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Preparation and anti-HIV activity of low-molecular-weight carrageenans and their sulfated derivatives

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Lambda-, kappa-, and iota-carrageenan were depolymerized in the presence of ferrous ions or ferrous ions plus ascorbic acid at room temperature. Tetrabutylammonium salts of lambda-, kappa- and iota-carrageenan were dissolved in *N,N*-dimethylformamide and sulfated by the addition of pyridine-sulfur trioxide in *N,N*-dimethylformamide. Then, the sulfated carrageenans were depolymerized by the incubation with ferrous ions and ascorbic acid. The anti-HIV activities of the various preparations were determined in a system in which MT-4 cells were infected with HTLV-IIIB. The activity of lambda-carrageenan was similar to that of dextran sulfate and the depolymerized lambda-carrageenan retained high activity. The activities of kappa- and iota-carrageenan and their depolymerized forms were about 6% of that of lambda-carrageenan. The sulfation of kappa- and iota-carrageenan increased the activities of these compounds to the same level as that of lambda-carrageenan. However, the sulfation of lambda-carrageenan did not affect its activity. Depolymerized sulfated kappa- and iota-carrageenan with mean molecular weights of 50,000 (81,000–46,000) had higher anti-HIV activity than dextran sulfate and all other preparations of carrageenan tested. © 1997 Elsevier Science Ltd

INTRODUCTION

Carrageenans (Rees *et al.*, 1982) are polysaccharides that can be extracted from certain marine red algae, such as *Chondrus* and *Gigartina*. Each is a sulfated galactan consisting of D-galactose and 3,6-anhydro-D-galactose. Carrageenans can be conveniently divided into three main types depending on the number and position of sulfate groups and the 3,6-anhydro-D-galactose content.

Recently, we reported a new method for the fractionation of lambda-carrageenan that was based on differences in solubility in a highly concentrated solution of ammonium sulfate. Using this method, we recovered a depolymerized species of lambda-carrageenan as a definite fraction (Yamada *et al.*, 1993). It became clear that the fragmentation of lambda-carrageenan was actually correlated with trace amounts of contaminating Fe(II)/Fe(III) ions in our solutions of ammonium sulfate. Therefore, in the present study, we tried to depolymerize lambda-carrageenan in the presence of ferrous ions and ascorbate.

Fragmentation of polysaccharides by active oxygen species has been known since the 1930s. In particular, that of hyaluronic acid has attracted attention because a decrease in its high molecular weight has been found to be associated with some diseases and with aging (Von Sonntag, 1980). The combination of metal ions and a reducing agent is well-known as a system for generating active oxygen, and ascorbate is very effective as such a reducing agent (Herp *et al.*, 1967). It has been proposed that the fragmentation of sugar chains is caused by hydroxy radicals that are generated by the action of local ferrous ions and that ascorbate serves to keep the ferrous ions in a reducted state.

Natural carrageenan has been reported to have anti-HIV (anti-human immunodeficiency virus) activity (Nakashima *et al.*, 1987a), but details of the relationship between its structural features and such activity are ambiguous. In general, anionic polysaccharides appear

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to interfere with an early event in HIV infection, namely, the adsorption of virus particles to the target lymphocytes (Mitsuya *et al.*, 1988). Mizumoto *et al.* (1988) revealed the important role of sulfate groups in the sugar chain by comparing the anti-HIV activities of various polysaccharides.

The present report describes the generation of low-molecular-weight preparations and sulfated products from three types of carrageenan by a combination of mild depolymerization and sulfation in a homogeneous reaction medium (Nagasawa *et al.*, 1986). The relationships between the degree of sulfation, the molecular weight and the anti-HIV activity of the various preparations were examined with the eventual goal being the development of anti-HIV drugs.

EXPERIMENTAL

Materials

Lambda-carrageenan (lot no. 96F-0441), kappa-carrageenan (lot no. 115F-0008), iota-carrageenan (lot no. 115F-0665), dextran sulfate (lot no. 77F-0634; avg. mol. wt. approx. 5,000), dextran sulfate (lot no. 78F-01631; avg. mol. wt. 500,000), D-fructose (lot no. 58F-0008) and heparin (lot no. 63F-0450) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Pullulan standards of various molecular weights were purchased from Showa Denko Co. (Tokyo, Japan).

