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Purification and Partial Characterization of an Acidic Polysaccharide with Complement Fixing Ability from the Stems of *Avicennia Marina*

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An acidic polysaccharide fraction that had high anti-complementary activity was isolated from the stems of Grey Mangrove in 0.15% yield. The final fractions was designated HAM-3-IIb-II. The polysaccharide fraction appeared to be homogenous by high performance size exclusion chromatography with an estimated molecular weight of 105 kDa. The isolated polysaccharide is more effective than polysaccharide K (PSK) in its anti-complementary activity at 58 μg/ml of PSK and 23 μg/ml of HAM-3-IIb-II that inhibit 50% of complement activity in the complement fixation assay. Structural studies indicated that HAM-3-IIb-II was rich in galacturonic acid along with arabinose, galactose and rhamnose, characterizing a pectin-type polysaccharide, which was also confirmed by FT-IR spectrum. The presence of rich neutral sugar side chains of arabinogalactans may have contributed to the expression of high activity. Traditionally, this mangrove plant is used for medicinal purposes and it appears to have some scientific applications.

Keywords: Anti-complementary activity, Arabinogalactans, *Avicennia marina*, Pectin, Polysaccharide

Introduction

Avicennia marina (Forsk.) Vierh. (Avicenniaceae, Common names: Grey Mangrove, Tivar, Timar), an evergreen shrub, is one of the most widespread mangrove species of the ancient world, extending from East Africa to Fiji in Polynesia and the North Island of New Zealand, occurring at the coldest regions in New Zealand, subtropical China and southeastern Australia. The plant is widely used for the treatment of skin diseases (Sharaf *et al.,* 2000). Researchers have so far focused their attentions on its medicinal functions. The extracts from the stems of this plant have been found to have some additional medicinal functions as they are believed to cure rheumatism, smallpox and ulcers (Bandaranayake, 2002). Besides, its anti-malaria activities and cytotoxicity of *A. marina* have been reported (Sharaf *et al.,* 2000).

Research findings on the low-molecular-weight constituents of *Avicennia marina* highlighted the following Iridoid glucosides, fatty acids, sterols, hydrocarbons and flavonoids (Sharaf *et al.,* 2000). Low molecular weight compounds have for a long time been considered as the active components of medicinal plants, but they often do not contribute to all of the clinical effects achieved. Among the high molecular weight components of medicinal herbs, several polysaccharides have been reported to elicit a wide range of anti-tumour (Chen *et al.,* 2004), anti-complementary (Diallo *et al.,* 2003), anti-inflammatory (Garbacki *et al.,* 1999), immunological (Garbacki *et al.,* 1999) and anti-viral activities (Premanathan *et al.,* 1999). The presence of such compounds could be partly responsible for the above-mentioned medicinal functions of *A. marina*. In general, complement system plays a vital role, such as primary defense against bacterial invasions and viral infections. This system is composed of over 20 serum proteins, which are activated by classical, alternative and lectin pathways. Immunomodulation associated with the activation of the complement system can have thymus dependant antibody response, regulation of specific cyclic antibody production, regulation of the IgG-IgM switch, induction of the suppressor or helper T-cells and modulation of T and B cell proliferation (Yamada and Kiyohara, 1999). The materials activating the complement system are therefore apparently considered to enhance the immunity of human body.

The isolation, purification and characterization of polysaccharides from *A. marina* and subsequent evaluation of biological activity (anti-complementary activity) are yet to be reported. Hence, in this study, an acidic polysaccharide was purified from hot water extract and further tested for its anti-complementary activity.

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Materials and Methods

Materials. Stems of A. marina were collected in Techeng Island, Xiashan district in September 2004. The specimen was identified and its holotype deposited in the herbarium of Shantou Agriculture and Forestry Bureau. Resins of DEAE-Cellulose were purchased from Shanghai Hengxin Chemical Reagent Co., Ltd.. DEAE-Sepharose Fast-flow and Sephacryl S-400 were purchased from Pharmacia Ltd.. Polymyxin B, lipopolysaccharide (LPS) of *E. coli* 0127:B8 and pectin standard (code no P9135) from citrus fruits with the degree of esterification (DE) of 60.97% were obtained from Sigma-Aldrich Co.. Human serum were drawn from 10 healthy volunteers by venue puncture with no additive; rabbit anti-sheep erythrocyte antibodies were obtained from Yuhuan Southern Reagent Factory, Sheep erythrocytes were obtained from Huisheng Reagent Co., Ltd.. The sample of Polysaccharide-K, PSK (Sakagami *et al.,* 1991) was obtained from China National Institute for the Control of Pharmaceutical and Biological Products. All other chemicals were of analytical reagent-grade.

Extraction, fractionation and purification of crude polysaccharides. The stems of the plant were air dried and powdered. Extraction, fractionation and purification details are shown in Fig. 1.

General analysis. Total carbohydrate, uronic acid and protein contents were assayed using the phenol-sulfuric acid (Dubois *et al.,* 1956), m-hydroxydiphenyl (Blumenkantz and Asboe-Hansen, 1973) and Lowry methods (Lowry *et al.,* 1951), respectively; arabinose, galacturonic acid and bovine serum albumin (BSA) were used as the respective standards. Monosaccharide compositions were determined by GC analysis and the sample preparation was carried out according to the method described by Blakeney *et al.,* (1983). GC was carried out with a Shimadzu GC-14A, using the following conditions: OV1701 column (30 × 0.25 mm) with column temperature 195-240°C, nitrogen gas carrier (1.0 ml/min.), FID temperature, 230°C and injection temperature 250°C. The specific optical rotation was determined in H_2O at 20°C using a WZZ-T polarimeter.

