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The distribution of carboxymethyl groups in *O*-(carboxymethyl)ated (1→3)-β-D-glucans and (1→3)-α-D-glucans*

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ABSTRACT

The distribution of the substituents in *O*-(carboxymethyl)ated, linear (1→3)-β-D-glucans, branched (1→3)-β-D-glucan, and (1→3)-α-D-glucans was analyzed by g.l.c. and g.l.c.–m.s. The results indicated that the distribution of the substituents in *O*-(carboxymethyl)-D-glucans was characteristic of glucan type, especially at position 2 on the D-glucosyl residues, and suggested that the conformation of the D-glucans affected the *O*-carboxymethylation pattern.

INTRODUCTION

(1→3)-D-Glucans are usually insoluble in water, and it has been reported[1,2] that carboxymethylation improves the solubility and antitumor activity against Sarcoma 180 in mice. We have elucidated the degree of substitution (d.s., the average number of carboxymethyl groups substituted per anhydroglucose unit) and the distribution of the *O*-(carboxymethyl) groups in carboxymethylcellulose (CMC) and carboxymethylstarch (CMS) consisting of (1→4)-linkages by a gas–liquid chromatographic (g.l.c.) method[3]. This assay involves the reduction of carboxymethyl groups to *O*-(hydroxyethyl) groups, hydrolysis of the resulting *O*-(hydroxyethyl)-D-glucan, and g.l.c. analysis of the hydrolyzate as the alditol acetates and as 1,2-*O*-ethylene-D-glucose derivatives[4–7].

In the present study, the distribution of the substituents in five samples of *O*-(carboxymethyl)ated (1→3)-D-glucans was investigated using the g.l.c. method. This paper deals with determination of d.s. and the distribution of *O*-(carboxymethyl) substituents in *O*-(carboxymethyl)ated products of linear (1→3)-β-D-glucans, a (1→6)-branched (1→3)-β-D-glucan, and a (1→3)-α-D-glucan by g.l.c. and g.l.c.–mass spectrometry (g.l.c.–m.s.).

EXPERIMENTAL

Materials. — Curdlan from *Alcaligenes faecalis* was purchased from Wako Pure Chemical Industries Co. (1→3)-β-D-Glucan (PS)[8] from *A. faecalis* var. *myxogenes* IFO

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13140, the *O*-(carboxymethyl)ation product of PS (CMPS)[1], and scleroglucan from *Sclerotium rolfsii* were kindly provided from Takeda Chemical Industries and San-ei Chemical Industries, respectively.

O-*(Carboxymethyl)ation.* — The glucans were *O*-(carboxymethyl)ated according to the method of Sasaki *et al.*[1] Briefly, the procedure is as follows: To a suspension of scleroglucan (1.50 g) in 2-propanol (40 mL), a 30% solution of sodium hydroxide (6 mL) was added with stirring for 1.5 h at room temperature. Then chloroacetic acid (1.8 g) was added to the mixture with stirring for 5 h at 60–70°. The suspended product was recovered by filtration, washed, and dialyzed against distilled water. The non-dialyzed fraction was lyophilized to *O*-(carboxymethyl)-D-glucan (1.56 g). (1→3)-α-D-Glucan (AG-AL) from *Agrocybe cylindracea* (Fr.) Maire was similarly *O*-(carboxymethyl)ated to give water-soluble (AG-AL-CMS) and gelatinous (AG-AL-CMI) products as previously described[9].

