JOURNAL OF Applied Polymer SCIENCE

Preparation and Potential *In Vivo* Anti-Influenza Virus Activity of Low Molecular-Weight κ-Carrageenans and Their Derivatives

Fengxiang Tang,[1] Fang Chen,[1] Feng Li[2]

[1]College of Chemistry and Chemical Engineering, Fuzhou University, Fuzhou 350108, Fujian, China

[2]Institute of Pharmaceutical Biotechnology and Engineering, Fuzhou University, Fuzhou 350108, Fujian, China

Correspondence to: F. Tang (E-mail: hellotfx@fzu.edu.cn)

ABSTRACT: Degradation of native κ-carrageenan was performed using acid hydrolysis aided with microwave heating. Combined with nonofiltration membrane (cut-off molecular weight 250 Da) separation, 1. 400 Da - 50 kDa low-molecular-weight (LMW) κ-carrageenans were obtained. Narrow molecular weight distribution of LMW κ-carrageenans could be prepared under pH 2.18 during the microwave power range investigated. The *in vivo* anti-influenza virus (IV) activity of three kinds of LMW κ-carrageenans (3, 5, and 10 kDa), their acetylated derivatives (acetylation degree of 1.5), as well as an acetylated and sulfated derivative of 3 kDa carrageenan (acetylation degree of 1.0 and sulfation degree of 2.4), were investigated using FM1-induced pulmonary oedema model. These LMW κ-carrageenans showed significant inhibition against FM1-induced pulmonary oedema as compared with the virus control, although their activities were inferior to that of positive control, Rabivirin. Introduction of acetyl groups greatly increased their anti-IV activity. The acetylated 3-kDa κ-carrageenan exhibited comparative activity with Rabivirin at both doses of 6 and 30 2. mg/kg·d, and the acetylated and sulfated derivative of 3 kDa carrageenan displayed higher activity than Rabivirin at the dose of 30 mg/kg·d. These results disclosed that 3 kDa κ-carrageenan with proper acetylation degree and sulfation degree was a potential candidate against influenza virus. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: degradation; polysaccharides; modification; biological applications of polymers; molecular weight distribution

Received 2 March 2011; accepted 15 February 2012; published online

DOI: 10.1002/app.37502

INTRODUCTION

Influenza viruses (IV) can cause acute respiratory diseases in humans and animals with high morbidity and morality rates. Several influenza pandemics in recorded history have infected and killed tens of millions of people all over the world. Even in the 21st century with advanced medical science, the outbreak of highly pathogenic avian influenza in 2003 and H1N1 in 2009 also brought people into deep panic, revealing the fact that there is still lacking amply and effective drugs when humans are confronted with constant mutations of surface proteins on IV. All of this underscores the necessity of looking for new drugs against IV.

Carrageenans (κ-, λ-, and τ-), a family of linear sulfated polysaccharides extracted from red seaweeds, are made up of galactans with alternating α(1→3) linkages, β(1→4) linkages, and occasional 3,6-anhydro-D-galactose units.[1] They have been shown to inhibit the replication of various enveloped viruses such as herpes simplex virus (HSV), human cytomegalovirus (HCMV), and human immunodeficiency virus (HIV).[2–7]

κ-Carrageenan is made up of α(1→4) D-galactose-4-sulfate (G4S) and β(1→3) 3,6-anhydro-D-galactose (AG).[1,8,9] Earlier report by Ehresmann et al. has shown that native κ-carrageenan can protect fertilized eggs against IV type B.[10] Our previous study also demonstrated that sulfated depolymerized κ-carrageenans with molecular weight (MW) of 50 kDa and 90 kDa were more effective against IV A than native κ-carrageenan, with three to six times the treatment index of amantadine hydrochloride in an embryonated chicken egg model,[11] implying that the anti-IV activity of κ-carrageenan could be potentiated by changing its sulfate content and MW. In fact, many evidences have demonstrated that the MW of sulfated polysaccharides was closely related to their biological activities.[12–17] However, the underlying mechanism has not been clarified so far.

