An Acidic Polysaccharide Having Activity on the Reticuloendothelial System from the Bark of *Eucommia ulmoides*

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An acidic polysaccharide, named eucomman A, was isolated from the dried bark of *Eucommia ulmoides* OLIV. It was homogeneous on electrophoresis and gel chromatography, and showed significant reticuloendothelial system-potentiating activity in a carbon clearance test. It is composed of L-arabinose : D-galactose : D-glucose : L-rhamnose : D-galacturonic acid in the molar ratio of 8 : 6 : 4 : 5 : 8 in addition to small amounts of peptide moiety. Its molecular mass was estimated to be 6.0×10^4. Methylation analysis, carbon-13 nuclear magnetic resonance and periodate oxidation studies enabled elucidation of its structural features.

Keywords *Eucommia ulmoides*; bark; eucomman A; acidic polysaccharide; polysaccharide structure; immunological activity; reticuloendothelial system; methylation analysis

The dried bark of *Eucommia ulmoides* OLIV. (Eucommiaceae) is a traditional Chinese crude drug used as a tonic, an analgesic and an antihypertensive agent under the name of Du-Zhong in China (Japanese name, Tochū).

As the constituents, many lignans, lignan glucosides, iridoid glucosides, phenolic compounds[1-5] and a polyprenoid[6] have been isolated. It has been reported that pinoresinol diglucoside is the major antihypertensive principle,[1] and epipinoresinol, glucosides of syringaresinol and medioresinol, and genipin are known as anti-complementary active compounds in this crude drug.[7] However, no study on the water-soluble organic polymers has been reported so far on both structure and biological activity. We have now isolated a pure acidic polysaccharide from this crude drug. This substance showed immunological activity on reticuloendothelial system (RES). Its properties and structural features are reported here.

Materials and Methods

Isolation of Polysaccharides The material was imported from China. The sliced bark (810 g) was extracted with hot water (8.1 l) while stirring for 30 min in a boiling water bath, then the filtrate was poured into two volumes of ethanol. After centrifugation and drying, the precipitate (4.2 g) was dissolved in 0.01% sodium sulfate (400 ml) and centrifuged. 10% cetyltrimethylammonium bromide (90 ml) was added to the supernatant. The precipitate was separated by centrifugation, then dissolved in 2 M sodium chloride (400 ml). After centrifugation, the supernatant was poured into two volumes of ethanol. The resulting precipitate was dissolved in water, then dialyzed and lyophilized. The yield was 1.77 g. The aqueous solution of this fraction was applied to a column (5 × 32 cm) of diethylaminoethyl (DEAE)-Sephadex A-25 (Pharmacia Co.). DEAE-Sephadex A-25 was used as the carbonate form in the manner described in a previous report.[8] After elution with water (560 ml), the column was eluted with 0.2 M ammonium carbonate. Fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method.[9] The eluates obtained from tubes 16 to 39 were combined, dialyzed, concentrated and applied to a column (5 × 87 cm) of Sephadex G-25. The column was eluted with water and fractions of 20 ml were combined, concentrated and lyophilized. Eucomman A (69.8 mg) was obtained as a white powder.

Polyacrylamide Gel Electrophoresis (PAGE) This was carried out in an apparatus with gel tubes (4 × 150 mm each) and 5 mM Tris–glycine buffer (pH 8.3) at 5 mA/tube for 40 min. Gels were stained by the periodate–Schiff (PAS) procedure and with Coomassie blue reagent. Eucomman A gave a clear band at a distance of 58 mm from the origin.

Gel Chromatography The sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a column (2.6 × 85 cm) of Sephacryl S-300, pre-equilibrated and developed with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method. Standard pullulans (Shōwa Denkō Co.) having known molecular masses were run on the column to obtain a calibration curve.

Phagocytic Activity This was measured as described in a previous report.[10] The samples and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved in physiological saline and dosed i.p. (40 mg/kg body weight) once a day. The phagocytic index, K, was calculated by means of the following equation:

$$K = (\ln OD_1 - \ln OD_2)/(t_2 - t_1)$$

where OD_1 and OD_2 are the optical densities at times t_1 and t_2, respectively. Results were expressed as the arithmetic mean ± S.D. of five male mice (ICR-SPF).

Qualitative Analysis of Component Sugars Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described in a previous report.[8] The configuration of component neutral sugars were identified by gas chromatography (GC) of trimethylsilylated α-methylbenzylaminoalditol derivatives.[11] GC was carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector.