Complement fixation activity. The complement fixation test is an in vitro test for the ability of the samples to interact with the complement cascade reaction. It was carried out according to the complement fixation assay (Method A) (Michaelsen *et al.,* 2000) with slight modifications using PSK as a positive control. This method will not distinguish between the classical and alternative pathways. Sheep erythrocytes were washed twice with 0.9% NaCl and centrifuged at 2000 rpm for 5 min and once with veronal buffer (VB) pH 7.2 containing 0.13 M NaCl, 0.2 nm Ca^{2+}, 0.8 nm Mg^{2+}, 2 mg/ml bovine serum albumin, VB/BSA. The washed sheep erythrocytes were sensitized with antibodies by adding rabbit anti-sheep erythrocyte antibodies to a final dilution 1 : 4000 and incubated at 37°C for 30 min. The sensitized sheep erythrocytes were then washed three times with VB/BSA, diluted to 1% suspension and used the same day. The human sera with intact complement proteins obtained from 10 healthy volunteers, were pre-treated for removal of antibodies against sheep erythrocytes as described by Michaelsen *et al.,* (1991). The serum was diluted with the buffer to a concentration of about 50% hemolysis of sheep erythrocytes; 50 µl serial diluted samples were mixed with 50 µl of diluted serum in triplicates to wells on a microplate and incubated on a shaker at 37°C. After 30 minutes, the sensitized sheep erythrocytes (50 µl) were added to each well and incubated further for 30 min at 37°C. After centrifugation at 3000 rpm for 10 min, 100 µl of supernatant was pipetted in an empty flat-bottom microplate for absorbency reading at 405 nm using by an ELISA reader (Multiskan MK3, Labsystems, Finland). 100% hemolysis was obtained with distilled water and sensitized sheep erythrocytes (A_{water}). VB/BSA, serum and sensitized sheep erythrocytes were used as control for the medium ($A_{control}$), while PSK was used as positive control. Inhibition of hemolysis induced by the test sample was calculated by the formula: $[(A_{control} - A_{sample})/A_{control}] \times 100\%$. A dose response curve was constructed to calculate the concentration of test sample that was able to give 50% inhibition of hemolysis (ICH_{50A}).

Pronase digestion and periodate oxidation of crude polysaccharides. Pronase digestion and periodate oxidation of hot water crude polysaccharides (HAM-0) were performed using the method described by Yamada *et al.* (1991). The control was neither treated with pronase nor with periodate.

Homogeneity and molecular weight determination. Homogeneity of the purified polysaccharide (HAM-3-IIb-II) was determined by high performance size exclusion chromatography (HPSEC) on a linked column of Ultrahydrogel™ Linear (300 mm × 7.8 mm) equipped with a Waters 600 HPSEC system, eluting 0.1 M $NaNO_3$ at a flow rate of 0.9 ml/min. And standard dextrans of T-2000 (2×10^6), T-580 (5.8×10^5), T-190 (1.88×10^5), T-70 (7×10^4), T-10 (1×10^4) and T-5 (4.6×10^3) were also applied to the same system. Molecular weight distribution of HAM-3-IIb-II was determined by comparison with the retention time of standard dextrans under the same conditions.

FT-IR spectra. HAM-3-IIb-II was incorporated into KBr (spectroscopic grade) and pressed into 1-mm pellet. Spectra were recorded at the absorbance mode from 4000 to 400 cm^{-1} on a Nicolet Nexus FT-IR spectrometer. For comparison purpose, a commercial pectin standard from citrus fruits was also used.

Determination of the degree of esterification (DE). DE of HAM-3-IIb-II was determined by the titrimetric method described in Food Chemical Codex with slight modification (Singthong *et al.,* 2004) using a commercial pectin standard from citrus fruits as control. 80 mg of sample (HAM-3-IIb-II) was transferred to a 250 ml flask, moistened with 2 ml of ethanol and dissolved in 100 ml of carbon dioxide-free water. After the sample was completely dissolved, five drops of phenolphthalein were added, the sample was then titrated with 0.01 M NaOH and the result recorded as the initial titer. Then 10 ml of 0.5 M NaOH was added, the sample was shaken vigorously, and allowed to stand for 15 min. 10 ml of 0.5 M HCl was added and the sample was shaken until the pink color disappeared. Three drops of phenolphthalein were added and the solution was titrated with 0.01 M NaOH to a faint pink color that persisted after vigorous shaking (end-point). This volume of titration was recorded as the saponification titer (the final titer). DE was calculated by the formula: %DE = the final titer/(the initial titer + the final titer) × 100.

Effect of polymyxin B on the anti-complementary activity. The effect of hemolysis by polymyxin B was examined using the procedure described by Morrison and Jacobs (1976a). Lipopolysaccharide and HAM-3-IIb-II fractions were incubated with an equal weight of polymyxin B and VB/BSA (100 µl) at 37°C for 30 min, 50 µl of solution was used for the assay of anti-complementary activity while the control sample was not treated with polymyxin B.

Discrimination between classical and alternative pathway of complement activation. EGTA will effciently block Ca^{2+} that is essential for classic pathway C3 convertase, while it will not block the added Mg^{2+} which is essential for alternative pathway C3 convertase, and EDTA will block both Ca^{2+} and Mg^{2+} essential for complement-derived hemolysis. Thus, to investigate an activation pathway by purified polysaccharide, HAM-3-IIb-II was assayed by ELISA in VB/BSA, VB/BSA-EGTA-Mg^{2+} (VB/BSA added 8 mM EGTA and 3 mM $MgCl_2$) and VB/BSA EDTA buffer (VB/BSA added 8 mM EDTA) respectively (Michaelsen *et al.,* 2000).

