# Synthesis and Anticoagulant Activity of Pectin Sulfates

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**ABSTRACT:** In this article, we describe the first use of trisulfonated sodium amine as a sulfating reagent for preparing pectin sulfate in aqueous solution. The main reaction parameters that were expected to affect the degree of substitution (DS) were studied. The optimal reaction conditions for the synthesis of pectin sulfate were found to be as follows: the pH of the reaction medium, the reaction temperature, the reaction time, and the ratio of the mole of sodium nitrite ($n_{NaNO2}$) to the mass of pectin ($m_{pectin}$) were 6, 60°C, 12 h, and 2.5/190 mol/g, respectively. Pectin and pectin sulfate were characterized by Fourier transform infrared (FTIR) spectroscopy and $^{13}$C-NMR. The FTIR spectra showed the characteristic absorptions of sulfate ester bonds at 1264 and 830 $cm^{-1}$. Furthermore, the anticoagulant activity of pectin sulfates with different DSs, concentrations, and molecular weights were investigated with respect to the activated partial thromboplastin time (APTT), thrombin time (TT) and prothombin time. The clotting assay indicated that the pectin sulfate prolonged APTT and TT through inhibition of the activity of antithrombin. These results suggest that the introduction of sulfate groups into the pectin structure improved its anticoagulant activity. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 2171–2178, 2012

**Key words:** pectin sulfate; sulfating reagent; trisulfonated sodium amine; anticoagulant activity

## INTRODUCTION

Pectin is a water-soluble biocompatible anionic polysaccharide extracted from the cell walls of higher plants. The main structure of pectin is the backbone of (1→4)-linked α-D-galacturonic acid (GalA) units, some of which are esterified by methoxyl groups.[1] Pectin is widely used as a gelling agent, thickening agent, and stabilizer in food industry.[2] Moreover, it can remove some toxic heavy metals from solution, reduce blood cholesterol levels, increase immunity, and delay gastric emptying and be used as a carrier for drug delivery in the pharmaceutical industry and many other industries.[3–6] However, many polysaccharides (e.g., chitosan, cellulose, alginate) with some chemical modifications brought about by the introduction of new functional groups onto the polymer backbone show more bioactivities that include anticoagulant, antiviral, antitumor, and other activities.[7–9]

Sulfated polysaccharides and their anticoagulant activities have attracted much attention in recent years.[10–14] Up to this point, sulfated polysaccharides have been prepared by the sulfating of the reagent in an organic solvent.[15–18] The main sulfating reagents are sulfuric acid, chlorosulfonic acid, sulfuryl chloride, sulfur trioxide, and sulfamic acid. Also, many organic solvents have been used as reaction media, including pyridine, dimethyl sulfoxide, trimethylamine, and toluene. These agents not only can result in hydrolytic or degradation of the polysaccharide chain during the reaction but also lead to serious pollution problems.[19] Usually, the sulfated polysaccharide exhibits anticoagulant activity because of the similar structure and anticoagulant mechanism to heparin in a way. Although heparin has been widely used for anticoagulant therapy for 60 years, it has some problems in clinical applications, such as its inefficacy in antithrombin-deficient patients, poor bioavailability, the risk of bleeding, and heparin-induced thrombocytopenia developing

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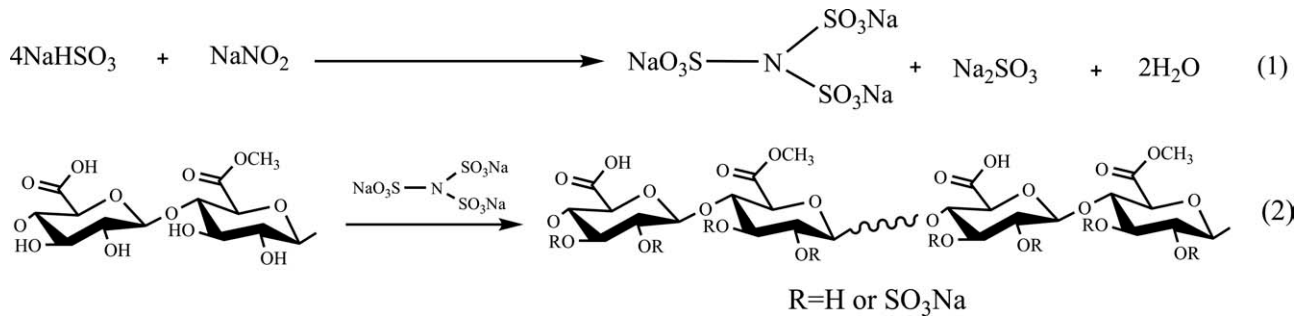


**Scheme 1** Synthesis route to the (1) sulfating reagent and (2) pectin sulfate.

in some heparin recipients.[20–23] Pectin sulfate and its anticoagulant activity have been reported previously,[24–26] but pectin sulfates were prepared by the traditional methods, which have the disadvantages discussed previously, and the degree of substitution (DS) value of 0.15 was very low in Bae et al.'s[24] research. In addition, the used pectin, whose source may affect its anticoagulant activity,[26] was from citrus or other plants but not apples. The anticoagulant activity was evaluated by the one or two factors, among them the DS, concentration, and molecular weight, but not all of them. We aim to create a new chemical method to prepare sulfated apple pectin and are attempting to overcome the previous problems to create a potential heparin alternative.