Determination of Components Neutral sugars were analyzed by GC after conversion of the hydrolyzate into alditol acetates as described in a previous report.[12] Galacturonic acid was determined by the *m*-hydroxybiphenyl method.[13] Peptide determination was performed by the method of Lowry *et al.*[14]

Nuclear Magnetic Resonance (NMR) NMR spectrum was recorded on a JEOL JNM-GX 270 FT NMR spectrometer in heavy water containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 30 °C.

Reduction of Carboxyl Groups This was carried out with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and sodium borohydride as described in a previous report.[15] The reduction was repeated three times under the same conditions. Yield was 19 mg from 50 mg of eucomman A.

Methylation Analysis Methylation was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide as described in a previous report.[16] The reaction was repeated three times under the same conditions. Yields were 11.3 mg from 12.3 mg of eucomman A, and 15.4 mg from 17.4 mg of the carboxyl-reduced derivative. The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described in a previous report.[17] The partially methylated alditol acetates obtained were analyzed by gas chromatography-mass spectrometry (GC-MS) using a fused silica capillary column (0.32 mm i.d. × 30 m) of SP-2330 (Supelco Co.) with a programmed temperature increase of 4 °C per min from 160 to 220 °C at a helium flow of 1 ml per min. GC-MS was performed with a JEOL JMS-GX mass spectrometer. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in GC and the main fragments in MS are listed in Table I.

Periodate Oxidation The sample (10.7 mg) was oxidized with 0.05 M sodium metaperiodate (5 ml) at 4 °C in the dark. The periodate consumption was measured by a spectrophotometric method.[18] The oxidation was completed after 3 d. The reaction mixture was successively treated with ethylene glycol (0.1 ml) at 4 °C for 1 h and sodium borohydride (30 mg) at 4 °C for 16 h, and was adjusted to pH 5.0 by addition of acetic acid. The solution was concentrated and applied to a column (2.6 × 95 cm) of Sephadex G-25. The column was eluted with water, and fractions of

TABLE I. Relative Retention Times on GC and Main Fragments in MS of Partially Methylated Alditol Acetates

	Rt_R [a]	Main fragments (m/z)
1,5-Ac_2-2,3,4-Me_3-L-rhamnitol	0.64	43, 101, 117, 131, 175
1,2,5-Ac_3-3,4-Me_2-L-rhamnitol	0.96	43, 89, 129, 131, 189
1,2,4,5-Ac_4-3-Me-L-rhamnitol	1.29	43, 87, 101, 129, 143, 189, 203
1,4-Ac_2-2,3,5-Me_3-L-arabinitol	0.69	43, 45, 71, 87, 101, 117, 129, 161
1,3,5-Ac_3-2,4-Me_2-L-arabinitol	1.04	43, 87, 113, 117, 233
1,4,5-Ac_3-2,3-Me_2-L-arabinitol	1.14	43, 87, 101, 117, 129, 189
1,5-Ac_2-2,3,4,6-Me_4-D-glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,4,5-Ac_3-2,3,6-Me_3-D-glucitol	1.47	43, 45, 87, 99, 101, 113, 117, 233
1,5-Ac_2-2,3,4,6-Me_4-D-galactitol	1.10	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,3,5-Ac_3-2,4,6-Me_3-D-galactitol	1.36	43, 45, 87, 101, 117, 129, 161
1,4,5-Ac_3-2,3,6-Me_3-D-galactitol	1.43	43, 45, 87, 99, 101, 113, 117, 233
1,5,6-Ac_3-2,3,4-Me_3-D-galactitol	1.59	43, 87, 99, 101, 117, 129, 161, 189
1,2,4,5-Ac_4-3,6-Me_2-D-galactitol	1.76	43, 45, 87, 99, 113, 129, 189, 233
1,3,5,6-Ac_4-2,4-Me_2-D-galactitol	2.00	43, 87, 117, 129, 189

a) Relative retention time. Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Abbreviations: Ac=acetyl; Me=methyl (*e.g.*, 1,5-Ac_2-2,3,4-Me_3-=1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-).

10 ml were collected. The eluates obtained from tubes 22 to 26 were combined, concentrated and lyophilized. Yield, 9.4 mg.

