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Antiviral polysaccharides isolated from Hong Kong brown seaweed *Hydroclathrus clathratus*

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Two relatively pure polysaccharides H3-a1 and H3-b1 had been isolated from the brown seaweed *Hydroclathrus clathratus*. They were characterized by HPLC, ultraviolet scanning, gas chromatography, infrared spectroscopy and elemental analysis, and shown to be two different sulfated polysaccharides with different monosaccharide content, but both with high relative molecular mass. They contained some proteins and uronic acid respectively. The sulfate content and bioactivity of these polysaccharides varied during purification. The fractions derived from the hot water extract also exhibited low anticoagulant effect. This is the first time that the antiherpetic and anticoagulant activities were evaluated for the polysaccharides from the Hong Kong brown seaweed *Hydroclathrus clathratus*.

Hong Kong seaweed, *Hydroclathrus clathratus*, antiviral polysaccharide, polysaccharide characterization

Polysaccharides are the main component of seaweed cell walls. All seaweed species produce at least one type of sulfated polysaccharide[1]. Due to the wide variations in their molecular weights, structural parameters and physiological characteristics, seaweed polysaccharides show diverse bioactivities, such as antiviral[2−4], antiproliferative or antitumor, anticoagulant, antioxidant, anti-inflammatory and anti-complementary effects[5−8].

Based on our previous screening experiments, the aqueous crude extract of *Hydroclathrus clathratus*, a brown seaweed collected from Hong Kong coastal water, exhibited high antiviral activity against HSV with low cytotoxicity to Vero and HEp-2 cells. Anticoagulant activity of these extracts was also detected. The present study was focused on the isolation, fractionation, purification and characterization of the antiviral polysaccharides from this seaweed.

1 Materials and methods

1.1 Seaweed sample, virus and cells

Hydroclathrus clathratus was collected from Wu Pai, Hong Kong, in March 2003 and stored at −20℃ until use.

Vero cell (ATCC CCL-81) and HEp-2 cell (ATCC CCL-23) were grown in growth medium which is Eagle's minimum essential medium (MEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 50 μg/mL gentamycin, 20 mmol/L HEPES and 2 mmol/L glutamine (Sigma), and cultured at 37℃ in 95% humidified atmosphere and 5% CO_2 incubator. Assay medium is MEM containing 1% FBS and the other components are the same as growth medium. Herpes simplex virus-2 (HSV-2) standard strain was kindly provided by Dr. Spence H.S. Lee (Dalhousie University, Halifax, N.S., Canada). It was propagated in Vero cell culture.

1.2 Bioassay-guided isolation of polysaccharides from *Hydroclathrus clathratus*

H. clathratus was extracted with hot water firstly, and then the residue was extracted with diluted acid and al-

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kali respectively following the protocol shown in Figure 1. All fractions were monitored by phenol-H_2SO_4 assay[9] and Lowry-Folin assay[10]. The fraction H-I-3, which showed the highest antiviral activity against HSV-2 among the crude extracts, was further fractionated by ion exchange chromatography on DEAE-cellulose (Sigma) column and then purified by gel filtration chromatography on Sepharose CL-4B (Sigma) column. All of the obtained fractions were examined for their antiviral activity *in vitro*.

1.3 Cellular toxicity

The cytotoxicity of polysaccharides on Vero and HEp-2 cells was determined by lactate dehydrogenase (LDH) release assay according to the instruction manual of cytotoxicity detection kit (LDH) (Roche Diagnostics GmbH, Germany). Briefly, cells in the 96-well plate (Iwaki, Japan) were covered by 100 μL fresh assay medium and 100 μL of a serial two-fold diluted sample solution. After 24 h incubation, 100 μL supernatant was mixed with 100 μL of fresh prepared reaction mixture in corresponding wells of another plate and incubated for 30 min at 25℃ in darkness followed by adding stop solution (50 μL/well). The absorbance of the reaction system was measured at 490 nm and a reference wavelength of 620 nm. Cytotoxicity was expressed as 50% cytotoxic concentration (CC_{50}), which is the concentration of substances that inhibits up to 50% of the viability of cells.

