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Water-Soluble Constituents of Rehmanniae Radix. I. Carbohydrates and Acids of *Rehmannia glutinosa* f. *hueichingensis*

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The water extract of the roots of *Rehmannia glutinosa* Libos. forma *hueichingensis* Hsiao was fractionated by the chromatographies on two types of ion-exchange resin. Fifteen amino acids and D-glucosamine were found and determined in the basic fraction. Phosphoric acid was detected and estimated as a component of the acidic fraction. Neutral fraction, the main fraction of the water extract, was mostly constituted with carbohydrates. D-Glucose, D-galactose, D-fructose, sucrose, raffinose, manninotriose, stachyose, verbascose and D-mannitol were identified and determined. It was found that stachyose is the main component of the water extract and the yield of the substance was 48.3% from the dried material.

The roots of *Rehmannia glutinosa* Libos. have long been used in Chinese medicine for the purpose of blood-making, analeptic and antipyretic. On the constituents of this crude drug, a few substances, that is, β-sitosterol,[2] mannitol, sucrose and catalpol[3] have been reported until present time.

We have now fractionated the water extract from the roots of *Rehmannia glutinosa* Libos. forma *hueichingensis* Hsiao, into three fractions by the chromatographies on Dowex 50 W and Dowex 2. The yields from the dried weight of the material were 74.4 % in neutral fraction, 3.3% in basic fraction and 7.2% in acidic fraction. These procedures are shown in Chart 1.

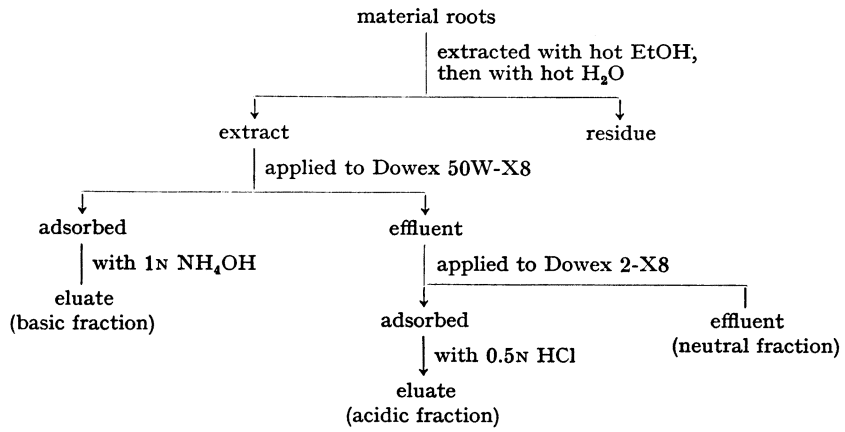

Chart 1. Fractionation of Water-Soluble Constituents

The neutral fraction was applied to a column of active charcoal and several fractions were eluted with water and further stepwise increments of ethanol. Each fraction was examined by cellulose thin-layer chromatography and component sugars were purified by repeated charcoal column chromatography and paper chromatography. D-Glucose, D-galactose, D-

1) Location: *6-3, Shibakōen, Minato-ku, Tokyo.*
2) Z. Sakura, *Toyama Yakusen Kiho*, **2**, 125 (1936).
3) I. Kitagawa, T. Nishimura, A. Kobayashi and I. Yosioka, *Yakugaku Zasshi*, **91**, 593 (1971).

fructose, sucrose(β-D-fructofuranosyl α-D-glucopyranoside), raffinose(β-D-fructofuranosyl O-α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside) and stachyose (β-D-fructofuranosyl O-α-D-galactopyranosyl-(1→6)-O-α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside) were identified. In addition to these monosaccharides and oligosaccharides, the presences of manninotriose (O-α-D-galactopyranosyl-(1→6)-O-α-D-galactopyranosyl-(1→6)-D-glucopyranose) and verbascose (β-D-fructofuranosyl O-α-D-galactopyranosyl-(1→6)-[O-α-D-galactopyranosyl-(1→6)]$_2$-α-D-glucopyranoside) were proved by determination of their component sugars, analyses of methylation products, periodate oxidation and enzymic transformation. From the results of gas–liquid chromatography of trimethylsilyl derivatives, D-mannitol was also detected and determined. The contents of them in the neutral fraction are shown in Table I. Analytical data by chromatographies showed the presences of several unknown carbohydrates in the neutral fraction, but they must be minor components.