In this study, we synthesized apple pectin sulfates in aqueous solution with a new mild sulfating agent, trisulfonated sodium amine [$N(SO_3Na)_3$], which was prepared by the reaction of $NaHSO_3$ and $NaNO_2$. The synthesis routine is shown in Scheme 1. The main reaction parameters, the pH of the reaction medium, reaction time, reaction temperature, and molar ratio, were investigated systematically by the DS. The structure of pectin and its sulfate were characterized by Fourier transform infrared (FTIR) spectroscopy and $^{13}$C-NMR spectroscopy. In addition, the anticoagulant activity of pectin sulfate *in vitro* was measured by activated partial thromboplastin time (APTT), thrombin time (TT), and prothombin time (PT). The mechanism of the anticoagulant activity was also studied with different DSs, molecular weights, and concentrations.

## EXPERIMENTAL

### Materials

Pectin [with 75% degree of esterification (DE) and without acetyl or amide groups] was supplied by Xiangfu Fine Chemical Co., Ltd. (Zhengzhou, China). APTT, PT, and TT were applied by Sun Biologic Co., Ltd. (Shanghai, China). Human plasma was supplied by Blood Center of Wuhan (Wuhan, China). All of the chemicals used in this work were analytical grade. They were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

### Preparation of the sulfating agent [$N(SO_3Na)_3$]

The sulfating agent was prepared by Cui and Liu,[27] as shown in Scheme 1. Briefly, a predetermined amount of $NaHSO_3$ was dissolved in 40 mL of distilled water in a three-necked flask, under agitation and heating in an oil bath. Then, the $NaNO_2$ previously dissolved in 10 mL of distilled water was added dropwise to the reactor under magnetic stirring at 90°C and reacted for 90 min. After that, $N(SO_3Na)_3$ was obtained.

### Synthesis of the pectin sulfates

Pectin sulfate was synthesized as follows: first, the sulfating agent solution was adjusted to suitable pH with sodium hydroxide (NaOH) or hydrochloric acid (HCl). Second, 5.0 g of pectin was added to the previous solution. The mixture was preserved at a preset temperature for a certain time. Third, the solution was dialyzed for 72 h against distilled water and concentrated by a rotary evaporator. Finally, the pectin sulfate was dried at 40°C for 2 days in a vacuum oven.

### FTIR measurements

The IR spectra were recorded with a Nicolet 170SX FTIR spectrometer (Nicolet Instrument Co., USA). All of the samples were prepared as potassium bromide (KBr) pellets and were scanned against a blank KBr pellet background.

### $^{13}$C-NMR spectra of the sodium pectin sulfates

The $^{13}$C-NMR spectra were recorded on a Bruker AMX-500 NMR spectrometer (Bruker Optics Co., Germany) operating at 400 MHz. The samples were dissolved in $D_2O$ at 30°C, and the concentration of the solution was about 5%.

### Measurement of DS

DE is defined as the number of carboxyl (COOH) group substituted perrepeating structural units in the pectin backbone. The DE of the apple pectin sample was determined by the previous FTIR

analysis with the Omnic 8.0 software package (Nicolet Instrument Co., USA):[28]

$$\mathrm{DE} = \frac{A_1}{A_1 + A_2} \times 100\%$$

where $A_1$ is the area of the esterified carboxyl groups (the separate peaks at 1745–1760 $cm^{-1}$) and $A_2$ is the area of the nonesterfied carboxyl groups (the separate peaks at 1620–1640 $cm^{-1}$).

DS is defined as the number of hydroxyl (OH) groups substituted per repeating structural unit of the pectin backbone. In this work, the DSs of the pectin sulfates were determined by the barium sulfate nephelometry method.[29] Pectin sulfate (0.030 g) was weighed accurately. HCl (10 mL, 0.1 mol/L) was added to hydrolyze the sulfate groups of pectin sulfate at 100°C for 8 h. After the obtained solution was evaporated to dryness, 10.00 mL of distilled water was added with stirring to dissolve the residue. A barium chloride ($BaCl_2$)–glutin solution (1.25 mL), which was prepared by the dissolution of 0.5 g of $BaCl_2$ and 0.5 g of glutin in 100 mL of distilled water at 60°C, and 0.70 mL of 18% (w/v) trichloroacetic acid ($Cl_3CCOOH$) were added to a quartz cuvette and allowed to set for 15–20 min to ensure that the reaction between the sulfate ions and barium chloride had gone to completion and that the barium sulfate precipitate was equally distributed in the quartz cuvette. The absorbency of barium sulfate ($BaSO_4$) was determined with a UV/vis 1700 spectrophotometer (Shanghai Phoenix Co, Ltd., Shanghai, China) at 360 nm. The same volume of distilled water was used as an internal standard. A standard curve was recorded with different concentrations of potassium sulfate ($K_2SO_4$) instead of a hydrolysis solution with the other agents in the same conditions given previously.[30] The DSs of pectin sulfates were determined by comparison with the standard curve and calculated with the following equation according to Lim et al.,[31] with some modification:

$$\mathrm{DS} = \frac{[\mathrm{DE} \times 176 + (1 - \mathrm{DE}) \times 162] \times S\%}{32 \times 100 - 102 \times S\%}$$

where 162 is the molecular weight (g/mol) of GalA, 176 is the molecular weight (g/mol) of galacturonan methyl ester, $S$% is the sulfur content (%) of pectin sulfate obtained from the calculation of the absorbancy, 32 is the sulfur atom mass (g/mol), and 102 is the $-SO_3Na$ group mass (g/mol).

### Degradation of the pectin sulfates

Different molecular weights of polysaccharide sulfates have different anticoagulant activities. In this work, three different molecular weight pectin sulfates were prepared. The oxidation degradation method was used to degrade the products, and the other parameters were the same for the research.[32] First, 0.5 g of pectin sulfates were weighed three times. Then, 50 mL of distilled water was added to dissolve each. Different quantities of hydrogen peroxide ($H_2O_2$) were added to the previous solution. Finally, the three oxide degradations were allowed to stand for 4 h at 50°C.