Different methods for depolymerizing carageenans such as acid hydrolysis,[13,18–24] enzymatic hydrolysis,[25–27] ultrasonic depolymerization,[28] γ-irradiation,[29] and microwave heating[30] have been reported. The traditional acid hydrolysis is cheap, simple,

and easy to stop. However, brown products resulting from long-period heating greatly limit the wide application of acid hydrolysis. Microwave heating can remarkably enhance the reaction rates in hydrolysis procedures, but a high pressure is needed.[30] In the current contribution, microwave-aided acid hydrolysis under atmosphere was developed as an alternative approach for depolymerizing native κ-carrageenan. κ-Carrageenans with MW less than 50 kDa were prepared in this method and some of them were acylated and /or sulfated. The potential and challenges of the anti-IV activities of these LMW κ-carrageenans and their derivatives were evaluated using FM1-induced pulmonary oedema model.

EXPERIMENTAL

Degradation of Native κ-Carrageenan

κ-Carrageenan of food grade was purchased from commercial resources in China. Five percent (wt %) κ-carrageenan solution was prepared in 1 L distilled water, and heated in a 800 W microwave oven (Glanz, China) at high fire for 7 min to obtain homogenous solution.

After the solution cooled down, 120 mL distilled water and certain amount of 2*M* sulfuric acid were added into the solution under vigorous stirring for adjusting its pH value (FiveEasy pH meter, FE20K, resolution 0.01, accuracy ±0.01, Mettler-Toledo). Then the solution was subjected to microwave heating at different fires (powers) for different minutes (as shown in Table I below). After the microwave heating, the reaction was terminated by titrating 1*M* sodium hydroxide (NaOH) in an ice-water bath until a pH value of 3.5 was reached. The solution was diluted with 5 L distilled water and then filtered using a 250 Da (cut-off MW) nanofiltration membrane system (Sundar Membrance Sci. technology Co. Ltd, Xiamen, China) to remove salts and monosaccharides. The cut-off solution was then concentrated and lyophilized to obtain white degraded κ-carrageenan powder.

Determination of Molecular Weight of Degraded κ-Carrageenans

The MW and MW distribution of these degraded κ-carrageenans were determined by high-performance size chromatography (HPSEC) on a Waters515 unit equipped with a refractive index detector and a TSK-GEL G3000PWXL (7.8 mm × 300 mm) column. The column temperature was set at 30°C. Dextran standards (Sigma-Aldrich Company, Shanghai, China) with MW ranging from 1000 to 50,000 were used to calibrate the system. Na_2SO_4 (0.7%) in deionized water was used as mobile phase at a flowrate of 0.6 mL/min. The eluate was monitored by refractometry.

Preparation of LMW κ-Carrageenan Acetylates and Sulfates

Two grams of LMW κ-carrageenan (corresponding to 14.1 mmol OH groups) was suspended in 30 mL pyridine, and 11 mL (78.9 mmol) triethylamine and 0.43 g (3.52 mmol) 4-dimethylaminopyridine (DMAP) were added together. Additionally, 1.32 g (4.99 mmol) 18-crown-6 (used as phase transfer catalyst) and acetic anhydride (in the case of 3 kDa, 3.5-fold moles of OH groups in the κ-carrageenan; in the case of 5 kDa, 6.5-fold; in the case of 10 kDa, 10.0-fold) were added to the solution.

Table I. Average Molecular Weights of Depolymerized κ-Carrageenans Under Different Depolymerization Conditions (Da)

	pH		
Heating model (HM)	2.08	2.18	2.30
20 min at middle-low fire (HM1)	4900	10,200	53,100
3 min at high fire and 10 min at middle-low fire (HM2)	2000	7900	30,400
3 min at high fire and 20 min at middle fire (HM3)	780	4500	9150
10 min at high fire and 10 min at middle fire (HM4)	570	3450	5200
20 min at high fire (HM5)	375	900	3900

The mixture was stirred continuously at room temperature for 20 h. After cooling in ice water, 5% sodium hydrogen carbonate was gradually added until the mixture is weak alkaline. The solution was concentrated *in vacuo* to remove pyridine, triethylamine, and most of water. The residue was dialyzed against distilled water for 2 days and the solution was then concentrated and lyophilized to give O-acetyl LMW (3, 5, and 10 kDa) κ-carrageenans.