1.4 Antiviral assays

Antiviral activity of polysaccharides was tested using cytopathic effect (CPE) reduction assay[11] and plaque reduction assay (PRA)[12]. In these assays, confluent

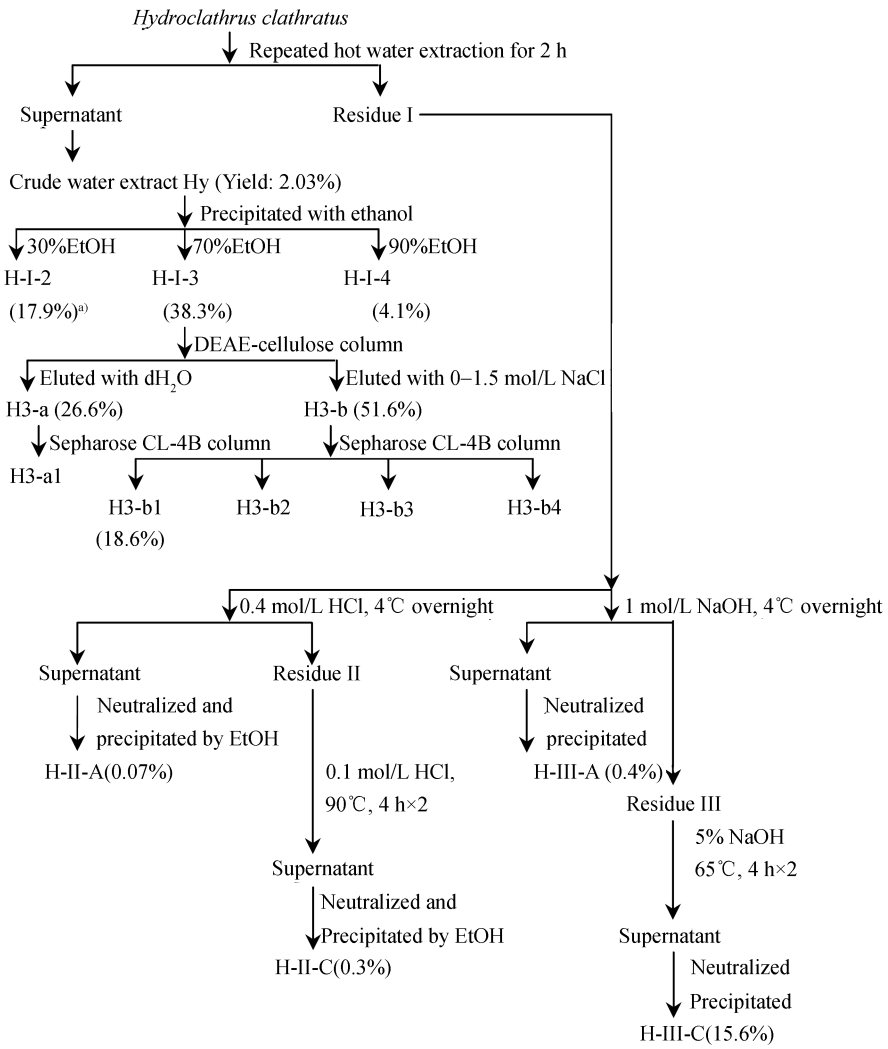

Figure 1 Flow chart of extraction, fractionation and purification of polysaccharides from *Hydroclathrus clathratus*. a) Each yield ratio was based on the upper component.

Vero cells were infected with HSV-2 at multiplicity of infection (MOI) of 10^3 $TCID_{50}$/mL or 80 plaque formation unit (PFU)/well respectively. The results were recorded on the 3rd day post infection. The 50% effective concentration (EC_{50}) is the concentration of test sample required to reduce CPE or viral plaques by 50% when compared with the virus control.

1.5 Anticoagulant activity assay

These experiments were based on the assay of thrombin time (TT) as described previously[13]. In brief, 0.2 mL of human plasma (purchased from the Red Cross of Hong Kong) and 0.1 mL of polysaccharide solution (from 0.98 to 500 μg/mL) were mixed in a small test tube and placed in water bath at 37℃ for 1 min. Then, 0.1 mL of 3－4 units/mL thrombin (Sigma) solution was added. The time taken for the solution to clot was recorded in seconds. To act as the controls, the polysaccharide sample was replaced by 0.1 mL heparin solution (Sigma) and phosphate buffer solution (PBS).

