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Preparation of *O*-acylated low-molecular-weight carrageenans with potent anti-HIV activity and low anticoagulant effect

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Abstract

Depolymerization of λ- and κ-carrageenan was performed using ultrasonication at room temperature in the presence of ferrous ions, ascorbic acid and ethylenediaminetetraacetic acid. Tetrabutylammonium (TBA) salts of each depolymerized carrageenan were dissolved in *N*,*N*-dimethylformamide (DMF) and acylated by carboxylic acid anhydride at room temperature in the presence of dimethylaminopyridine and tributylamine as catalysts. The TBA salts of acylated carrageenans were dissolved in DMF and sulfated by pyridine–sulfur trioxide. The anti-HIV activities of each preparation were determined in a system in which MT-4 cells were infected with HTLV-III B. Sulfated samples prepared from low butanoylated or low hexanoylated derivatives had higher anti-HIV activities than dextran sulfate used as reference. Anticoagulant activity of these preparations was determined from the measurement of activated partial thromboplastin time (APTT). The butanoylated derivatives of λ-carrageenan with a degree of substitution of 1.1–1.5 had anticoagulant activities of 6.7–8.5 unit/mg, which were distinctly lower than that of dextran sulfate (avg. mol. wt. 0.7×10^4), 23.4 unit/mg. This result indicated clearly the decreasing effect of the substituted acyl group on anticoagulant activity. Hemolytic activity of these preparations was negligible.

Keywords: Carrageenan; Depolymerization; Sulfation; Acylation; Anti-HIV activity; Anticoagulant activity

1. Introduction

The λ-carrageenan family extracted from marine algae was the first natural sulfated polysaccharide found to have anti-HIV (anti-human immunodeficiency virus) activity (Nakashima, Kido, Kobayashi, Motoki, Neushul & Yamamoto, 1987). It was also reported that λ- and κ-carrageenans, used as control substances, showed strong anti-HIV activities. Since then, many natural and synthetic sulfated polysaccharides have been shown to have anti-HIV activities, and the relationship between their structures and activities were examined (Hatanaka et al., 1991a and 1991b). Among them, dextran sulfate received attention due to its potentiality as a therapeutic drug for AIDS (Ueno & Kuno, 1987) and, this was examined up to the clinical trial stage. However, many problems for its practical application were indicated by this trial.

The sulfated polysaccharides were found to interfere with the initial stage of infection when HIV adsorbs to CD4 on the T-cell surface (Mitsuya, Looney, Kuno, Ueno, Wong-Staal & Broder, 1988). On the other hand, as represented by heparin and dextran sulfate, sulfated polysaccharides have strong anticoagulant activity. This activity is considered to be an adverse reaction when sulfated polysaccharides are used as therapeutic drug for AIDS. Therefore, development of preparations with lower activity is essential.

In our research for effective drugs for prevention and medical treatment of AIDS, we examined natural polysaccharides as starting materials. We have previously reported the depolymerization of carrageenans and the chemical methods to prepare sulfated depolymerized carrageenans with different molecular weights and sulfate contents. These studies showed that anti-HIV activity was increased by depolymerization and sulfation (Yamada, Ogamo, Saito, Watanabe, Uchiyama & Nakagawa, 1997), and suggested that anti-HIV activity would be potentiated by changing the constituent sugar chain, sulfate content and molecular weight of carrageenans. However, as the anticoagulant activities of carrageenans remained similar to the levels in dextran sulfate, effort aimed to reduce this activity was necessary.

Petitou et al. (1992) introduced acyl groups into heparin

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and its fragments. Barzu et al. (1993) measured anti-HIV and anticoagulant activities of these compounds, and reported that they exhibited lower anticoagulant activities although their anti-HIV activities did not differ markedly from that of dextran sulfate. Uryu et al. (1992) synthesized sulfated alkyl oligosaccharides by introducing alkyl groups to the reducing terminal of the oligosaccharide and sulfate groups to sugar chains. Introduction of alkyl groups to sugar chains increased anti-HIV activity.

In the present study, introduction of an acyl group into low-molecular-weight carrageenans was carried out to reduce the anticoagulant activity and other adverse reactions such as hemolytic activity. Preparations were made by introducing straight chain carboxylic acids of different length (C4, C6 and C12), then by sulfation of the remaining unsubstituted hydroxy groups of the sugar chains. Effects of chain length of the substituents, the degree of substitution and sulfate content on anti-HIV activity were examined.

