Synthesis and Anticoagulant activity of Sulfated alginate

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Abstract

Alginate is a natural polysaccharide containing carboxyl groups and (1-4) linkage between β-D-mannuronic acid and α-L-guluronic acid. Applying of sulfated modification for alginate could generate heparin-like structure containing sulfate, carboxyl group and uronic units. This research aimed to apply chemical modification using reaction between sodium alginate and sulfurochloridic acid (ClSO$_3$H) in formamide to generate product possessing anticoagulant activity which was evaluated by activated partial thrombosis time (APTT) and prothrombin time (PT). The success of sulfation was determined by sulfate content measurement and verified by FTIR spectra. The alginate sulfates (AlgS) and alginate sulfate fragments (AlgHS) obtained containing sulfate content of 14.9 and 12.9 % with degree of sulfation (DS) of 1.75 and 1.35, respectively. The existence of sulfate groups was confirmed by two strong peaks at 797 and 1244 cm^{-1}. The AlgS and AlgHS yielded APTT at approximately 287 and 102 seconds, respectively, at 75 g ml^{-1} but showed no increase of PT compared to the control assay. Sulfated alginate could be used for potential application such as anticoagulant.

Keywords: sulfated alginate, anticoagulant, APTT

1. Introduction

Alginate is majorly derived from brown seaweeds. It is made up of a linear block copolymer of α-L-guluronic acid (G-block) and β-D-mannuronic acid (M-block) arranged in random blockwise pattern of MM, MG, GM and GG. M-Block contributes linear and flexible conformation resulted from β-(1-4) linkages whereas G-block contributes folded and rigid conformation resulted from α-(1-4) linkages (1). The blocks can also be varied in size. Alginate has been used widely in several industrial applications spanning from food, cosmetics and pharmaceuticals. It has been currently considered as a source of potential bioactive materials and novel applications such as anticoagulant, antiviral, antiproliferation and anticancer according to its biocompatibility, non-toxic, non-immugenic and biodegradable properties (2). The alginate structure is closely to heparin which had been commonly used for anticoagulant therapy for decades. Heparin is a linear polysaccharide with a disaccharide repeating unit containing preponderantly of α-D-glucosamine alternating with α-L-iduronic acid. Sulfate and carboxyl groups that comprised in heparin structure contribute high negative charge density resulting in anticoagulation efficacy. In addition, alginate structure offers numerous free hydroxyl and carboxyl groups distributed along the backbone of alginate. Structural

alginate can be altered through via chemical and enzymatic modifications (3-7). After the modification, certain properties of alginate can be altered such as solubility, hydrophobicity, physicochemical and biological characteristics.

In this research, the alginate sulfates (AlgS) and alginate sulfate fragments (AlgHS) were prepared from sodium alginate through sulfation reaction using $ClSO_3H$ in formamide and hydrolyzation using 5M HCl. Anticoagulant activities (APTT and PT) of alginate sulfates and hydrolyzed alginate sulfates were evaluated.

2. Materials and Methods

Sodium alginate was commercial reagent (CARLO ERBA). Sulfurochloridic acid ($ClSO_3H$; Merck), Barium chloride ($BaCl_2$; SIGMA), analytical grade of Gelatin (Fluka) used in this work. APTT reagent (C.K. PREST) and PT reagent (NEOPLASTINE Cl PLUS) were used for determination of the anticoagulant activity by coagulation analyzer at Thanarat hospital, Thailand.

2.1 Hydrolysis of alginate sample

One gram alginate was dissolved in 90 ml of distilled water. Then, 10 ml of 5.0 M HCl was added to the solution and the hydrolysis reaction was subsequently allowed to occur at 100 ^{o}C for 2 hours (8). Later, the mixture was abruptly cooled down in order to stop the hydrolysis and following by neutralization with NaOH. The hydrolyzed alginate was then precipitated with 95% (v/v) ethanol and collected by centrifugation. The precipitate was dried at 37 ^{o}C. The dried hydrolyzed alginate was redissolved with sterile deionized water prior desalting and size exclusion using Sephadex G-50 packed column chromatography. The eluent was finally precipitated with ethanol and dried at 37 ^{o}C for 2 days.