1.6 Characterization of active polysaccharides

1.6.1 Determination of relative molecular mass (*M*r) of H3-a1 and H3-b1 by HPLC. Average molecular mass of relatively pure polysaccharide was determined by HPLC using Hewlett Packard series 1100 HPLC system equipped with a TSK-GEL G5000PW column [7.5 mm (i.d.) × 30.0 cm (L), particle size: 17 μm] (Toshohaas Co., Japan). Samples were dissolved in 0.5 mol/L NaCl and eluted with the same buffer. The eluted component was detected with a Refractive Index detector (HP 1047A RI Detector). Dextran standards (Fluka) of MW 25, 80, 270 and 670 kDa were used as molecular markers to estimate the average *M*r of tested polysaccharides.

1.6.2 Ultraviolet scanning. The test samples were dissolved in 0.1 mol/L NaCl at the concentration of about 1 mg/mL and scanned at 200－800 nm using Milton Roy Sptronic 3000 (Milton Ray, USA). UV absorption was measured with the spectrophotometer.

1.6.3 Analysis of monosaccharides by gas chromatography (GC). Firstly, the polysaccharides were subjected to acid hydrolysis. The hydrolysate was used to prepare alditol acetates of neutral sugars according to the method described by Blakeney[14]. Determination of the alditol acetates of the neutral sugar by gas chromatography (GC) was carried out using an Alltech DB-225 capillary column [15 m (L) × 0.25 mm (i.d.), 0.25 μm film thickness; Alltech Associates, Inc., Deerfield] on a Hewlett Packard HP6890 series GC system (USA). The oven temperature program was: initial temperature, 180℃ with 4℃/min rise to 220℃ and held at 220℃ for 30 min. Helium gas was used as a carrier. The inject sample volume was 2 μL and the neutral sugar in the sample was detected by flame ionization.

1.6.4 Determination of uronic acid content. The uronic acid content in test samples was determined according to the Official Methods of Analysis (AOAC, 1996). In this experiment, 2 mol/L sulfuric acid acted as blank. D-galacturonic acid (6.25－400 μg/mL) solution was used as a standard. The uronic acid content present in each sample was expressed as proportion by weight (% w/w).

1.6.5 Determination of sulfate content. The sulfate content of polysaccharide was measured according to the $BaCl_2$ turbidimetric method described previously[15] but with some modification. Briefly, an accurately weighed sample (20 mg) was hydrolyzed in 0.5 mL of 2 N HCl and N_2 for 2 h at 100℃ in a sealed glass tube. Then the cooled hydrolysate was made to the final volume of 10 mL by adding distilled water. After being decolorized by activated charcoal, 1 mL of the colorless solution, 0.5 mL of 6 mol/L HCl, 2.5 mL of 70% sorbitol and 0.5 g $BaCl_2 \cdot H_2O$ were mixed one by one in a test tube. The absorbance of the suspension was measured at 470 nm 15－20 min later. The standard curve was made of potassium sulfate solution with the series concentrations.

1.6.6 Infrared (IR) spectroscopy. This study was performed on Magna-IR 560 spectrophotometer system following the previous method[16] but with some modification. Pellets for infrared analysis were obtained by carefully grinding a mixture of about 1 mg of dry polysaccharide sample with dry potassium bromide. The mixture was pressed into a disc to form a film and then applied to scan. The transform infrared spectra were obtained in the whole IR spectrum region (400－4500 cm^{-1}).

1.6.7 Elemental analysis. Elemental analysis for polysaccharide sample was conducted by MEDAC Ltd (UK). Data were given in percentage for elements of carbon, hydrogen, nitrogen and sulfur.

2 Results

2.1 Bioassay-guided isolation of polysaccharides

The sequential extraction of seaweed polysaccharides from *H. clathratus* was mainly based on their different solubility following the procedure shown in Figure 1. All fractions obtained were evaluated for antiviral activities against HSV-2 and the fraction that showed the best effect was purified further. H-I-3, derived from hot water extract, was fractionated by ion exchange chromatography on DEAE-cellulose column and eluted firstly by distilled water and then by 0—1.5 mol/L NaCl. According to the positive result of Phenol-H_2SO_4 assay, two fractions were obtained and designated as H3-a and H3-b respectively (Figure 2). The H3-a and H3-b were further purified by gel filtration chromatography on Sepharose CL-4B column. Only one main fraction from H3-a, namely H3-a1, was obtained (Figure 3(a)), while four fractions were isolated from H3-b and designated as H3-b1, H3-b2, H3-b3 and H3-b4 (Figure 3 (b)).

