Synthesis and Anticoagulant activity of Sulfated pectin

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Abstract

Pectin, a heteropolysaccharide made up of three major components; homogalacturonan (HG), rhamno-galacturonan type I (RG-I) and rhamnogalacturonan type II (RG-II), is derived from cell wall of higher terrestrial plants. The pectin was submitted to chemical modification specifically sulfation and anticoagulant activity of the sulfated pectin was investigated. Successful replacement of hydroxyl groups of pectin with sulfate groups was confirmed by degree of sulfation (DS) and Fourier fransform infrared spectroscopy (FTIR) spectra of absorption bands at 830 (S=O) and 1242 (C-O-S) cm^{-1}. Sulfated pectin (PecS) and hydrolyzed sulfated pectin (PecHS) demonstrated significantly prolonging activated partial thrombosis time (APTT) of 179 and 63.5 seconds, respectively, at a concentration of 50 μg ml^{-1}. The PecS elevated prothrombin time (PT) to 54 seconds at a concentration of 250 μg ml^{-1} compared to native pectin that had no significant anticoagulant effect. The result indicated the inhibition of intrinsic pathway according to the prolonged APTT. The extrinsic coagulation pathways could be inhibited with PecS at high concentrations according to an increase of prolonged PT.

Keywords: sulfated pectin, sulfation, anticoagulant, APTT, PT

1. Introduction

Pectin is a water-soluble anionic polysaccharide that has the main linear backbone structure of (1-4) linked alpha-D-galacturonic acid (GalA) units. It has been mainly used in food industrial applications as a gelling agent, thickening agent and stabilizer. Recently, chemical modification of many polysaccharides including pectin demonstrated new bioactivities such as anticoagulant, antioxidant, antiviral, antitumor, antiproliferation and immunomodulation (1-2). Among those chemical modifi-cations, sulfation of polysaccharides in which hydroxyl groups along the polysaccharide structure are substituted by sulfate groups has been often employed for enhancement of intrinsic biological properties or generating new functional properties of polysaccharides particularly anticoagulant and anticancer activities (3-4). Many studies suggested that anticoagulant efficiency of sulfated polymers depended on several factors including the structure, degree of substitution by sulfate and molecular weight of polymers (5-9). There are several sulfating reagents commonly used such as chlorosulfonic acids, sulfuric acid, sulfur trioxide and sulfamic acids. In addition, pyridine, dim ethyl sulfoxide, trimethylamine and toluene have been used as media for the reaction.

In this study, sulfated pectin (PecS) and sulfated hydrolyzed pectin (PecHS) were synthesized with a common sulfating reagent, chlorosulfonic acid, and pyridine as media for sulfating reaction. The molecular size, structure and anticoagulant activity of pectin sulfates and its derivatives were investigated by polyacrylamide gel electrophoresis (PAGE), degree of substitution (DS) assay, FTIR, APTT and PT, respectively.

2. Materials and Methods

Pectin was a commercial reagent (EAC Co., Thailand). Chlorosulfuric acid (ClSO$_3$H; Merck), Barium chloride (BaCl$_2$; SIGMA), Gelatin (Fluka) used in this work were analytical grade. 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 pectin sample

One gram of pectin was hydrolyzed in 100 ml of 0.3 M HCl at 80 ^{o}C for 8 h. Then, the reaction was placed in an ice-water bath to cease the hydrolysis and neutralized to pH 7.0 by adding 0.1 M NaOH. The hydrolyzed pectin was precipitated with 3 volumes of 95% (v/v) ethanol overnight. The precipitate was collected by centrifuging at 8000 rpm for 5 min to obtain hydrolyzed pectin. Finally, hydrolyzed pectin was dried at 37°C for 2 days (6). The dried hydrolyzed pectin was redissolved in sterile deionized water. The hydrolyzed pectin was then desalted and size exclusive separated using Sephadex G-50 packed column chromatography. The eluate was finally precipitated with ethanol and dried at 37 ^{o}C for 2 days.

2.2 Preparation of pectin sulfate

The mixture of 10 ml formamide and 10 ml pyridine was used to dissolve 0.5 g pectin. Then, certain volume of chlorosulfonic acid (in a proportion of 10 mole of chlorosulfonic acid per mole of free hydroxyl) was slowly dropped to the mixture under magnetic stirring while the flask was being incubated on an ice bath. The mixture was ensured to be homogeneous and the reaction was maintained at 50°C for 4 h (9). Later, the reaction solution was precipitated overnight with 3 volumes of 95% ethanol and centrifuged at 8000 rpm for 5 min. Then, the precipitate was resuspended in distilled water and dialyzed for 72 h. Next, centrifugation at 8000 rpm for 5 min was adopted to collect precipitated sulfated polysaccharide. Finally, the sulfated polysaccharide was dried at 37°C for 2 days. The dried sulfated pectin was kept in a desiccator for further examination.