2.2 Preparation of alginate sulfate

The sufating reagent was prepared by slowly interval dropping of 2 ml of sulfurochloridic acid to 6 ml of formamide (6). Then 1 g of native sodium alginate or hydrolyzed alginate was added to the sulfating reagent and kept temperature of reaction at 60 ^{o}C for 4 hours. The mixture was subsequently precipitated with 20 ml of cold acetone and centrifuged at 7000 rpm. Later, the precipitate was redissolved with sterile MilliQ water and adjusted pH to 10-11 by 1M NaOH. The solution was applied to dialysis tubing with MWCO of 3.5 kDa for 72 hours. The sulfated alginate with molecular weight above 3.5 kDa obtained from dialysis was then precipitated with cold 95% (v/v) ethanol at volume ratio of 1:3 (sodium sulfated alginate : cold ethanol) for 12 hours. The precip1itate was collected by centrifugation at 8000 rpm for 10 minutes and subsequently dried at 37 ^{o}C for 2 days in the incubator. The dried sulfated alginate was kept in desiccator until further use)

2.3 Measurement of degree of substitution (DS)

The DS of native and sulfated alginate were examined by the barium sulfate nephelometry method with slight modification (9). The obtained sodium alginate sulfate 0.03 g was hydrolyzed with 10 ml of 0.1 M HCl for 8 hours at 100 ^{o}C to release sulfate groups of the alginate sulfate (4). Then the solution was evaporated until dried and the residue was dissolved with 10 ml of MilliQ water. Later, 1.0 ml of MilliQ water was added to 250 μl of the dissolved residue following by adding 625 μl of glutin–barium chloride solution (combining of 5% $BaCl_2$ w/v and 5% w/v glutin in distilled water at 60 ^{o}C). The amount of 350 μl of 8% (w/v) trichloroacetic acid (Cl_3CCOOH) was subjected to the mixture and ensured well mixing prior setting for 20 minutes. This resulted in complete reaction between sulfate ions and barium chloride including equal distribution of the barium sulfate precipitate. The absorbency of barium sulfate ($BaSO_4$) was measured with T70 UV-Vis spectrophotometer (PG Instruments Ltd., England) at 360 nm. Different concentration of potassium sulfate (K_2PO_4) was employed instead of examined sample to obtain standard curve

whereas MilliQ water was used as blank with the same manner to the potassium sulfate solution. The DS which referred to the average number of sulfate groups on each anhydroglucose unit, was calculated from sulfur content and described as the following equation:

$$DS = \frac{198[S]}{3200 - 102[S]}$$

where [S] was the sulfur content (%) of sodium sulfate.

2.4 Characterization of the alginate sulfate by polyacrylamide gel electrophoresis (PAGE) and Fourier transform infrared spectroscopy (FTIR)

Polyacrylamide-gel electrophoresis of native alginate and sulfated alginate was performed by the method of Pulsawat and Thongmalee (7) in order to estimate their approximated molecular weight (MW). The IR spectrum of alginate sulfates was determined by a Fourier transform infrared spectrophotometer, FTIR (Spectrum 100, Perkin Elmer). The amount of 0.01 g of each sample was prepared as KBr pellets and scanned in the frequency range of 4000-400 cm^{-1} against a blank KBr pellet background.

2.5 In vitro coagulation assay

Determination of anticoagulant activities of sulfated alginates were conducted through classical coagulation assays with slight modification to determine APTT and PT (3). The results were compared to unfractionation heparin as a reference compound. For APTT assay, the amount of 50 μl of citrated normal human plasma was mixed with equal volume of sample solution at concentration ranged 0-250 μg ml^{-1}. The mixture was incubated at 37 ^{o}C for 3 minutes. Then the amount of

50 μl of APTT reagent was added to the mixture and subsequently followed by incubation at 37 ^{o}C for 3 minutes. Afterward, 50 μl of 0.025 M CaCl$_2$ was added to the mixture and the clotting time was then recorded. The PT assay was performed by mixing between citrate normal human plasma and alginate sulfate similar to the first step in APTT assay. The mixture was incubated at 37 ^{o}C for 3 minutes. Next, 100 μl of pre-incubated PT reagent (37 ^{o}C for 10 minutes) was added to the mixture and the clotting time was recorded.