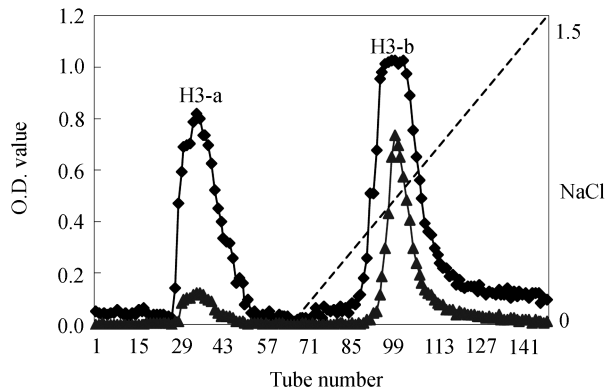

Figure 2 Ion exchange chromatography of H-I-3 on DEAE-cellulose column. H-I-3 was dissolved in distilled water and applied on column. The column was eluted by distilled water firstly and then by 0—1.5 mol/L NaCl buffer (30 mL/h, 6 mL/tube). The carbohydrates in each fraction were determined by phenol-H_2SO_4 assay and the O.D. values were tested at 490 nm (◆); the content of protein in each fraction was determined by Lowry-Folin assay at 750 nm (▲).

All of the fractions obtained were used to detect the antiviral activities against HSV-2 by CPE reduction assay. As indicated in Table 1, the hot acid and alkali extracts, H-II-C and H-III-C, showed no anti-HSV-2 activity at the concentration of 50 μg/mL. However, the other fractions, especially the hot water extract and those derived from it inhibited the generation of CPE induced by virus. Moreover, the water extract Hy showed higher antiviral effect than that of the acid and alkali extracts. The main fraction H-I-3 of Hy exhibited potent anti-HSV-2 activity with lower EC_{50} value (6.25 μg/mL) than those of the other two fractions. After further fractionation through DEAE-cellulose, H3-a and H3-b showed no higher anti-HSV effect than that of H-I-3. However, after purification by gel filtration, the antiviral activities of the relatively pure fractions, i.e. H3-a1 and H3-b1, were increased slightly. H3-b3 and H3-b4 showed only weak or no antiviral activity respectively.

The antiviral assay showed that both H3-a1 and H3-b1 exhibited excellent antiviral activity against HSV-2 standard strain in a dose-dependent manner (Figure 4). Their EC_{50} values against HSV-2 were 1.7 and 1.67 μg/mL respectively when detected by plaque reduction assay. Because these two compounds were almost non-toxic to Vero and HEp-2 cells when tested by LDH method, they possessed a high selective index against HSV-2 (Table 2). This indicated that both H3-a1 and H3-b1 might be potent substances for treating HSV-2 infections.

2.2 Anticoagulant activity of polysaccharides

During the TT measurement, if the plasma did not form clot even 5 min after thrombin addition, it was consid-ered as completely inhibiting of coagulation and the coagulating time was recorded as >300 s. In this assay, the seaweed polysaccharides isolated were all observed to be concentration-related in prolonging the clotting time, similar to heparin and DS_{10k}. However, coagulation was completely inhibited by these fractions at concentrations of at least 125 μg/mL while heparin and DS_{10k} inhibited coagulation completely at concentrations of 1.95 and 31.25 μg/mL respectively. To prolong the coagulation time to double of that of the baseline value (coagulating time of PBS, 21.3 s) in the TT assay, the required concentration of H3-a1 and H3-b1 (about 35 μg/mL) was 32-fold higher than that of heparin (about 1.1 μg/mL) and 2.7-fold higher than that of DS_{10k} (about 13.2 μg/mL).

