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Preparation and biological properties of dicarboxy-glucomannan: enzymatic degradation and stimulating activity against cultured macrophages

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Recently, it has been reported that some polysaccharides showed interesting biological or immunological activities. Glucomannan (GM) purified from *Amorphophallus Konjac* is a copolymer of 1,4-linked β-D-glucose and β-D-mannose, which has some mono-residual D-glucose or D-mannose branches at the 3-position of D-mannose units of the main chain. We prepared dicarboxy-glucomannan (DC-GM), a carboxylic acid derivative of glucomannan, by IO^{4-}/ClO^{2-} oxidation. The number-average molecular weight (M_n) of the DC-GM obtained was about $2{\cdot}0 \times 10^4$. The chemical structure of DC-GM was investigated by gas-chromatography. It showed high water-solubility and enzymatic degradation behavior by cellulase and β-D-glucosidase. The enzymatic degradation rate of the DC-GM was dependent on the degree of introduction of carboxylic acid group. The immunological enhancement activity of the DC-GM was evaluated *in vitro* by testing glucose consumption and β-D-glucuronidase activity against cultured macrophages, PMA (phorbol-12-myristate-13-acetate)-differentiated HL-60 (*human promyelocytic leukemia*) or U937 (*human monoblast leukemia*) cells. The DC-GM showed higher stimulating effects against such cultured macrophages than the other polysaccharide derivatives.

INTRODUCTION

Many polysaccharides are non-toxic, non-immunogenic and show good biocompatibility. For example, dextran has been used as a plasma expander. Polysaccharides are relatively stable against non-enzymatic hydrolysis, however, they are easily degraded in certain manners by specific enzymes. Therefore, they are expected to apply for biomaterials which can be degraded under specific conditions. Recently, it has been reported that some polysaccharides showed interesting biological or immunological activities. Mannan from yeast (Suzuki *et al.*, 1969, 1971), chitin (Sirica & Woodman, 1971) and some kinds of 1,3-β-D-glucan, such as lentinan (Maeda *et al.*, 1971), were reported to show antitumor activity by immunological pathway. The biological activities of naturally occurring polysaccharides are also attractive for biomedical application.

However, most of the polysaccharides have no functional group except for the hydroxy group, and they do not always show water-solubility because of their high crystallity and the formation of hydrogen bonds. In order to prepare a chemically modified polysaccharide, the introduction of highly reactive and polar functional groups, such as carboxylic acid group or primary amino group, should be effective for enhancement of reactivity and solubility.

Saccharides play important roles in biological recognition on cell surfaces. In many cases, recognizable oligosaccharides have very complex branched structures. However, a few terminal saccharide residues play a critical role in biological recognition. For example, liver parenchymal cells recognize galactose or *N*-acetylgalactosamine (Hudgin & Ashwell, 1974). Macrophages have receptors for mannose (Wileman *et al.*, 1985). From the standpoint of glycotechnology, many studies apply such recognition abilities of saccharides to biologically functional materials. Akaike *et al.* (1989) reported that a polystyrene derivative having lactose residues could be recognized by liver parenchymal cells and so use of this polymer as a cell culture matrix or liver-specific drug carrier was a possibility (Akaike *et al.*, 1989; Goto *et al.*, 1992). A technique for chemical syntheses of naturally occurring branched oligosacchar-

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ide chains has been developed. But, it is not possible yet to synthesize freely branched oligosaccharides having various sequences. Most naturally occurring polysaccharides possess a certain degree of branched structure. The existence of this branched structure means that they possess many terminal saccharide residues. Such branched polysaccharides are interesting for biological recognition.

It was reported that some kinds of glucan, such as grifolan (Takeyama *et al.*, 1987), lentinan (Dennert & Tucker, 1973) and curdlan (Sasaki *et al.*, 1979), showed stimulating activity of immunocompetent cells or antitumor activities. Glucomannan (GM) purified from root of plant, *Amorphophallus Konjac*, is a copolymer of 1,4-linked β-D-glucose and β-D-mannose, which has some mono-residual D-glucose or D-mannose branches at the 3-position of D-mannose units of main chain (Maeda *et al.*, 1980) (Fig. 1). The immunological activity of GM having mannose branches is interesting because it is expected to show an affinity for macrophages, having a receptor for mannose. However, it did not show high water-solubility and the biological activity of GM was not clearly revealed.