2. Experimental

2.1. Materials

λ-Carrageenan (lot no. 96F-0441), κ-carrageenan (lot no. 115F-0008), dextran sulfate (lot no. 77F-0634; avg. mol. wt. approx. 5000), dextran sulfate (lot no. 78F-01631; avg. mol. wt. 500,000), were obtained from Sigma Chemical Company (St. Louis, MO, USA). Pullulan standards of various molecular weights were purchased from Showa Denko Company (Tokyo, Japan).

2.2. Analytical methods

The sulfur content of samples was determined using the method of Dodgson and Price (1962).

The molecular sizes of depolymerized λ-, κ-carrageenan, the corresponding acylated carrageenans, and each of the sulfated derivatives were determined using HPLC with reference to the calibration curve obtained using standard pullulans of known molecular weight, as described previously (Yamada, Ogamo & Nakagawa, 1993).

The acyl group content was determined as follows. The sample to be analyzed (2 mg) was dissolved in a solution of butanol (250 μl). Concentrated sulfuric acid (50 μl) was added and the tube was sealed and heated for 1 h at 100°C. After cooling, the contents of the tube were diluted with chloroform (5 ml), and were added to a solution of butanol (250 μl) containing 20 mM *n*-butyl *n*-caprylate as an internal standard. After washing with water (20 ml × 3), the solution was made up to 10 ml with chloroform, and 2 μl was injected into the gas chromatograph.

The anti-HIV activity of each preparation was measured using the microtiter plate method, as described previously (Yamada et al., 1997).

The anticoagulant activity of each preparation was determined from the measurement of activated partial thromboplastin time (APTT), as described previously (Yamada et al., 1997).

The hemolytic activity was determined as follows. Washed erythrocytes from a rabbit were suspended in 10-fold saline. This solution (0.2 ml) was added to each sample solution (80 μg/ml saline) (5 ml) and allowed to stand for 30 min at 37°C. After cooling, the solution was centrifuged, the absorbance of the obtained supernatant was measured at 540 nm to determine the rate of hemolysis (%).

2.3. Depolymerization of λ- and κ-carrageenan

A solution of each carrageenan (600 mg) in distilled water (100 ml) was heated for 5 min at 80°C. After cooling at room temperature, 10 mM ferrous sulfate (5 ml), 100 mM ascorbic acid (5 ml), and 50 mM ethylenediaminetetraacetic acid disodium salt (EDTA) (5 ml) were added to these solutions. The reaction mixtures were treated with an ultrasonic generator for 1 h at 4°C (in ice water).

Each solution was passed through a column (30 mm i.d. × 65 mm, 46 ml) of Dowex 50W-× 2 (Na^+, 50–100 mesh; The Dow Chemical Company, USA). The eluate and washings (220 ml total) were adjusted to pH 6.5–7.0 with 0.1 M NaOH. The solution was dialyzed against distilled water (20 l × 3) for 1 day, concentrated in vacuo to about 50 ml, and re-dialyzed against distilled water (20 l × 3) for 1 day at 20–25°C. The dialyzed solutions were adjusted to pH 6.5–7.0 with 0.1 M NaOH and filtered through a DISMIC-25cs membrane (0.45 μm; ADVANTEC TOYO Company, Tokyo, Japan). The filtered solutions were concentrated in vacuo to a small volume and then each of the low-molecular-weight products was lyophilized.

2.4. O-acylated derivatives of low-molecular-weight carrageenans

A solution of low-molecular-weight carrageenans sodium salt (200 mg) in distilled water (100 ml) was passed through a column of Dowex 50W-× 2 (tetrabutylammonium form, 50–100 mesh, 10 ml), at a flow rate of 3–4 ml/min. The pH of the effluent was adjusted to 10.0 by the addition of 10% tetrabutylammonium hydroxide (TBA). The solution was concentrated in vacuo to a small volume and then lyophilized to give the TBA salt.