Analytical methods

The 3,6-anhydro-D-galactose content of samples was determined by the method of Yaphe and Arsenault using D-fructose as a reference sugar (Yaphe & Arsenault, 1965), and the sulfur content of samples was determined by the method of Dodgson and Price (1962). The molecular sizes of lambda-, kappa-, iota-carrageenan, the corresponding sulfated carrageenans, and each of the low-molecular-weight derivatives were determined after HPLC by reference to a calibration curve obtained with the standard pullulans of known molecular weight, as described previously (Yamada *et al.*, 1993).

The anti-HIV activity of each preparation was measured by the microtiter plate method. Inhibition was monitored of the cytopathic effect (CPE) of infection of MT-4 cells (established to human T-cell line by integration with gene of HTLV-I) by HIV (Nakashima *et al.*, 1986). MT-4 cells were exposed to HIV (HTLV-IIIB) at 100 $TCID_{50}$ (50% tissue culture infectious dose) and incubated for 60 min at 37°C. After adsorption of the virus, the cells were suspended in fresh medium at a concentration of 1.5×10^5 cells per ml. This suspension of infected cells was then supplemented with preparations of carrageenans at various concentrations and cells were cultured in a CO_2 incubator. The HIV-induced cytopathic effect was observed under the light microscope.

The anticoagulant activity of carrageenans was determined by the measurement of the activated partial thromboplastin time (APTT). Assays were performed with NKK Hema Trace 2 (Tokyo, Japan), using human plasma (Ortho Plasma Coagulation Control Level 1, Ortho-Clinical Diagnostics K. K., USA), and partial thromboplastin with an activator (Activated Thrombofax Reagent-Optimized, Ortho-C.D., USA) and a solution of calcium chloride (Ortho-C.D., USA). The activities were expressed relative to that of standard heparin (Novo heparin; 1 ml = 1000 Japanese Pharmacopoeia units; Novo-Nordics A-S, Denmark).

Depolymerization of carrageenans by treatment with ferrous ions and with ferrous ions plus ascorbic acid

A solution of each carrageenan (30 mg) in water (30 ml) was heated for 5 min at 80°C. Three ml of 1 mM ferrous sulfate were added to these solutions, to give a final concentration of 91 μM, and the reaction mixture (Reaction Fe) was allowed to stand for 24 h at 20–25°C. Similarly, an identical solution of carrageenan (30 mg) in water (30 ml) was supplemented with 1 mM ferrous sulfate (3 ml), to give a final concentration of 83 μM, and 2.5 mM ascorbic acid (3 ml), to give a final concentration of 208 μM. This reaction mixture (Reaction Fe-AsA-1) was also allowed to stand for 24 h at 20–25°C.

Each solution was dialyzed against distilled water (20 l×6) for 3 days, concentrated *in vacuo* to about 20 ml, and re-dialyzed against distilled water (20 l×4) for 2 days at 20–25°C. The solution was passed through a column (15 mm i.d.×40 mm) of Dowex 50W-×8 (K^+, 50–100 mesh; The Dow Chemical Co., USA). The eluate and washings (30 ml total) were adjusted to pH 6.5–7.0 with 0.01 M KOH and filtered through a DISMIC-25cs membrane (0.45 μm; ADVANTEC TOYO Co., Tokyo, Japan). The filtered solutions were concentrated *in vacuo* to a small volume and then each was lyophilized to give Product Fe or Product Fe-AsA-1, respectively.

A second product (Fe-AsA-2) was obtained when the above procedure was repeated with Fe-AsA-1.

Sulfation of lambda-, kappa- and iota-carrageenan

A solution of each carrageenan (3 g) in water (3 l) was passed through a column (20 mm i.d.×170 mm; 55 ml) of Dowex 50W-×2 (H^+, 50–100 mesh), at a flow rate of 3–4 ml/min, and the pH of the effluent was adjusted to 10.0 by the addition of a 10% solution of tetrabutylammonium hydroxide (TBA) in an ice-water bath. The solution was concentrated *in vacuo* to a small volume and then lyophilized to give the TBA salt as a white powder.

A solution of the TBA salt of each carrageenan (2 g)

in *N,N*-dimethylformamide (240 ml) was added to a solution of pyridine-sulfur trioxide (7.6 g) in *N,N*-dimethylformamide (120 ml), and the mixture was stirred for 1 h at room temperature. The reaction mixture was poured into cold water (332 ml) and the pH of the solution was adjusted to 9.0–9.5 with 1 M NaOH. The solution was diluted with ethanol (2.4 l) that had been saturated with sodium acetate and kept for overnight at 4° to give a white precipitate. The precipitate was collected by centrifugation at 900×**g** for 5 min and dissolved in 500 ml of distilled water. The solution was dialyzed against distilled water (20 l×6) for 3 days at room temperature, concentrated *in vacuo* to a small volume and re-dialyzed against distilled water (20 l×4) for 2 days. 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), evaporated *in vacuo* to a small volume and lyophilized.