The acetylated and sulfated derivative of 3-kDa carrageenan was prepared as follows. Then 3-kDa carrageenan was first acetylated with acetic anhydride corresponding to 2.2-fold moles of OH groups in the carrageenan as mentioned above. In this way, acetylated 3 kDa carrageenan with acetylation degree of 1.0 was obtained. This acetylated derivative was further sulfated as below. About 6.5 mL of chlorosulfonic acid was slowly dropped into 50 mL pyridine (PY) under vigorous stirring and cooling in an ice-water bath and thus the sulfation agent, SO_3·PY was obtained. Then 1.0 g lyophilized acetylated 3 kDa carrageenan was added to the sulfation agent and the mixture was stirred at 80°C for 4 h. After reaction, the mixture was cooled to room temperature by an ice bath, neutralized with 30% NaOH solution, and concentrated under reduced pressure to evaporate the solvent. The concentrate was dialyzed against distilled water for 2 days and the solution was then concentrated and lyophilized to give O-acetyl and O-sulfate LMW κ-carrageenan.

Determination of Acylation and Sulfation Degrees and IR Spectra

The acyl group content was determined according to the method described by Yamada et al.[28] The GC analysis was conducted on a Varian 3300 gas chromatograph equipped with a flame ionization detector (FID), using a fused silica capillary column (30 m × 0.25 mm) coated with SE-30. Chromatography was run according to column temperature program: 80°C (1 min), 20°C/min to 210°C, 30°C/min to 260°C. Nitrogen was used as the carrier gas at a flow rate of 1 mL/min and a split ratio of 80 : 1. The acetylation degree stood for acetyl moles per mole of repeating disaccharide in κ-carrageenan.

The sulfate content was determined according to the method described by Kawai,[31] and the sulfation degree was defined as sulfate moles per mole of repeating disaccharide in κ-carrageenan.

The infrared spectra of LMW κ-carrageenans and their derivatives in KBr pellets were determined with a FTIR Spectrometer (PerkinElmer, SPECTRUM 2000) in the range of 500–4000 cm^{-1}.

Virus and Animals

Mouse adapted influenza virus A/FM/1/47(H1N1), hereafter referred to as FM1, was provided by Institute of Virology, Chinese Academy of Preventive Medicine, and preserved at −80°C. Before infection, the virus was inoculated into allantoic fluid of 9-day chicken embryos to cultivate for 48 h at 37°C. At the end of culture, the allantoic fluid containing FM1 was collected and its virulence was evaluated using agglutination. ICR mice (SPF grade) of either sex were purchased from Shanghai Laboratory Animal Center. The test mice were anesthetized by aether (Shanghai Chemicals Inc, China) and challenged with 40 μL FM1 virus suspension with titer 1 : 640 ($10LD_{50}$) by nasal drip.

Mice Treatment

Mice were randomly divided into following groups: virus control group (virus+distilled water), normal control group (distilled water), Ribavirin group, and LMW κ-carrageenan or its derivative groups. Each group consisted of eight mice.

Solutions of LMW κ-carrageenans and their derivatives were prepared in concentrations of 1, 5 mg/mL with deionized water and sterilized. Ribavirin (made by Fuzhou Haiwang Fuyao Pharmaceuticals Co. Ltd, National Drug Approval No H19993231) in concentrations of 1, 5 mg/mL was prepared too. Mice received 40 μL of LMW κ-carrageenans or their derivatives or Ribavirin or distilled water three times daily by nasal drip for 7 days. Viral infection was performed on the second day. At the end of each treatment the test mice were sacrificed and their lungs were taken out for the assay of inhibition on FM1-induced pneumonia.

Inhibition of FM1-Induced Pneumonia

FM1-induced pneumonia was indicated by the pulmonary oedema, which was described as lung index (LI). The LI was calculated according to the formula LI = LW (mg)/BW (g), where LW was the lung weight and BW the body weight of the test mouse. The inhibition rate on FM1-induced pneumonia was calculated according to $(LI_{\text{virus control}} - LI_{\text{test}})/LI_{\text{virus control}} \times 100\%$.[32]

RESULTS AND DISCUSSIONS

Degradation of Native κ-Carrageenan

It was reported that κ-carrageenan was resistant to desulphation under acid (pH 2) hydrolysis,[23] and microwave degradation almost did not change the structure and constitutions of the λ-carrageenan under low stress (<15 atm).[15] Therefore, acid hydrolysis aided with microwave heating under atmosphere is suitable and applicable for the degradation of native κ-carrageenan.