Results

The crude polysaccharide fraction (fr. CPS) was isolated from the bark of *Eucommia ulmoides* by hot water extraction followed by precipitation with ethanol, then dissolved in dilute sodium sulfate. The solution was treated with cetyltrimethylammonium bromide, and the resulting precipitate was dissolved in sodium chloride solution and treated with ethanol. The final precipitate was purified by ion-exchange chromatography with DEAE-Sephadex A-25 and gel chromatography with Sephadex G-25. A pure polysaccharide designated as eucomman A was obtained.

The polysaccharide gave a singlet band on PAGE, after staining with periodate–Schiff and Coomassie blue reagents. It gave a single peak on gel chromatography, and the result gave a value of 6.0×10^4 for its molecular mass. Eucomman A showed a positive specific rotation ($[\alpha]_D^{24}$ +25.0° in 0.1 M NH_4OH, $c=0.1$).

The effects of eucomman A and fr. CPS on the RES were demonstrated by a modification[10] of the *in vivo* carbon clearance test[19] using zymosan as a positive control. As shown in Fig. 1, the phagocytic index was significantly increased, suggesting the activation of RES by i.p. injection of eucomman A. On the other hand, fr. CPS showed no RES activity.

Quantitative analyses showed that eucomman A contained 21.3% L-arabinose, 19.5% D-galactose, 13.0% D-glucose, 14.9% L-rhamnose, 28.3% D-galacturonic acid and 3.1% peptide moiety. The molar ratio of these component sugars was 8:6:4:5:8.

The carbon-13 NMR (^{13}C-NMR) spectrum of eucomman A showed five signals due to anomeric carbons at δ 100.37, 101.01, 101.53, 106.19, 107.06 and 110.22 ppm. Among these, it was evident that those at δ 101.01 and 101.53 ppm were attributable to anomeric carbons of α-D-galactopyranosyluronic acid and α-L-rhamnopyranose,[20] those at δ 100.37 and 106.19 to anomeric carbons of α-D-glucopyranose and β-D-galactopyranose,[21] and those at δ 107.06 and 110.22 to anomeric carbons of α-L-arabinopyranose and α-L-arabinofuranose.[22] No signals suggesting the presence of acetyl groups or carboxylic acid methyl esters were observed.

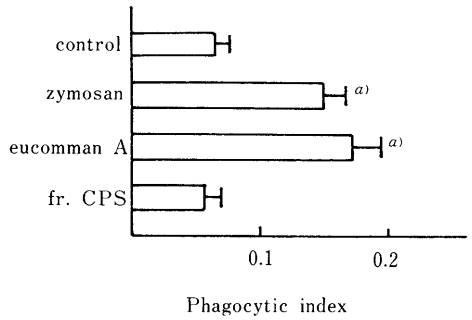

Fig. 1. Effects of Eucomman A and Fr. CPS on Carbon Clearance Index in ICR Mice

Significantly different from the control, a) $p<0.001$.

The carboxyl groups of galacturonic acid residues in the polysaccharide were reduced to give the corresponding neutral sugar residues.[23] Both the original polysaccharide and the carboxyl-reduced derivative were methylated with methylsulfinyl carbanion and methyl iodide in dimethylsulfoxide.[24] The methylated products were hydrolyzed, then converted into the partially methylated alditol acetates. Methyl ethers of galacturonic acid were removed from the hydrolyzate of the original polysaccharide by treatment with an anion-exchange resin. GC-MS[25] showed the presence of 2,3,5-tri-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-galactose, 3,6-di-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-galactose, 2,3,4,6-tetra-*O*-methyl-D-glucose, 2,3,6-tri-*O*-methyl-D-glucose, 2,3,4-tri-*O*-methyl-L-rhamnose, 3,4-di-*O*-methyl-L-rhamnose and 3-*O*-methyl-L-rhamnose in both methylated products. The molar ratio was 3:2:11:3:2:3:1:2:1:2:6:1:5:4 as the products from the original methylated polysaccharide, and was 3:2:11:3:2:17:1:4:1:2:6:1:5:4 as the products from the methylated derivative of the carboxyl-reduced product.