Table I. Contents of Carbohydrates in the Neutral Fraction

Carbohydrates	Contents (%)	Carbohydrates	Contents (%)
Glucose	3.1	Raffinose	4.9
Galactose	0.7	Manninotriose	4.4
Fructose	1.7	Stachyose	64.9
Mannitol	1.8	Verbascose	4.2
Sucrose	5.2		

The basic fraction was examined by two dimensional cellulose thin-layer chromatography and fifteen amino acids were detected. The analyses and determinations of amino acids were also performed by the use of an amino acid analyzer. In addition to these amino acids, D-glucosamine was detected by thin-layer chromatography and by gas chromatography of its trimethylsilyl derivative and it was estimated colorimetrically. The results are shown in Table II.

Table II. Contents of Amino Acids and Sugar in the Basic Fraction

Components	Contents (%)	Components	Contents (%)
Lysine	0.3	Alanine	0.9
Histidine	0.4	Valine	0.7
Arginine	8.7	Isoleucine	0.9
Aspartic acid	3.9	Leucine	0.5
Glutamic acid	5.3	Tyrosine	0.3
Threonine	1.1	Phenylalanine	0.6
Serine	0.9	γ-Amino butyric acid	0.4
Glycine	0.1	Glucosamine	1.4

The acidic fraction was examined by cellulose thin-layer chromatography and phosphoric acid was detected. The result of colorimetric determination of this acid showed the content in the fraction is only 1.30%.

In conclusion, it was revealed that stachyose is the chief element of the material and the other several oligosaccharides belonging to "raffinose family" and monosaccharides composing these oligosaccharides were also found as secondary main components. Although

stachyose has been isolated from different plant species more than forty[4a-c], the root of *Rehmannia glutinosa* f. *hueichingensis* must be one of the richest source of the tetrasaccharide.

Experimental

All melting points are uncorrected. Specific rotations were measured by the use of JASCO model DIP-SL automatic polarimeter. The determination of free amino acids was performed by the use of Hitachi KLA-3B amino acid analyzer. Solutions were evaporated at 40° or below with rotary evaporators under reduced pressure.

Extraction and Fractionation of Water-Soluble Constituents——The material was obtained in the beginning of 1970 from the plants cultivated in Nara prefecture. The fresh roots (100 g), which contain 78.5% of water, were crushed, then extracted with hot ethanol (300 ml) for 30 min. After suction filtration, the residue was further extracted with hot water (200 ml) for 30 min in boiling water bath, followed by filtration and repetition of the process twice. All extracts were collected and concentrated to 100 ml, then applied to a column (2×25 cm) of Dowex 50W-X8 (H^+, 50 to 100 mesh). After washing with 200 ml of water, the eluates and washings were collected and concentrated, then applied to a column (2×20 cm) of Dowex 2-X8 (OH^-, 50 to 100 mesh). After washing with 300 ml of water, the eluates and washings were collected and concentrated, followed by lyophilization. The yield of the neutral fraction was 16.02 g. The basic fraction was obtained by the elution with 1 N NH_4OH (400 ml) from the column of Dowex 50W and the acidic fraction was obtained by the elution with 0.5 N HCl (400 ml) from the column of Dowex 2. Ammonium hydroxide or hydrochloric acid were respectively removed by repeated rotary evaporation. The yields were 0.71 g in the former and 1.56 g in the latter.