RESULTS AND DISCUSSION

The presence of ferrous ions in aqueous solutions of lambda-, kappa- and iota-carrageenan for 24 h at room temperature resulted in the depolymerization of each carrageenan to give sugar chains with molecular weights of about 12.8×10^4–31.0×10^4 (samples indicated as Fe in Table 1). Furthermore, ascorbate accelerated the effects of ferrous ions. Thirty mg of each carrageenan were depolymerized under the same conditions for 24 h and samples with molecular weights of 4.6×10^4–9.6×10^4 were obtained (samples indicated as Fe-AsA-1 in Table 1). Repeated treatment of depolymerized samples by the same procedure (with ferrous ions and ascorbate) gave preparations with molecular weights of 0.7×10^4–1.5×10^4 (samples indicated as Fe-AsA-2 in Table 1).

Analysis of each sample (Table 1) indicated that the sulfate esters of Fe and Fe-AsA-1 samples were stable, whereas a small decrease in sulfate content was observed when samples were treated twice with ferrous ions and ascorbate. The levels of anhydro galactose, a constituent sugar of kappa- and iota-carrageenan, did not change during the depolymerization procedure.

The ability of these carrageenans and depolymerized carrageenans to inhibit replication of HIV was examined. The activity of lambda-carrageenan was high (7.8 μg/ml; concentration of lambda-carrageenan inhibited perfectly HIV) and similar to that of dextran sulfate, which was used as a positive control. The sample of depolymerized lambda-carrageenan retained this high activity, while a preparation with the relatively low molecular weight of 0.7×10^4 had no anti-HIV activity. Therefore, sugar chains of a certain length (containing endogenous sulfate groups) might be essential for the activity of lambda-carrageenan. The activities of kappa- and iota-carrageenan were difficult to determine because of the gelation of corresponding aqueous solutions. The activities of depolymerized kappa- and iota-carrageenan (molecular weights, 12.8×10^4 and 31.0×10^4, respectively) were only about 6% of that of lambda-carrageenan, and that of low-molecular-weight samples (1.5×10^4 and 0.9×10^4, respectively) was almost negligible. In general, the biological activities of sulfated polysaccharides are thought to be due to their polyanionic characteristics and to be dependent on the sulfate content. However, we found a marked difference between the anti-HIV activities of lambda-carrageenan and of iota-carrageenan despite the similar sulfate content of the two samples. These findings suggest the significance of structural features of the sugar chain in the anti-HIV activity.

As represented by heparin, sulfated polysaccharides are known to have anticoagulant activity. Such activity

Table 1. Analytical data and biological activities of the products of depolymerization of three types of carrageenan

Carrageenans and their products	Mol. wt.† (× 10^4)	S (moles per mole of disaccharide)	3,6-anGal (moles per mole of disaccharide)	Anti-HIV (IC_{100}, μg/ml)	APTT (unit/mg)
λ	170	2.4	0.18	7.8	31.6
λ Fe	28.0	2.4	0.15	3.9	33.1
λ Fe-AsA-1	4.6	2.2	0.16	3.9	25.0
λ Fe-AsA-2*	0.7	1.9	0.14	250	6.6
κ	100	1.5	1.01	#	5.9
κ Fe	12.8	1.3	0.96	62.5	4.1
κ Fe-AsA-1	4.7	1.4	0.98	125	3.5
κ Fe-AaA-2*	1.5	1.2	0.94	>250	0.5
ι	103	2.2	0.94	#	23.4
ι Fe	31.0	2.0	0.97	62.5	31.0
ι Fe-AsA-1	9.6	2.1	0.84	250	31.4
ι Fe-AsA-2*	0.9	1.7	0.86	>250	1.6

#, Not determined because of gelation.
*, The depolymerization procedure was repeated.
†, Relative value obtained by size-exclusion chromatography using standard pullulan as a reference.
3,6-anGal, 3,6-anhydro-D-galactose.

of effective anti-HIV drugs should be low. As shown in Table 1, the anticoagulant activities of lambda-carrageenan and of iota-carrageenan, which had a high sulfur content, were similar to that of dextran sulfate, while kappa-carrageenan, a poorly sulfated preparation, had lower activity (10%). The activities of low-molecular-weight preparations of lambda-carrageenan and iota-carrageenan (less than 1.0×10^4) were almost negligible.