### Determination of the molecular weights

The weight-average molecular weight of the pectin sulfates was determined with static light scattering. The light-scattering intensities were measured with a modified commercial light-scattering spectrometer (ALV/SP-125) (ALV Co., Germany) equipped with an ALV-5000/E multi-τ digital time correlator and an He–Ne laser ($\lambda = 632.8$ nm) in an angular range from 30 to 150° at 10° intervals at 25°C. The pectin sulfates were dissolved in aqueous solution (0.1 mol/L NaCl) and made optically clean by filtration through 0.22-μm Millipore filters. The specific refractive-index increment of the pectin sulfates in a 0.1 mol/L NaCl aqueous solution was measured on an Optilab refractometer (Wyatt Technology Co., USA) at 632.8 nm and 25°C and was found to be 0.140 $cm^3/g$.

### *In vitro* coagulation assay

The anticoagulant activities of all the samples were investigated by the classical coagulation assays PT, TT, and APTT with unfractionated heparin as a reference compound. The PT assay was carried out as follows: citrated normal human plasma was mixed with a solution of pectin sulfates and incubated for 3 min. Then, 0.2 mL of the PT assay reagent, preincubated for 10 min at 37°C, was added, and we recorded the clotting time. For the TT assay, citrated normal human plasma was mixed with a solution of pectin sulfates and incubated for 3 min. Then, 0.2 mL of the TT assay reagent was added, and the clotting time was recorded. The TT assay reagent did not need incubate at 15–25°C. For the APTT assay, citrated normal human plasma was mixed with a solution of samples and APTT assay reagent, and then, the mixture was incubated for 5 min at 37°C. Afterward, 0.1 mL of 0.025 mol/L $CaCl_2$ was added, and we recorded the clotting time.[33]

## RESULTS AND DISCUSSION

### Structural characterization

#### FTIR spectra of the pectin and pectin sulfate

The FTIR spectra of the pectin and pectin sulfate are illustrated in Figure 1. The absorption peaks at 3388, 2936, 1748, and 1638 $cm^{-1}$ of pectin contributed to the —OH, —CH, and C=O of ester and acid

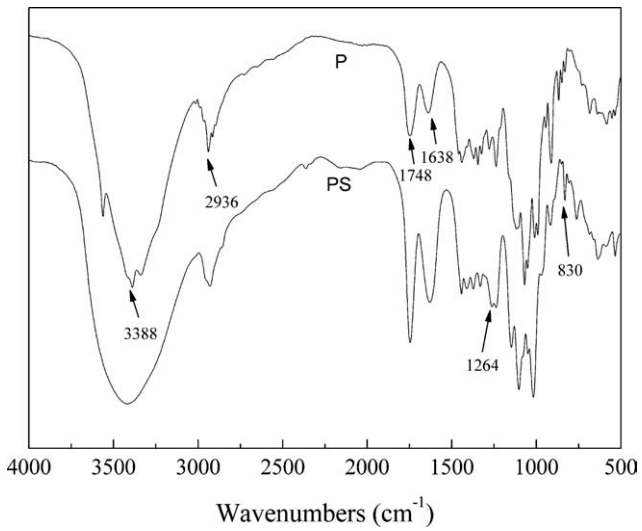


**Figure 1** FTIR spectra of pectin (P) and pectin sulfate (PS).

stretching of the galactouronic acid, respectively.[34] Compared with the pectin spectrum, two new absorption peaks appeared at 830 and 1264 $cm^{-1}$ for the pectin sulfate due to the presence of the C—O—S and S=O bonds, respectively;[35,36] these indicated that the pectin sulfate was synthesized successfully.

### $^{13}$C-NMR spectra of the pectin and pectin sulfate

The structure of pectin is very difficult to determine because pectin can change during isolation in plants, storage, and the processing of plant material. At present, pectin is thought to consist mainly of GalA units joined in chains by means of α-(1-4) glycosidic linkages. However, rhamnose is a minor component of the pectin backbone and introduces a kink into the straight chain, and other neutral sugars, such as arabinose, galactose, and xylose, may occur in the side chains. Figure 2 shows the $^{13}$C-NMR spectra of the pectin and pectin sulfate. In the spectrum of pectin, the weak signal at 171.26 ppm was assigned to the C-6 of COOH groups of α-D-GalA. The signals at 100.49, 68.15, 69.26, 79.25, and 71.10 ppm were assigned to the C-1, C-2, C-3, C-4, and C-5 of α-D-GalA. This was in good agreement with the results of Cipriani et al.[25] The signal at 92.25 ppm was from the reducing end units (C-1′) of α-D-GalA. The spectrum had an intense resonance at 52.97 ppm, which was the methyl carbon of the methyl of the ester ($COOCH_3$) and indicated that the pectin was highly methyl-esterified.[37] Furthermore, the big resonance at 60.75 ppm indicated that most of the C-6 of α-D-GalA was substituted.[38]

Compared with pectin spectrum, pectin sulfate gave clearer resonance signals. The signals at 104.36, 81.43, 76.42, 73.98, and 72.56, which were assigned to the characteristic resonances of galactose,[39] disappeared, and others peaks were enhanced. This result suggests that some side chains including galactose were removed from the backbone of the α-D-GalA residues during the process of preparation of pectin sulfate. The peaks at 99.98, 79.12, 73.49, 71.39, 69.07,