A solution of each carrageenan TBA salt (200 mg, λ- corresponds to 0.47 mmol of OH groups per disaccharide unit, κ- corresponds to 0.96 mmol, similarly) in *N*,*N*-dimethylformamide (DMF) (5 ml) was added to a 4-dimethylaminopyridine (in the case of λ-; 14.6 mg, 0.12 mmol, in the case of κ-; 29.3 mg, 0.24 mmol), tributylamine (in the case of λ-; 0.6 ml, 2.7 mmol, in the case of κ-; 1.2 ml, 5.3 mmol). Acid anhydride (butanoic, hexanoic, dodecanoic), corresponding to 0.5-, 1.0-, 2.5-, 5.0-fold moles of OH groups per disaccharide unit of each carrageenan, was added to this solution. The reaction mixture was stirred continuously for 20 h at 20–25°C. After cooling in

Table 1
Analytical data and biological activities of sulfation products of acylated λ-carrageenan

Acyl derivatives or reference substances	Acyl (moles per mole of disaccharide)	S (moles per mole of disaccharide)	Molecular weight[a] ($\times 10^4$)	Anti-HIV (IC_{100}, μg/ml)	APTT (unit/mg)	Hemolysis (%)
No acylation	0	5.0	1.6	7.8	58.0	0
Butanoylate	0.3	4.4	2.1	3.9	17.3	0
	1.1	3.1	2.8	3.9	8.5	0
	1.4	3.0	2.7	3.9	6.7	0
	1.5	2.7	2.6	7.8	7.8	0
Hexanoylate	0.3	5.5	2.4	3.9	8.4	0
	1.1	4.1	2.3	3.9	12.5	23.1
	1.4	3.4	2.0	7.8	11.5	23.4
	1.8	3.1	1.8	15.6	10.3	13.4
Dodecanoylate	0.1	5.4	2.9	3.9	4.8	0
	1.1	4.6	2.5	15.6	6.6	25.0
	1.2	2.4	[b]	[b]	[b]	[b]
	1.4	1.9	[b]	[b]	[b]	[b]
Dextran sulfate	–	4.6	0.7	7.8	23.4	0.2
	–	4.8	43	7.8	51.1	0.2

[a] Relative value obtained by size-exclusion chromatography using standard pullulan as a reference.
[b] Not determined because of insolubility in water.

ice water, 5% sodium hydrogen carbonate (10 ml) was gradually added, and the mixture was stirred for 1 h at room temperature. After neutralization with 1 N HCl, the solution was added to cold ethanol (300 ml) and allowed to stand for 1 h at 4°C. After decantation, the precipitate was centrifuged, washed with ethanol (20 ml × 2), and dissolved in distilled water (40 ml). The solution was passed through a column of Dowex 50W- × 2 (H^+, 50–100 mesh, 3 ml) and adjusted to pH 6.5–7.0 with 0.1 M NaOH and filtered through a DISMIC-25cs (0.45 μm) membrane. The solution was lyophilized to give the *O*-acylated carrageenans as a white powder.

2.5. Sulfation of O-acylated low-molecular-weight carrageenans

The TBA salt of *O*-acylated low-molecular-weight carrageenans was prepared following the same procedure as for the previous *O*-acylation item, using a column of Dowex 50W- × 2 (TBA form).

A solution of the TBA salt of each carrageenan (200 mg) in *N*,*N*-dimethylformamide (DMF) (24 ml) was added to a solution of pyridine-sulfur trioxide (760 mg) in DMF (12 ml), and the mixture was stirred for 1 h at 0, 20 or

Table 2
Analytical data and biological activities of sulfation products of acylated κ-carrageenan

Acyl derivatives or reference substances	Acyl (moles per mole of disaccharide)	S (moles per mole of disaccharide)	Molecular weight[a] ($\times 10^4$)	Anti-HIV (IC_{100}, μg/ml)	APTT (unit/mg)	Hemolysis (%)
No acylation	0	4.1	1.7	7.8	29.7	0
Butanoylate	0.3	3.8	3.0	3.9	22.4	0
	1.3	2.6	1.9	3.9	20.6	0
	2.3	2.0	1.9	3.9	11.3	0
	2.9	1.4	[b]	15.6	[b]	0
Hexanoylate	0.1	4.1	2.6	3.9	12.8	0
	0.9	2.6	2.5	3.9	15.4	12.1
	2.3	1.7	3.5	15.6	13.6	96.0
	2.4	1.8	1.6	15.6	9.8	42.5
Dodecanoylate	0.7	1.8	2.6	15.6	12.4	2.3
	2.5	1.5	[b]	15.6	9.5	25.9
	2.9	1.4	[b]	[b]	[b]	[b]
Dextran sulfate	–	4.6	0.7	7.8	23.4	0.2
	–	4.8	43	7.8	51.1	0.2

[a] Relative value obtained by size-exclusion chromatography using standard pullulan as a reference.
[b] Not determined because of insolubility in water.

