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WATER-SOLUBLE GLUCANS FROM THE SEED OF *COIX LACRYMA-JOBI* VAR. *MA-YUEN*

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Key Word Index—*Coix lacryma-jobi* var. *ma-yuen*; Gramineae; polysaccharide; glucan; HPLC; iodine binding; anti-complementary activity.

Abstract—The water-soluble major polysaccharides from the seed of *Coix lacryma-jobi* var. *ma-yuen* eluted as a broad peak by gel filtration on Sepharose CL-2B. The mixture (CS-Glucan) was resolved into 7 glucans by HPLC on the column of Asahi-Pak GS-510 + GS-320. Similarities were observed between M_r shown in the gel filtration profile and the elution volume in HPLC. Methylation analysis indicated that the ethanol-fractionated CS-glucan contained 4-*O*- and 4,6-di-*O*-substituted glucosyl residues. ^1H and ^{13}C NMR data accorded with the results of methylation analysis, and the glycosidic linkages were shown to have an α-configuration. Thus, CS-glucan contained (1 → 4) linked α-D-glucans to which are attached glucosyl side chains at O-6 of the main chain in a similar way to amylopectin. Each purified glucan was shown to have different absorption maxima (> 550 nm or 530 nm) in the iodine reaction. The results of the methylation analysis and of the pullulanase digestion suggest that the 550 nm-glucan has a lower branching frequency and shorter side chains than the 530 nm-glucan. Although CS-glucan was found to have weak anti-complementary activity, HPLC-purified > 550 nm-glucan was found to be more potent than the 530 nm-glucan. Thus CS-glucan is highly heterogeneous, and the glucans which form a tight complex when tested with iodine, generally tend to have considerable anti-complementary activity.

INTRODUCTION

The seed of *Coix lacryma-jobi* L. var. *ma-yuen* Stapf without the husks, has been used in a Chinese herbal medicine. It is known that *C. lacryma-jobi* contains 3,4-dihydroxybenzaldehyde glycoside [1], triterpenoids such as friedelin [2] and isoarborinol [2], fatty acids [3] such as palmitic acid and myristic acid, coixenolide [4] and some amino acids [5]. However, very few studies have been undertaken on the polysaccharides. In a study of polysaccharide components in Chinese herbs, those from *C. lacryma-jobi* were fractionated and a water-soluble glucan was obtained as a major material. The present paper deals with its purification and chemical properties.

RESULTS AND DISCUSSION

The crude polysaccharide, CS-1, was obtained by EtOH precipitation and dialysis from the hot water extract of *C. lacryma-jobi*. CS-1 was further fractionated into an unabsorbed and absorbed fractions by column chromatography on DEAE-Sepharose CL-6B (Cl^- form). The absorbed fraction was obtained by the stepwise elution of 0.2 M NaCl and 0.5 M NaCl solutions. The unabsorbed fraction (I) was the major polysaccharide and consisted of only glucose whereas the absorbed fractions IIa and IIb were minor polysaccharides which consisted of glucose, arabinose, galactose and xylose (and trace of mannose and rhamnose). Because the hydrolysates of the neutral fraction (CS-glucan) were completely oxidised by D-glucose oxidase, it was estimated that CS-glucan consisted of D-glucose. CS-glucan was eluted by gel filtration on Sepharose CL-2B as a wide band, suggesting heterogeneity. Since the iodine reaction of CS-glucan was positive, each fraction eluted from Sepharose CL-2B was tested with iodine. The CS-glucan was heterogeneous, since the absorption maximum of the color produced by iodine was different for each fraction. The fractions eluted in the void volume and the lower M_r fraction (M_r < 100 000) showed maxima at 550–570 nm whereas other fractions (M_r > 100 000) showed maxima at 530 nm. The heterogeneity of CS-glucan was further analysed by HPLC. CS-Glucan was resolved six peaks on a combined Asahi-Pak GS-510 and GS-320 column in H_2O, but the glucan was not well resolved on TSK-PW-5000 + PW-4000 columns or on Shodex OH Pak B-806, B-804 and B-803 columns. When CS-glucan was developed in 0.01 M NaCl solution on Asahi-Pak columns, the glucan was resolved into three peaks. CS-Glucan was also not well resolved by the other columns even when eluted with salt solution. The elution volume of each glucan separated by Asahi-Pak column was dependent on the MW of the fraction by gel filtration on Sepharose CL-2B. To purify each glucan, we attempted fractionation by EtOH precipitation. CS-Glucan contained at least seven glucans from the result of HPLC of the 15% EtOH ppt. A (Fig. 1). Ppt. A, B-2, C and E were still glucan mixtures but their proportions differed. The properties of precipitates, A, B-2, C and E were then analysed (Table 1). These glucan fractions were found to have a high hexose content and only ppt. A contained significant amounts of protein and phosphate. Ppt. A showed the lowest rotation and E showed the highest. Iodine assay showed Ppt. A and E to have absorption maxima at 570–560 nm and B-2 and C at 530 nm. These glucan fractions were methylated by the method of Hakomori, and the fully methylated product