Statistical analysis. All results are expressed as the mean ± S.D. The significance of any differences between means was evaluated by Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Purification of anti-complementary polysaccharides. As the procedure described in Fig. 1, ground-dried stems (500 g) were first extracted 2 times with ethanol (2.5 L) and centrifuged at 3,000 rpm for 10 min. Subsequently, cold water (2.5 L) was added to the dried ethanol extracted residue, and stirred at room temperature for 24 h and centrifuged at 3,000 rpm for 10 min. After 2 times extractions with cold water, the air-dried residue was extracted twice with hot water (10 L) at 100°C for 2 h to obtain hot water extract. The extract was then concentrated under reduced pressure at 50°C and then precipitated by the addition of 4 times the volume of ethanol. The resulting gel-like precipitate was dissolved in distilled water and centrifuged at 3,000 rpm for 10 min to remove any insoluble materials. The supernatant was lyophilized and a black crumbly mass was obtained (HAM-0, yield: 1.84%w/w; calculated from dried and ground stems). HAM-0 was re-dissolved in distilled water and fractionated by ultra-filtration with a 3 kDa and 10 kDa cellulose cartridge (Shanghai Yadong Nuclear Grade Resins Co., Ltd.) and lyophilized. A brown crumbly mass HAM-3 (yield: 0.96% w/w; calculated from dried and ground stems). HAM-3 was then de-proteinated by trichloroacetic acid (TCA), the samples were mixed with 100 ml of 0.4 M TCA, left for 12 h and centrifuged at 3,000 rpm for 10 min. The supernatant was dialyzed with a membrane cut-off of 10 kDa, non-dialyzable portion was lyophilized and successively purified by column chromatography with active fractions as follows: The samples were applied to a column (2.6 × 30 cm) of DEAE-Cellulose in chloride form, and eluted stepwise with distilled water followed by a NaCl gradient (0-1.0 M) at a flow rate of 1.5 ml/min. Each fraction was collected and monitored for the presence of the carbohydrate using phenol-sulfuric acid assay (Dubois *et al.*, 1956). Fractions containing carbohydrate were pooled, dialyzed and lyophilized. The absorbed fraction of HAM-3-II (yield: 0.24% w/w; calculated from dried and ground stems) was further loaded on a column (2.6 × 20 cm) of DEAE-Sepharose Fast-flow, eluting stepwise with distilled water and a NaCl gradient (0-0.8 M) at a flow rate of 1.6 ml/min. Fractions containing carbohydrate were also pooled, dialyzed and lyophilized. HAM-3-II was separated into HAM-3-IIa and HAM-3-IIb, the major fraction (HAM-3-IIb, yield: 0.16% w/w; calculated from dried and ground stems) was then purified by size exclusion chromatography on a column (1.6 × 100 cm) of Sephacryl S-400 and eluted with 10 mM NaCl at a flow rate of 19.6 ml/h, thereby obtaining the purified anti-complementary polysaccharide (HAM-3-IIb-II, yield: 0.15% w/w; calculated from dried and ground stems).

The evaluation of each sub-fraction on the complement system was carried out using PSK as a positive control. As shown in Table 1, HAM-0 expressed a high anti-complementary activity of 50% inhibition of hemolysis at 52 µg/ml, while EAM (ethanol extraction) exhibited less than 50% inhibition of hemolysis at the highest concentration test (500 µg/ml), and CAM-0 (cold water crude polysaccharides) exhibited 50% inhibition of hemolysis at 125 µg/ml. HAM-0 was further fractionated by ultra-filtration with a 3 kDa and 10 kDa membrane cartridge, obtaining three fractions (HAM-1, molecular weight < 3 kDa; HAM-2, 3 kDa < molecular weight < 10 kDa; HAM-3, molecular weight > 10 kDa). Of the three fractions, HAM-3 showed the highest potent anti-complementary activity of 50% inhibition of hemolysis at very low concentrations of 41 µg/ml compared with positive control (PSK). PSK typically showed a 50% inhibition of hemolysis at 58 µg/ml. HAM-3 was de-proteinated by TCA and further separated into three fractions (HAM-3-I, HAM-3-II and HAM-3-III) after elution on the DEAE Cellulose column as shown in Fig. 2. HAM-3-I was eluted with water whereas HAM-3-II and HAM-3-III were eluted in the range of 0-0.5 M and 0.5-1.0 M NaCl solutions, respectively. The major fraction, HAM-3-II exhibited higher effects on complement system although its anti-complementary activity was not significantly increased compared to HAM-3. HAM-3-II was then purified by DEAE-Sepharose Fast-flow, resulting into two fractions, HAM-3-IIa and HAM-3-IIb (Fig. 3). Both fractions showed more potent activities (ICH_{50} < 48 µg/ml) than PSK. The acidic fraction (HAM-3-IIb) contained approximately 96.8% of carbohydrate and its anti-complementary activity was higher than HAM-3-IIa and HAM-3. HAM-3-IIb was collected and further applied on size exclusion chromatography of Sephacryl S-400 and three fractions (HAM-3-IIb-I, HAM-3-IIb-II and HAM-3-IIb-III) were obtained (Fig. 4). Fraction B (HAM-3-IIb-II, yield: 0.15%) was obtained as a major intermediate purified material. Among the three fractions obtained, HAM-3-IIb-II showed the highest anti-complementary activities and yield. Evaluation of