The MW of native κ-carrageenan reaches up to 4000–5000 kDa. When the concentration of native κ-carrageenan was 5.0%, the system became heterogeneous and showed some gel characteristics because of high viscosity of native κ-carrageenan.

κ-Carrageenan was degraded under different microwave heating models (HM) and pH values as shown in Table I. From HM1 to HM5, heating was intensified gradually. Obviously, the average MW of depolymerized κ-carrageenan increased with the increase of pH value, whereas decreased with the increase of heat intensity. For example, when the pH value decreased from 2.18 to 2.08, the average MW dropped by half under HM1, by four times under HM2, and by five times under HM3, respectively. On the other hand, when the pH value increased from 2.18 to 2.30, the average MW increased nearly five times under HM1, four times under HM2, and two times under HM3, respectively. As shown in Table I, the lowest average MW (375 kDa) approached the MW (424 kDa) of repeating disaccharide in κ-carrageenan, implying that nanofiltration had removed molecules with MW less than that of disaccharide.

When only acid hydrolysis was carried out, the hydrolytic reaction was very slow. For instance, 4,800 kDa κ-carrageenan was degraded for 120 min by acid (pH 2) hydrolysis at 35 and 55°C, and 4000 and 510 kDa κ-carrageenans were obtained, respectively.[23] In contrast, the required degradation time by acid hydrolysis aided with microwave heating in our study was much shorter, approximately several tens of minutes (Table I). Although microwave degradation in airtight environment was much quicker, as was shown by Zhou et al.,[15] who reported that 652 kDa λ-carrageenan was degraded into 9.3 kDa by microwave under 15 atm for 6 min, a special high-pressure equipment was needed and was difficult to operate. Since acid hydrolysis aided with microwave heating can be performed in a domestic microwave oven, so it is superior to microwave degradation in airtight environment.

Figure 1 presents the MW distributions of depolymerized κ-carrageenans under different depolymerization conditions. The distributions under pH 2.08 and 2.30 tended to bimodal or multimodal. Narrower MW distribution can be observed at pH 2.18. Generally, lower pH easily leads to broader MW distribution. The multimodal distribution under higher pH may attribute to the nonhomogeneous degradation because of high viscosity of native or primarily degraded κ-carrageenans. In comparison to mild acid hydrolysis[22] and enzyme specific hydrolysis[27], the LMW κ-carrageenans obtained by microwave-aided acid hydrolysis combined with nanofiltration had narrower MW distribution.

It was also observed that 3–10 kDa LMW κ-carrageenans with narrow MW distribution can be obtained under pH 2.18. To investigate the anti-IV activity of LMW κ-carrageenans, products produced under pH 2.18 by HM4, HM3, and HM1 were selected and designated as 3, 5, and 10 KDa carrageenans in the following discussion, respectively.

Infrared Spectroscopy

As shown in Figure 2(a), the typical IR spectra of LMW κ-carrageenan displayed the characteristic peaks of native carrageenan at 3457, 1250, 1042, 930, and 848 cm^{-1},[33] verifying that acid hydrolysis with microwave heating might not change the chemical components. The broad band at 3457 cm^{-1} was because of stretching of —OH groups in κ-carrageenan. The bands observed at 1250, 1042, 930, 848 cm^{-1} can be attributed to the