Table 1. Chemical properties and methylation analysis of EtOH fractionated CS-glucans

Glucan fraction	Hexose (%) (as Glc)	Protein (%) (as BSA)	Phosphate (%) (as Pi)	$[\alpha]_D$	Iodine reaction $\lambda_{max(nm)}$
A	95.8	1.8	0.025	154.8	570
B-2	99.4	n.d.*	0.007	162.0	530
C	99.0	n.d.*	0.004	161.8	530
E	99.5	n.d.*	0.002	167.2	560

Methylated alditol acetate derivatives	Mol. %					Major mass spectral fragments (m/z)	Linkage
	T†	A	B-2	C	E		
2,3,4,6-tetra-O-Me Glc	1.0	5.7	5.7	7.8	8.0	43, 45, 71, 87, 101, 117 129, 145, 161, 205	$Glc^1 \rightarrow$
2,3,6-tri-O-Me Glc	2.3	90.7	91.0	88.4	88.1	43, 45, 87, 99, 101, 113 117, 233	$\rightarrow {}^4Glc^1 \rightarrow$
2,3-di-O-Me Glc	4.5	3.6	3.3	3.8	3.9	43, 101, 117, 261	$\rightarrow {}^4_6Glc^1 \rightarrow$

*Not detected.
†1% OV-225 170°.

was hydrolysed with acid and then converted into alditol acetates. The partially methylated alditol acetates were analysed by GC and GC/MS, and 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylglucitol and 1,4,5,6-tetra-O-acetyl-2,3-di-O-methylglucitol were identified in different proportions. The results of methylation analysis indicated that CS-glucan contained mainly (1 → 4) linked glucosyl residues and branching points at 0–6 of (1 → 4) linked glucosyl residues. ^1H NMR of ppt. C in D_2O showed the signals of anomeric protons due to α-configuration at δ4.91 and 5.30, and the spectrum was similar to those of AR-glucan [6] and potato amylopectin [6]. ^{13}C NMR spectrum of ppt. C showed a signal at δ102.8 in the region of the anomeric carbons. From comparison with the spectrum of AR-glucan [6], other signals of ppt. C at δ80.0, 76.4, 74.5, 74.2 and 63.5 were assigned to C-4, C-3, C-2, C-5 and C-6, respectively. The results of the NMR studies corresponded well with those of the methylation analysis. Thus CS-glucan is similar to amylopectin, and its heterogeneity is related to the difference in MW, branching frequency and chain length. Each glucan in CS-glucan was purified by preparative HPLC on Asahi-Pak GS-510 + GS-320, and seven purified glucans were eventually obtained. Glucans 1 and 7 showed absorption maxima at 595–580 nm by iodine-assay. Analysis of the results of methylation study and iodine assay for glucans 2, 6 and 7 suggests that the iodine-binding capacity is related to the low branching frequency because there was an increase in the non-reducing terminal that corresponded to glucan-2 > glucan-6 > glucan-7 and the absorption maximum of the color produced by iodine assay was such that there were longer wavelengths that corresponded to glucan-7 > glucan-6 > glucan-2 (Table 2). CS-Glucan mixture was found to have very weak anti-complementary activity. The order of the anti-complementary activity of each purified glucan was also shown to have the same tendency as that of iodine binding ability (Table 2). To determine the length of the side chain of CS-glucan, glucans 2, 6 and 7 were treated with pullulanase that specifically hydrolyses α (1 → 6) glucosidic linkages. After enzyme digestion, glucans 2 and 6 yielded significant amounts of water-insoluble material as well as amylopectin, but glucan-7 yielded no insoluble material. It is suggested that glucans 2 and 6 release long-chain amylose-like material but glucan-7 releases only short chain molecules. The water-soluble material of the reaction products was fractionated by the column of Bio-gel P-4, and various higher oligosaccharides bigger than hexasaccharide were obtained from these purified glucans, but only glucan-7 was found to have released various lower oligosaccharides in addition to these higher oligosaccharide.