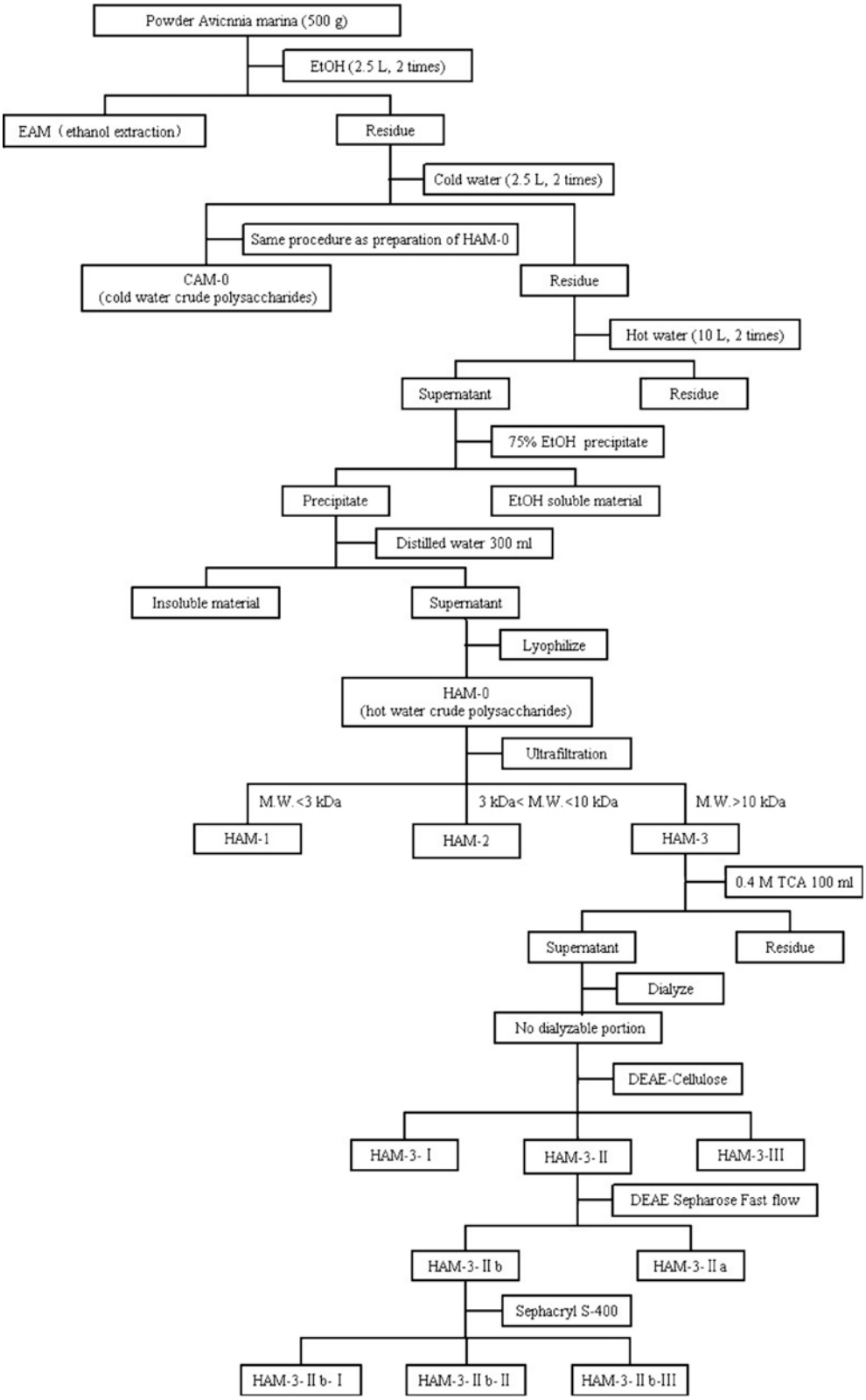

Fig. 1. Purification procedures of anti-complementary polysaccharide from stems of *Avicennia marina*.

Table 1. Anti-complementary activities of sub-fractions in each purification step

	Sample	Anti-complementary activity (ICH_{50})[a]
		Concentration (μg/ml)
Control	PSK	58 ± 2
Ethanol extract	EAM	n.d.[b]
Cold water polysaccharides	CAM-0	125 ± 5
Ethanol precipitation	HAM-0	52 ± 2
Ultra-filtration	HAM-1	248 ± 7
	HAM-2	155 ± 5
	HAM-3	41 ± 1*
DEAE-Cellulose	HAM-3-I	79 ± 3
	HAM-3-II	35 ± 2*
	HAM-3-III	161 ± 4
DEAE-Sepharose Fast-flow	HAM-3-IIa	47 ± 1
	HAM-3-IIb	25 ± 1*
Sephacryl S-400	HAM-3-IIb-I	25 ± 1*
	HAM-3-IIb-II	23 ± 2*
	HAM-3-IIb-III	79 ± 1

[a]ICH_{50} (μg/ml): Concentration inhibiting the hemolysis by 50%
[b]n.d.: Not determined
*Significantly different from sub-fractions with the highest anti-complementary activity sample of previous purification ($p < 0.05$)

the anti-complementary activities of these fractions showed that HAM-3-IIb-II expressed the most potent activity among all the sub-fractions during each purification step.