Introduction of carboxylic acid groups into polysaccharide is one of the most general methods to give polysaccharides water-solubility. Carboxymethylation and ring-opening oxidation are the methods generally used to introduce carboxylic acid groups to polysaccharides. It was reported that some carboxylic acid derivatives of glucan, such as grifolan (Adachi *et al.*, 1989) or curdlan (Sasaki *et al.*, 1979), also showed immunological enhancement activity or antitumor activities. In the carboxymethylation method, control at the site of introduction of the carboxymethyl group is not easy. However, in the ring-opening IO^{4-}/ClO^{2-} oxidation method, polysaccharides containing only 1,4-linked glucose and mannose units are selectively oxidized at the C-2 and C-3 position (diol group), and the structure of the product should be, chemically, as expected.

In this study, we report on the synthesis of a water-soluble carboxylic acid derivative of GM, dicarboxyglucomannan (DC-GM), by IO^{4-}/ClO^{2-} oxidation, structural analysis by gas-chromatography and some biological properties. In order to evaluate DC-GM as a biodegradable material, we investigated its degradation behavior by some enzymes *in vitro*. However, it is reported that some polyanions could activate macrophages (Donaruma *et al.*, 1980). The DC-GM, anionic derivative of glucan, having mannose branches is expected to show a high activation effect against macrophages having mannose receptors. So, we evaluate the stimulation effect of DC-GM against cultured macrophages, PMA(phorbol-12-myristate-13-acetate)-differentiated HL-60 (*human promyelocytic leukemia*) or U937 (*human monoblast leukemia*) cells, *in vitro* by testing glucose consumption and β-glucuronidase (a kind of lysosome enzyme) activity. Further, we discuss the relationship between their chemical structures and stimulating activities against cultured macrophages *in vitro*.

EXPERIMENTAL

Materials

Glucomannan (GM) obtained from *Amorphophallus Konjac* was purified according to the method described in Nishida and Hashima (1930). Cellulase from *Trichoderma viride* (paper broken activity 8300 units per mg solid) was purchased from Wako Pure Chemical Industry. β-D-Glucosidase from almond (activity 4800 units per gram solid) was purchased from Sigma. 6-*O*-Carboxymethylchitin (CM-chitin), degree of introduction of carboxymethyl group (DCM) = 60 mol%/sugar unit, $M_n = 5 \times 10^3$, was supplied from Katokichi Co. RPMI-1640 medium (Moore *et al.*, 1966) was purchased from Nissui Pharmaceutical Co., Ltd. The other reagents were commercial grade and used without further purification.

Oxidation of GM

Preparation of DC-GM was carried out according to the reference (Matsumura *et al.*, 1991) with minor modifications, and is described as a typical example (Scheme I). GM (300 mg) was suspended in 0.050 M aqueous sodium periodate (8.70 ml) and stirred at 0°C for 1 h in the dark, the reaction suspension was then filtered. The filtrate was dialyzed using cellulose tube in distilled water for 3 days and freeze-dried to give dialdehyde-glucomannan in 252 mg yield (84.3 wt%) as a white powder. The generation of aldehyde group was confirmed by precipitation formation after treatment with phenylhydrazine and appearance of a C=O group in IR spectra (1650 cm^{-1}) and ^{13}C-NMR spectra (δ = 185 ppm). The obtained dialdehyde-glucomannan (252 mg) was dissolved in