2.3 Characterization of H3-a1 and H3-b1

2.3.1 Purification and relative molecular mass (*M*r) determination. The purity of H3-a1 and H3-b1 was determined by HPLC analysis. It was revealed that the fraction H3-b1 had only one peak while H3-a1 had a small peak beside the main one. The relative molecular mass of H3-a1 and H3-b1 was estimated by comparing with the standard dextran molecular marker. They were two large molecular compounds. The average *M*r of

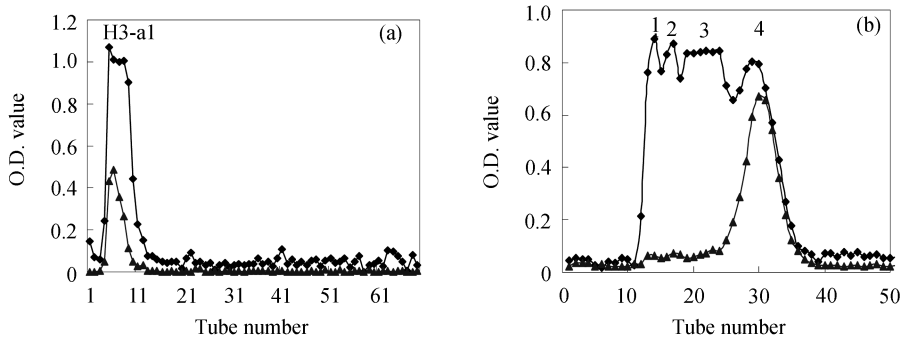

Figure 3 Gel filtration chromatography of H3-a (a) and H3-b (b) on Sepharose CL-4B column. The column was eluted with dH_2O and 0.1 mol/L NaCl buffer (30 mL/h, 2 mL/tube) respectively. Each fraction was determined by Phenol-H_2SO_4 assay at 490 nm (◆) and Lowry-Folin assay at 750 nm (▲) at the same time. The four peaks in (b) are the four obtained fractions from H3-b, namely H3-b1, H3-b2, H3-b3 and H3-b4.

Table 1 Antiviral activity of fractions against HSV-2 tested by cytopathic effect reduction assay in Vero cells

Fractions	EC_{50}[a)] (μg/mL)
	HSV-2 standard strain
Hy (crude extract)	12.5
H-II-A	25
H-II-C	–[b)]
H-III-A	18
H-III-C	–
H-I-2	12.5
H-I-3	6.25
H-I-4	12.5
H3-a	6.25
H3-b	6.25
H3-a1	3.13
H3-b1	2.3
H3-b2	12.5
H3-b3	>25
H3-b4	–
DS_{10k}	2.3
Acyclovir	2.0

Quadruplicate confluent monolayers of Vero cells in 96-well plate were covered with virus suspension (10^3 $TCID_{50}$/mL) and equal volume of serial twofold diluted test sample solutions. The CPE was scored on day 3 post infection. a) EC_{50}, 50% antiviral effective concentration; b) —, no antiviral effect at the concentration of 50 μg/mL.

H3-b1 was 3.9×10^6, and that for H3-a1 was $(0.34—4.7)\times10^6$.

2.3.2 Chemical properties of H3-a1 and H3-b1. To investigate the chemical properties of the active polysaccharides H3-a1 and H3-b1, their total carbohydrate, protein, structural sugar contents, and the sulfate and uronic acid contents were examined. The UV spectrum gave the result that both H3-b1 and H3-a1 had prominent absorbance around 200 nm. H3-a1 also showed a little absorbance at 275 nm while H3-b1 had very slight absorbance at this wavelength. It seems that H3-a1 contains a little more protein than H3-b1. The H3-b1 and H3-a1 showed purple red polysaccharide band only in Schiff's reagent stained gel but no band in gel which was stained by Coomassie blue dye when detected by