Analysis of O-*(carboxymethyl)*-D-*glucans by g.l.c. and g.l.c.–m.s.* — Reduction of *O*-(carboxymethyl)-D-glucans and preparation for g.l.c. and g.l.c.–m.s. were performed as previously described[3]. Each *O*-(carboxymethyl)-D-glucan (100 mg) was dissolved in water (30 mL), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (1 g) was added. The pH of the reaction mixture was maintained at pH 4.75 by titration with 0.1M hydrochloric acid with stirring for 2 h. Then, 2M sodium borohydride (10 mL) was gradually added to the reaction mixture, and the pH was maintained at pH 7.0 by addition of 4M hydrochloric acid. The solution was dialyzed, and the product of the non-dialyzable fraction was reduced two additional times under the same conditions. The final product *O*-(hydroxyethyl)-D-glucan was successively hydrolyzed with 90% formic acid for 5 h at 100° and with 2M trifluoroacetic acid for 4 h at 100°. The hydrolyzate was reduced with sodium borohydride and acetylated with 1:1 acetic anhydride–pyridine for 2 h at 95°. The resulting derivatives were analyzed by g.l.c. and g.l.c.–m.s. G.l.c. was performed with either a Shimadzu 4CM gas chromatograph equipped with a hydrogen flame-ionization detector and a glass column (1.5 m × 0.3 cm) packed with 2% EGSS-X on Chromosorb W AW DMCS (60–80 mesh) or with a Shimadzu 15A instrument with a CP-Sil 88 FS-WCOT fused-silica capillary column (25 m × 0.25 mm), as previously described[3]. Peak areas and retention times were measured by use of a Shimadzu Chromatopac C-R5A integrator. G.l.c.–m.s. was performed with a JEOL JMS-D 300 apparatus equipped with a glass column (1 m × 0.2 cm) packed with 2% EGSS-X and operated at 185°. The mass spectra were recorded at an ionizing potential of 70 eV, an ionizing current of 50 μA, and an ion-source temperature of 220°.

RESULTS AND DISCUSSION

The original β-D-glucans of *O*-(carboxymethyl)ated D-glucans were curdlan (d.p. 450) and PS (d.p. 540) from *Alcaligenes faecalis* as a linear (1→3)-β-D-glucan, and scleroglucan (d.p. 800) from *Sclerotium rolfsii* as a β-(1→6)-branched (1→3)-β-D-glucan (one branch per every three residues of the main chain). AG-AL (d.p. 3400) was a linear (1→3)-α-D-glucan isolated from the fruiting bodies of *Agrocybe cylindracea*. These

TABLE I

G.l.c. and g.l.c.–m.s. spectroscopy of D-glucose derivatives from hydrolyzates of reduced *O*-(carboxymethyl)-D-glucans

Peak number	*Relative retention time*[a]	*Sugar derivatives*[b] *(as acetates)*	*Prominent fragments* (m/z)	*Percentage of total*				
				CMCD	*CMPS*	*CMSG*	*AG-AL-CMS*	*AG-AL-CMI*
1	0.62	1,2-*O*-etn-α-D-Glc*f*	43,73,127,170,187,273	—	—	0.7	1.4	0.9
2	0.77	1,2-*O*-etn-α-D-Glc*p*	43,73,86,157,170,199,230,272	—	—	2.1	7.3	5.0
3	1.00	D-Glc-ol	43,73,145,217,289,361,375	16.4	44.2	48.0	39.1	41.6
4	1.64	1,2-*O*-etn-β-D-Glc*p*	43,73,86,157,170,199,230,272	—	—	0.9	1.2	1.8
5	1.94	1,2-*O*-etn-6-*O*-he-α-D-Glc*f*	43,73,87,127,187	—	—	0.6	1.4	2.6
6	2.54	1,2-*O*-etn-6-*O*-he-α-D-Glc*p*	43,73,86,157,170,199,259,316	—	—	1.1	2.9	2.0
7	2.93	6-*O*-he-D-Glc-ol	43,87,117,170,375	36.7	29.5	14.8	14.8	9.3
8[c]	*3.33*	*2-O*-he-D-Glc-ol	43,73,87,189,375,405	—	—	2.4	9.8	7.6
9[c]		*3-O*-he-D-Glc-ol	43,87,115,261,333,375	—	—	2.1	—	—
10	3.73	4-*O*-he-D-Glc-ol	43,87,115,261,333,375	14.3	12.7	16.0	7.3	9.7
11	4.55	1,2-*O*-etn-4-*O*-he-D-Glc*p*	43,73,115,145,173,245,275,317	—	—	—	—	4.7
12	4.99	1,2-*O*-etn-6-*O*-he-β-D-Glc*p*	43,73,86,157,170,199,259,316	—	—	—	5.5	10.1
13	9.99	4,6-*O*-dihe-D-Glc-ol	43,73,87,117,305,333,419	32.6	13.6	11.5[d]	—	—
14	10.09	2,4-*O*-dihe-D-Glc-ol	43,73,87,189,261,333,375	—	—	—	9.6[e]	4.6[e]