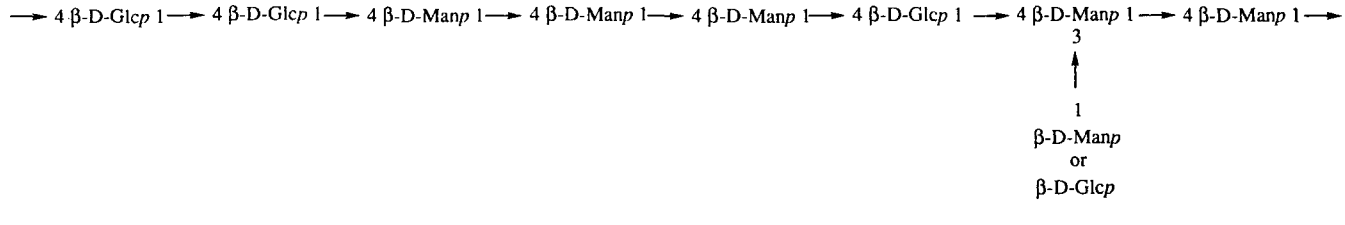
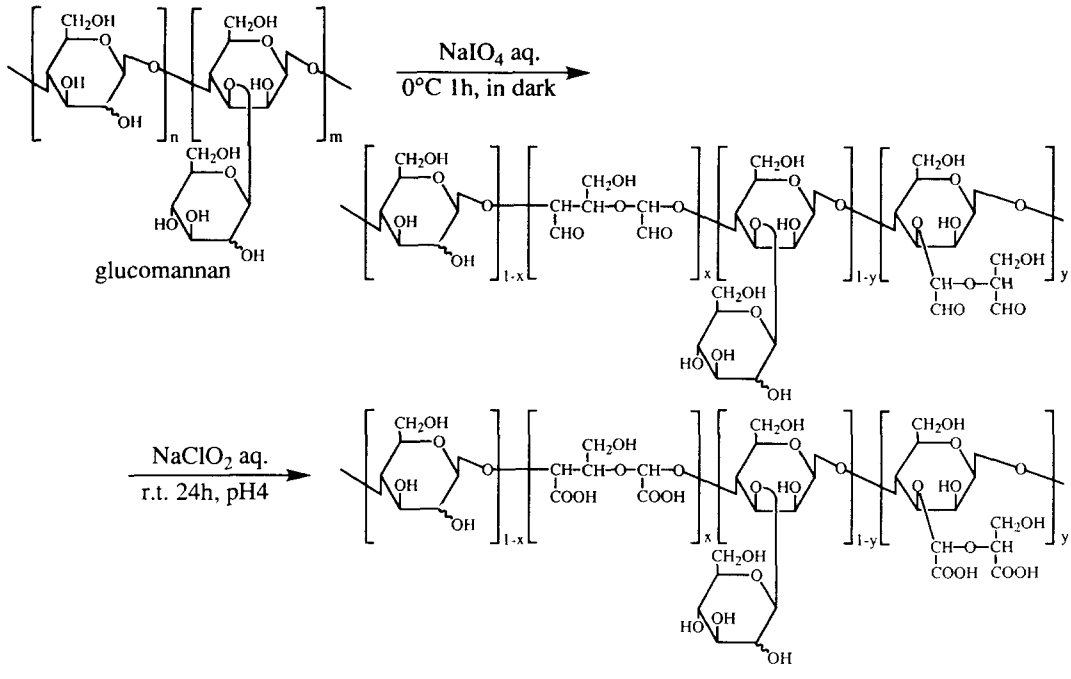


Fig. 1. Assumed unit sequence of glucomannan (GM).



Scheme I

3.2 ml of aqueous sodium chlorite (3·32 mol/l). The pH of the solution was adjusted to 4 by addition of acetic acid. The reaction mixture was then stirred at room temperature for 24 h, nitrogen was then passed through the solution until a colorless solution was obtained. After the pH of the solution was raised to 9 with 1 M aqueous NaOH, the solution was dialyzed in distilled water for 7 days. The obtained solution was freeze-dried to give the sodium salt of DC-GM in 253 mg yield (93.4 wt%) as a white powder. The obtained sodium salt of DC-GM (250 mg) was dissolved in 20 ml of water and the pH of the solution was adjusted to 2.5 with 3 M HCl. This solution was ultrafiltrated for 3 h and reprecipitated in acetone. The precipitate obtained was dissolved in water, ultrafiltrated for 3 h again and freeze-dried to give DC-GM in 197 mg yield (78.8 wt%) as a white powder.

The degree of introduction of carboxylic acid group (DCA) (mol%/sugar unit) measured by a colloid titration method (Toei & Kohara, 1976) was 28 mol%/sugar unit, which means the molar content of opened ring is equal to 14 mol%/sugar unit. Number-average molecular weight (M_n) of the DC-GM was determined to be $2{\cdot}29 \times 10^4$, and the ratio of weight-average molecular weight (M_w) to M_n was determined to be 2·10 by gel-permeation chromatography (GPC) using the data processing system, Labchart 180 (System Instruments Co.). The following spectral data of DC-GM confirmed the structure. IR (KBr Disk): absorptions at 3385 (OH), 2936 (CH_2), 1740 (COOH), 1027 cm^{-1} (C–O–C). ^{13}C-NMR (D_2O): δ 62.7–63.2 (CH_2), 70.4–76.3 ($CH(CH_2OH)$), 103.0–105.5 (OCHO), 172.6–178 ppm (COOH).

Dicarboxy-dextran (DC-Dex) was prepared by similar procedures to the ones described above. The M_n and DCA for DC-dex were determined to be $1{\cdot}95 \times 10^4$ and 10 mol%, respectively.