Charcoal Column Chromatography——Active charcoal (for chromatographic use, Wako-Junyaku Co.) was treated before use with hot 15% AcOH, followed by washing with water. The water solution of the neutral fraction (10 g) was applied to the top of the charcoal column (4×20 cm), followed by successive elution with water (600 ml), 5% ethanol (1200 ml), 10% ethanol (1200 ml), 15% ethanol (1000 ml) and 25% ethanol (1000 ml). Fractions were collected at 50 ml and carbohydrates in eluates were measured by phenol–sulfuric acid method.[5] The eluates obtained from the column were divided into eight groups: Frac. 1, tubes 4 to 10; Frac. 2, tubes 11 to 17; Frac. 3, tubes 18 to 23; Frac. 4, tubes 24 to 32; Frac. 5, tubes 33 to 42; Frac. 6, tubes 43 to 65; Frac. 7, tubes 66 to 84; Frac. 8, tubes 85 to 100. The yields were 725 mg in Frac. 1, 269 mg in Frac. 2, 1334 mg in Frac. 3, 1870 mg in Frac. 4, 1298 mg in Frac. 5, 3102 mg in Frac. 6, 677 mg in Frac. 7 and 120 mg in Frac. 8.

Thin-Layer Chromatography——Avicel SF cellulose (20 g) were mixed well with water (90 ml) in a homogenizer and glass plates were coated with 0.25 mm thick layer by the use of an applicator. The plates were stored overnight at room temperature before use. For the purpose of carbohydrate analysis, following solvent systems were used: A, AcOEt: pyridine: AcOH: H_2O (5:5:1:3); B, BuOH: pyridine: AcOH: H_2O (3:2:1:2); C, BuOH: pyridine: H_2O (1:1:1). Monosaccharides and oligosaccharides were revealed with silver nitrate,[6] naphthoresorcinol–phosphoric acid[7] and diphenylamine–aniline[8] reagents. The results are shown in Table III.

Two dimensional cellulose thin-layer chromatography was carried out for the qualitative analysis of amino acids. Primary solvent system (D) was BuOH: AcOH: H_2O (12:3:5) and the secondary (E) was PhOH: 0.3% NH_4OH (4:1). Ninhydrin reagent was used for detection. The results are shown in Fig. 1.

Analysis of Phosphoric Acid——Avicel SF cellulose (5 g) were mixed well with 0.01 N KOH (22 ml) and thin-layer plates were prepared as described above. Three solvent systems were used for the analysis of acids: F, EtOH: 25% NH_4OH: H_2O (8:2:1); G, PhOH: HCOOH: H_2O (3:1:1); H, BuOH: HCOOH: H_2O (4:1:1). Only one spot corresponding to phosphoric acid is detected in each condition by bromocresol green reagent.[9] *Rf* values were 0.02 in solvent F, 0.41 in solvent G and 0.31 in solvent H. Colorimetric determination of phosphoric acid was carried out according to the method of Chen, *et al.*[10]

4) *a*) D. French, *Adv. Carbohyd. Chem.*, **9**, 149 (1954); *b*) M.L. Wolfrom and A. Thompson, "Methods in Carbohyd. Chem.," I, Academic Press, New York and London, 1962, p. 368; *c*) J.H. Pazur, "The Carbohydrates, Chemistry and Biochemistry," IIA, ed. by W. Pigman and D. Horton, Academic Press, New York and London, 1970, p. 69.
5) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
6) W.E. Trevelyan, D.P. Procter and J.S. Harrison, *Nature*, **166**, 444 (1950).
7) V. Prey, H. Berbalk and M. Kausz, *Mikrochim. Acta*, **1961**, 968.
8) R.W. Bailey and E.J. Bourne, *J. Chromatog.*, **4**, 206 (1960).
9) D. Braun and H. Geenen, *J. Chromatog.*, **7**, 56 (1962).
10) P.S. Chen, Jr., T.Y. Toribara and H. Warner, *Anal. Chem.*, **28**, 1756 (1956).