2.3 Measurement of degree of substitution (DS)

This nephelometry method using the barium sulfate was carried out to determine the DS of sulfated polysaccharides (10). Pectin sulfate and its derivatives (0.03 g) were weighed accurately and dissolved in 10 mL of distilled water. Then 1.0 ml of distilled water, 625 μl of barium chloride (BaCl$_2$)–glutin solution and 350 μl of 8% trichloroacetic acid were added to 250 μl of pectin sulfate or pectin sulfate derivatives solution and the mixture was stirred for 1 min. After that the reaction was allowed to set for 20 min. The absorbance of barium sulfated was measured by UV-Vis spectrophotometer (model T70, PG Instruments Ltd., England) at 360 nm. A standard curve was constructed by using different concentration of potassium sulfate (K$_2$SO$_4$) instead of pectin sulfate (distilled water was used as blank). The DS of the sulfated pectin was determined by comparison with the standard curve and calculating according to the following equation (11);

$$DS = \frac{MWU[S]}{3200 - MWG[S]}$$

Where [S] was the sulfur content (%) of sulfated pectin, MWU was the molecular weight of a sugar unit and MWG was the molecular weight of a sulfate group.

2.4 Characterization of the pectin sulfate by poly-acrylamide gel electrophoresis (PAGE) and Fourier transform infrared spectroscopy (FTIR)

The estimate of the approximated molecular weight (MW) of native alginate and sulfated alginate was performed by polyacrylamide-gel electrophoresis (PAGE) according to Pulsawat and Thongmalee (12).

IR spectra of samples were evaluated by a Perkin Elmer precisely Spectrum 100 Fourier transform infrared spectrometer. 0.01 g of each sample was prepared by the KBr-disk method and scanned in the frequency range of 4000–400 cm^{-1} against a blank KBr pellet background.

2.5 *In vitro* coagulation assay

Anticoagulation activity of all samples was evaluated with respect to the activated partial thromboplastin time (APTT) and prothrombin time (PT). These assays were performed by semi-automated coagulation analyzer. For APTT assay, 50 μl of citrated normal human plasma was mixed with 50 μl sample solution at concentration of 5-80 μg ml^{-1} and subsequently incubated at 37°C for 3 min. Then, 50 μl pre-incubated APTT determination reagent (C.K. Prest® 5, Diagnostica Stago S.A.S) was added to the mixture and incubated at 37°C for 3 min. Next, 50μl pre-incubated 0.025 mol/L CaCl$_2$ was added and the clotting time was recorded. For PT assay, 50 μl of citrated normal human plasma was mixed with 50 μl sample and incubated at 37°C for 3 min. Later, 100 μl pre-incubated neoplastine reagent was added and the clotting time was recorded.

3. Results and discussion

3.1 Chemical characteristics of the pectin sulfate and pectin sulfate fragments

Molecular weight (MW), % yield and degree of substitution (DS) of obtained pectin sulfates and its derivatives were examined after the sulfation and hydrolyzation of native pectin as illustrated in Table 1. Yield of pectin sulfates without hydrolysis was 82% (w/w) whereas it was 68% and 44% for hydrolysed pectin (PecH) and hydrolyzed pectin sulfate (PecHS), respectively. The electrophoretic mobility exhibited by each oligosaccharide correlated with its relative molecular size as displayed in Figure 1. The molecular weight (MW) of PecH (Lane 4 and 5) and PecHS (Lane 6 and 7) was less than 50 kDa. The size exclusive PecH as shown in Lane 5 exhibited marginally higher intensity of the dye staining color compared to that of the PecH. This could be due to the higher concentration of size exclusive PecH which consisted of the MW between 3.5 and 50 kDa in the loading sample for PAGE. The hydrolysis of PecH could occur through the sulfation process therefore the maximum molecular weight of PecHS was noticeably less than that of PecH. Degree of substitution (DS) of 1.5 and 1.3 were achieved from PecS and PecHS, respectively while the sulfate content of Pec and PecH were not detected. The noteworthy high DS of sulfate pectin was obtained in this study compared to other reports (7-8, 13).