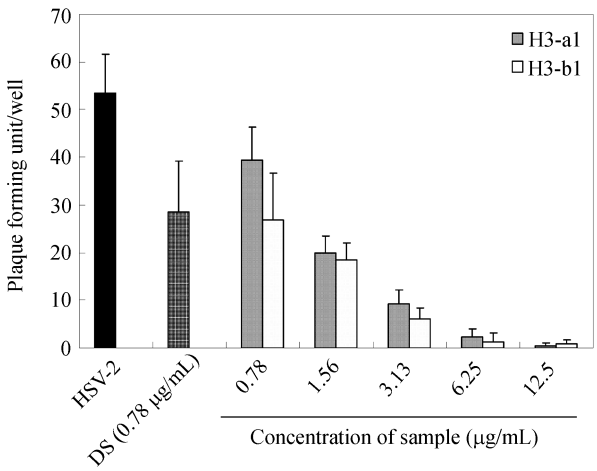

Figure 4 Antiviral activities of H3-a1 and H3-b1 against HSV-2 in Vero cells. Vero cells were infected with HSV-2 standard strain (80 PFU/well) with the absence (virus control) or presence of serially diluted samples or dextran sulfate (DS_{10k}, positive control) at 37℃ for 1 h. The unabsorbed virus was removed by washing with PBS. The infected cells were incubated with corresponding samples at 37℃ for 72 h and then fixed, stained, and the number of plaques was counted.

Table 2 Antiviral activity of H3-a1 and H3-b1 against HSV-2 standard strain

Compounds	CC_{50}[a)] (μg/mL)		EC_{50}[b)] (μg/mL) (HSV-2)	SI[c)]
	Vero cells	HEp-2 cells		
H3-a1	>1000	>1000	1.7	>588
H3-b1	>1000	>1000	1.67	>598

a) CC_{50}, the concentration of the sample that showed cytotoxic effect on the growth of cells by 50% as determined by LDH assay; b) EC_{50}, the concentration of the sample that inhibited the growth of HSV by 50% in Vero cells as determined by plaque formation reduction assay. c) SI, selective index = CC_{50}/EC_{50}.

SDS-PAGE (data not shown). These results suggested that H3-b1 and H3-a1 contained no or little protein. Using phenol-H_2SO_4 assay and Lowry-Folin method, H3-b1 was found to consist of a high proportion of carbohydrate (42.7%) and only 0.69% of protein. H3-a1 contained a lower percentage of carbohydrate (27.8%) and more protein (8.6%) than H3-b1.

GC assay showed that besides fucose (6.2%), polysaccharide in H3-b1 contained mainly rhamnose (as high as 44.7%) and then glucose, mannose, galactose, ribose, galactosamine and a little xylose (Table 3). The monosaccharide contents of polysaccharide H3-a1 were quite different from those found in H3-b1. It had only 0.27% of fucose and the main monosaccharide was galactose (26.0%) followed by glucose, mannose, glucosamine, rhamnose, arabinose and a little ribose. This polysaccharide contained arabinose and glucosamine which were not present in H3-b1, while galactosamine and xylose were only found in H3-b1.

Table 3 Relative amount of monosaccharide contents of polysaccharides in H3-a1 and H3-b1

Monosaccharide	Relative amount (%)	
	H3-a1	H3-b1
Arabinose	3.27±0.07	–
Fucose	0.27±0.01	6.16±0.11
Galactosamine	–	1.47±0.03
Galactose	26.01±0.18	9.19±0.02
Glucosamine	14.91±0.16	–
Glucose	22.23±0.02	15.17±0.18
Mannose	18.59±1.84	10.45±0.63
Rhamnose	9.61±0.05	44.70±0.32
Ribose	0.61±0.04	9.02±0.25
Xylose	–	0.90±0.23
Others	4.5	2.94

During purification, the sulfate contents of these polysaccharide fractions changed from lower than 20% (Hy and H3-b) to higher than 26% (H-I-3) and eventually reached 32.8% (H3-a1) and 47.5% (H3-b1) (Table 4). Elemental analysis showed that the proportion of four elements in H3-b1 was carbon (23.61%), hydrogen (4.3%), low nitrogen (0.63%) and relatively high sulfur content (8.28%). In other words, the proportion of carbon, hydrogen, nitrogen and sulfur in H3-b1 was about 1∶2∶0.023∶0.13, suggesting that there was one sulfate in every 1.28 glycosyl residue. This confirmed that H3-b1 was of carbohydrate nature with high content of sulfate group.

Table 4 Sulfate and uronic acid content in different polysaccharide fractions from the brown seaweed *Hydroclathrus clathratus*

Fraction	Content (%)	
	sulfate	uronic acid
Hy	17.05±0.34	/ [a]
H-I-3	26.33±0.42	11.75±0.45
H3-b	19.86±2.20	23.05±0.49
H3-a1	32.81±0.21	10.29±0.06
H3-b1	47.50±1.35	0.74±0.03

Results are expressed in means ± S.D. as wt. % of three different experiments. a) Not determined.

