose with a small amount of xylose. They differed in their contents of mineral matter. The high glucose content of the hydrolysates and the IR spectra of the polysaccharides showed the presence of a glucan of the cellulose type. There was 2.5 times more of it in the stems than in the leaves, which is due to the different functional roles played by the morphological parts of the plant. The protopectins of the leaves and stems of *Symphytum asperum* L. — the polysaccharides isolated by means of 0.5% ammonium oxalate — were studied in more detail. The preparations obtained formed viscous solutions on being dissolved in water.

The carbohydrate composition of the polysaccharide was determined after acid hydrolysis by PC and GLC. The protopectins obtained were heteropolysaccharides containing not only galacturonic acid residues but also the residues of neutral sugars, among which rhamnose and

galactose predominated. An unidentified sugar chromatographically more mobile than O-Me-xylose was found in both hydrolysates. The depth of hydrolysis was 37.0% for the protopectin of the stems and 414% for that of the leaves.

As the result of hydrolysis with 2 N H_2SO_4, partial decarboxylation of the galacturonic acid took place, and therefore its amount was determined in parallel by the carbazole and titrimetric methods.

The protopectins of the leaves and of the stems differed only slightly from one another in their amounts of functional groups. The amount of impurities of noncarbohydrate nature (ash, nitrogen) in the preparation isolated from the leaves was twice as great as that from the stems. Below we give the characteristics of the protopectins of *Symphytum asperum* (% on the weight of the absolute dry substance):

Object of investigation	Amount of galacturonic acid determined by the following methods		Nitrogen	Ash	$[\alpha]_D^{20}$	Molecular mass	K_f	K_e	OCH_3
	carbazole	titrimetric							
Leaves	66.33	66.98	1.09	1.93	222	17000	16.91	5.08	2.95
Stems	69.44	71.24	0.55	0.70	228	20000	17.88	6.24	3.41

The heterogeneity of the polysaccharides isolated was determined by chromatography on DEAE-cellulose in the phosphate form. The polysaccharides were separated into fractions by using gradient elution with water, sodium dihydrogen phosphate, and NaOH. Elution with phosphate buffer gave a single fraction and removed a small amount of polysaccharide. About 80% of the polysaccharide was eluted with the aid of increasing concentrations of NaOH (Table 2).

The fractions of acidic polysaccharides obtained differed in their amounts of galacturonic residues. Neutral sugars predominated in the fractions isolated by 0.5 M NaH_2PO_4, and galacturonic acid in the fractions isolated by NaOH.

The gel chromatography of the polysaccharides on a column of Sephadex G-200 showed their polydispersity. When the protopectins were separated according to their molecular masses, two fractions were obtained: a low-molecular-mass fraction (elution volume v_e = 28 ml) and a high-molecular-mass fraction (volume v_e = 40 ml). The high-molecular-mass fraction of the protopectin of the stems amounted to 91.7%, and that of the leaves to 97.2%.

Partial acid degradation of the protopectin yielded a fraction consisting of D-galacturonic acid residues. The yield of polygalacturonan from the stems was twice that from the leaves. The polyuronide fragments contained 81.27 and 80.01% of galacturonic acid, 3.12 and 3.07% of methoxy groups, and 23.72 and 21.90% of free carboxy groups for the stems and leaves, respectively. Their molecular mass was half that of the initial polysaccharides.

In the galacturonan, the D-galacturonic acid residues were present in the pyranose form and were linked by α-glycosidic bonds, as was shown by the results of IR spectroscopy and the high positive specific rotation.

Saponification of the pectin substances formed pectinic acids. Their yields were: for the leaves, 18.63%, and for the stems 49.93%; the values of $[\alpha]_D^{20}$ were, respectively +217° and +213°.

The complete hydrolysis of the pectic acids showed the presence of galacturonic acid and the same monosaccharides as those from which the initial protopectins were constructed.

EXPERIMENTAL

Preparation of the Raw Material. The plant was gathered in the Botanical Garden of the Academy of Sciences of the USSR at the budding stage. The comminuted herbage was defatted with ether in a Soxhlet apparatus, and the free sugars were eliminated by treatment with 82% ethanol on heating. Then the raw material was dried in the air and analyzed.

Fractionation of the Polysaccharides. The raw material (10 g) after the elimination of ether- and ethanol-soluble substances was stirred several times with cold water (ratio 50). The solid phase was separated by centrifugation, and the extract was evaporated under vacuum and treated with ethanol in a ratio of 1:4. The precipitate was treated similarly with hot water. The solution was separated off, and the polysaccharides were extracted from the residue with a 0.5% solution of ammonium oxalate at 80°C. Then extraction was carried out with 10% KOH. The resulting solution was cooled rapidly with ice water and was acidified

with acetic acid to pH 4-5. The precipitate of HMC A that deposited was separated off by centrifugation, and the solution was poured into ethanol. Treatment with 18% NaOH in the presence of 4% boric acid was carried out in the same way. The residue after all the extractions was washed with water until the wash-waters were neutral and was dried. The completeness of extraction was checked by the anthrone method.

Isolation of the Protopectin. The polysaccharides isolated by 0.5% ammonium oxalate at 80°C were purified by two reprecipitations from ammonium oxalate solution and were washed with ethanol acidified with hydrochloric acid to pH 2.5-3.0, and then with 96% ethanol, and were dried over P_2O_5.