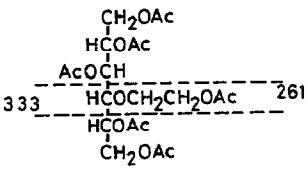
[a] Relative to hexa-*O*-acetyl-D-glucitol on a packed column (EGSS-X). [b] etn, ethylene; he, hydroxyethyl; dihe, dihydroxyethyl. [c] Peak numbers 8 and 9 can be separated with a capillary column (CP-Sil 88) as described in a previous paper[3]. [d] Overlapping peak with 2,4-*O*-dihe-D-Glc-ol. [e] Overlapping peak with 2,6-*O*-dihe-D-Glc-ol.

glucans were carboxymethylated by the method using chloroacetic acid in 2-propanol and 30% sodium hydroxide in which the reaction mixtures were kept in suspension. Free hydroxyl groups of the D-glucosyl residues were only partially carboxymethylated by this method. The native glucans were both water-insoluble and alkali-soluble, but all *O*-(carboxymethyl)ated β-D-glucans (CMCD from curdlan, CMPS from PS, and CMSG from scleroglucan) were soluble in water. Water-soluble (AG-AL-CMS) and gelatinous (AG-AL-CMI) products were obtained by *O*-(carboxymethyl)ation of (1→3)-α-D-glucan (AG-AL).

The *O*-(carboxymethyl)-D-glucans were analyzed as follows: They were first reacted with 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide, and the resulting carbodiimide-activated carboxymethylated D-glucans were reduced with sodium borohydride to yield the hydroxyethylglucans. The products were hydrolyzed with acid and the hydrolyzates were reduced with sodium borohydride and then acetylated. The derivatives thus obtained were analyzed by g.l.c. with both CP-Sil 88 capillary and 3% EGSS-X columns and by g.l.c.–m.s. and identified by comparing their retention times with those of authentic samples and/or by m.s. analyses[5]. 2-*O*-(Hydroxyethyl)-D-glucose derived from 2-*O*-(carboxymethyl)-D-glucose was partially converted into 1,2-*O*-(ethylene)-D-glucose derivatives in the acid hydrolysis. These components gave the various peaks on g.l.c. as described in the following paragraph.

The results of the g.l.c. and g.l.c.–m.s. analyses are shown in Table I. Peak 10 was identified as 4-*O*-acetoxyethyl-1,2,3,5,6-penta-*O*-acetyl-D-glucitol (**1**) by the mass fragments at *m/z* 261 resulting from C-3–C-4 cleavage and *m/z* 333 from cleavage between C-4 and C-5. From the intense peaks at *m/z* 305, 333, and 117 arising from C-3–C-4, C-4–C-5, and C-5–C-6, peak 13 was identified as 4,6-di-*O*-(acetoxyethyl)-1,2,3,5-tetra-*O*-acetyl-D-glucitol (**2**). 2,4-Di-*O*-(acetoxyethyl)-1,3,5,6-tetra-*O*-acetyl-D-glucitol (**3**) (peak 14) was also identified by characteristic mass fragments at *m/z* 189 and 333 from C-2–C-3 cleavage, and at *m/z* 261 from cleavage between C-3 and C-4.

The dissociation pattern of peak 6 or 12 [6-*O*-(acetoxyethyl)-3,4-di-*O*-acetyl-1,2-*O*-ethylene-α- or -β-D-glucopyranose] were as follows: Dissociation proceeded successively by elimination of CH_3COOH, $CH_2=CO$ and $CH_2OCH_2CH_2OCOCH_3$ to *m/z* 316 [376(M^+) − CH_3COOH], to *m/z* 272 (316 − $HOCH=CH_2$), to *m/z* 230 (272 − $CH_2=CO$), to *m/z* 170 (230 − CH_3COOH), then to *m/z* 86; or to *m/z* 259 (376 − $CH_2OCH_2CH_2OCOCH_3$), to *m/z* 199 (259 − CH_3COOH), to *m/z* 157 (199 − $CH_2=CO$), then to *m/z* 86. This pattern is similar to that of peak 2 (3,4,6-tri-*O*-acetyl-1,2-*O*-ethylene-α- or -β-D-glucopyranose) as previously reported[5]. On the other



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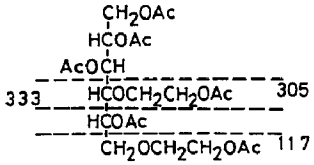

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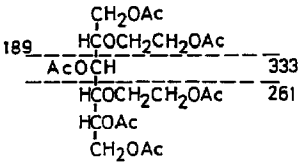

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hand, the dissociation of peak 11 is different from that of either peak 6 or 12. The dissociation proceeded successively by elimination of CH_3COO, CH_2CO, OCH_2CH_2O, CH_2O, CH_2CH_2 and CHO, to *m/z* 317 [376(M^+) − CH_3COO], to *m/z* 275 (317 − CH_2 = CO), to *m/z* 245 (275 − CH_2O), to *m/z* 173 (275 − CH_2 = CO − OCH_2CH_2O), to *m/z* 145 (173 − CH_2CH_2), to *m/z* 115 (145 − CH_2O), then to *m/z* 86 (115 − CHO). Peak 11 was assigned tentatively to be 4-*O*-(acetoxyethyl)-3,6-di-*O*-acetyl-1,2-*O*-ethylene-D-glucopyranose.