Structural analysis by gas-chromatography

The content ratios of D-mannose to D-glucose for GM and DC-GM were determined by gas-chromatography analysis after acidic hydrolysis. GM (30·0 mg) or DC-GM (30·0 mg) were dissolved in 1 M aqueous HCl, and the solution was kept at 100°C until the start spot (R_f = 0·00) disappeared using thin-layer chromatography (developing solvent: 1-propanol/ammonia/water = 6/2/1). The solution was cooled to room temperature and evaporated under reduced pressure. Distilled water was added to the precipitate and evaporated again to remove hydrochloride. These procedures were repeated until hydrochloride was completely removed. The obtained acidic hydrolysates (10 mg) were converted to the trimethylsilyl ethers by the methods previously described (Sweeley *et al.*, 1963). The gas-chromatography analysis was performed by using the fused capillary silica column containing an OV-101 (25 m × 0·2 mm (i.d.) stainless column, Hewlett-Packard Co. & Ltd) with an ascending thermal column at 140–270°C (initial temperature was 140°C for 3 min, ascending temperature was 10°C/min for 13 min and the final temperature was 270°C for 5 min). The gas-chromatography unit was a Hewlett-Packard Model HP5890A incorporating a hydrogen frame detector operated under the following conditions, carrier gas: He, He pressure: 28·45 psi, H_2 pressure: 17·07 psi, air pressure: 31·29 psi. The trimethylsilyl ethers of D-glucose and D-mannose prepared by the same methods described

above were used as reference derivatives. The ratio of the sum of the α and β anomer of D-mannose to that of D-glucose was calculated.

The branched structures of GM and DC-GM were investigated by direct permethylation and gas-chromatography analysis. GM or DC-GM were methylated by the Haworth method (Haworth, 1915). After isolation of partially methylated products, they were permethylated completely with methyl iodine by the Purdie method (Jones, 1947). The fact that the permethylations proceeded completely was confirmed by disappearance of the hydroxy group absorption in the IR spectra. The permethylated polysaccharides were hydrolysed with 90%(v/v) aqueous formic acid in an oil bath (100°C) for 1 h. The acidic hydrolysate of permethylated polysaccharide was added to sodium borohydride and stirred for 2 h at room temperature for reduction to take place. Excess reagents were deactivated with 0.1 M hydrochloric acid, and sodium ion was removed by passing through Amberlite IR-120 (H^+) ion exchange resin. Then, the obtained solution was evaporated under reduced pressure. The residue was dissolved in methanol and evaporated again to remove boric acid as trimethylborate. These procedures were repeated three times. The obtained alditols were acetylated with an excess amount of acetic anhydride in pyridine at 100°C for 2 h. The solution was cooled to room temperature and evaporated under reduced pressure to give partially methylated alditol acetate derivatives. They were analysed by gas-chromatography under the same conditions described above. Partially methylated alditol acetate derivatives were chemically synthesized according to the method reported previously (Hirst & Macbeth, 1926) and used as references.

Enzymatic degradation of DC-GM

The obtained DC-GM is a polysaccharide derivative containing β-1,4-oligoglucose unit. Kato *et al.* (1970) reported the degradation of GM by cellulase. Therefore, we investigated degradation behavior of DC-GM by cellulase and β-D-glucosidase in aqueous solution. DC-GM (5.00 mg) and cellulase (1·00 mg) were dissolved in 1/15 M phosphate buffer (pH 4·5, 5·0 ml) and stirred at 40°C. DC-GM (5·00 mg), β-glucosidase (20·0 mg) and 1/15 M phosphate buffer (pH 5·0, 5·0 ml) were stirred at 37°C. A buffer solution of DC-GM without enzymes was stirred at the same conditions and used as a blank. The degradation rate was evaluated by the decrease of M_n, which was measured by GPC.

Assay of glucose consumption activity

The assay of glucose consumption activity was carried out according to the methods described previously (Ohya *et al.*, 1993). The HL-60 (*human promyelocytic leukemia*) cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum with kanamycin at 37°C in a 5% CO_2 atmosphere (Collins *et al.*, 1977). HL-60 (1.0×10^5 cells/well) were cultured for 5 days at 37°C after treatment with 40 nM PMA to differentiate macrophage-like cells (Huberman *et al.*, 1982; Harris & Ralph, 1985). After macrophage-like HL-60 cells were activated by DC-GM or other samples, glucose remaining in the culture supernatant was measured by the use of the Glucose B-test Wako (Adachi *et al.*, 1990; Suzuki *et al.*, 1991). The supernatants (20 μl) obtained from the culture sampled for 48 h at 37°C were incubated with 300 μl of color reagent for 20 min at 37°C. The optical density at 505 nm of the solution was determined from a calibration curve with standard glucose solution. The results were expressed as percentage glucose consumption, calculated from the following formula:

$$\text{Relative activity } (\%) = [(B - A)/(B - A_0)] \times 100.$$

A_0, glucose content in culture medium cultured with PMA-differentiated HL-60 cells; A, glucose content in culture medium cultured with PMA-differentiated HL-60 cells and test samples; B, glucose content in culture medium without cells.