Pronase digestion and periodate oxidation of crude polysaccharides. HAM-0 was digested with pronase (yield 75.5% w/w; calculated from dried HAM-0) and oxidized with sodium periodate (yield 76.4% w/w; calculated from dried HAM-0) to identify the real active moiety. As shown in Fig. 5, the anti-complementary activity of HAM-0 was not affected by pronase digestion, instead a significant decrease in anti-complementary activity by periodate oxidation was observed. These results indicated that periodate labile part of the fraction was essential for anti-complementary activity. The crude polysaccharides (HAM-0) were thus considered as anti-complementary polysaccharides.

Physicochemical properties of anti-complementary polysaccharides. The physicochemical properties of anti-complementary fractions are summarized in Table 2. The carbohydrate content of polysaccharides was significantly increased to 99.4% whereas the protein content was significantly decreased to 0.5% during the subsequent purification procedures. The purified polysaccharide (HAM-3-IIb-II) comprised 48.3% galacturonic acid and 51.7% neutral sugars. The main neutral sugars were Rhamnose, Arabinose and Galactose in the molar ratio of 1 : 2.9 : 2.6, while Xylose and Mannose were present in trace amounts.

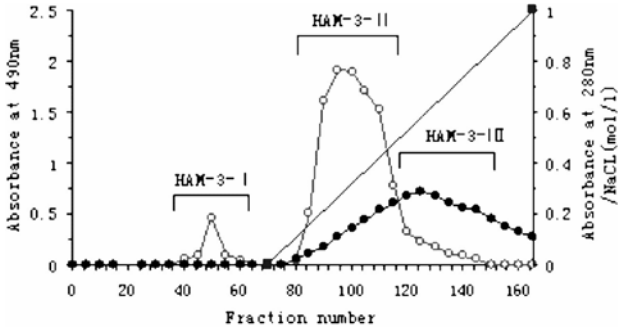

Fig. 2. Anion-exchange chromatography of HAM-3 on DEAE-cellulose. 0.2 g of sample was applied on the column (2.6 × 30 cm), eluted stepwise with distilled water followed by a NaCl gradient (0-1.0 M). ○: Carbohydrate (490 nm); ●: Protein (280 nm); ■: NaCl (mol/L).

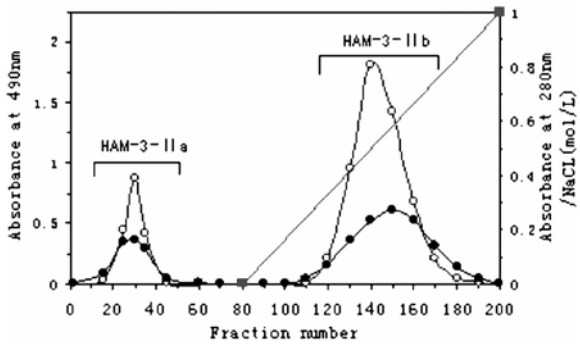

Fig. 3. Anion-exchange chromatography of HAM-3-II on DEAE-Sepharose Fast flow. 0.1 g of sample was loaded on the column (2.6 × 20 cm), eluted stepwise with distilled water and a NaCl gradient (0-1.0 M). ○: Carbohydrate (490 nm); ●: Protein (280 nm); ■: NaCl (mol/L).

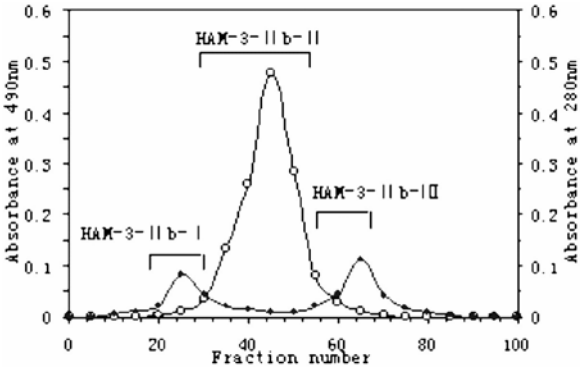

Fig. 4. Size exclusion chromatography of HAM-3-IIb on Sephacryl S-400. 3 ml of 0.5 % sample was loaded on a column (1.6 × 100 cm) and eluted with 10 mM NaCl. ○: Carbohydrate (490 nm); ●: Protein (280 nm).

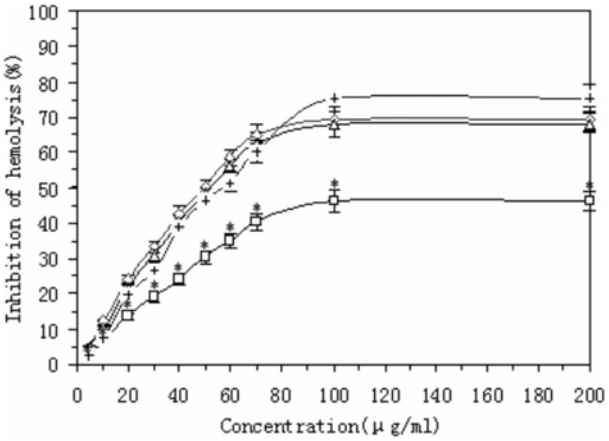

Fig. 5. Anti-complementary activity of HAM-0 after treatments of periodate oxidation and pronase digestion. +: PSK (Polysaccharide-K) as a positive control; △: HAM-0 (Refer to Fig. 1.); ◇: treated with pronase; □: treated with periodate oxidation. *Significantly different from non-treated HAM-0 ($p < 0.05$).