TABLE III. *Rf* Values and Colorations of Sugars in the Neutral Fraction

Sugars[a]	*Rf* values			Colorations[b]		
	Solvent A	Solvent B	Solvent C	S.N.[c]	N.P.[d]	D.A.[e]
Fructose	0.51	0.45	0.60	br	r	o-y
Glucose	0.46	0.40	0.56	br	bl	g-o
Galactose	0.40	0.37	0.51	br	bl	g-o
Sucrose	0.41	0.33	0.53	—	r	o
Raffinose	0.24	0.22	0.41	—	r	o-r
Manninotriose	0.13	0.16	0.30	br	bl-v	o
Stachyose	0.12	0.12	0.30	—	r	o-r
Verbascose	0.09	0.09	0.25	—	r	o-r
X-1[f]	0.61	0.51	0.65	br	r	o-y
X-2	0.25	0.26	0.43	br	v	o
X-3	0.21	0.23	0.37	br	v	o
X-4	0.07	0.06	0.20	—	r	o-r
X-5	0.72	0.57	0.75	—	y	o-r
X-6	0.76	0.66	0.80	—	r-v	o-y
X-7	0.47	0.36	0.58	—	y	o-r
X-8	0.36	0.27	0.50	—	bl-v	o-v

a) Charcoal column Fr. 1 contains fructose, glucose and galactose; Fr. 2 contains sucrose, manninotriose, X-1, -2 and 3; Fr. 3 contains sucrose, manninotriose, raffinose and stachyose; Fr. 4 and 5 contain raffinose and stachyose; Fr. 6 contains stachyose; Fr. 7 contains stachyose, verbascose, X-4 and 5; Fr. 8 contains verbascose, X-6, 7 and 8.
b) Abbreviations for colorations are as follows: br=brown; r=red; bl=blue; bl-v=bluish violet; v=violet; y=yellow; r-v=reddish violet; o-y=orange yellow; g-o=grayish orange; o=orange; o-r=orange red; o-v=orange violet.
c) S.N.=silver nitrate reagent
d) N.P.=naphthoresorcinol-phosphoric acid reagent
e) D.A.=diphenylamine-aniline reagent
f) X-1 to X-8 show unknown spots.

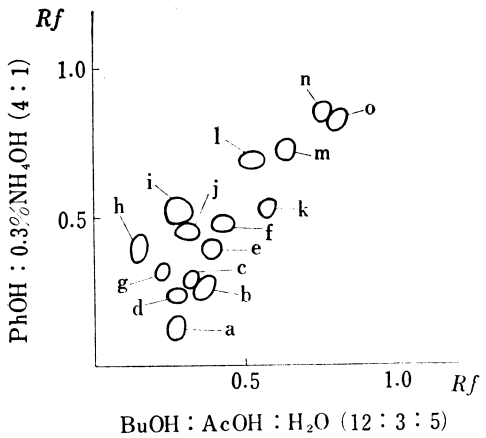

Fig. 1. Two Dimensional Thin-Layer Chromatogram of the Basic Fraction

a, aspartic acid; b, glutamic acid; c, glycine; d, serine; e, threonine; f, alanine; g, histidine; h, lysine; i, arginine; j, glucosamine HCl; k, tyrosine; l, γ-amino butyric acid; m, valine; n, phenylalanine; o, leucine & isoleucine

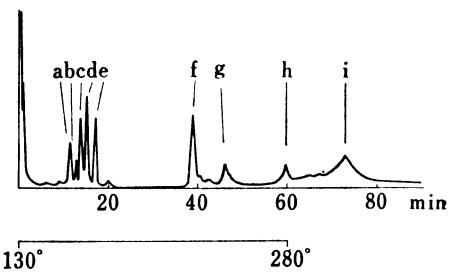

Fig. 2. Gas Chromatogram of TMS Derivative of the Neutral Fraction

a, fructose; b, galactose; c, α-glucose; d, mannitol; e, β-glucose; f, sucrose; g, manninotriose; h, raffinose; i, stachyose