Applied Polymer SCIENCE

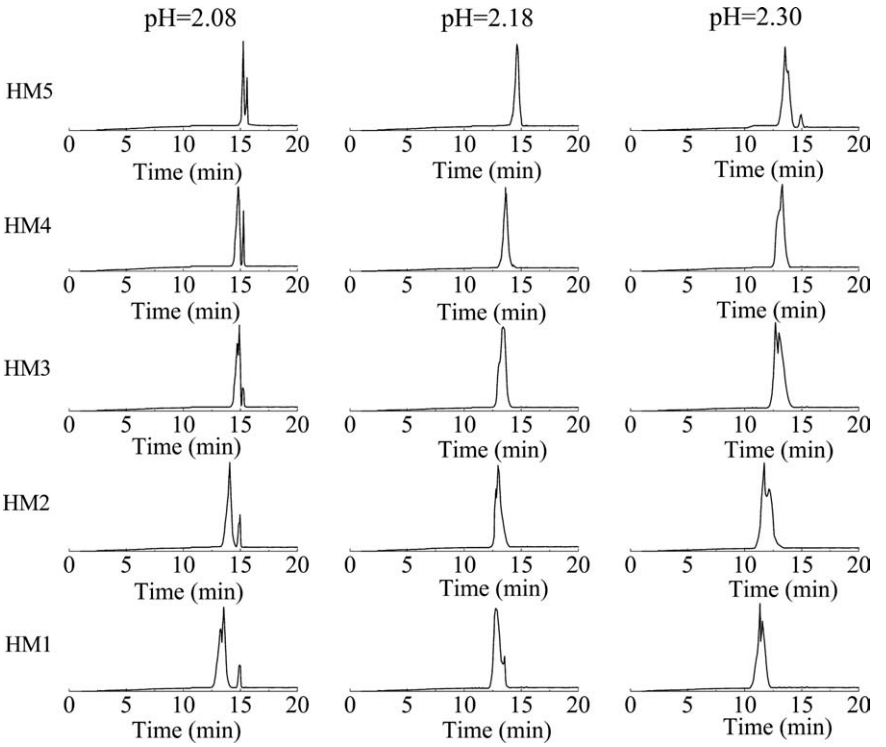

Figure 1. MW distributions of depolymerized κ-carrageenans under different depolymerization conditions.

asymmetric stretching of S=O, glycosidic linkage, 3,6-andydro-D-galactose, and D-galactose-4-sulfate, respectively. The structural difference between the typical acetylated LMW κ-carrageenan [Figure 2(b), 3 kDa, its acetylation degree, Da, was 1.5] and LMW κ-carrageenan [Figure 2(a), 3 kDa] was illustrated by the new sharp bands at 1747 and 1357 cm^{-1}, which arose from carbonyl stretching and symmetrical bending vibration of methyl in the introduced acetyl groups, confirming the formation of acetylated products. All other acetylated LMW κ-carrageenans displayed similar IR spectra modes.

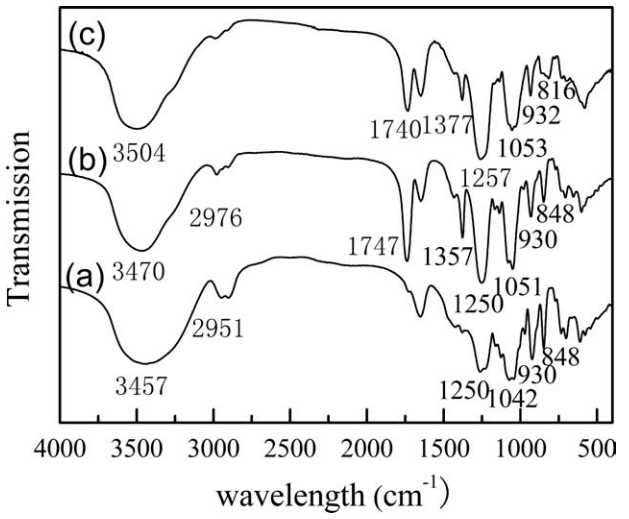

Figure 2. Typical IR spectra of (a) LMW κ-carrageenan, (b) acetylated LMW κ-carrageenan, and (c) acetylated and sulfated LMW κ-carrageenan.