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

Pectin / derivatives	% Yield (dry wt.)	MW (kDa)	DS
Pec	100	> 90	0
PecS	82	> 90	1.5
PecH	68	< 50	0
PecHS	44	< 50	1.3

Note: Pec: native pectin, PecS: obtained sulfated pectin, PecH: obtained hydrolyzed pectin, PecHS: the hydrolyzed and subsequently sulfated pectin.
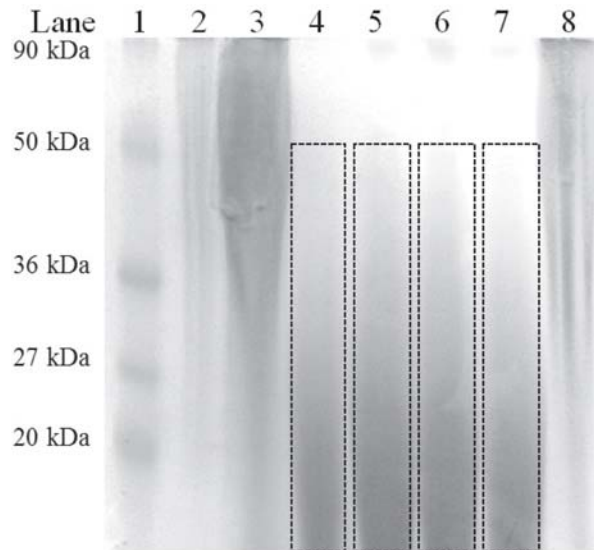
Figure 1.   Estimation of molecular weight of Pec, PecH, PecS and PecSH by polyacrylamide gel electrophoresis (PAGE).

Note: Lane (1) marker, (2) Pec, (3 and 8) PecS, (4) PecH, (5) PecH with size exclusion and (6 and 7) PecHS. Lane (4-7) shown in dash line box present the modified pectin with molecular weight lower than 50 kDa.
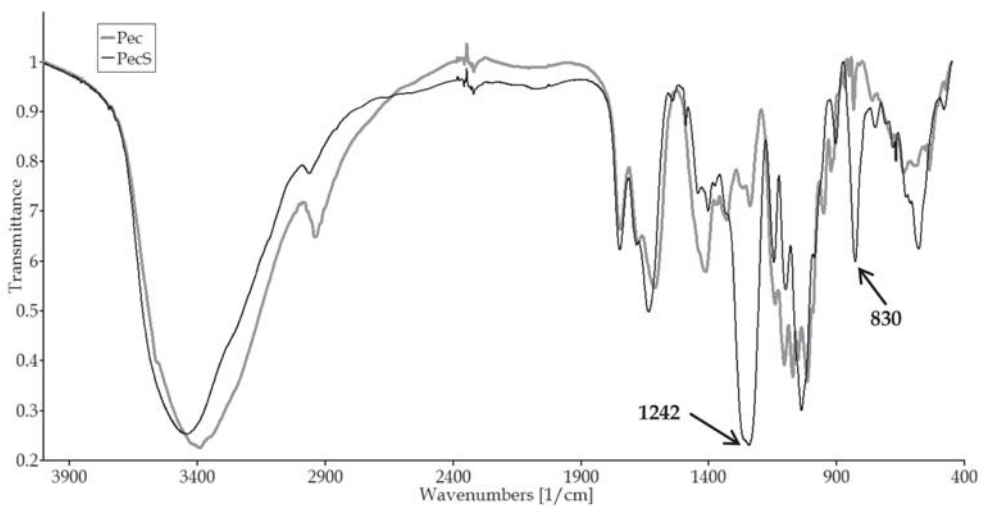

Figure 2.   FTIR Spectra of pectin (Pec, grey line) and Pectin sulfate (PecS, black line).

The hydroxyl groups along the pectin backbone structure were substituted by sulfate groups when pectin was submitted to sulfation. FTIR spectra of pectin sulfate as illustrated in Figure 2 confirmed the success of the pectin sulfation. Two new peaks detected at 830 and 1242 cm^{-1} were corresponding to characteristic vibrations for C–O–S and S=O, respectively. The signal at 3390, 2941, 1746 and 1676 cm^{-1} of pectin denotes the –OH, –CH, C=O of ester and C=O of acid stretching of galacturonic acid, respectively.

3.2 Anticoagulant activity of the pectin sulfate

Anticoagulant activity of PecS and PecHS were

evaluated by APTT and PT assays. The high DS values of pectin sulfate and its derivatives as a result of an increase in sulfate group substitution were speculated to attribute to anticoagulant activity. High negative charge density resulted from high sulfate group content could neutralize the positively charged amino acid residues comprised of the antithrombin, thus enhancing the anticoagulation activity. The anticoagulant activity of native pectin was not observed due to the results of APTT and PT assay found in the normal range (22-38 s and 10-14 s, respectively) (7). The anticoagulant activity of PecS and PecHS was 824 and 139 s at the concentration of 80 μg ml^{-1} (Figure 3:A) which were 22.6 and 3.8 times