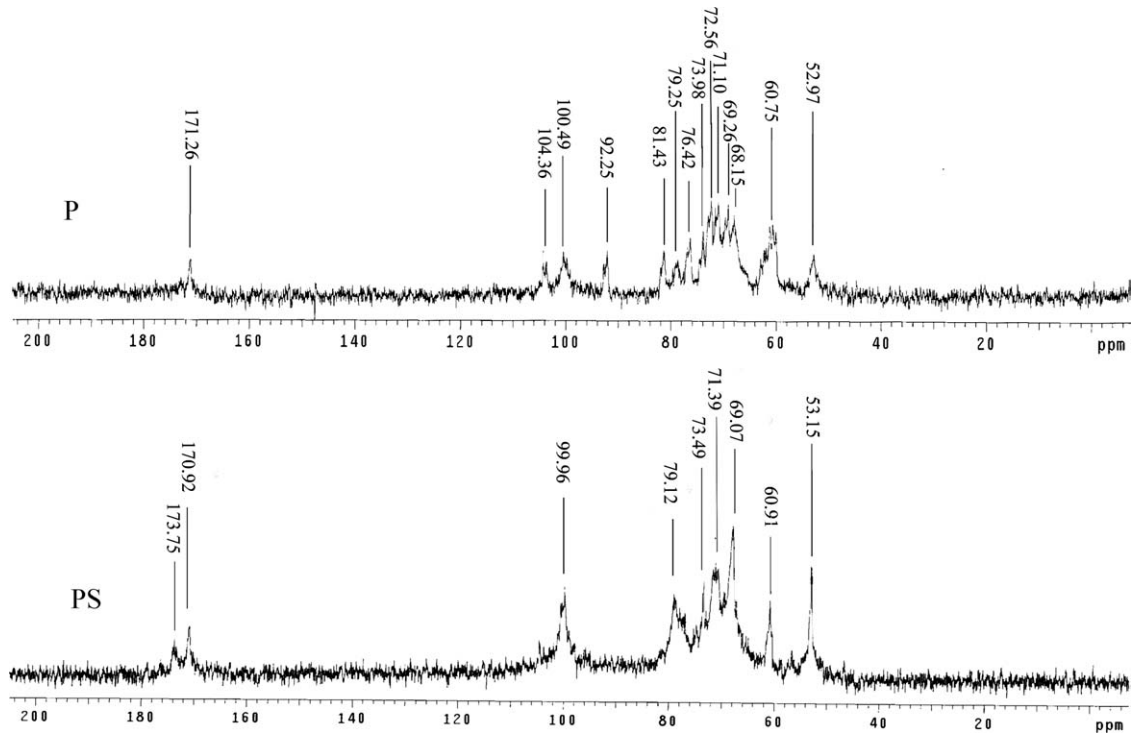


**Figure 2** $^{13}$C-NMR spectra of pectin (P) and pectin sulfate (PS).

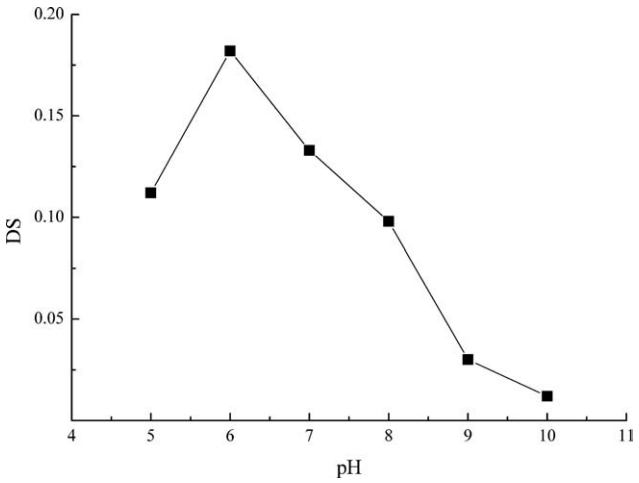


**Figure 3** Effect of different pHs on the DS values of the pectin sulfates (reaction temperature = 40°C, reaction time = 4 h, ratio of $n_{NaNO2}$ to $m_{pectin}$ = 2.0/190 mol/g).

and 53.15 ppm were assigned, respectively, to C-1, C-4, C-5, C-3, C-2, and the methyl carbon of the methyl ester ($COOCH_3$). The signals at 170.92 and 173.75 ppm were attributed to the carboxyl groups bound by methyl groups ($COOCH_3$) and the ionic carboxyl groups ($COO^-$), respectively. However, some differences were also found in the spectrum of pectin sulfate. Both C-2 and C-3 shifted the signals to the lower field. Also, C-4 shifted the signal to a higher field; this was influenced by the presence of sulfate groups at the C-2 and C-3.[40] These results demonstrate that sulfate groups were linked to the C-2 and C-3 atoms of the pectin structure.

### Optimization of the reaction conditions of the prepared sulfating agent

The sulfating agent was prepared by the reaction of $NaHSO_3$ and $NaNO_2$, as shown in Scheme 1. Because this reaction was exothermic and $NaHSO_3$ decomposes to $SO_2$ gas at temperatures greater than 60°C, the $NaNO_2$ solution should be dropped slowly, and more $NaHSO_3$ should be added. However, this reaction requires a higher temperature to trigger, so we decided to adopt a molar ratio of $NaHSO_3$ to $NaNO_2$ of 4.25, which was greater than theoretical value 4. From this, the optimal reaction conditions for the preparation of the sulfating agent were as follows: the molar ratio of $NaHSO_3$ to $NaNO_2$ was 4.25, the reaction temperature was 90°C, and the reaction time was 90 min.