40°C. The reaction mixture was poured gradually into cold water (35 ml) and the pH of the solution was adjusted to 9.0–9.5 with 1 M NaOH. The solution was diluted with cold ethanol (240 ml) that had been saturated with sodium acetate and kept for 1 h at 4°C to give a white precipitate. The precipitate was collected after centrifugation at $900 \times g$ for 10 min and dissolved in 50 ml of distilled water. The solution was dialyzed against distilled water (20 l × 3) for 1 day at room temperature, concentrated in vacuo to a small volume and re-dialyzed against distilled water (20 l × 3) for 1 day. After adjustment of the pH to 6.5–7.0 with 0.1 M NaOH, the solution was filtered through a DISMIC-25cs membrane (0.45 μm), concentrated in vacuo to a small volume, and lyophilized.

3. Results and discussion

3.1. Characterization of sulfated O-acylated preparations of low-molecular-weight carrageenan

Depolymerization of carrageenans using an active oxygen species, as reported previously, was attained by reaction with ferrous ions or ferrous ions plus ascorbic acid for 24 h at room temperature (Yamada et al., 1997). This reaction condition required a considerable time. In the present study, depolymerization was attained within a short time by combination with ultrasonication. Namely, 1 h ultrasonic treatment of a carrageenan solution in the presence of ferrous ions, ascorbic acid and EDTA resulted in the depolymerization to give molecular weight of 20,000–30,000.

Low-molecular-weight λ- and κ-carrageenans obtained using the above method were acylated. Acylation was carried out by the addition of three different chain lengths of carboxylic acid anhydrides, butanoic (C4), hexanoic (C6), dodecanoic anhydride (C12). The degree of acyl substitution of preparations was controlled by the alteration of the added amount of each anhydride. Table 1 shows the degree of acylation of λ-carrageenans expressed in molar ratio to a constituent disaccharide unit, and Table 2 shows that of κ-carrageenans. The highest degree of substitution was different between the two types of carrageenan. In λ-carrageenan the highest degree was 1.8 irrespective of the type of acyl groups, whereas in κ-carrageenan this degree was 2.9.

This difference was considered to be related to the three-dimensional structures of λ- and κ-carrageenan. In λ-carrageenan, D-galactoses are linked alternatively by β-(1 → 4) and α-(1 → 3) bonds, and both of them has C1, C1 chair conformation. This disaccharide unit already has three sulfate ester groups, leaving three substitutable hydroxyl groups per disaccharide unit. However, the steric distance between the bulky 2-sulfate group linked to the α-(1 → 3)-galactose and 3-hydroxyl group linked to β-(1 → 4)-galactose is very close. Therefore, acylation of this 3-hydroxyl group has been suggested to be difficult. Thus, two other substitutable hydroxyl groups were acylated and given the maximum degree of acylation of 1.8 as indicated in the experimental result. Rees (1969) carried out conformation analysis using a computer based on X-ray data, and suggested the possibility that, in a molecular model of λ-carrageenan, the foregoing 2-sulfate group and the 3-hydroxyl group approached each other to form a hydrogen bond.

In κ-carrageenan, in contrast, the 3-hydroxyl group in the β-(1 → 4)-linked 3,6-anhydro-D-galactose are absent. Therefore, in the case of κ-carrageenan, there are three free hydroxyl groups, all of which are considered to be acylated. This estimation agrees well with the maximum degree of substitution of 2.9 in the experimental result.

Solubility of an acylated derivative in water is influenced by the type and degree of the acyl groups. Thus, λ-carrageenan with high substitution by a long-chain dodecanoyl group was insoluble in water and the lypophilic character of the acyl group was found to have priority over the property of a sugar chain. The highly substituted derivatives of κ-carrageenan with dodecanoyl and butanoyl groups were also found to be insoluble in water.

The acylated and non-acylated low-molecular-weight carrageenan were sulfated, the first being the non-acylated λ-carrageenan. Although the maximum degree of substitution in λ-carrageenan was calculated to be 6.0 (total number of sulfate ester groups and non-substituted hydroxyl groups), introduced maximum sulfate groups were limited to 5.0, which was similar to the case of acylation. However, the maximum degree of substitution was obtained, 4.1, by sulfating non-acylated κ-carrageenan (theoretical number was 4.0). This suggests that a non-substituted hydroxyl group remained in λ-carrageenan, whereas there was no free hydroxyl group left in κ-carrageenan in which sulfation progressed easily.