3. Results

3.1 Chemical characteristics of the pectin sulfate and alginate sulfate fragments

The sulfation and hydrolysis of native alginate demonstrated influences on molecular weight (MW), % sulfate content and degree of substitution (DS) of alginate sulfates and alginate sulfate fragments as shown in Table 1. The molecular weight of alginate and its derivatives obtained in this study were investigated using polyacrylamide gel electrophoresis (PAGE) as illustrated in Figure 1. MW of alginate processed through sulfation was similar to the native alginate whereas it was decreased lower than 36 and 27 kDa for hydrolyzed alginate and sulfated alginate fragments, respectively. Once the native alginate and hydrolyzed alginate were subjected to sulfation, the exhibition of % sulfate content of 14.9 and 12.9 % for alginate sulfate (AlgS) and the alginate sulfate fragments (AlgHS), respectively, was obtained. This was also true

Table 1. The effect of sulfation and hydrolization on molecular weight, degree of substitution (DS) and % sulfate content of alginate derivative.

Alginate / derivatives	Yield (dry wt., g)	MW (kDa)	DS
Alg	100 %	> 118	0
AlgS	8.7%	< 36	1.75
AlgHS	5.4%	< 27	1.35

Note;   Alg : sodium alginate, AlgS: sulfated alginate, AlgHS : hydrolyzed and subsequently sulfated alginate.
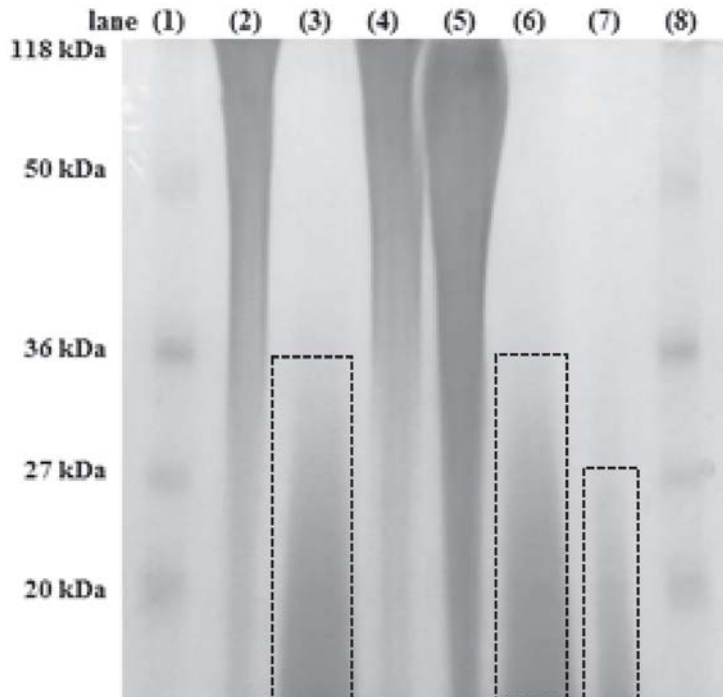
Figure 1. Estimation of molecular weight of Alg, AlgS and AlgHS by polyacrylamide gel electrophoresis (PAGE). Lane (1) and (8) are marker. Lane (2) and (4) are native sodium alginate. Lane (3) and (6) are AlgH. Lane (5) is AlgS. Lane (7) is AlgHS. Lane (3, 6 and 7) shown in dash line box present AlgH and AlgHS with molecular weight lower than 36 and 27 kDa, respectively.
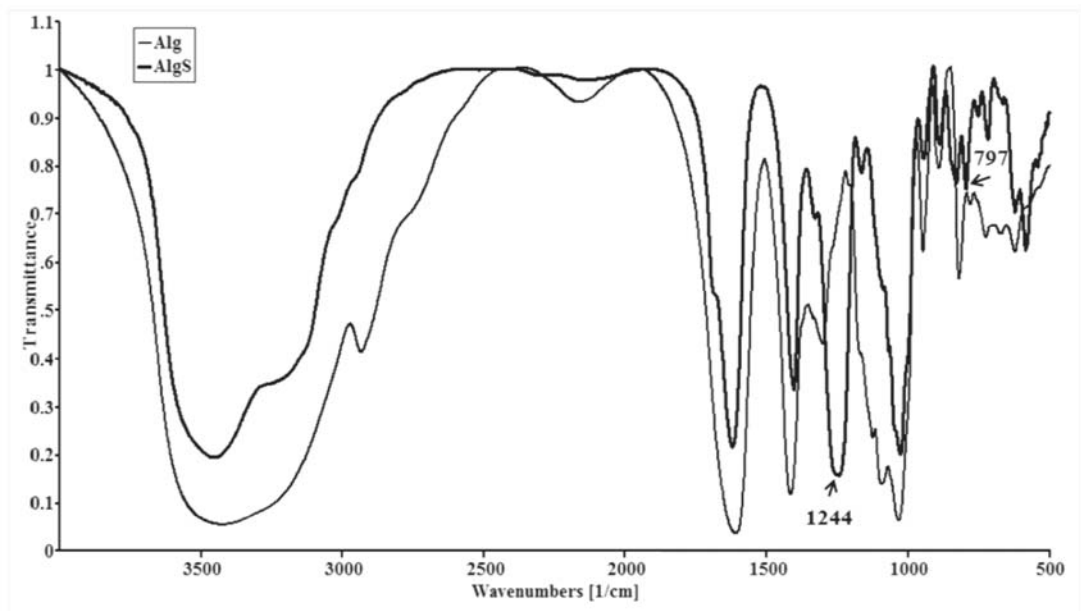