### Optimization of the reaction conditions of the synthesized pectin sulfates

#### Influence of various pHs of the reaction medium on the DS

The influence of different pHs (5–10) on the DS are shown in Figure 3. It was clear that DS increased as the pH of the reaction medium increased from 5 to 6. The DS had a maximum at pH 6 (DS = 0.133). However, beyond 6, the DS decreased obviously. It could be seen that the pH of the reaction medium was an important factor in the preparation of pectin sulfate. On one hand, ionization of the carboxylate groups of pectin were suppressed so that its solubility decreased in water. On the other hand, pectin was rapidly deesterified and degraded at alkaline pH values. These two adverse effects made it hard to obtain pectin sulfate with a high DS. From this analysis, we came to the conclusion that pH 6 was the optimal pH value.

#### Influence of various reaction temperatures on DS

The influence of different reaction temperatures (25–70°C) on DS was also investigated. Figure 4 shows that the DS values increased with the reaction temperature from 25 to 60°C. It reached a maximum value (0.247) at 60°C. However, a reaction temperature higher than 60°C prompted DS to decrease. An increase in the temperature facilitated both the swelling of the pectin and the diffusion of the reactants. However, the rate of degradation sped up at elevated temperatures. In addition, this sulfonation reaction was not only an exothermic reaction but also a reversible reaction. The DS values became lower and lower if the reaction proceeded at high temperature. Therefore, we concluded that the optimal temperature was 60°C.

#### Influence of various reaction times on DS

The effect of the reaction time on DS is presented in Figure 5. The DS increased with reaction time from 2 to 12 h and decreased slowly with further

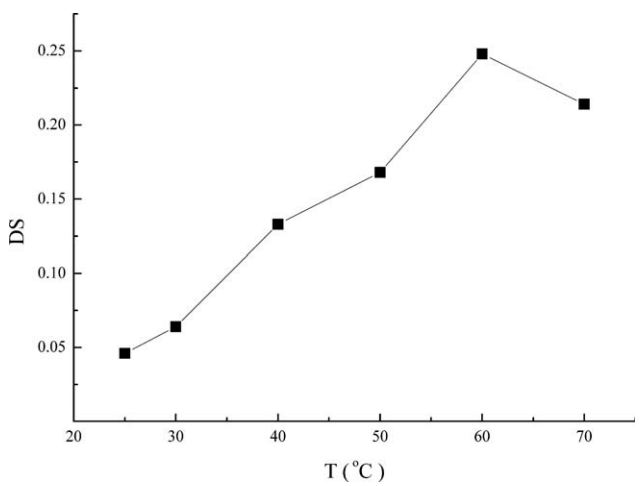


**Figure 4** Effect of the reaction temperature ($T$) on the DS values of the pectin sulfates (pH of the reaction medium = 7, reaction time = 4 h, ratio of $n_{NaNO2}$ to $m_{pectin}$ = 2.0/190 mol/g).

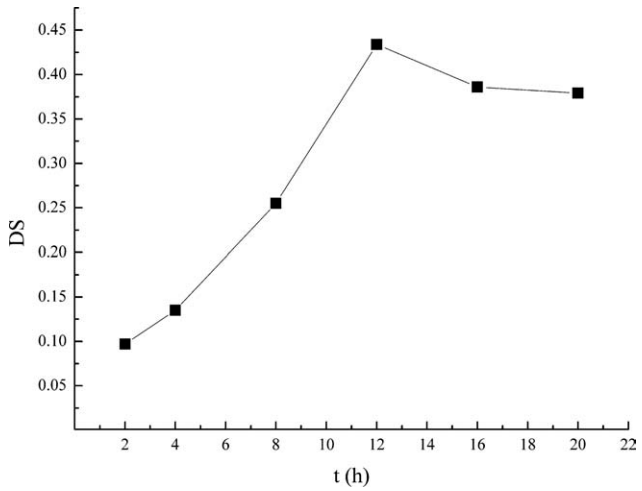


**Figure 5** Effect of the reaction time ($t$) on the DS values of the pectin sulfates (pH of reaction medium = 7, reaction temperature = 40°C, ratio of $n_{NaNO2}$ to $m_{pectin}$ = 2.0/190 mol/g).

increases in the reaction time. A maximum DS of 0.43 was obtained in 12 h. The increase of the DS with time was a result of the enhanced period of contact of the sulfating reagent with the pectin molecules. It was also reasonable that the longer reaction time enhanced pectin swelling and, ultimately, improved the homogeneity of the reactants. However, there was no remarkable further increase in DS after 12 h of reaction, because of the low activity of the sulfating agent. Moreover, byproducts and a low-molecular compound were obtained. From this analysis, we came to the conclusion that the optimal reaction time was 12 h.

### Influence of various ratios of the sulfating reagent to pectin on the DS

To investigate the various ratios of reagent to pectin in the synthesis of pectin sulfate on the DS, galacturonate methyl ester was taken as the structure of the high methoxyl pectin unit. That was because most of GalA units are esterified by methyl groups in high-methoxyl pectin. The influence of various ratios of $NaNO_2$ to pectin (molar ratio of $NaNO_2$ to the mass of pectin in mol/g) on the DS was also studied, as shown in Figure 6.