The derivatives of the various peak numbers are assigned as follows: peak no. 1 (1,2-*O*-ethylene-α-D-glucofuranose), no. 2 (1,2-*O*-ethylene-α-D-glucopyranose), no. 4 (1,2-*O*-ethylene-β-D-glucopyranose), and no. 8 [2-*O*-(hydroxyethyl)-D-glucitol] from the substituents at O-2; that of peak no. 9 [3-*O*-(hydroxyethyl)-D-glucitol] from the substituents at O-3; that of peak no. 10 [4-*O*-(hydroxyethyl)-D-glucitol] from the substituents at O-4; that of peak no. 7 [6-*O*-(hydroxyethyl)-D-glucitol] from the substituents at O-6; those of peak nos. 5 [1,2-*O*-ethylene-6-*O*-(hydroxyethyl)-α-D-glucofuranose], 6 [1,2-*O*-ethylene-6-*O*-(hydroxyethyl)-α-D-glucopyranose], and 12 [1,2-*O*-ethylene-6-*O*-(hydroxyethyl)-β-D-glucopyranose] from the substituents at O-2 and O-6; those of peak nos. 11 (1,2-*O*-ethylene-4-*O*-(hydroxyethyl)-D-glucopyranose] and 14 (2,4-di-*O*-(hydroxyethyl)-D-glucitol) from the substituents at O-2 and O-4, and that of peak no. 13 [4,6-di-*O*-(hydroxyethyl)-D-glucitol] from the substituents at O-4 and O-6. Hexa-*O*-acetyl-D-glucitol of peak no. 3 was derived from unsubstituted residues.

In *O*-(carboxymethyl)ated linear (1→3)-β-D-glucans, CMCD and CMPS, only four peaks were observed (Fig. 1), *i.e.*, 6-*O*-(hydroxyethyl)- and 4-*O*-(hydroxyethyl)-D-glucitol acetates as monosubstituted units, 4,6-di-*O*-(hydroxyethyl)-D-glucitol acetate as disubstituted units, and D-glucitol acetate were indentified, but the derivatives from D-glucose substituted at O-2 were not detected. In *O*-(carboxymethyl)ated (1→6)-

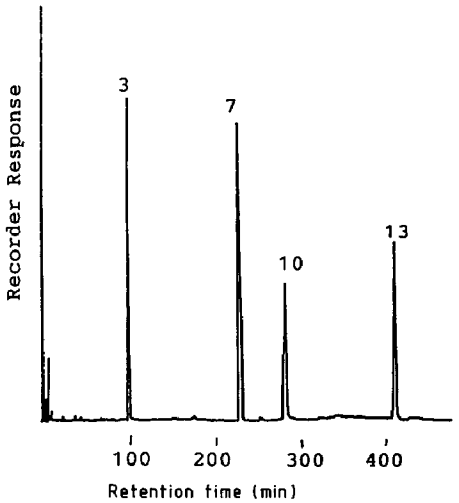

Fig. 1. G.l.c. of the alditol acetates from the acid hydrolyzate of reduced CMCD. Conditions: CP-Sil 88 capillary column at 180°→220°. For identification of each peak, see Table I.