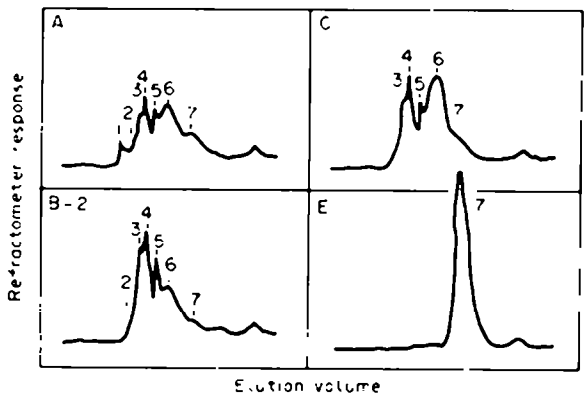

Fig. 1. HPLC resolutions of EtOH fractionated CS-glucans.

Thus, the structure of the major water-soluble polysaccharide, CS-glucan, involves an α(1 → 4) linked glucan,

Table 2. Properties of HPLC purified CS-glucans

HPLC peak	Mol. % Glc¹→	→⁶Glc¹→	→³,⁶Glc¹→	Iodine reaction λ_{max} (nm)	Anti-complementary activity (%) concentration (μg/ml) 1000	500	100
1	n.e.*	n.e.*	n.e.*	595	38.6	33.3	31.4
2	8.5	87.5	4.0	530	14.5	18.1	0
3	6.0	90.7	3.3	530	6.5	3.3	0.2
4	6.0	89.6	4.4	530	13.7	14.9	8.0
5	7.3	89.3	3.4	530	15.4	6.3	0.4
6	6.8	89.1	4.1	555	17.3	15.1	1.6
7	5.7	90.7	3.6	580	22.2	21.8	4.4

*Not examined because of the small quantity available.

to which different sized glucooligosaccharides are attached at position 6. The structure is similar to amylopectin.

EXPERIMENTAL

Materials. The seed of *Coix lacryma-jobi* var. *ma-yuen* without husks (Yoku-inin), from China, was purchased from Tochimoto Tenkaido Co. Ltd., Osaka, Japan. *Enterobactor aerogenes* pullulanase was purchased from Sigma. DEAE-Sepharose CL-6B, Sepharose CL-2B and Sephadex LH-20 were purchased from Pharmacia and Bio-gel P-4 (−400 mesh) from Bio-Rad.

General methods. Component sugar were determined by TLC of the acid hydrolysates (1 M TFA, 100°, 4 hr) [7], and by GC of the alditol acetate derivatives prepared from hydrolysates [8]. TLC of monosaccharides was performed on cellulose-coated plastic sheets (Merck, 5577) in EtOAc–pyridine HOAc–H_2O (5:5:1:3), and sugars were detected by using alkaline $AgNO_3$ [9]. GC (Shimadzu GC-6A) was equipped with FID and a glass column (0.3 × 200 cm) of 3% ECNSS-M or 1% silicone OV-225 on Uniport Hp at 190° or 180°, respectively. N_2 was used as a carrier gas at flow rate of 60 ml/min. HPLC was conducted by using Waters Model ALC/GPC 244 equipped with columns (0.76 × 50 cm) of Asahi-Pak GS-510 + GS-320 (Asahi Chemical Industry Co., Ltd., Japan) and developed with H_2O. Total carbohydrate was determined by PhOH H_2SO_4 method [10] with glucose as the standard. D-Glucose in CS-glucan was determined by the action of D-glucose oxidase [11]. Phosphorous content was measured as described by Chen *et al.* [12] with Pi as the standard. Protein was assayed by the method of Lowry *et al.* [13] with bovine serum albumin as the standard. Optical rotations were determined at 25°.