As shown, DS increased with the increasing ratio from 1/190 to 2.5/190. However, beyond 2.5/190, DS decreased slightly. With regard to the sulfating reaction equation, the stoichiometric proportion of sulfating agent [$N(SO_3Na)_3$] to the hydroxyl groups in the anhydrogalactose units was 1 : 2, and each anhydrogalactose unit in pectin contained two hydroxyls. Thus, according to the stoichiometric proportion, each molecule of the sulfating agent reacted with one anhydrogalactose unit. As the molecular weight of anhydrogalactose units in galacturonate methyl ester was 190, the theoretical value of the mole of sodium nitrite ($n_{NaNO2}$)/$m_{pectin}$ was 1/190 mol/g. However, the sulfating agent was moderate, and $N(SO_3Na)_3$ decomposed into other compounds at high temperature. Also, the weight of actual $N(SO_3Na)_3$ should have been in excess to make it react with pectin completely. Therefore, the required amount of $NaNO_2$ was higher than the theoretical value. However, there were steric effects and electrostatic repulsion between the sulfate substituents and hydroxyl groups in the pectin sulfates, so it was impossible that unreacted hydroxyls could be further sulfonated.[27] As a combination of these effects, the best condition was 2.5/190 mol/g.

### Calculation of the molecular weight

To investigate influence of the molecular weight on the anticoagulant activities of the pectin sulfates, the oxidation method was used to obtain products with different molecular weights, and the other parameters were the same for this research. The molecular weight was measured by static light scattering. The molecular weights were $2.3 \times 10^4$, $2.0 \times 10^4$, and $1.6 \times 10^4$.

### Anticoagulant activity

#### Effect of DS on the pectin sulfate anticoagulant activity

The anticoagulant activity of pectin sulfate largely results from its high level of negative charge density, which is produced by the sulfate groups. So, the sulfate group plays an important role in anticoagulant activity. We adopted DS values of pectin sulfates of 0.12, 0.21, and 0.37 as anticoagulants. The TT, PT, and APTT results are shown in Figure 7.

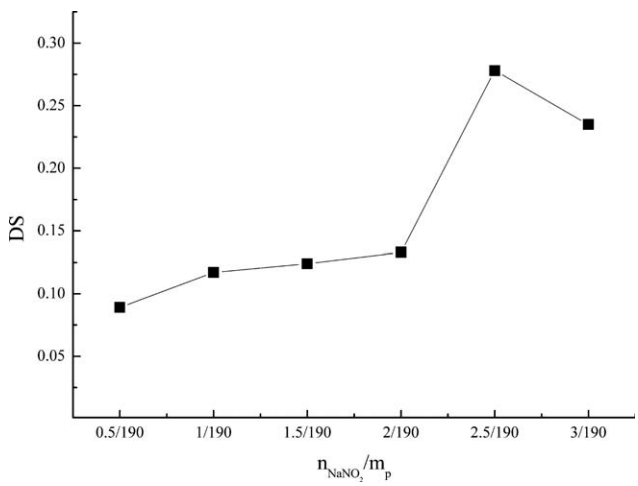


**Figure 6** Effect of the moles of ($n_{NaNO2}$) to the mass of pectin ($m_p$ or $m_{pectin}$) on the DS of the pectin sulfates (pH of the reaction medium 7, reaction time 4 h, reaction temperature 40°C).

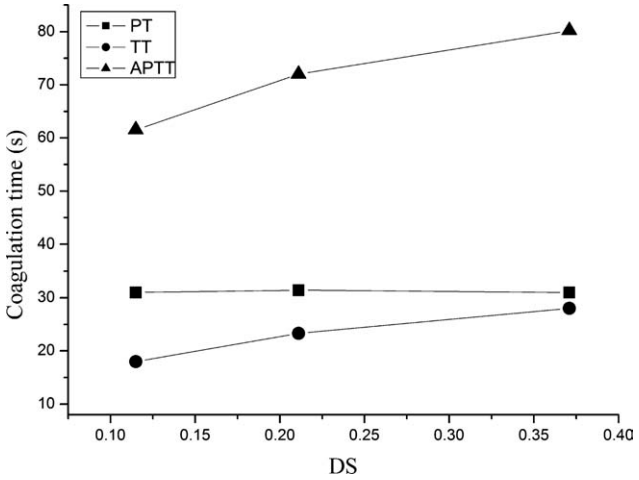


**Figure 7** Curve of the DS effect on the coagulation time (molecular weight = 23,000, concentration = 50 μg/mL).

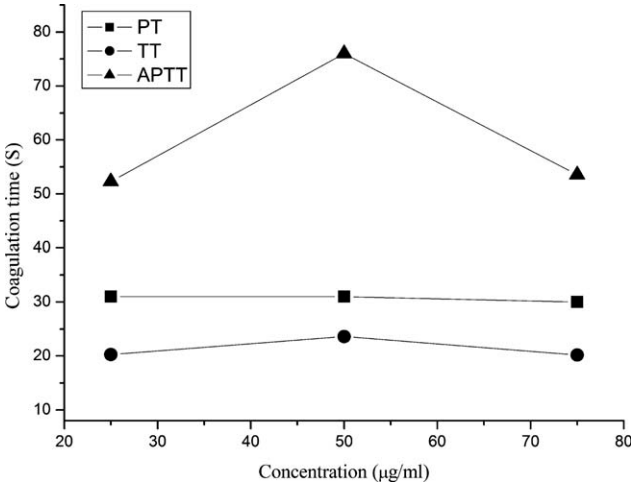


**Figure 8** Curve of the concentration effect on coagulation time (molecular weight = 23,000, DS = 0.37).

### Effect of the concentration on the pectin sulfate anticoagulant activity

The concentration of the pectin sulfates has an effect on the blood anticoagulant activity. We adopted concentrations of pectin sulfates of 25, 50, and 75 μg/mL samples for the anticoagulants. The anticoagulant results are shown in Figure 8.