Figure 2. FTIR Spectra of alginate (Alg) and alginate sulfates (AlgS).

for degree substitution (DS), DS of 1.75 and 1.35 were obtained for AlgS and AlgHS, respectively. Yield of of AlgS and AlgHS was significantly low at 8.7 and 5.4 % according to initial amount of sodium alginate.

The IR spectrum of AlgS was compared with Alg spectra as illustrated in Figure 2. The signals at 797 and 1244 cm^{-1} were resulted from the presence of bending vibration of the C-O-S in axial position and stretching vibration of S=O bonds, respectively. These indicated success synthesized alginate sulfate. The frequencies at 947, 892 and 820 cm^{-1} of sodium alginate indicated containing of guluronic acids and mannuronic acids, respectively.
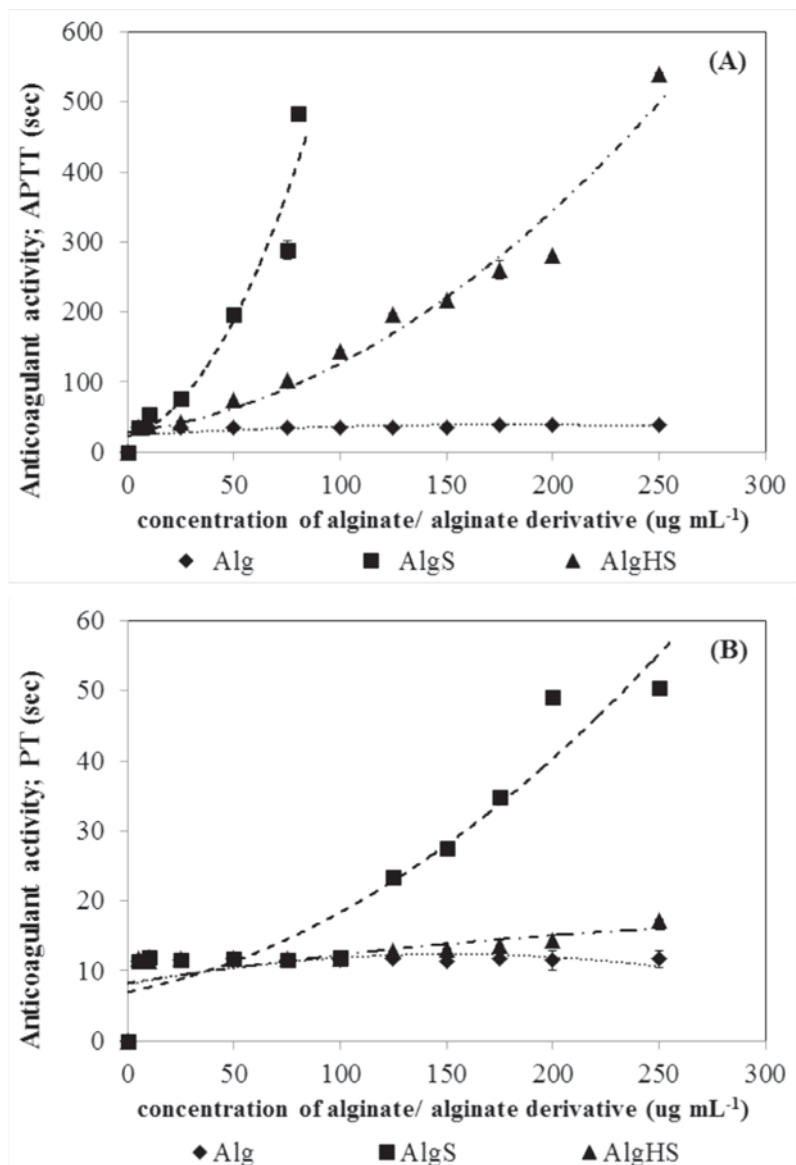
Figure 3. Anticoagulant activities of Alg, AlgS and AlgHS with respect to APTT (A) and PT (B). (Note; Normal APTT = 22-38 seconds, normal PT = 10-14 seconds, heparin APTT = 174 seconds; 5 μg ml^{-1})