The glucose content was 2.4%, but gave a negative reaction for starch when a solution of I_2/KI was added. From these structural monosaccharide contents, it was assumed that HAM-3-IIb-II was mostly like a pectic polysaccharide. On HPSEC profile in Fig. 6, the purified polysaccharide appeared as a symmetric peak to prove its homogeneity. The molecular mass was calculated to be 105 kDa and $[\alpha]_D$ at 20°C = +27.54 ($_C$0.1; H_2O).

Partial characterization of purified polysaccharide (Pectin identification assay). The FT-IR spectrum of HAM-3-IIb-II is shown in Fig. 7. This spectrum was compared with commercial pectin standard. It was found that the spectrum shape of HAM-3-IIb-II is similar to that of pectin standard, coinciding with the initial supposition that HAM-3-IIb-II was a pectic polysaccharide. Both polysaccharides show high absorbance in the region 1200-850 cm^{-1}, which is considered as the so-called "finger print" region, where the position and intensity of the bands are specific for pectic polysaccharides. This region is dominated by ring vibrations overlapped with stretching vibrations of (C-OH) side groups and the (C-O-C) glycosidic bond vibration (Kacuráková *et al.,* 2000). For galacturonic acid in pectic polysaccharides, the main absorbance regions are at 1,150, 1,100 and 1,020 cm^{-1} (Coimbra *et al.,* 1998). HAM-3-IIb-II and pectin sample rich in uronic acid revealed intense peaks at 1,144, 1,098 and 1,020 cm^{-1} which are also characteristic peaks of pectic polysaccharides. The bands occurring at around 1,730 cm^{-1} and 1,600 cm^{-1} refer to the ester carbonyl groups (COOR) and free carboxylate groups (COO^-), respectively (Gnanasambandam and Proctor, 2000). The pectin sample shows intense peak at 1,739 cm^{-1}

Table 2. Physicochemical properties of crude and purified anti-complementary polysaccharides

	HAM-0	HAM-3	HAM-3-II	HAM-3-IIb	HAM-3-IIb-II
Appearance	Black, fluffy	Brown, fluffy	Cream, fluffy	White, fluffy	White, fluffy
Carbohydrate[a] (%)	35.3 ± 2.1	43.2 ± 1.7	96.2 ± 1.5	96.8 ± 1.1	99.4 ± 0.8
Uronic acid[b] (%)	28.1 ± 0.8	35.3 ± 0.7	46.9 ± 0.5	47.9 ± 0.5	48.3 ± 0.4
Protein[c] (%)	25.1 ± 1.1	19.9 ± 0.6	3.8 ± 0.2	3.2 ± 0.1	0.5 ± 0.1
Molecular weight (kDa)	n.d.	n.d.	n.d.	n.d.	105
Sugar composition (mol%)					
Rhamnose	n.d.	n.d.	7.6	7.4	7.5
Arainose	n.d.	n.d.	22.4	21.7	21.6
Xylose	n.d.	n.d.	0.8	0.7	0.7
Mannose	n.d.	n.d.	0.4	0.4	0.3
Galactose	n.d.	n.d.	19.7	19.8	19.4
Glucose	n.d.	n.d.	2.2	2.0	2.2

[a]α-D-Glucose as a reference
[b]α-D-Galacturonic acid as a reference
[c]Bovine serum albumin as a reference

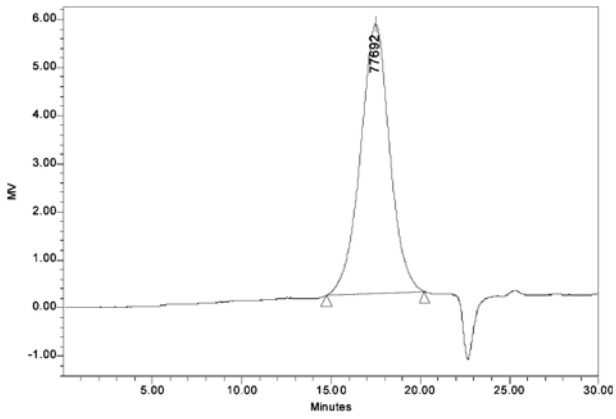

Fig. 6. HPSEC profile of the purified polysaccharide from *Avicennia marina*. HAM-3-IIb-II was injected into a column of Ultrahydrogel™ Linear (300 mm × 7 mm id × 2), and then eluted with 0.1 M $NaNO_3$, and the HPSEC system was Waters 600 HPSEC series equipped with a reflective index (RI) detector.

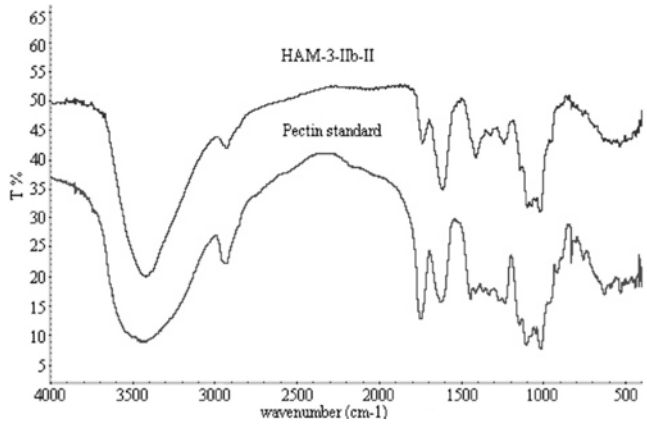

Fig. 7. Fourier transform infrared spectra of HAM-3-IIb-II fraction and commercial pectin standard from citrus fruits. Both polysaccharides show high absorbance in the region 1200-850 cm^{-1}, which is considered as the so-called "finger print" region specific for pectic polysaccharides. The bands occurring at around 1,730 cm^{-1} and 1,600 cm^{-1} refer to the ester carbonyl groups (COOR) and free carboxylate groups (COO^-), respectively. The pectin sample shows intense peak at 1739 cm^{-1} whereas HAM-3-IIb-II shows 1740 cm^{-1} peak with intermediate intensity indicate high and low methoxyl pectin, respectively.