### Effect of the molecular weight on the pectin sulfate anticoagulant activity

The molecular weight of pectin sulfate is also an important parameter influencing the anticoagulant activity. Generally, a suitable molecular weight is $2.6 \times 10^4$.[31] We adopted molecular weights of pectin sulfates of $2.3 \times 10^4$, $2.0 \times 10^4$, and $1.6 \times 10^4$ for the anticoagulants. The results are illustrated in Figure 9.

### Effect of the pectin sulfates on the anticoagulant activity

The normal range of PT is 10–14 s, that of TT is 10–16 s, and that of APTT is 22–38 s. Standard therapeutic heparin showed an APTT of 48 s at 0.4 μg/mL and a TT as 23 s at 0.4 μg/mL.[41] In this work, pectin showed the PT as 11 s at 75 μg/mL, the TT as 10 s at 75 μg/mL, and the APTT as 24 s at 75 μg/mL. We concluded that the anticoagulant activity of the pectin was very weak.

From these three pictures, pectin sulfate was more effective for prolonging APTT and TT than for prolonging PT; this did not change at all with different DSs, molecular weights, or concentrations. According to Matsubara et al.,[42] we concluded that pectin sulfate had strong anticoagulant activity through inhibition of the intrinsic coagulation pathway and thrombin-mediated fibrin formation not through inhibition of the extrinsic coagulation pathway.

Moreover, we found three main results. The first one was that the anticoagulant activity of pectin sulfates promoted the increase of DS. This might have been due to the negative charge of the sulfate groups of pectin sulfate neutralizing the positively charged amino acid residues, which, in the antithrombin, improved the anticoagulant activity. The higher DS increased the density of negative charges to inhibit the activity of antithrombin.

The second one was that the pectin sulfate with a molecular weight of $2.0 \times 10^4$ had the best anticoagulant activity. Although sulfated polysaccharide reportedly inhibits the coagulation cascade by forming a complex with antithrombin III, the molecular weight as a major factor could determine the rate of the inhibited process. In general, the anticoagulation activity of sulfated polysaccharide is higher at a higher molecular weight. However, a high molecular weight could limit the diffusion and uptake of pectin sulfate fractions into the blood stream, so the optimal molecular weight was $2.0 \times 10^4$.

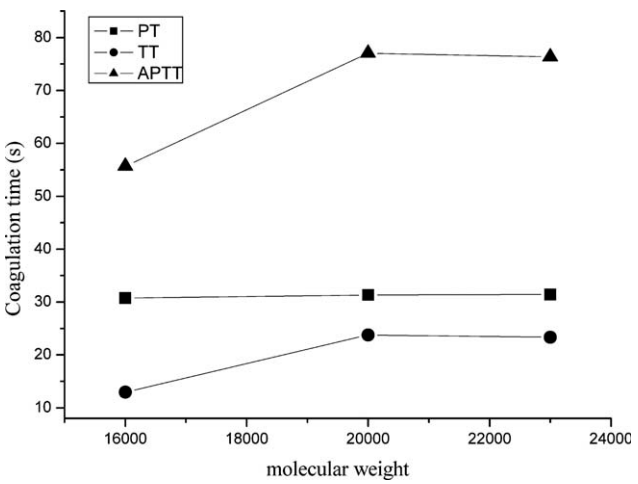


**Figure 9** Curve of the molecular weight effect on the coagulation time (DS = 0.37, concentration = 50 μg/mL).

The third result was that the best anticoagulant concentration of pectin sulfate was 50 μg/mL. The main mechanism by which nonfractionated heparin exerted its anticoagulant activity was by accelerating a plasma serine proteinase inhibitor, such as the IIa factor ("II" is the symbol of prothrombin, which is one of coagulation factor. Its active form is "IIa") or Xa factor ("X" is the symbol of Stuart-Prower factor, its active form is "Xa"). The anticoagulant activity was higher when the concentration of pectin sulfate increased. However, in our work, the result in concentration was adverse to previous studies with regard to sulfated polysaccharide.[10,41] According to Mansour et al,[43] this may have been influenced by the source of pectin or the complicated structure of pectin. Thus, it was reasonable to conclude that the optimum anticoagulant concentration of pectin sulfate was 50 μg/mL.

## CONCLUSIONS

We used a simple and effective method to synthesize pectin sulfate and evaluated its anticoagulant activity. The anticoagulant results demonstrated that the introduction of sulfate groups into the pectin structure improved its anticoagulant activity. Pectin sulfate has potential for biomedical application as a heparin alternative and as intermediates for further chemical modifications.