Sulfated derivatives were prepared from various acylated products of different degrees of substitution. As shown in Tables 1 and 2, the type of the substituted acyl groups exerted an influence on the sulfation pattern. Total degree of sulfation and acylation of the butanoylated λ-carrageenan did not exceed 5, whereas the hexanoylated derivative was greater than this value. It can be seen that the latter compound contained a sulfate group even at the 3-hydroxyl group of the β-(1 → 4)-linked galactose. This position of non-substituted λ-carrageenan accepts substitution with difficulty due to steric hindrance. In dodecanoylated derivatives, those with a low degree of substitution are readily sulfated. This is similar to hexanoylated derivatives, but the degree of sulfate substitution was repressed as the degree of dodecanoyl increased. In other words, shorter alkyl chain, butanoyl and hexanoyl groups make the sulfate substitution easy, but the bulky dodecanoyl groups repress the sulfate substitution. However, in the substituted κ-carrageenans, the chain length of the substituted acyl groups exerted no influence on sulfation.

3.2. Anti-HIV activity and activities related to adverse reactions in the prepared compounds

Tables 1 and 2 show anti-HIV activities and related activities to adverse reactions of the respective preparations. As shown by dextran sulfate, many sulfated polysaccharides have anticoagulant properties. In the present study, preparation with low anticoagulant activity was desirable. Anti-HIV activities and anticoagulant activities of various derivatives were assayed. Hemolytic activity was also determined to investigate the interactions of the acylated derivatives with erythrocytes.

Influence of acylation on anti-HIV activity and anticoagulant activity was clearly shown. Acylated λ- and κ-carrageenan with a sulfate content showed higher anti-HIV activities than the highly sulfated non-acylated preparations. Anticoagulant activities of these carrageenans were clearly decreased compared with non-acylated preparations, but the effect of the acyl groups of preparation on the anti-HIV activity differed between λ- and κ-carrageenans. Thus, a high anti-HIV activity was observed in the butanoylated carrageenan, provided that the degree of sulfation was above certain degree (3.0 in λ-derivatives and 2.0 in κ-derivatives). A decrease in anticoagulant activity of λ-derivatives was eminent in contrast to κ-derivatives. When both activities of butanoylated preparations were compared, λ-carrageenans with a degree of butanoylation of 1.1 ∼ 1.4 and degree of sulfation of around 3.0 were most appropriate because of the high anti-HIV activity and low anticoagulant activity. Among the hexanoylated preparations, λ-carrageenan with a degree of hexanoylation of 0.3 and with a degree of sulfation of 5.5 was desirable. In preparations linked with the long-chain dodecanoyl group, λ-carrageenan with the lowest degree of substitution of 0.1 alone showed a high anti-HIV activity and a fairly low anticoagulant activity among the tested preparatives. Therefore, the degree of acylation had a greater influence on anti-HIV and anti-coagulant activities than the chain length of the acyl group and, thus low acylations followed by high sulfation are rather desirable. In the case of butanoylated derivatives, however, an excess degree of sulfation resulted in a high anticoagulant activity. This activity was suppressed in hexanoylated and dodecanoylated derivatives.

To study the effects of these preparations on erythrocytes, hemolytic activities were measured. The butanoylated λ-and κ-carrageenans did not show hemolytic activities and the interactions on the erythrocyte membrane appeared to be weak. However, hemolytic activity was present in the hexanoylated and dodecanoylated derivatives when the degree of substitution was increased. This result showed the increase in membrane affinity by acylation, and hexanoyl and dodecanoyl groups were found to have higher affinities than butanoyl group.

4. Conclusions

Acylation of low-molecular-weight carrageenans followed by sulfation resulted in potentiation of anti-HIV activity and reduction of anticoagulant activity. It was demonstrated that anti-HIV and anticoagulant activities varied greatly depending on the balance between the lipophilic group, such as the type and degree of substitution of the acyl group, and the hydrophilic group, such as degree of sulfation. Comparison of activities of all the samples prepared in the present study showed that the low-molecular-weight carrageenans with a degree of butanoylation of 1.1 ∼ 1.4 and a degree of sulfation of around 3.0 had a high anti-HIV activity and low anticoagulant activity without hemolytic activity, and their potential as anti-HIV drugs is anticipated.

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