whereas HAM-3-IIb-II shows 1,740 cm^{-1} peak with intermediate intensity. DE of these two polysaccharides was determined by the titrimetric method and the values were calculated to be 65% and 25%, respectively. The results suggest that HAM-3-IIb-II was low methoxyl pectin. This is in perfect agreement with the findings of other researchers. These two bands are important to evaluate DE of pectic polysaccharides as the intensity of the absorbance of the ester carboxylate groups increased with an increase in DE (Singthong *et al.*, 2004).

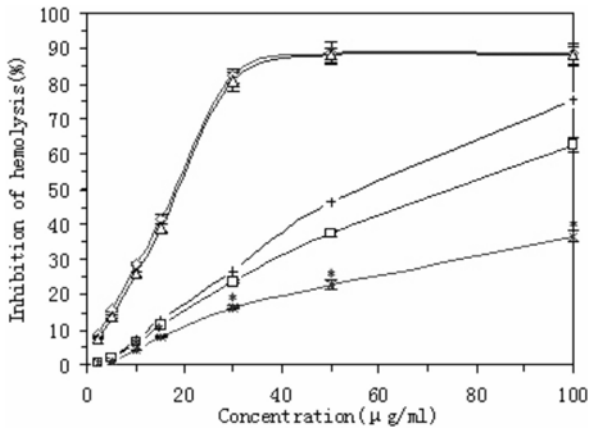

Fig. 8. Effect of polymyxin B on anti-complementary activity of LPS and HAM-3-IIb-II fraction. +: Positive control (PSK); ◇: HAM-3-IIb-II (Refer to Fig.1.), before treatment of polymyxin B; △: HAM-3-IIb-II, after treatment of polymyxin B; □: LPS (Lipopolysaccharide), before treatment of polymyxin B; ×: LPS, after treatment of polymyxin B. *Significantly different from LPS before treatment of polymyxin B ($p < 0.05$).

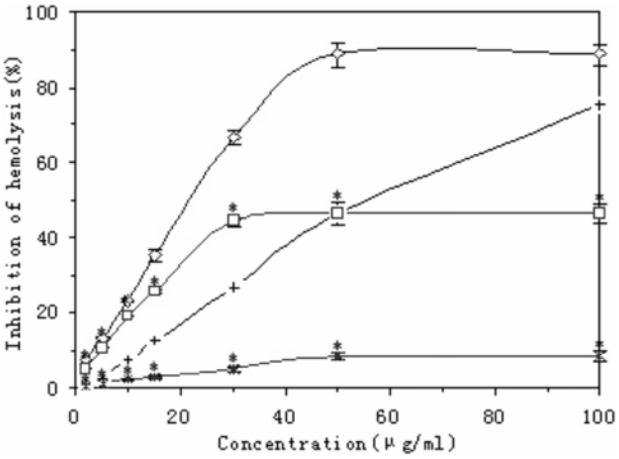

Fig. 9. Anti-complementary activity of HAM-3-IIb-II in VB/BSA buffer with or without metal ions. HAM-3-IIb-II exhibited approximately half anti-complementary activities in Mg^{2+}-EGTA-VB/BSA buffer and almost no anti-complementary activities in EDTA-VB/BSA buffer. The results indicated that HAM-3-IIb-II is both an activator of classical as well as the alternative pathway. +: Positive control (PSK); ◇: HAM-3-IIb-II; ×: EDTA-VB/BSA; □: EGTA-VB/BSA-Mg^{2+}. *Significantly different from non-treated HAM-3-IIb-II ($p < 0.05$).

Effect of polymyxin B on the anti-complementary activity. Lipopolysaccharide was reported as an activator of classical and alternative complement pathway (Morrison and Jacobs, 1976b). Polymyxin B inhibited the complementary activity of LPS. Therefore, the possibility of contamination by LPS was examined for the purified polysaccharide (HAM-3-IIb-II). As could be seen in Fig. 8, a remarkable decrease in activity was

observed only in LPS, while there was no change in activity for HAM-3-IIb-II treated with polymyxin B. This indicated that the modes of complement activation by HAM-3-IIb-II was not due to the presence of a microbial contamination during the purification processes.

Discrimination between classical and alternative pathway of complement activation. The complement system is activated via classical and alternative pathway. It is a well-known fact that Ca^{2+} and Mg^{2+} are needed for activating the classical pathway. Further, Mg^{2+} is needed for the alternative pathway. As shown in Fig. 9, HAM-3-IIb-II showed higher activity than PSK and the activity was concentration dependent at the range of low concentrations studied. Maximum concentration was achieved at 50 μg/ml, with an ICH_{50} of approximately 23 μg/ml, compared with approximately 58 μg/ml obtained for PSK. However, HAM-3-IIb-II exhibited approximately half anti-complementary activities in Mg^{2+}-EGTA-VB/BSA buffer and almost no anti-complementary activities in EDTA-VB/BSA buffer. The results indicated that the complement of activation of HAM-3-IIb-II is a complement activator both on the classical and alternative pathways.