H3-b1 and H3-a1 had similar IR spectrum in this IR spectroscopy study (Figure 5). They both had a large absorption band about 3400 cm^{-1} which was caused by a large amount of —OH stretching. About other absorption bands, there were C—H stretching at 2930 cm^{-1}, intense absorbance around 1640 cm^{-1} for stretching vibration of the carboxylic groups, symmetric CH_3 bending at 1380 cm^{-1}, and S=O stretching at 1254 cm^{-1}. Absorbance bands were also observed at lower frequencies below 900 cm^{-1}. The absorbance at ~840 cm^{-1} indicated the presence of sulfate esters linking secondary hydroxyl group. The difference between H3-a1 and H3-b1 was observed with the absorbance band at 1542 cm^{-1} in

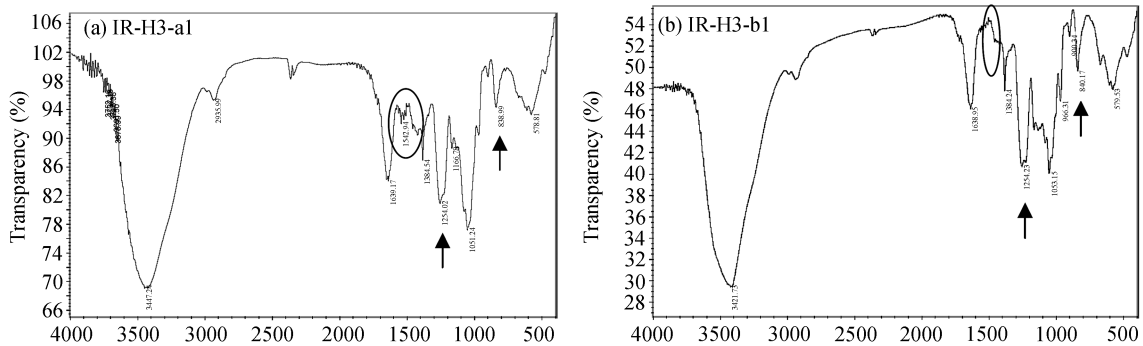

Figure 5 Infrared spectra of H3-a1 and H3-b1 from *Hydroclathrus clathratus*. Arrows show the S=O (1254 cm^{-1}) and C—O—S (~840 cm^{-1}) stretching absorption bands. The circles on the curve indicate the NH_2 bending absorption band.

H3-a1 (Figure 5(a)) but not in H3-b1 (Figure 5(b)). Theband near 1540 cm^{-1} was assigned to the amide -II band resulting from the coupling between the NH_2 deformation mode and CN stretching vibration[16]. This result further confirmed the fact that H3-a1 contains protein while H3-b1 does not.

3 Discussion

In our study, the seaweed was extracted by hot water or by acid and alkali at 4℃ or heated. The yield was higher at high treated temperature, but the hot acidic and alkali extracts showed no antiviral activity. In fact, almost all the polysaccharides depolymerized in solution and more rapidly in acid than in neutral or alkaline media[17]. Heating always promoted the reactions. The increase in yield that was associated with a drop in their bioactivity maybe due to the change in the properties of the polysaccharide such as solubility, structure and viscosity and so on. Therefore, hot water extract was the best way to extract seaweed polysaccharides for their antiviral activities.

Many seaweed-derived polysaccharides show polyanionic characteristics[18]. Based on their differences in ion strength, these polysaccharides were separated into different fractions by ion exchange chromatography. The fact that H3-b could bind to DEAE-cellulose and then be eluted with NaCl solution suggested that H3-b is anionic in nature. But the fraction H3-a did not bind to the anion exchanger DEAE-cellulose and was washed out by distilled water. It was a neutral compound with pH value of 7.0. Moreover, the polysaccharides H3-b1 and H3-a1 derived from these two fractions were also an acidic and a neutral polysaccharide respectively. Results of an experimental test showed that these fractions were all sulfated polysaccharides. It could therefore be suggested that because of the strong negative charge present in H3-a and H3-a1 molecule at pH 7, the electrostatically inert behavior of the anionic polymers might arise from a steric hindrance between the large anionic polysaccharide molecule and the polysaccharide matrix of the anion exchanger that counteracts the interionic binding. Alternatively, the investigated polysaccharide may exhibit neutral properties[19]. A possible explanation for the neutral character of the sulfated polysaccharide could be an additional esterification of the $R-O-SO_3^-$ groups. This may happen intramolecularly with free OH groups of the polysaccharide, or also intermolecularly with the precipitating EtOH during fractionation[19].