Gas-Liquid Chromatography——Trimethylsilyl derivatives of the neutral fraction were prepared by the method of Sweeley, *et al.*,[11] followed by removal of pyridine as reported by Yamakawa, *et al.*[12] The chloro-

11) C.C. Sweeley, R. Bentley, M. Makita and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
12) T. Yamakawa and N. Ueta, *Japan J. Exp. Med.*, **34**, 37 (1964).

form solution was applied to Hitachi model F6D gas chromatograph with hydrogen flame ion detector by the use of a column (0.3 cm inner diameter × 1 m long U-shape stainless steel) packed with 3% SE52 on Chromosorb W (80 to 100 mesh) and with a flow of 20 ml per min of nitrogen. The programmed temperature was increased 2.5° per min from 130 to 280°. The chromatogram is shown in Fig. 2.

For the detection of trimethylsilyl D-glucosamine hydrochloride from the basic fraction, a glass column (0.4 cm × 2 m long) packed with 2% OV17 on Chromosorb W (80 to 100 mesh) was used at 180°; carrier gas, N_2 (20 ml per min); t_R, 8.8, 10.2.

Determinations of Carbohydrates——Monosaccharides were determined by the gas–liquid chromatography. Most of the oligosaccharides were purified respectively by the repetition of charcoal column chromatography, but raffinose and verbascose were further purified by ascending paper chromatography with Tōyō-Roshi No. 50 and solvent A, followed by extraction with water. The recoveries were corrected according to the colorimetric determination of ketose by resorcinol method.[13] D-Glucosamine was estimated by the modification of Elson–Morgan reaction.[14]

Identifications of Three Monosaccharides and Three Oligosaccharides——Monosaccharides were separated from the charcoal column fraction by ascending paper chromatography with Tōyō-Roshi No. 50 and solvent A, then extracted respectively with water. Oligosaccharides were obtained as mentioned above.

D-Glucose was derived to *p*-nitrophenylhydrazone, orange yellow plates, mp 187—188° (decomp.).

D-Galactose was derived to methylphenylhydrazone, colorless plates, mp 191° (decomp.).

D-Fructose was derived to methylphenylosazone,[15] orange yellow needles, mp 158° (decomp.).

Sucrose was derived to octaacetate, colorless needles, mp 86°.

Raffinose was recrystallized from EtOH–H_2O to give colorless prisms (pentahydrate), mp 78—80°, $[\alpha]_D^{24}$ +105° (c=2, H_2O).

Stachyose was recrystallized from EtOH–H_2O to give colorless granules, mp 168—170°, $[\alpha]_D^{26}$ +149° (c=5.6, H_2O).

Each sample was identified by comparing with the respective authentic sample by mixing mp, TLC and IR spectra.

Identification of Manninotriose——The sample gave $[\alpha]_D^{26}$ +172° (c=1.7, H_2O) and this is almost the same value as manninotriose.[16] Stachyose (5 mg) and β-D-fructofuranoside fructohydrolase (Boehringer Co., Mannheim, Germany) (0.5 mg) were dissolved in 0.05 ml of water, and stood overnight at 40°. The solution was applied to cellulose thin-layer chromatography by the use of solvent system B, then the products were detected with silver nitrate reagent. The sample and manninotriose prepared from stachyose by the enzyme action showed the same *Rf* value (0.16). After hydrolysis with 1 N H_2SO_4 for 6 hr followed by neutralization with barium carbonate, trimethylsilyl derivative of the hydrolysate was applied to the gas chromatography with 3% SE52 on Chromosorb W and with a flow of 20 ml per min of nitrogen. The programmed temperature was increased 1.25° per min from 150 to 175°. D-Ribose was used as an intrinsic standard for quantitative determination of sugar components and the result showed that the sample is composed of 65.6% of D-galactose and 31.3% of D-glucose.