Comparison between the IR spectra of the acetylated and sulfated derivative [Figure 2(c), 3 kDa, its acetylation degree, Da, and sulfation degree, Ds, were 1.0 and 2.4, respectively] and the acetylated LMW κ-carrageenan [Figure 2(b)] revealed that the S=O vibration band at 1250 cm^{-1} of the sulfated derivative became wider and stronger. Instead of the sharp peak originally appearing at 848 cm^{-1} in Figure 2(b), a wide band was detected at 760–880 cm^{-1} [at the position of 816 cm^{-1} in Figure 2(c)],

Table II. Inhibitory Effects of LMW κ-Carrageenans Against FM1-Induced Pulmonary Oedema

Groups	Dose (mg/(kg d))	Lung index	Inhibition rate (%)
Normal control		0.88 ± 0.13	-
Virus control		2.33 ± 0.61[a]	-
Ribavirin control	6	1.04 ± 0.15[b]	55.4
	30	0.89 ± 0.21[b]	61.9
3 kDa κ-carrageenan	6	1.38 ± 0.43[b]	40.7
	30	1.40 ± 0.50[b]	39.8
5 kDa κ-carrageenan	6	1.64 ± 0.63[b]	29.7
	30	1.54 ± 0.67[b]	33.8
10 kDa κ-carrageenan	6	1.74 ± 0.45[b]	25.4
	30	1.51 ± 0.20[b]	35.3

[a]$P < 0.01$ verus normal control, [b]$P < 0.01$ verus virus control.

Table III. Inhibitory Effects of LMW κ-Carrageenan Derivatives Against FM1-Induced Pulmonary Oedema

Groups	Dose (mg/(kg d))	Lung index	Inhibition rate (%)
Normal control		0.99 ± 0.27	-
Virus control		2.79 ± 0.87[a]	-
Ribavirin control	6	1.09 ± 0.37[b]	60.8
	30	0.89 ± 0.21[b]	61.9
3 kDa κ-carrageenan (Da = 1.5)	6	1.17 ± 0.42[b,c]	57.8
	30	1.06 ± 0.35[b,c]	62.1
5 kDa κ-carrageenan (Da = 1.5)	6	1.85 ± 0.55[b,d]	33.8[c]
	30	1.08 ± 0.39[b,c]	61.1
10 kDa κ-carrageenan (Da = 1.5)	6	1.99 ± 0.94[b,d]	28.5
	30	1.12 ± 0.35[b,c]	59.7
3 kDa κ-carrageenan (Da = 1.0, Ds = 2.4)	6	0.80 ± 0.10[b,c]	61.7
	30	0.89 ± 0.16[b,d]	65.7

[a] $P < 0.01$ verus normal control, [b] $P < 0.01$ verus virus control, [c] $P > 0.05$ verus Ribavirin, [d] $P < 0.01$ verus Ribavirin.

which might cover the peaks corresponding to the already existing 4-sulfate on G4S (848 cm^{-1}), newly formed 2-sulfate on G4S C-2 (830 cm^{-1}) and 2-sulfate on AG C-2 (805 cm^{-1}).[17]

Activity Against FM1-Induced Pulmonary Oedema

Although there have been many researches on the anti-virus activities of carrageenans, the related study on LMW or oligo- carrageenans was comparatively deficient. Only Yamada et al.[28] investigated the anti-HIV activity of LMW κ-carrageenans (MW > 10^4). Recently, κ-carrageenan oligosaccharides were reported to behave as elicitors in the cell–cell recognition process that involve host–pathogen interactions in marine plants.[33] In this study, LMW carrageenans such as 3, 5, and 10 kDa carrageenans (can be called oligo- κ-carrageenans) were tested for their inhibitory effects against FM1-induced pulmonary oedem. It was apparent that the lung index of the virus control group was much higher (2.33 ± 0.61) than that of the normal control group (0.88 ± 0.13) (Table II). Ribavirin treatment at doses of 6 and 30 mg/kg·d greatly reduced the lung index to 1.04 ± 0.15 and 0.89 ± 0.21, with corresponding inhibition rates of 55.37% and 61.87%, respectively. As for the LMW κ-carrageenans, all of them produced lower (versus Ribavirin control) but obvious inhibition on pulmonary oedema as compared with the virus control ($P < 0.01$). All these results showed that these LMW κ-carrageenans were potential agents against anti-IV.