higher than the normal APTT, respectively. At 90 and 250 μg ml^{-1} of PecS and PecHS, the APTTs detected were over 900 s. High molecular weight of polysaccharide sulfate contributing enhancement of anticoagulant activity was reported (12, 14). The DS of sulfated pectin demonstrated the enhancement of anticoagulant activity for intrinsic pathway (according to APTT) with the increase in its concentration. Exerted anticoagulation activity of pectin sulfate was mainly resulted from the accelerated plasma serine proteinase inhibitor such as IIa factor or Xa factor (8). The highly molecular size and negative charged PecS can acts at multiple sites in the coagulation cascade. The specific binding between
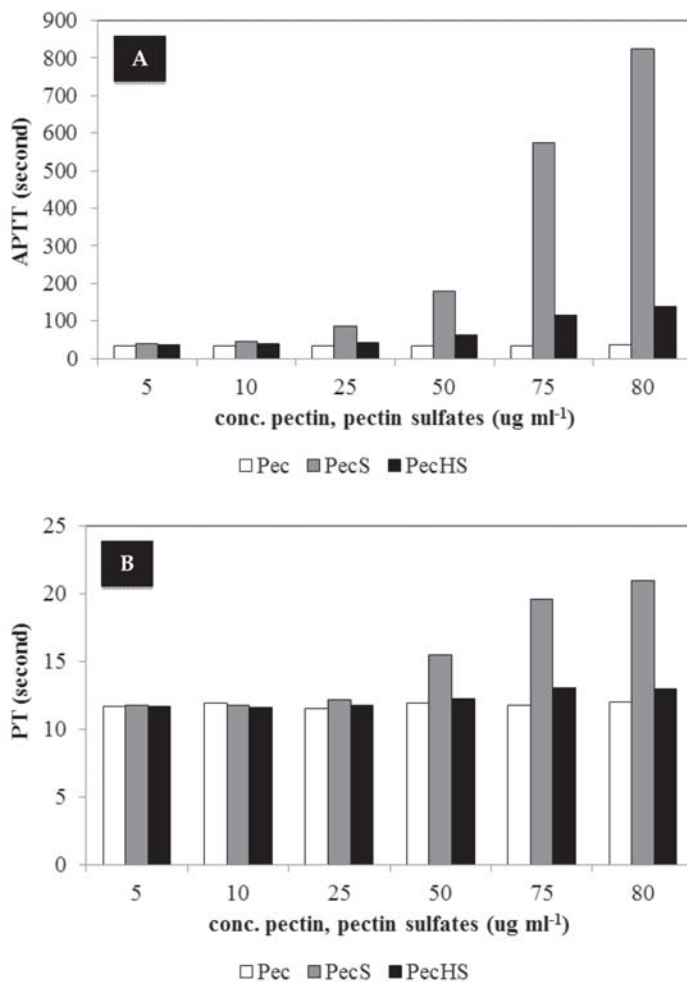

Figure 3.   Anticoagulant activities of Pec, PecS and PecHS with respect to APTT (A) and PT (B).

Note: Normal APTT = 22-38 s, normal PT = 10-14 s, heparin APTT = 174 s, 5 μg ml^{-1}

sulfate and carboxylate ions in sulfated pectin arginine and arginine and lysine cations in the antithrombin III accelerates the circulating antithrombin III. This contributes prolonged blood clotting. In addition, antithrombin III forms a stable 1:1 complex with both thrombin and factor Xa with slow rate but binding is accelerated more than 2000-fold when heparin is presented (15). With all mentioned before, these could be the reasons to explain that PecS contributed the higher APTT than of PecHS.

The PT of PecS was slightly increased with increasing of sulfated pectin concentration. There was no or very weak significant observed influences for extrinsic pathways at low and high doses of pectin sulfate according to PT. The PT of Pec and PecHS were in the normal range at the concentrations between 5 and 200 μg ml^{-1} (Figure 3;B) excepted for the PecS with concentrations above 50 μg ml^{-1} (PT = 16 s and 54 s at 200 μg ml^{-1}).

4. Conclusion

Pectin sulfate and its derivatives were obtained by employing a simple and effective chemical synthesis. The obtained yield of PecS (82%, w/w) and PecHS (44%, w/w) after the synthesis was markedly high. The MW of PecH and PecHS preliminary determined were approximately less than 50 kDa. The existence of intensive FITR spectra at 830 and 1242 cm^{-1} of C-O-S and S=O vibration bonding, respectively, confirmed successful introducing of sulfate groups to pectin structure. The anticoagulant activities were evaluated and demonstrated the potential for biomedical applications as a heparin alternative.

5. Acknowledgement

6. References

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