Hydrolysis and Identification of the Monosaccharides. The polysaccharides were hydrolyzed with 2 N H_2SO_4 in sealed tubes at 100°C for 16 h. The monosaccharides were identified by the GLC and PC methods using the following solvent systems: 1) 1-butanol–benzene–pyridine–water (5:1:3:3), 2) ethyl acetate–acetic acid–formic acid–water (18:3:1:4); and 3) ethyl acetate–acetic acid (4:3:1).

GLC was performed on a Chrom chromatograph with a flame-ionization detector. The stationary phase was 5% of XE-60 on a Chromaton N-AW-DMCS (0.2-0.25 mm) in steel columns (1200 × 3 mm). The rate of flow of carrier gas (helium) was 35 ml/min. The temperature was programmed at 2°C per minute in the range from 140 to 220°C. The monosaccharides were analyzed in the form of the alditol acetates [5].

The amylolysis was carried out as described by Kozhina and Mamatova [6].

Gel Chromatography on G-200. A 0.8-1.0% solution of the polysaccharides was deposited on a 40 × 2 cm column filled with Sephadex and was eluted with the corresponding solvent. The eluates were collected in 3-ml portions and the carbohydrates were analyzed by the anthrone method and the protein substances by Lowry's method.

IR spectra were taken on a UR-20 instrument in the wavelength intervals of 4000-2000 cm^{-1} (LiF prism) and 2000-700 cm^{-1} (NaCl prism). The samples were prepared by molding the polysaccharides in KBr [7].

Fractionation was performed on DEAE-cellulose treated as described by Neukom et al. [8]. The fractions were monitored by the anthrone and carbazole methods [9]. The eluates belonging to a single peak were combined, dialyzed against water to neutrality, and hydrolyzed with 2 N H_2SO_4. The qualitative and quantitative composition of the monosaccharides and the amount of galacturonic acid in the hydrolysates were determined.

The isolation of the polyuronide and the preparation of the pectic acid were carried out as described by Solov'eva et al. [10]. The polysaccharides were hydrolyzed with 1 N H_2SO_4 at 100°C for 6 h.

SUMMARY

The carbohydrates of the leaves and stems of *Symphytum asperum* L. contain water-soluble polysaccharides (of the type of arabinogalactan and pectin substances), protopectin, hemicelluloses, a glucomannan, and cellulose. The HMCs consist of a complex group of polysaccharides including xylose, glucuronic acid, glucose, arabinose, and galactose residues.

The protopectin forming a component of the carbohydrate complex of *Symphytum asperum* L. is itself a complex of high-molecular-mass acidic products. The macromolecule is based on a fragment constructed of D-galacturonic acid residues linked with one another by α-glycosidic bonds. Among the neutral sugars, rhamnose, galactose, and arabinose predominate.

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AMPLIFICATION METHOD FOR THE TITRIMETRIC DETERMINATION OF L-RHAMNOSE BY PERIODATE OXIDATION

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The stoichiometry of the oxidation of rhamnose by periodate has been studied using a method based on the determination of iodate. Conditions have been found under which one mole of rhamnose stoichiometrically reduces six moles of periodate. An amplification method for the titrimetric determination of rhamnose is proposed in which one mole of rhamnose reacts with 6 moles of periodate with the formation of six moles of iodate, which is equivalent to 18 moles of triiodide, in the titration of which 36 gram-equivalents of sodium thiosulfate are consumed. The method is distinguished by high sensitivity and accuracy. The relative standard deviation in the determination of 2-3.5 mg of rhamnose does not exceed 0.5-1%.

L-Rhamnose (6-deoxy-D-mannose) is a methylpentose and is present as a component of numerous plant and bacterial polysaccharides and also of plant glycosides. In contrast to other aldoses and ketoses, in an aqueous solution rhamnose mutarotation is completed rapidly — in 10-60 min at 20°C — and a first-order kinetic equation is applicable to the rate of mutarotation of rhamnose in water [1]. The latter fact indicates that in a solution of rhamnose of appreciable concentration not more than two tautomeric forms of the substance are present. Consequently, it was to be expected that the periodate oxidation of rhamnose would take place more rapidly and more simply than in the case of other aldoses and ketoses [2, 3].

The information in the literature on the periodate oxidation of rhamnose is sparse and, at the same time, extremely contradictory, which can be explained by differences in the conditions of performing periodate oxidation and the methods of final determination. Formic acid is determined by visual titration [4, 5] — 1 mole of rhamnose yields 4 moles of HCOOH in 20 min at 100°C and in 45 h at room temperature. On the basis of a determination of the excess of periodate it has been established that in 3-24 h 3.56-3.64 moles of periodate [6] and at pH 3.6 and room temperature after 20 h 3.52 moles of periodate [7] is consumed per 1 mole of rhamnose. The excess of periodate is found by thermometric titration with a solution of hydrazine sulfate [8]. A method has been proposed for determining rhamnose which is based on the determination of acetaldehyde by the bisulfite method [9, 10].

It appeared of interest to ascertain the possibility of determining rhamnose by the amplification method [11] proposed for the determination of other aldoses and ketoses [2, 3]. In the amplification method, after the end of the periodate oxidation reaction the excess of periodate is masked by the addition of an excess of molybdate and the iodate is converted into the equivalent amount of triiodide.

$$IO_3^- + 8I^- + 6H^+ \rightarrow 3I_3^- + 3H_2O, \quad (1)$$

which is titrated with a solution of sodium thiosulfate [1].

The development of methods of determining monosaccharides and their derivatives based on the determination of iodate [2, 3] requires a careful study of the stoichiometry in order to choose the optimum conditions of oxidation.

We have been unable to find in the literature a stoichiometric equation for the oxidation of rhamnose by periodate. We have experimentally found linear sections on the curves

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