DC-dex (DCA = 10 mol%/sugar unit) and CM-chitin (DCM = 60 mol%/sugar unit) were used as reference carboxylic acid derivatives of polysaccharides.

Assay of β-D-glucuronidase activity

β-D-Glucuronidase activity in the cell lysate was measured by hydrolysis of 4-nitrophenyl β-D-glucuronide (Adachi *et al.*, 1990; Greenberger *et al.*, 1978; Ohya *et al.*, 1994). The U937 cells were cultured in RPMI-1640 medium containing 10% fetal calf serum with kanamycin at 37°C in a 5% CO_2 atmosphere. U937 cells (1×10^6 cells/well) were cultured at 37°C for 5 days after treatment with 40 nM PMA to differentiate macrophage-like cells (Harris & Ralph, 1985; Ralph *et al.*, 1982). After macrophage-like U937 cells were activated by DC-GM or other samples for 24 h, the macrophage-like U937 cells lysate prepared with 40 μl of 10% Triton X-100 was mixed with 100 μl of 6 M 4-nitrophenyl β-D-glucuronide in 0.1 M citrate buffer (pH 5.0). After incubation at 37°C for 2 h, the reaction was halted with 100 μl of 0.2 M borate buffer (pH 9.8). The *p*-nitrophenol released by the enzyme-dependent hydrolysis of the substrate was quantified spectrophotometrically by measuring optical density at 405 nm using a Corona MTP-120 microplate reader. The values of β-D-glucuronidase activity were calculated by comparing with the value obtained for the control experiment without treatment.

Table 1. Preparation of dicarboxy-glucomannan (DC-GM)

Run	Oxidation of glucomannan to dialdehyde-glucomannan by aqueous $NaIO_4$			Oxidation of dialdehyde-glucomannan to dicarboxy-glucomannan by aqueous $NaClO_2$[a]			
	Time (h)	Concentration of $NaIO_4$ (mol/liter)	Yield (wt%)	Yield (wt%)	DCA[b] (mol%/sugar unit)	M_n[c]	M_w/M_n[c]
1	1	0·230	91·6	92·7	62	$1·79 \times 10^4$	2·28
2	1	0·115	81·2	92·5	46	$1·93 \times 10^4$	2·05
3	1	0·077	87·6	90·1	42	$2·03 \times 10^4$	2·15
4	1	0·050	84·3	93·4	28	$2·29 \times 10^4$	2·10
5	1	0·040	79·8	97·3	16	$1·80 \times 10^4$	2·07
6	5	0·050	87·7	80·2	74	$0·96 \times 10^4$	1·98

[a] Concentration of $NaClO_2$ = 3·32 mol/l, reaction time = 24 h.
[b] Degree of introduction of carboxylic acid group per sugar unit.
[c] Estimated by GPC.

Measurement

Molecular weight of polymers was measured with a gel-permeation chromatography (GPC) system (column: Shodex OHpack B-805, Showa Denko K.K., eluent: 1/15 M phosphate buffer, standard: pullulan) with a data processing system, Labchart 180 (System Instruments Co.). IR spectra were measured on a Perkin-Elmer 1600 Series FT-IR spectrometer. ^1H- and ^{13}C-NMR spectra in D_2O were measured with a JEOL GSX-400 spectrometer using DSS as the initial reference. The gas-chromatography measurement was carried out using a Hewlett-Packard Model HP5890A. The column used was the fused capillary silica column containing an OV-101 (25 m × 0.2 mm (i.d.) stainless column, Hewlett-Packard Co. & Ltd). The detailed conditions of gas-chromatography were as described previously.

RESULTS AND DISCUSSIONS

Preparation of DC-GM

DC-GMs having various DCA values could be prepared by the ring-opening IO^{4-}/ClO^{2-} oxidation method. The reaction condition, DCA values and molecular weights of the DC-GMs obtained are summarized in Table 1. The DCA values could be controlled by varying the concentration of sodium periodate at the first step of the reaction. The M_n of the DC-GMs was about 2×10^4. When the reaction time of the first step was longer (5 h), DC-GM having a high DCA value was obtained, however, the M_n value became relatively low. This is because the degradation reaction was dependent on reaction time. All of the DC-GMs obtained showed high water solubility.