### References


1. Ridley, B. L.; O'Neill, M. A.; Mohnen, D. Phytochemistry 2001, 57, 929.
2. Thakur, B.; Singh, R.; Handa, A.; Rao, M. A. Crit Rev Food Sci Nutr 1997, 37, 47.
3. Kartel, M. T.; Kupchik, L. A.; Veisov, B. K. Chemosphere 1999, 38, 2591.
4. Garcia Rojas, E. E.; dos Reis Coimbra, J. S.; Minim, L. A.; Freitas, J. F. Carbohydr Polym 2007, 69, 72.
5. Sanaka, M.; Yamamoto, T.; Anjiki, H.; Nagasawa, K.; Kuyama, Y. Clin Exp Pharmacol Physiol 2007, 34, 1151.
6. Vaidya, A.; Jain, A.; Khare, P.; Agrawal, R. K.; Jain, S. K. J Pharm Sci 2009, 98, 4229.
7. Zhao, X.; Yu, G.; Guan, H.; Yue, N.; Zhang, Z.; Li, H. Carbohydr Polym 2007, 69, 272.
8. Lei, W. X.; Yang, Q. H.; Jia, X. G.; Zhang, T. Y. J Appl Polym Sci 2010, 118, 3453.
9. Xing, R.; Liu, S.; Yu, H.; Guo, Z.; Li, Z.; Li, P. Carbohydr Polym 2005, 61, 148.
10. Groth, T.; Wagenknecht, W. Biomaterials 2001, 22, 2719.
11. Huang, R.; Du, Y.; Yang, J.; Fan, L. Carbohydr Res 2003, 338, 483.
12. Vongchan, P.; Sajomsang, W.; Subyen, D.; Bach, H. Carbohydr Res 2002, 337, 1239.
13. Vikhoreva, G.; Bannikova, G.; Stolbushkina, P.; Panov, A.; Drozd, N.; Makarov, V.; Varlamov, V.; Gal'braikh, L. Carbohydr Polym 2005, 62, 327.
14. Wang, Z.-M.; Xiao, K.-J.; Li, L.; Wu, J.-Y. Cellulose 2010, 17, 953.
15. Jayakumar, R.; Nwe, N.; Tokura, S.; Tamura, H. Int J Biol Macromol 2007, 40, 175.
16. Zou, C.; Du, Y.; Li, Y.; Yang, J.; Feng, T.; Zhang, L.; Kennedy, J. F. Carbohydr Polym 2008, 73, 322.
17. Suwan, J.; Zhang, Z.; Li, B.; Vongchan, P.; Meepowpan, P.; Zhang, F.; Mousa, S. A.; Mousa, S.; Premanode, B.; Kongtawelert, P. Carbohydr Res 2009, 344, 1190.
18. Feng, Y.; Li, W.; Wu, X.; He, X.; Ma, S. Carbohydr Polym 2010, 82, 605.
19. Morris, G. A.; Ralet, M.-C.; Bonnin, E.; Thibault, J.-F.; Harding, S. E. Carbohydr Polym 2010, 82, 1161.
20. Beijering, R. J. R.; ten Cate, H.; ten Cate, J. W. Ann Hematol 1996, 72, 177.
21. Sun, Z.; He, Y.; Liang, Z.; Zhouand, W.; Niu, T. Carbohydr Polym 2009, 77, 628.
22. Martinichen-Herrero, J. C.; Carbonero, E. R.; Gorin, P. A. J.; Iacomini, M. Carbohydr Polym 2005, 60, 7.
23. Alban, S.; Schauerte, A.; Franz, G. Carbohydr Polym 2002, 47, 267.
24. Bae, I. Y.; Joe, Y. N.; Rha, H. J.; Lee, S.; Yoo, S.-H.; Lee, H. G. Food Hydrocolloids 2009, 23, 1980.
25. Cipriani, T. R.; Gracher, A. H.; de Souza, L. M.; Fonseca, R. J.; Belmiro, C. L.; Gorin, P. A.; Sassaki, G. L.; Iacomini, M. Thromb Haemost 2009, 101, 860.
26. Vityazev, F. V.; Golovchenko, V. V.; Patova, O. A.; Drozd, N. N.; Makarov, V. A.; Shashkov, A. S.; Ovodov, Y. S. Biochemistry (Moscow) 2010, 75, 759.
27. Cui, D.; Liu, M.; Liang, R.; Bi, Y. Starch-Stärke 2007, 59, 91.
28. Monsoor, U. K. M. A.; Proctor, A. J Agric Food Chem 2001, 49, 2756.
29. Schöniger, W. Microchim Acta 1956, 44, 869.
30. Cui, D.; Liu, M.; Wu, L.; Bi, Y. Int J Biol Macromol 2009, 44, 294.
31. Lim, D. W.; Whang, H. S.; Yoon, K. J.; Ko, S. W. J Appl Polym Sci 2001, 79, 1423.
32. Li, X.; Xu, A.; Xie, H.; Yu, W.; Xie, W.; Ma, X. Carbohydr Polym 2010, 79, 660.
33. Yang, J.; Du, Y.; Huang, R.; Wan, Y.; Li, T. Int J Biol Macromol 2002, 31, 55.
34. Kumar, A.; Chauhan, G. S. Carbohydr Polym 2010, 82, 454.
35. Koshy, K. M.; Boggs, J. M. Carbohydr Res 1997, 297, 93.
36. Sun, Y. J.; Ye, X. Q.; Pang, J.; Li, J.; Lu, Y. Chin J Struct Chem 2009, 28, 439.
37. Hokputsa, S. Carbohydr Res 2004, 339, 753.
38. Zhao, Z.; Li, J.; Wu, X.; Dai, H.; Gao, X.; Liu, M.; Tu, P. Food Res Int 2006, 39, 917.
39. Maaheimo, H.; Räbinä, J.; Renkonen, O. Carbohydr Res 1997, 297, 145.
40. Tao, Y.; Zhang, L.; Cheung, P. C. K. Carbohydr Res 2006, 341, 2261.
41. Park, P.-J.; Je, J.-Y.; Jung, W.-K.; Ahn, C.-B.; Kim, S.-K. Eur Food Res Technol 2004, 219, 529.
42. Matsubara, K.; Matsuura, Y.; Bacic, A.; Liao, M.-L.; Hori, K.; Miyazawa, K. Int J Biol Macromol 2001, 28, 395.
43. Mansour, M. B.; Dhahri, M.; Hassine, M.; Ajzenberg, N.; Venisse, L.; Ollivier, V.; Chaubet, F.; Jandrot-Perrus, M.; Maaroufi, R. M. Comp Biochem Physiol B 2010, 156, 206.