It has been reported that sulfated polysaccharides are generally endowed with anticoagulant properties[20]. On one hand, the seaweed polysaccharides are sometimes used as modulators of coagulation and as alternatives to heparin which is the anticoagulant used as medicine[21]. On the other hand, the anticoagulant properties of polysaccharides also hamper their usefulness as antiviral drugs. The polysaccharides isolated from *H. clathratus* showed moderate anticoagulant effect. However, at the EC_{50} value (1.56－12.5 μg/mL), these polysaccharides did not significantly change the thrombin time. Only at concentration of 62.5 μg/mL, which is 7－12.5 times higher than the EC_{50} against HSV-2, could the polysaccharides prolong the TT. Therefore, it can be concluded that polysaccharides isolated from *H. clathratus* exhibited relatively weak anticoagulant activities. With respect to their possible use as an antiviral drug especially of the pure compound H3-b1, this potential side effect can be neglected.

Previous studies proved that the biologically active polysaccharides from plant extracts are macromolecules and their molecular masses range from thousands to millions of Daltons[22]. The differences in molecular weight and potent bioactivities of seaweed polysaccharides could be due to the extraction method or to seasonal or geographical variations[23]. The present results suggested that the hot water extract of *H. clathratus* consisted of different water-soluble polysaccharide fractions with a wide range of molecular mass.

Furthermore, the sulfated polysaccharides from many types of brown seaweed are unique, not occurring in other seaweeds or land plants. They are composed mainly of fucose and sulfate, with smaller portions of uronic acid, galactose, mannose and xylose[24]. However, in the present study based on GC assay, both H3-b1 and H3-a1 contained some different monosaccharide components and both had low content of fucose. The high ratio of rhamnose in H3-b1 was similar to that of the rhamnan sulfate, which was isolated from a species of Chlorophyta and composed of large amounts of rhamnose residues[4]. This rhamnan sulfate also showed high antiviral activity against HSV-1, HCMV and HIV-1 with very low EC_{50} value. In fact, due to the large molecular mass property and the process of purification, seaweed polysaccharides sometimes contain large amounts of

sugars other than fucose, such as galactose, mannose, xylose, or uronic acid, and sometimes even proteins[21]. Moreover, their composition could change depending on the algal species, the extraction process, the season of harvest and the local climatic conditions.

The antiviral assay showed that both H3-a1 and H3-b1 exhibited the highest anti-HSV effect. The H-I-3 exhibited higher antiherpetic activity than Hy and H3-b but a little lower than H3-a1 and H3-b1. In this experiment, it seems that the higher the sulfate content the polysaccharides contained, the greater the antiviral effect they would exhibit. Meanwhile, the anticoagulant activity of these polysaccharides increased with the increase of their sulfate content in this study. Thus the sulfate groups play an important role in the pharmaceutical activities of *H. clathratus* polysaccharides. This is in agreement with the previous study on other algal polysaccharides[25].

IR analysis had previously been used in detecting brown seaweed polysaccharide[26] and earlier results suggested that the absorption at 840 cm^{-1} was due to sulfate groups at the axial C-4 position[27]. Sulfate groups at the equatorial C-2 and C-3 positions should give a small shoulder of absorption at 820 cm^{-1}. Thus based on the IR spectrum in this study, most sulfate groups in the polysaccharides should be connected at the C-4 of the sugar units of H3-a1 and H3-b1.

Although the bioactivities of seaweed polysaccharides are related to their charge contents, the relationship between bioactivities and uronic acid, which is another charged component present in these polysaccharides, was not clear in the present study. Their molecular structures as well as the structural basis of the biological properties still remain to be established. With high antiviral activity against HSV, the two sulfated polysaccharides, H3-a1 and H3-b1 isolated from *H. clathratus*, may be potent substances for treating herpes virus infections.

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