3.2 Anticoagulant activity of the alginate sulfate

Anticoagulant activity of the sulfated alginate and alginate sulfate fragments were evaluated with respect to APTT and PT. The anticoagulant activity of alginate sulfate and alginate sulfate fragments was majorly resulted from their high level of negative charge density generated by the sulfate groups. The obtained anticoagulant activities were corresponding to % sulfate content and DS of AlgS and AlgHS. Native alginate did not generate anticoagulant activity according to its contribution APTT and PT in the normal range (22-38 and 10-14 seconds, respectively). The APTT of AlgS and AlgHS were 288 and 102 seconds at the concentration of 75 μg ml^{-1} (Figure 3:A) which were higher than Alg approximately 8.1 and 2.9 folds, respectively. At 80 μg ml^{-1} of AlgS, the APTT reached 483 seconds compared to 540 seconds that was contributed by AlgHS at concentration of 250 μg ml^{-1}.

The PT was slightly increased with an increase of the sulfated alginate concentration. Nevertheless, obtained PT of Alg, AlgS and AlgHS were in the normal range at the concentration of 5-200 μg ml^{-1} (Figure 3:B) excepted of the AlgS with concentration above 125 μg ml^{-1} (PT = 23 seconds and PT = 50 seconds at 250 μg ml^{-1}).

4. Discussions

The hydrolysis of alginate using 5M HCl at 100 ^{o}C for 2 hours which was considered as extreme condition could cause low % yield of depolymerized alginate which was subsequently subjected to sulfation. In addition, high amount of the smaller alginate including monosaccharide, disaccharides and trisaccharides produced by the hydrolysis depolymerization was not precipitated by 95% (v/v) ethanol. The MW of AlgS and AlgHS were decreased to approximately lower than 36 and 27 kDa which were accounted for 15% and 11% compared to initial MW of sodium alginate (240 kDa). In comparison, yield (13%) of depolymerized alginate obtained from the oxidation reaction using 30% H$_2$O$_2$ (v/v) at 50 ^{o}C for 2 hours was slightly higher than one obtained in this study (10). The DS of AlgS and AlgHS were significantly higher than of alginate sulfates (DS = 1.25) obtained from synthesis using trisulfonated sodium amine (N(SO$_3$Na)$_3$) as sulfating agent. In addition, the efficiency for prolonging blood clotting of the AlgS (according to the APTT) at concentration of 75 μg ml^{-1} was 1.7 fold of one reported by Fan et al. (4).

The presence of the peak 1244 cm^{-1} of FTIR of alginate sulfate (AlgS) which is describing S=O stretching bond vibration was corresponding to many reports (5-6,11). This signal at 1244 cm^{-1} was compared to signal of sulfated MG-block (1250 cm^{-1}) and sulfated MM-block (1260 cm^{-1}). This indicated that sulfate groups preferred to substitute hydroxyl group of MG-block than MM-block (7)

According to the result of anticoagulant activity assays, AlgS and AlgHS could hardly prolong PT similar to most of sulfated polysaccharides. The anticoagulant activity of sulfated alginate was closely dependent on the sulfate groups as the APTT was markedly increased with an increase of substituted sulfate groups of alginate structure. APTT is a performance indicator measuring the efficacy of both intrinsic and the common coagulation pathways. Sulfated alginate with higher molecular weight had significantly higher anticoagulation activity (APTT). This suggested that the molecular size profoundly effect on the anticoagulant activity.

5. Conclusion

Chemically synthesis and hydrolysis of sodium alginate were adopted to modify alginate structure for anticoagulant activity. The significant anticoagulant activity of alginate sulfates indicated successful introduction of sulfate groups into the alginate structure. Alginate sulfates obtained has potential for biomedical applications as alternative for anticoagulant and for other related applications such as anticancer, antiviral and antiproliferation.

6. Acknowledgement

7. References

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