Discussion

From the investigation of the anti-complementary activity of several medicinal mangrove plants, hot water extract of *Avicennia marina* and *Acanthus illicifolius* appeared to be potent anti-complementary materials. The current study focuses on the isolation, purification and characterization of polysaccharides from the stems of *Avicennia marina* since crude polysaccharides extracted from this plant expressed more potent anti-complementary activity than that of *Acanthus illicifolius*. *Avicennia marina* (Forsk) Vierh is a small tree used in traditional medicine (Sharaf *et al.,* 2000; Bandaranayake, 2002). Its special use for the treatment of chronic wound has been a great impetus to evaluate the complement fixing ability of extracts from this plant.

To optimize the extraction conditions for anti-complementary materials, several solvent extracts were prepared to compare the activities, and hot water extraction was selected as the optimal extraction condition in spite of observation of potent anti-complementary activities in organic solvent extracts and cold water extracts (data not shown). Extracts with organic solvents were suggested to be low molecular compounds: anti-complementary phytosterols, stigastane, cholestane and pregnone (Chun *et al.,* 2002). On the other hand, hot water extracts were postulated to be macromolecules such as polysaccharides. Various reports show that polysaccharides from different plants can be responsible for the effects associated with the healing of wounds; some of these have an influence on the immune system and are often called immunomodulators when the complement system is involved. Examples of such plant polysaccharides in different parts of the world are those from the Chinese and Japanese herbs *Glycyrrhiza uralensis* (Zhao *et al.,* 1991) *Bupleurum falcatum* L (Yamada *et al.,* 1989) and *Angelica acultiloba* (Kiyohara *et al.,* 1986). Various other reports have shown that pectins have influence on the complement system, examples include polysaccharides from; *Trichilia emetica* (Diallo *et al.,* 2003), *Vernonia kotschyana* (Nergard *et al.,* 2005), and *Plantago major* (Samuelsen *et al.,* 1998). All are coming from plants that traditionally have been used in the treatment of wounds. Therefore, it is possible to use pectic polysaccharides for medicinal purposes. Although HAM-0 showed a potent and dose-dependent anti-complementary activity, it contained high amount of protein. Since it was not known whether protein and carbohydrate moiety contributed to the expression of this activity, HAM-0 was therefore oxidized and digested with sodium periodate and pronase, respectively. The results indicated that its carbohydrate moiety was responsible for the activity, and that the resistance glycosidic linkage to periodate oxidation existed because the activity was not completely inactivated. HAM-0 was further purified by several purification steps. After evaluation of the anti-complementary activities of the fractions during each step, the fraction of HAM-3-IIb-II showed the most potent anti-complementary activity. The average molecular mass of HAM-3-IIb-II was 105 kDa. It was concluded that bioactive polysaccharides from plant extracts are macromolecules and their molecular masses disperse from thousands to a million Daltons (Kiyohara *et al.,* 1986). Interestingly, HAM-3-IIb-II was placed under this category and showed a potent anti-complementary activity.

To be able to discuss the chemical properties of the purified polysaccharide, carbohydrate, uronic acid, protein and monosaccharide compositions were examined. It was characterized by a high content of galacturonic acid (48.3%), and consisted mainly of arabinose (21.6%), galactose (19.4%) and rhamnose (7.5%), featuring a pectin-type polysaccharide. Samuelsen et al., (1998) highlighted the fact that rhamnose and galacturonic acid may constitute a rhamnogalacturonan backbone that can be part of pectin which is either associated with or might be covalently linked to arabinogalactan. Therefore, we confirmed the purified polysaccharide as a pectic polysaccharide using FT-IR spectrum. Many pectic polysaccharide isolated from medicinal plants have shown biological activity (Yamada, 1994; Zhu *et al.,* 2005). However, not all pectins have the effect on the complement system. It has been summarized that bioactive pectins (with the effect on the complement system) are the pectins rich in neutral sugar side chains of arabinogalactans containing β-(16) linkage (Yamada *et al.,* 1999). These monosaccharide contents of HAM-3-II were compared to those of anti-complementary polysaccharides Vk100A2a from *Vernonia kotschyana* (Nergard *et al.,* 2005), which were grouped in arabinogalactan. Pectic polysaccharide Vk100A2a contains 31.3% arabinose and 24.4% galactose, whereas PMII from *Plantago major* (Samuelsen *et al.,* 1996) contains 8.8% arabinose and 8% galactose. Another polysaccharide (A1002)

exhibiting similar activity obtained from *Acanthus ebracteatus* (Hokputsa *et al.,* 2004), contained 7.3% arabinose and 16.2% galactose. It is clearly seen that the arabinose and galactose content of HAM-3-IIb-II is higher than PMII and A1002, but slightly lower than Vk100A2a. The very high activity of HAM-3-IIb-II may be explained by its high length of neutral sugar side chains and similar findings were reported with an anti-complementary pectic polysaccharide isolated from the roots of *Bupleurum falcatum* L. Pectins having the effect on the complement system depend on the neutral carbohydrate side-chains and rhamnogalacturonan backbone (Yamada *et al.,* 1989).

An acidic polysaccharide with complement fixing ability from the stems of *Avicennia marina* was purified and partially characterized. However, we need a deeper understanding of the relationship between structure and activity of the purified polysaccharide, and further structural analysis and structure-activity determinations are in progress.

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