Eucomman A was subjected to periodate oxidation followed by reduction. The component sugar analysis of the product showed that one-eighth of the arabinose units, one-fourth of the galactose units, one-fifth of rhamnose

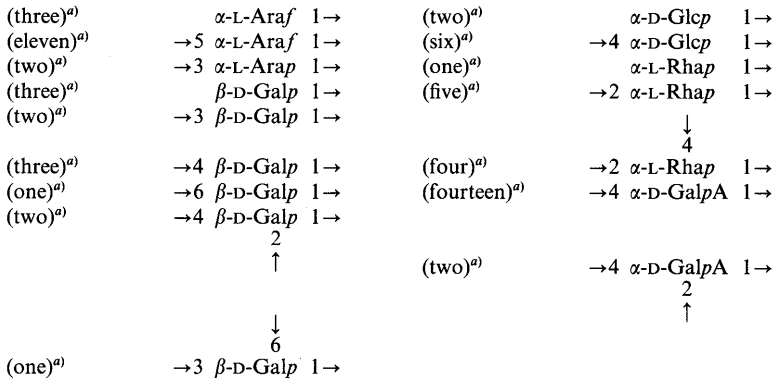

Chart 1. Component Sugar Residues in the Minimal Unit in the Structure of Eucomman A

a) Number of residues. Ara*f*, arabinofuranose; Ara*p*, arabinopyranose; Gal*p*, galactopyranose; Glc*p*, glucopyranose; Rha*p*, rhamnopyranose; Gal*p*A, galactopyranosyluronic acid.

units and one-eighth of galacturonic acid units survived after periodate oxidation. No glucose was found in the product. In conjunction with the results of methylation analysis, these observations suggest that the minimal unit of eucomman A is composed of sixteen kinds of component sugar units, as shown in Chart 1.

Discussion

As the examples of RES-activating polysaccharides from Oriental crude drugs, sanchinan-A has been obtained from the root of *Panax notoginseng*,[26] and it belongs to an arabino-3,6-galactan type substance. Recently, two polysaccharides named tochibanan-A and -B were isolated from the rhizome of *Panax japonicus*.[27] They belong to β-1,4-linked D-galactan and partially 2,3,6-branched β-1,4-linked D-galactan, respectively.

We have reported the RES activities and structural features of saposhnikovans A and C from the root and rhizome of *Saposhnikovia divaricata*,[12,28] MVS-IIIA and -IVA from the seed of *Malva verticillata*,[29,30] cinnaman AX from the bark of *Cinnamomum cassia*,[10] ukonans A and B from the rhizome of *Curcuma longa*,[31,32] and of glycyrrhizans UA and UB from the root of *Glycyrrhiza uralensis*.[33] Saposhnikovan A has a β-1,4-linked D-galacturonan backbone bearing arabino-3,6-galactan type side chains. Saposhnikovan C possesses an rhamnogalacturonan backbone with α-3,5-branched L-arabinan and β-3,4-branched D-galactan side chains. MVS-IIIA and -IVA are mainly made up of arabino-3,6-galactan structure with α-1,3-linked L-arabinopyranosyl, β-1,4-linked D-xylosyl and α-1,4-linked D-galacturonan units. Further, MVS-IVA possesses additional α-1,2-linked L-rhamnosyl units. Cinnaman AX has a β-1,4-linked D-xylan backbone bearing terminal β-L-arabinopyranosyl units and α-L-arabinofuranosyl-(1→3)-β-L-arabinopyranose side chains. Ukonans A and B are basically arabino-3,6-galactans having a rhamnogalacturonan backbone. They belong to polysaccharides similar to MVS-IVA, though ukonans A and B have additional terminal and α-1,4-linked D-glucosyl residues. The main part of glycyrrhizan UA is occupied by arabino-3,6-galactan type units with additional α-1,3-linked L-arabinopyranosyl, β-1,4-linked D-galactosyl residues and branched rhamnogalacturonan units. Glycyrrhizan UB has a basically similar structural type to UA, though branched rhamnogalacturonan units occupy the major part in this polysaccharide with additional β-2,4-branched D-galactosyl and terminal α-D-glucosyl units.

Eucomman A has similar arabinogalactan and rhamnogalacturonan units to those in glycyrrhizan UB. Fourteen structural units are observed commonly in the both polysaccharides. However, D-glucose occupies only terminal residues as a minor component sugar in glycyrrhizan UB. On the other hand, eucomman A possesses many additional α-1,4-linked D-glucosyl units, and it has no terminal L-arabinopyranose residues. The presence of terminal α-L-rhamnopyranosyl units is especially characteristic of this polysaccharide among the RES-activating substances described above.