LMW κ-carrageenans have better water-solubility than native κ-carrageenan due to the great decrease in MW. To further increase their bioavailability, all of them were acetylated with an acetylation degree of 1.5 (Da = 1.5). Table III presents the inhibitory effects of these acetylated LMW κ-carrageenans against FM1-induced pulmonary oedema. As compared with Table II, the anti-IV activity of LMW κ-carrageenans was noticeably improved after acetylation in most cases. Compared to the virus control, most treatments produced remarkable inhibitory effects on pulmonary oedema. In comparison with Rabivirn control, the acetylated 3 kDa κ-carrageenan exhibited comparative inhibition rates at both doses of 6 mg/kg d and 30 mg/kg·d, implying that acetylated 3 kDa κ-carrageenan could be an effective inhibitor against pulmonary oedema. These findings supported the necessity of acetylation of the sugar chain in improving its anti-IV activity.

The original sulfation degree of 3 kDa κ-carrageenan was only 0.9 (a little lower than the sulfation degree of native κ-carrageenan, 1.0), which kept almost the same after acetylation. Since sulfate groups contribute to the activity of sulfated polysaccharides, a special 3 kDa κ-carrageenan with an acetylation degree of 1.0 and a sulfation degree of 2.4 was prepared. As shown in Table III, its anti-IV activity was obviously increased at the dose of 30 mg/kg·d in comparison to Rabivirn. This suggested that increase of sulfate content was necessary for enhancing the anti-IV activity of 3 kDa κ-carrageenan.

In spite of the good anti-IV activity achieved by these LMW κ-carrageenans and their derivatives, their mode of action is not clear at present. Further study is needed to clarify the relationship between chemical structure, properties, and anti-IV activity, and to elucidate the mode of action.

CONCLUSIONS

1. 400 Da–50 kDa LMW κ-carrageenans can be readily prepared by microwave-aided acid hydrolysis combined with nanofiltration. The average MW of LMW κ-carrageenans decreased with increasing microwave heating intensity and decreasing pH value. Narrow distributions of LMW κ-carrageenans ranging from 3 to 10 kDa can be obtained under pH 2.18 using a domestic microwave oven.
2. LMW κ-carrageenans displayed identical IR characteristics as that of native κ-carrageenan, which suggested that hydrolysis aided with microwave treatment might not change the structure and constitutions of κ-carrageenan. The IR spectra of the acetylated and/or sulfated derivatives confirmed the introduction of acetyl and sulfate groups.
3. κ-Carrageenans (3, 5, and 10 kDa) manifested obvious inhibition of pulmonary oedema as compared with the virus control and their anti-IV activity was noticeably intensified after acetylation in most cases. The acetylated 3 kDa κ-carrageenan (Da = 1.5) showed similar anti-IV activity with control drug, Ribavirin at both doses of 6 and 30 mg/kg·d. The acetylated and sulfated 3 kDa κ-carrageenan (Da = 1.0, Ds = 2.4) presented higher anti-IV activity than Ribavirin at the dose of 30 mg/kg·d.

ACKNOWLEDGMENTS

The project was supported by the Natural Science Foundation of Fujian Province of China (No.C0310007). The authors thank Miss Zheng Huayang for her help in English language-editing.