Sulfated preparations, obtained by sulfation of naturally occurring polysaccharides, have been reported to have anti-HIV activity (Nakashima *et al.*, 1987b; Yoshida *et al.*, 1988; Kaneko *et al.*, 1990). However, relevant structural features, in particular the contribution of the molecular weight and sulfate content to the anti-HIV activity, remain to be identified. To evaluate the effects of the extent of sulfation and the molecular weight on the activity of each carrageenan, we prepared sulfated samples from three types of carrageenan by a method, that allows sulfate groups to be introduced uniformly among sugar chains, and then we measured their activities (Table 2). Among the three sulfated carrageenans, the sulfate content of lambda-carrageenan was highest, since lambda-carrageenan itself has the highest level of hydroxyl groups and a low anhydro-galactose content. Even though kappa- and iota-carrageenan contain fewer hydroxyl groups because of their high anhydro-galactose content, sulfate groups could be introduced into these carrageenans nearly to saturation. The relative anti-HIV activities of the various samples were affected in different ways. Namely, sulfation had no effect on the activity of lambda-carrageenan, but it inceased the activities of kappa- and iota-carrageenan to levels similar to that of lambda-carrageenan.

Next, we prepared a low-molecular-weight sulfated samples from each sulfated sample by the depolymerization procedure, and measured their activities. There was no change in the activity of depolymerized sulfated lambda-carrageenan, but clear increases in activity were noted with depolymerized sulfated kappa- and iota-carrageenan. In particular, the activities of the samples with molecular weights of 51,000 and 54,000, respectively, were higher than those of sulfated lambda-carrageenan and dextran sulfate, which was evaluated as a positive control.

The activities did not increase simply with increases in sulfate content. However, samples with high activities were found to have a definite range of sulfate contents and molecular weights regardless of particular type of sulfated carrageenan. To confirm these effects on anti-HIV activity, a test of the inhibition of giant cells was performed with all samples, together with the test of anti-HIV activity. For all samples, the inhibitory concentration closely reflected the anti-HIV activity. The anticoagulant activities of the depolymerized sulfated samples were lower than those of the starting sulfated carrageenans prior to depolymerization. Furthermore, we tried to desulfate the original three types of carrageenan by a previously reported procedure (Nagasawa *et al.*, 1977). All samples lost their anti-HIV activity and their anticoagulant activity (data not shown).

The number of sulfate groups, as well as the molecular weight, was shown to play an important role in the anti-HIV activity of the sulfated carrageenans. Since the activities shown in Tables 1 and 2 were not entirely dependent on sulfate content, the positions and densities of the sulfate groups on the sugar chains can be assumed to be important factors in the interactions between carrageenans and the outer capsule glycoprotein, gp120, of HIV.

Table 2. Analytical data and biological activities of the products of depolymerization of three types of sulfated carrageenan

Sulfated carrageenans and their products	Mol. wt.† ($\times 10^4$)	S (moles per mole of disaccharide)	3,6-anGal (moles per mole of disaccharide)	Anti-HIV (IC_{100}, μg/ml)	APTT (unit/mg)
Sλ	189	5.1	0.19	7.8	67.0
Sλ Fe	31.0	4.2	0.15	7.8	65.4
Sλ Fe-AsA-1	10.2	4.4	0.16	7.8	56.1
Sλ Fe-AsA-2*	1.6	5.0	0.19	7.8	58.0
Sκ	108	4.3	1.05	7.8	50.2
Sκ Fe	8.1	3.9	0.97	3.9	38.1
Sκ Fe-AsA-1	5.1	3.8	0.92	2.0	28.6
Sκ Fe-AaA-2*	1.2	4.1	0.89	7.8	29.7
Sι	105	4.3	1.00	7.8	49.6
Sι Fe	5.4	3.4	0.91	1.0	26.5
Sι Fe-AsA-1	4.6	3.8	0.86	3.9	25.6
Sι Fe-AsA-2*	1.1	3.1	0.71	7.8	21.8
Dextran sulfate 5000	0.7	4.6	—	7.8	23.4
Dextran sulfate 500,000	43	4.8	—	7.8	59.7
Heparin	1.5	2.3	—	62.5	173.3

*, The depolymerization procedure was repeated.
†, Relative value obtained by size-exclusion chromatography using standard pullulan as a reference.
3,6-anGal, 3,6-Anhydro-D-galactose.

In conclusion, the main factors that influenced the anti-HIV activity of carrageenans were the type of sugar chain, the degree of sulfation, and the molecular weight. The most active preparations had a sulfate content of 3.4–3.8 mole/mole of disaccharide and a molecular weight of 51,000–54,000.

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