TABLE II

Patterns of O-(carboxymethyl) substitution on D-glucose

Compound	*Percentage of total*								*D.s.*[c]
	Glc	*2-CM-Glc*[a]	*3-CM-Glc*	*4-CM-Glc*	*6-CM-Glc*	*4,6-diCM-Glc*[b]	*2,4-diCM-Glc*	*2,6-diCM-Glc*	
CMCD	16.4	—	—	14.3	36.7	32.7	—	—	1.16
CMPS	44.2	—	—	12.7	29.5	13.6	—	—	0.68
CMSG	48.0	6.1	2.1	16.0	14.8	11.5[d]	—	1.7	0.65
AG-AL-CMS	39.1	19.7	—	7.3	14.8	—	—, 9.6[e]	9.8	0.80
AG-AL-CMT	41.6	15.4	—	9.7	9.3	—	4.7, 4.6[e]	14.7	0.82

[a] 2-CM-Glc = 2-*O*-(carboxymethyl)-D-glucose. [b] 4,6-diCM-Glc = 4,6-di-*O*-(carboxymethyl)-D-glucose, *etc.* [c] Degree of substitution. [d] Contains small amounts of 2,4-diCM-D-Glc-ol. [e] Mixture of 2,4-diCM-D-Glc-ol and 2,6-diCM-D-Glc-ol.

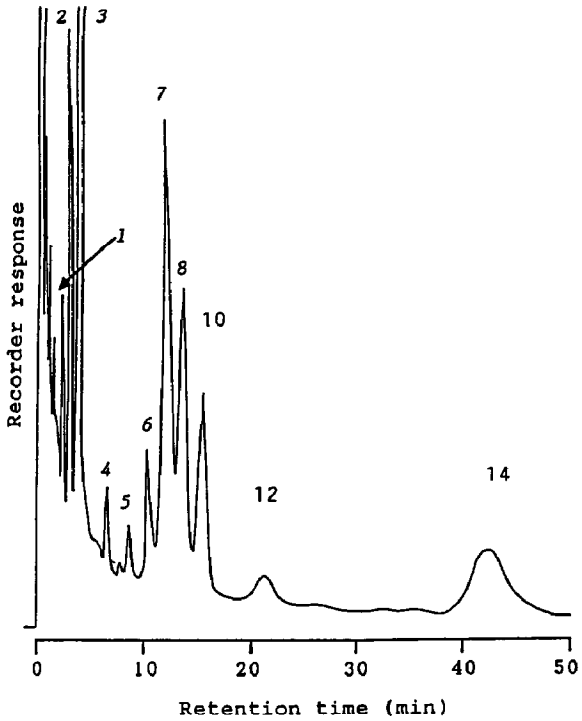

Fig. 2. G.l.c. of the alditol acetates from the acid hydrolyzate of reduced AG-AL-CMS. Conditions: 2% EGSS-X on Chromosorb-W at 215°.

branched (1→3)-β-D-glucan, CMSG, small amounts of other derivatives (peak nos. 1, 2, 4, 5, 6, 8, 9, and 14), in addition to the above derivatives, were detected because non-reducing terminal residues attached to O-6 of the residues in the main chain may be easily (carboxymethyl)ated. D.s. values were calculated from the individual peak areas on g.l.c. to be 1.16 for CMCD, 0.68 for CMPS, and 0.65 for CMSG (see Table II).

On the other hand, many peaks were observed on g.l.c. of *O*-(carboxymethyl)ated (1→3)-α-D-glucans (AG-AL-CMS and AG-AL-CMI) as shown in Fig. 2. The results showed that carboxymethyl groups were located at O-2, at O-4, at O-6, at O-2 and O-4, and at O-2 and O-6 on D-glucose, although the substituents at O-4 and O-6 observed in *O*-(carboxymethyl)ated (1→3)-β-D-glucans are absent. Water-soluble, AG-AL-CMS (d.s. 0.80) had more the substituents at O-2 and at O-6 than gelatinous AG-AL-CMI (d.s. 0.82). Thus the difference in the distribution of the *O*-carboxymethyl groups may affect the solubility.

The conformation of (1→3)-β-D-glucans has been reported to be of a helical structure[10,11] with position-2 of the D-glucosyl units inside the helix, while the (1→3)-α-D-glucan is a ribbon-like structure[10] on which any of the free hydroxyl groups in the α-D-glucan can be *O*-(carboxymethyl)ated. Since substituents at O-2 in the (1→3)-β-D-glucans were not observed, the glucans were shown to retain their conformation during *O*-(carboxymethyl)ation in 2-propanol. The substituents at O-2 in *O*-(carboxymethyl)-

ated (1→3)-α-D-glucans were located. Thus the substituents exhibited a characteristic distribution pattern between *O*-(carboxymethyl)ated (1→3)-β-D-glucans and (1→3)-α-D-glucans. As it has been reported that d.s. and distribution of carboxymethyl groups is related to antitumor activity against Sarcoma 180 in mice[1,2], these analytical data should give interesting information.

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