Structural analysis of DC-GM

The content ratio of D-mannose to D-glucose (Man/Glc) for GM was determined to be approximately 1·5 by gas-chromatography analysis after acidic hydrolysis of glucomannan, which reflects fairly well the contents obtained in previous reports (Kato *et al.*, 1969; Maeda *et al.*, 1980). We investigated the content ratio of D-mannose to D-glucose (Man/Glc) for DC-GMs prepared. Figure 2 shows the relationship between Man/Glc ratio and DCA value of DC-GM. The Man/Glc ratio in DC-GM decreased with an increase in DCA value. These results show that the ring-opening oxidation reaction proceeded at mannose having *cis*-diol group in preference to glucose. However, the information on the branched structure of DC-GM cannot be obtained from this experiment. Then, we investigated the branched structures of DC-GMs by direct methylation. Table 2 shows the results of this analysis. 1,5-$(Ac)_2$-2,3,4,6-$(Me)_4$-Glucitol and 1,5-$(Ac)_2$-2,3,4,6-$(Me)_4$-mannitol were derived from nonreducing terminal glucose and mannose, respectively. The other three derivatives were derived from saccharides consisting mostly of main chain. The decrease in ratio of nonreducing terminal saccharides was larger than that of main chain saccharides. These results suggest that the ring-opening oxidation reaction proceeded at a nonreducing terminal in preference to a main chain. Although no terminal mannose residue was detected in the

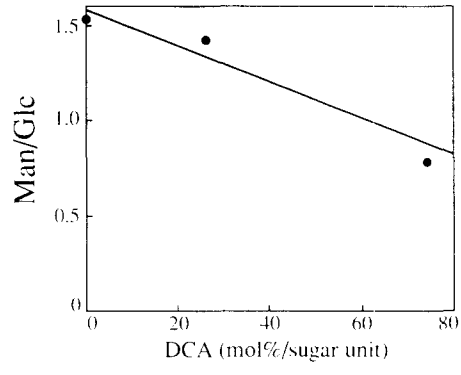


Fig. 2. Relationship between mannose/glucose ratio and DCA value in DC-GM. DCA: degree of introduction of carboxylic acid group.

Table 2. Gas-chromatographic analysis of partially methylated alditol acetates obtained by hydrolysis of permethylated GM and DC-GM

Identification of alditol acetate	R_t[a] (min)	Relative amount (%)			Parent sugar type
		GM[b]	DC-GM(26)[c]	DC-GM(74)[d]	
1,5-$(Ac)_2$-2,3,4,6-$(Me)_4$-glucitol	5·85	7·14	5·57	0·79	Glc 1-
1,5-$(Ac)_2$-2,3,4,6-$(Me)_4$-mannitol	5·95	8·79	5·84	0·00	Man 1-
1,4,5-$(Ac)_3$-2,3,6-$(Me)_3$-glucitol	6·20	32·47	30·23	35·08	-1 Glc 4-
1,4,5-$(Ac)_3$-2,3,6-$(Me)_3$-mannitol	6·29	37·31	33·18	15·47	-1 Man 4-
1,3,4,5-$(Ac)_4$-2,6-$(Me)_2$-mannitol	10·11	14·29	12·18	11·66	-1 Man 4-3

[a] Retention time.
[b] GM: Glucomannan.
[c] DC-GM(26): Dicarboxy-glucomannan (DCA = 26 mol%/sugar unit).
[d] DC-GM(74): Dicarboxy-glucomannan (DCA = 74 mol%/sugar unit).
DCA: degree of introduction of carboxylic acid group.

DC-GM having high DCA value (74 mol%/sugar unit), the DC-GM having low DCA value (26 mol%/sugar unit) kept a certain amount (5.84%) of branched or terminal mannose residue.

Enzymatic degradation behavior of DC-GM

The obtained DC-GMs contain a certain degree of unreacted sugar units. So, it is expected that they can be hydrolysed by some glycosidases. Figure 3 shows typical examples for the GPC profile of DC-GM after enzymatic hydrolysis by cellulase. This confirmed that DC-GM was biodegraded by cellulase. The relationship between the DCA values and the degradation rates was investigated. Figure 4 shows the relationship between the DCA values of DC-GMs and their molecular weight reduction (%), which was calculated from the following formula:

$$\text{Molecular weight reduction (\%)} = (M_{n0} - M_n)/M_{n0} \times 100$$

where M_{n0} is the initial molecular weight, and M_n is the molecular weight after enzymatic degradation for 1 h.