On the other hand, sodium hydride (100 mg) was mixed with $(CH_3)_2SO$ (4 ml) and the mixture was stirred at 70° for 1 hr. The sample (10 mg) in $(CH_3)_2SO$ (4 ml) was added into this solution. After 10 min stirring, CH_3I (4 ml) was added and the reaction mixture was stirred overnight at room temperature. The procedures were carried out in nitrogen atmosphere. After dilution with water, the mixture was extracted with $CHCl_3$. The extract was dried and the solvent was evaporated *in vacuo*, and the residue was methylated again under the same condition. The final product was heated with 0.5 N methanolic HCl (1 ml) in a sealed tube at 70° for 16 hr. After cooling, the solution was treated with Amberlite IR4B (OH^-) to remove HCl, then evaporated *in vacuo*. The chloroform solution of the residue was applied to a gas chromatograph.

GLC: column, 15% Poly-butane 1,4-diol succinate on Chromosorb W (80 to 100 mesh) (0.3 cm × 1 m long); column temperature, 175°; carrier gas, N_2 (20 ml per min); t_R, methyl 2,3,4,6-tetra-O-methyl-D-galactopyranoside 8.3; methyl 2,3,4-tri-O-methyl-D-glucopyranoside 11.9, 16.6; methyl 2,3,4-tri-O-methyl-D-galactopyranoside 30.7.

Furthermore, the sample (5 mg) was oxidized with 10 ml of 0.05 M sodium metaperiodate in 0.2 M acetate buffer (pH 4.4) at 5° in a dark place. The periodate consumption was measured by a spectrophotometric method[17] and the liberated formic acid was titrated with 0.01 N NaOH. As the result of the oxidation, 7.9 mole of periodate per one mole of the sample (for M.W. 504) was consumed with 6.2 mole of formic acid liberation.

From these all results, it is concluded that the sample must be manninotriose.

13) R.G. Kurka, *Biochem. J.*, **63**, 542 (1956).
14) C.J.M. Rondle and W.T.J. Morgan, *Biochem. J.*, **61**, 586 (1955).
15) C. Neuberg and I. Mandl, *Arch. Biochem.*, **11**, 451 (1946).
16) C. Tanret, *Compt. rend.*, **134**, 1586 (1902).
17) J.S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954).

Identification of Verbascose——The sample gave $[\alpha]_D^{21}+168°$ ($c=0.6$, H_2O) and this is almost the same value as verbascose.[18] Component hexoses of the sample were determined similarly to the case of manninotriose by the gas–liquid chromatography. Component fructose was estimated by resorcinol method.[13] The result showed that the sample is composed of 58.4% of D-galactose, 21.6% of D-glucose and 21.0% of D-fructose.

Methylation of the sample was carried out in the same way as the case of manninotriose and the product was hydrolysed with 76% methanol containing 1% oxalic acid at 75° for 18 hr, then methanolysed with 0.5 N methanolic HCl at 60° for 2 hr. Gas chromatography was performed in the same condition as described above; t_R, methyl 1,3,4,6-tetra-O-methyl-D-fructofuranoside 5.2, 6.2; methyl 2,3,4,6-tetra-O-methyl-D-galactopyranoside 8.3, 9.3; methyl 2,3,4-tri-O-methyl-D-glucoside 11.9, 16.6, 18.1; methyl 2,3,4-tri-O-methyl-D-galactopyranoside 30.7.

Periodate oxidation was also done as mentioned above, and finally, 9.2 mole of periodate per one mole of the sample (for M.W. 828) was consumed with 4.1 mole of formic acid liberation.

From these all results, it is concluded that the sample must be verbascose.

Acknowledgement The authors are indebted to Dr. M. Maeda, Department of Biochemistry, Faculty of Engineering and Science, Saitama University, for his kind supply of stachyose isolated from *Stachys tuberifera*, and Mr. K. Asaoka, Department of Biochemistry and Biophysics, Faculty of Science, University of Tokyo, for the determination of amino acids, and Prof. I. Yosioka, Faculty of Pharmaceutical Sciences, Osaka University, for his advice.

18) E. Bourquelot and M. Bridel, *Comp. Rend.*, **151**, 760 (1910).