Isolation of water-soluble polysaccharides. C. lacryma-jobi (300 g) was decocted with 6 l. H_2O to half vol. and the residual material was further decocted with 3 l. H_2O as above. The extracts were combined and centrifuged (7500 rpm, 20 min) to remove insoluble material. The supernatant was lyophilized to give the water-soluble extract (CS-O, yield 13.5 g). CS-O was refluxed with 1.4 l. MeOH for 1 hr (3 times) and centrifuged to give a MeOH-insoluble ppt. The ppt. was dissolved in H_2O and then 4 vols of EtOH were added. The resulting ppt. was collected by centrifugation. This ppt. was redissolved in H_2O and dialysed against running H_2O for 3 days. The non-dialysable portion was centrifuged to remove H_2O-insoluble material and the supernatant was lyophilized (CS-1, yield 9 g).

Ion exchange chromatography of CS-1. CS-1 (2 g) was applied to a column (3.0 × 33.0 cm) of DEAE-Sepharose CL-6B (Cl^- form) equilibrated with H_2O. Material was eluted first with H_2O until sugar was no longer detected, and then the absorbed polysaccharide fractions were recovered by stepwise elutions of 0.2 M NaCl (500 ml) and 0.5 M NaCl (500 ml). The unabsorbed fraction was lyophilized to obtain CS-glucan (yield 1.63 g).

Gel filtration chromatography. CS-Glucan (50 mg) was applied to a column (2.6 × 87 cm) of Sepharose CL-2B, equilibrated with 0.2 M NaCl, and carbohydrates were eluted with 0.2 M NaCl.

EtOH *fractionation of CS-glucan.* CS-Glucan was fractionated by sequential EtOH precipitation. EtOH was added to the water soln of CS-glucan (2 g/10 ml) at the final concentrations of 15%, 20%, 30%, 40% and 50%, and the ppt. A (382 mg), B (878 mg), C (380 mg), D (52.6 mg) and E (20.8 mg) and the remaining sup. were obtained. Major ppt. B (878 mg) was further fractionated into B-1 (500 mg), B-2 (171 mg) and B-3 (74 mg) by the difference of solubility against H_2O.

Methylation analysis. EtOH fractionated glucans and HPLC purified glucans were methylated × 2–3 by the Hakomori procedure [14] and completeness of methylation was checked using triphenylmethane [15]. The methylated glucans were purified by Sephadex LH-20 column chromatography in $CHCl_3$/MeOH (1:1) [16]. The fully methylated glucan was heated with 2 M TFA at 121° for 1 hr [17]. The hydrolysate was converted into alditol acetates [18], and analysed by GC/MS. GC/MS was performed on a Hitachi M-80 mass spectrometer equipped with a glass column packed with 1% of Silicone OV-225 on Chromosorb W at 170° and operated at an ionisation voltage of 70 eV and an ion source at 180°.

NMR studies. ^1H NMR spectrum of EtOH fractionated glucan (CS-glucan c) was obtained for 3% soln in D_2O at 400.13 MHz and at 80°. Chemical shifts were expressed relative to that of sodium 3-(trimethylsilyl) propane-1-sulfonate (TSP). ^{13}C NMR spectrum of same glucan fraction was obtained at 100.16 MHz and room temp. with complete proton decoupling. Chemical shifts were expressed as δ values (ppm) from the signal of TSP.

Iodine reaction of glucans. Iodine reagent (0.02% I_2 containing 0.2% KI soln) (300 μl) was added to the glucan solns (500 μl). Then, absorption maximum or absorbance at 550 nm was measured.

Enzymic hydrolysis. HPLC-purified glucan (1.4–1.3 mg) was dissolved in 1 ml 10 mM citrate buffer (pH 5.0), pullulanase (1.0 unit) was added, and the mixture was incubated in the presence of one drop of toluene at 37° for 2 days. The incubation mixture was heated at 100° for 5 min and centrifuged to remove

the insoluble residue. The supernatant was lyophilized and lyophilizate was applied to a column (1 × 40 cm) of Bio-gel P-4 (−400 mesh).

Anti-complementary activity. The anti-complementary activity was measured according to the previously described procedure [19], except H_2O was used for dilutions.

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