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References

1) C. J. Sih, P. R. Ravikumar, F.-C. Huang, C. Buckner and H. Whitlock, Jr., *J. Am. Chem. Soc.*, **98**, 5412 (1976).
2) T. Deyama, *Chem. Pharm. Bull.*, **31**, 2993 (1983).
3) T. Deyama, T. Ikawa and S. Nishibe, *Chem. Pharm. Bull.*, **33**, 3651 (1985).
4) T. Deyama, T. Ikawa, S. Kitagawa and S. Nishibe, *Chem. Pharm. Bull.*, **34**, 523, 4933 (1986).
5) T. Deyama, T. Ikawa, S. Kitagawa and S. Nishibe, *Chem. Pharm. Bull.*, **35**, 1803, 1785 (1987).
6) Z. Horii, Y. Ozaki, K. Nagao and S.-W. Kim, *Tetrahedron Lett.*, **1978**, 5015.
7) Y. Oshima, S. Takata, H. Hikino, T. Deyama and G. Kinoshita, *J. Ethnopharmacol.*, **23**, 159 (1988).
8) M. Tomoda, S. Kaneko, M. Ebashi and T. Nagakura, *Chem. Pharm. Bull.*, **25**, 1357 (1977).
9) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
10) M. Kanari, M. Tomoda, R. Gonda, N. Shimizu, M. Kimura, M. Kawaguchi and C. Kawabe, *Chem. Pharm. Bull.*, **37**, 3191 (1989).
11) R. Oshima, J. Kumanotani and C. Watanabe, *J. Chromatogr.*, **259**, 159 (1983).
12) N. Shimizu, M. Tomoda, R. Gonda, M. Kanari, N. Takanashi and N. Takahashi, *Chem. Pharm. Bull.*, **37**, 1329 (1989).
13) N. Blumenkrantz and G. Asboe-Hansen, *Anal. Biochem.*, **54**, 484 (1973).
14) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
15) M. Tomoda and M. Ichikawa, *Chem. Pharm. Bull.*, **35**, 2360 (1987).
16) N. Shimizu, M. Tomoda and M. Adachi, *Chem. Pharm. Bull.*, **34**, 4133 (1986).
17) M. Tomoda, K. Shimada, Y. Saito and M. Sugi, *Chem. Pharm. Bull.*,

28, 2933 (1980).
18) G. O. Aspinall and R. J. Ferrier, *Chem. Ind.* (London), **1957**, 1216.
19) G. Biozzi, B. Benacerraf and B. N. Halpern, *Br. J. Exp. Pathol.*, **34**, 441 (1953).
20) N. Shimizu and M. Tomoda, *Chem. Pharm. Bull.*, **33**, 5539 (1985).
21) K. Bock, C. Pedersen and H. Pedersen, "Advances in Carbohydrate Chemistry and Biochemistry," Vol. 42, ed. by R. S. Tipson and D. Horton, Academic Press, Inc., Orland, 1984, pp. 193—214.
22) J.-P. Joseleau, G. Chambat, M. Vignon and F. Barnoud, *Carbohydr. Res.*, **58**, 165 (1977).
23) R. L. Taylor and H. E. Conrad, *Biochemistry*, **11**, 1383 (1972).
24) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).
25) H. Björndall, B. Lindberg and S. Svensson, *Carbohydr. Res.*, **5**, 433 (1967).
26) K. Ohtani, K. Mizutani, S. Hatono, R. Kasai, R. Sumino , T. Shiota, M. Ushijima, J. Zhou, T. Fuwa and O. Tanaka, *Planta Medica*, **53**, 166 (1987).
27) K. Ohtani, S. Hatono, K. Mizutani, R. Kasai and O. Tanaka, *Chem. Pharm. Bull.*, **37**, 2587 (1989).
28) N. Shimizu, M. Tomoda, R. Gonda, M. Kanari, Aki. Kubota and Ake. Kubota, *Chem. Pharm. Bull.*, **37**, 3054 (1989).
29) R. Gonda, M. Tomoda, N. Shimizu and M. Kanari, *Planta Medica*, **56**, 73 (1990).
30) M. Tomoda, M. Kanari, R. Gonda and N. Shimizu, *Phytochemistry*, **28**, 2609 (1989).
31) M. Tomoda, R. Gonda, N. Shimizu, M. Kanari and M. Kimura, *Phytochemistry*, **29**, 1083 (1990).
32) R. Gonda, M. Tomoda, N. Shimizu and M. Kanari, *Chem. Pharm. Bull.*, **38**, 482 (1990).
33) M. Tomoda, N. Shimizu, M. Kanari, R. Gonda, S. Arai and Y. Okuda, *Chem. Pharm. Bull.*, **38**, 1667 (1990).