REFERENCES

1. Bayley, S. T. *Biochim. Biophys. Acta.* **1955,** *17*, 194.

2. Witvrouw, M.; Desmyter, J.; De Clercq, E. *Antiviral Chem. Chemother.* **1994,** *5*, 345.
3. Carlucci, M. J.; Pujol, C. A.; Ciancia, M.; Noseda, M. D.; Matulewicz, M. C.; Damonte, E. B.; Cerezo, A. S. *Int. J. Biol. Macromol.* **1997,** *20*, 97.
4. Baba, M.; Snoeck, R.; Pauwels, R; De Clercq, E. *Antimicrob. Agents. Chemother.* **1988,** *32*, 1742.
5. Carlucci, M. J.; Ciancia, M.; Matulewicz, M. C.; Cerezo, A. S.; Damonte, E. B. *Antiviral Res.* **1999,** *43*, 93.
6. deS.; F-Tischer, P. C.; Talarico, L. B.; Noseda, M. D.; Guimarães, S. M. P. B.; Damonte, E. B.; Duarte, M. E. R. *Carbohydr. Polym.* **2006,** *63*, 459.
7. Carlucci, M. J.; Scolaro, L. A.; Noseda, M. D.; Cerezo, A. S.; Damonte, E. B. *Antiviral. Res.* **2004,** *64*, 137.
8. O'Neill, A. N. *J. Am. Chem. Soc.* **1955,** *77*, 6324.
9. Weigl, J.; Yaphe, W. *Can. J. Microbiol.* **1966,** *12*, 939.
10. Ehresmann, D. W.; Deig, E. F.; Hatch, M. T. In Marine Algae in Pharmaceutical Science; Hoppe, H. A., Levring, T., Tanaka, R., Eds.; W. de Gruyter: New York, **1979.**
11. Li, F. M. Sc. Thesis, Fuzhou Univerisity, (Fuzhou), China, **2003.**
12. Franz, G.; Alban, S. *Int. J. Biol. Macromol.* **1995,** *17*, 311.
13. Yamada, T.; Ogamo, A.; SaitoT; Wantanabe, J.; Uchiyama, H.; Nakagawa, Y. *Carbohydr. Polym.* **1997,** *32*, 51.
14. Petitou, M.; Coudert, C.; Level, M.; Lormeau, J. C.; Zuber, M.; Simenel, C.; Fournier, J. P.; Choay, J. *Carbohydr. Res.* **1992,** *236*, 107.
15. Zhou, G.; Sun, Y.; Xin, H.; Zhang, Y.; Li, Z.; Xu, Z. *Pharmacol. Res.* **2004,** *50*, 47.
16. Zhou, G.; Xin, H.; Sheng, W.; Sun, Y.; Li, Z.; Xu, Z. *Pharmacol. Res.* **2005,** *51*, 153.
17. Yuan, H. M.; Zhang, W. W.; Li, X. G.; Lü, X. X.; Li, N.; Gao, X. L.; Song, J. M. *Carbohydr. Res.* **2005,** *340*, 685.
18. Ekström, L. G.; Kuivinen, J.; Johansson, G. *Carbohydr. Res.* **1983,** *116*, 89.
19. Singh, S. K.; Jacobsson, S. P. *Carbohydr. Polym.* **1994,** *23*, 89.
20. Jiang, Y. P.; Guo, X. K. *Carbohydr. Polym.* **2005,** *61*, 441.
21. Caram-Lelham, N.; Sundelöf, L. O.; Andersson, T. *Carbohydr. Res. 1995, 273*, 71.
22. Yu, G.; Guan, H.; Ioanoviciu, A. S.; Sikkander, S. A.; Thanawiroon, C.; Tobacman, J. K.; Toida, T.; Linhardt, R. J. *Carbohydr. Res.* **2002,** *337*, 433.
23. Karlsson, A.; Singh, S. K. *Carbohydr. Polym.* **1999,** *38*, 7.
24. Myslabodski, D. E.; Stancioff, D.; Heckert, R. A. *Carbohydr. Polym.* **1996,** *31*, 83.
25. Sarwar, G.; Matayoshi, S.; Oda, H. *Microbiol. Immunol.* **1987,** *31*, 869.
26. Potin, P.; Sanseau, A.; Le Gall, Y.; Rochas, C.; Kloareg, B. *Eur. J. Biochem.* **1991,** *201*, 241.
27. Knutsen, S. H.; Sletmoen, M.; Kristensen, T.; Barbeyron, T.; Kloareg, B.; Potin, P. *Carbohydr. Res.* **2001,** *331*, 101.
28. Yamada, T.; Ogamo, A.; Saito, T.; Uchiyama, H.; Nakagawa, Y. *Carbohydr. Polym.* **2000,** *41*, 115.
29. Relleve, L.; Nagasawa, N.;Luan L. Q.Yagi, T.; Aranilla, C.; Abad, L.; Kume, T.; Yoshii, F.; dela Rosa, A. *Polym. Degrad. Stab.* **2005,** *87*, 403.
30. Zhou, G. F.; Yao, W. H.; Wang, C. H. *Carbohydr. Polym.* **2006,** *64*, 73.
31. Kawai, Y. *Anal. Chem.* **1969,** *32*, 314.
32. Boyd, M. R.; Beeson, M. F. *J. Antimicrob. Chemother.* **1975,** *1*, 43.
33. Antonopoulos, A.; Favetta, P.; Helbert, W.; Lafosse, M. *Carbohydr. Res.* **2004,** *339*, 1301.