From the results of Fig. 4, molecular weight reduction by cellulase was dependent on the content of unreacted pyranose groups. It is suggested that the cellulase can recognize an unreacted sugar moiety and hydrolyze at such a position. Moreover, it was confirmed that DC-GM was also degraded by β-D-glucosidase (Fig. 5). However, the degradation rate of DC-GM by β-D-glucosidase was very low. This is because β-D-glucosidase is an exo-type hydrolase and the degradation should occur only at a terminal glucose unit and stop at terminal oxidized residues or mannose units. None of the DC-GMs obtained were degraded at all without enzymes in aqueous solution (pH = 7·4, 37°C).

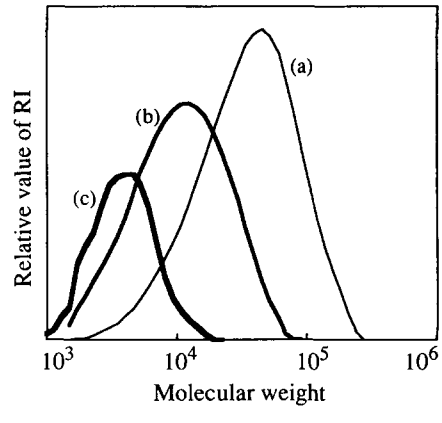


Fig. 3. Enzymatic degradation of DC-GM (DCA = 28 mol%/sugar unit) by cellulase: (a) before degradation, (b) after 5 min degradation, (c) after 1 h degradation. DCA: degree of introduction of carboxylic acid group.

Stimulating effect against cultured macrophages *in vitro*

In order to evaluate immunological enhancement of DC-GM activity, the stimulating effect of DC-GM against macrophage-like cells was investigated. The tests were carried out by measuring glucose consumption of PMA-differentiated HL-60 cells and measuring β-D-glucuronidase (a kind of lysosome enzyme) activity of PMA-differentiated U937 cells *in vitro*. These tests were carried out using DC-GMs, in which DCA = 16 or 62 mol%/sugar unit. CM-chitin (DCM = 60 mol%/sugar unit) and DC-Dex (DCA = 10 mol%/sugar unit) were also used as reference carboxylic acid derivatives of polysaccharides. Figure 6 shows the results of glucose

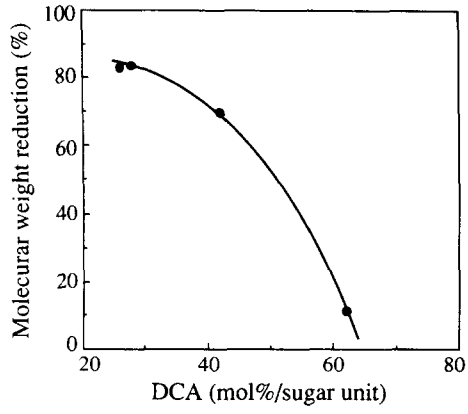


Fig. 4. The relationship between DCA values and the degradation rates of DC-GMs by cellulase. DCA: degree of introduction of carboxylic acid group.

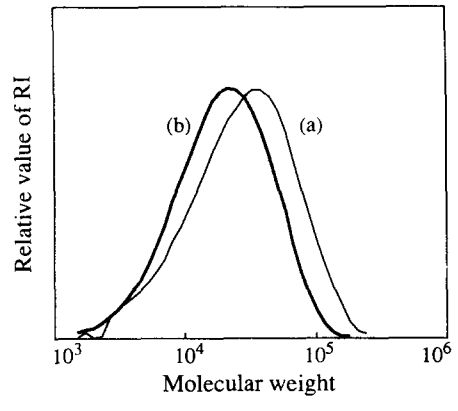


Fig. 5. Enzymatic degradation of DC-GM (DCA = 28 mol%/sugar unit) by *B*-D-glucosidase: (a) before degradation, (b) after 6 days degradation. DCA: degree of introduction of carboxylic acid group.

consumption activity. All samples used in this test showed a certain level of stimulating effect against PMA-differentiated HL-60 cells. Among these four samples, the DC-GM having low DCA value (16 mol%/sugar unit) showed the highest stimulating effect. The DC-GM having high DCA value (62 mol%/sugar unit) showed the same level of stimulating effect as the other polysaccharide derivatives. Figure 7 shows the results of β-D-glucuronidase activity. All samples used in this test also showed a certain level of stimulating effect against PMA-differentiated U937 cells. Among these four samples, the DC-GM having low DCA value (16 mol%/sugar unit) showed the highest stimulating effect. These results suggest that the introduction of carboxylic acid groups by ring-opening oxidation inhibits the stimulating effect of GM. From the results of structural analysis, the DC-GM having a low DCA value keeps a larger amount of unreacted terminal (branched) mannose residues than that of DC-GM having a high DCA value. It can be assumed that this difference in stimulating effects of DC-GMs is derived from the difference

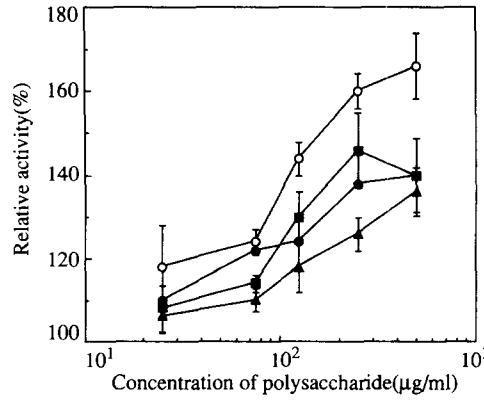


Fig. 6. Stimulating effect of DC-GM, CM-chitin and DC-Dex on glucose consumption of PMA-differentiated HL-60 cells *in vitro*. ○: DC-GM (DCA = 16 mol%/sugar unit), ▲: DC-GM (DCA = 62 mol%/sugar unit), ●: CM-chitin (DCM = 60 mol%/sugar unit), ■: DC-Dex (DCA = 10 mol%/sugar unit). DCA: degree of introduction of carboxylic acid group; DCM: degree of introduction of carboxymethyl group.

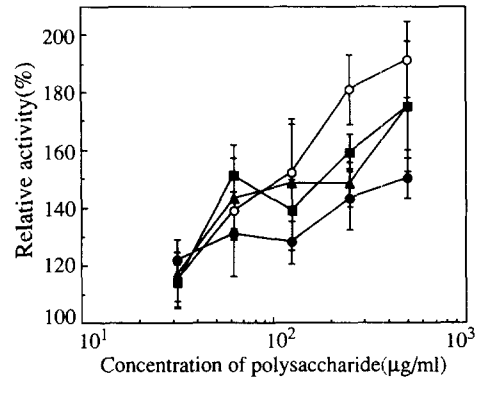


Fig. 7. Stimulating effect of DC-GM, CM-chitin and DC-Dex on β-D-glucuronidase activity of PMA-differentiated U937 cells *in vitro*. ○: DC-GM (DCA = 16 mol%/sugar unit), ▲: DC-GM (DCA = 62 mol%/sugar unit), ●: CM-chitin (DCM = 60 mol%/sugar unit), ■: DC-Dex (DCA = 10 mol%/sugar unit). DCA: degree of introduction of carboxylic acid group; DCM: degree of introduction of carboxymethyl group.

in the amount of terminal mannose residues, which allow mannose receptor-mediated uptake into cultured macrophages. We could not investigate the stimulaing effect of GM because of its low water-solubility.

Moreover, we investigated the effect of molecular weight of DC-GM on its stimulation effect against cultured macropharges. Figure 8 shows the stimulating effect of DC-GMs having three different M_n values (18000, 7700 and 3600) on glucose consumption of PMA-differentiated HL-60 cells *in vitro*. The high-molecular-weight DC-GMs showed slightly higher stimulating effect than low-molecular-weight DC-GMs. These results suggest that the stimulating effect of DC-GM was dependent on molecular weight in the M_n range tested. However, we should only conclude this

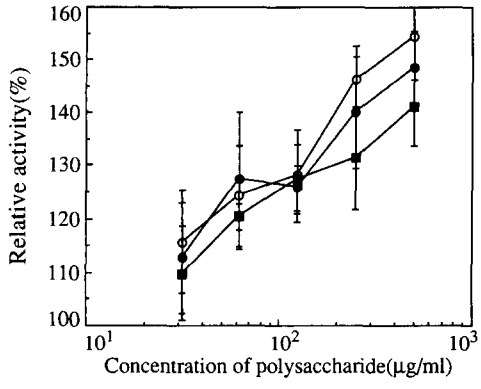


Fig. 8. Stimulating effects of DC-GM having various molecular weights on glucose consumption from PMA-differentiated HL-60 cells *in vitro*. (○): DC-GM (DCA = 16 mol%/sugar unit) M_n = 18000, (●): DC-GM (DCA = 16 mol%/sugar unit) M_n = 7700, (■): DC-GM (DCA = 16 mol%/sugar unit) M_n = 3600. DCA: degree of introduction of carboxylic acid group.

subject after investigating stimulating effects of DC-GMs having higher M_n than 20000.

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