Sulfation of Silk Fibroin by Sulfuric Acid and Anticoagulant Activity

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ABSTRACT: Silk fibroin (*Bombyx mori*) was sulfated with an aqueous sulfuric acid solution. The sulfated fibroin was characterized by Fourier transform infrared spectroscopy (FTIR), amino acid analysis, and gel filtration chromatography (GFC). Maximum yield was obtained at around 2–4 h, and it decreased at 6 h and more. The molecular size decreased and dispersed with sulfation, and the molecular weight was estimated at around 10,000 by GFC using protein standards. The amino acid composition indicated that the crystal region of the fibroin molecule remained in sulfated fibroin until a sulfation reaction time of 4 h. The incorporation of sulfate groups was confirmed by FTIR and the amount of sulfate groups introduced for 4 h sulfation was estimated in 0.3 mmol/g by acidimetric titration. The efficiency of sulfation was calculated at 15.7%. Blood coagulation was prevented by 20 mg of sulfated fibroin in 1 mL of blood, while original fibroin did not show the effect. This result indicates that sulfate group introduction results in addition of anticoagulant function to silk fibroin. Although a variety of polymer backbones have been used for synthesis of sulfated polymers as anticoagulant materials, no reports are available concerning a sulfated polymer based on a protein backbone. Sulfated fibroin is a new type of anticoagulant material having a protein backbone. © 2003 Wiley Periodicals, Inc. J Appl Polym Sci 87: 2377–2382, 2003

INTRODUCTION

Silk fibroin is a natural polymer, produced by silk worms, that can be mass produced, and has been used as a textile fiber. Recently, many studies have reported silk use in nontextile fields, especially the biomedical field, as a substrate for enzyme immobilization,[1,2] bone-compatible materials,[3,4] antithrombogenic material,[5,6] wound dressing,[7,8] drug carrier,[9] and as a cell culture substrate.[10–13] These findings indicate that silk fibroin will be a good basic component for new biomedical material development.

Heparin, a mucopolysaccharide with 5000–50,000 molecular weight, is known as a natural anticoagulant and is applied widely in the medical field. Many investigators have attempted to develop a new synthetic anticoagulant or antithrombogenic material having heparin-like structure that mimics heparin activity. Most of these comprise ionic polymers containing sulfate, sulfamide, and carboxylic acid groups because heparin anticoagulant activity is associated with the presence of these ionic functional groups.[14] Lovelock et al.[15] and Gregor[16] reported polystyrene sulfonate anticoagulant activity in blood. Jozefonvics et al.[17,18] and Jozefowicz et al.[19,20] reported that modification of polystyrene and crosslinked dextrans by incorporation of sulfonate and carboxylate moieties produced heparin-like anticoagulant activity when these insoluble polymers contacted with blood. Cooper et al.[21–24] synthesized soluble and insoluble sulfonated polyurethanes, and reported that these sulfonated polyurethanes had excellent antithrombogenic activity. Csomor et al.[25] indicated that sulfonated poly(vinyl alcohol–acrylic acid) and sulfated polyvinyl alcohol could inhibit blood coagulation under in vitro and in vivo conditions; also, plasmin activity inhibition by the polymer was reported by Voros et al.[26] Tamada et al. reported anticoagulant activity of sulfonated polyisoprene[27]; its mechanism was explained by complex formation with fibrinogen and interference with fibroin polymerization.[28] Han et al.[29] reported a heparin-like anticoagulant effect of sulfonated poly(ethylene oxide). Groth and Wagenknecht showed that substitution of cellulose with sulfate produced superior anticoagulant activity in comparison with phosphate by the regioselective derivatization technique.[30] These reports indicate definitively that sulfate and sulfonate group incorporation into polymers affects polymer anticoagulant activity.

Although a variety of polymer backbones have been used for synthesis of sulfated polymers as an anticoagulant material, no reports are available concerning a

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sulfated polymer based on a protein backbone. Study of sulfation of silk fibroin polymer and anticoagulant activity is beneficial both for creating a novel application of silk as a natural polymer and developing a new type of anticoagulant material.

This study concerns silk fibroin sulfation by sulfuric acid and anticoagulant effects to develop a new anticoagulant material based on silk. Characterization of sulfated fibroin molecules was performed by FTIR, amino acid analysis, and liquid chromatography; anticoagulant activity was determined by human blood recalcification time.

METHOD AND MATERIALS

Silk fibroin

Silk fibroin was prepared from a *Bombyx mori* cocoon. The cocoon was cut into small pieces and put into boiling 0.5% sodium carbonate solution to degum it. After thorough washing with hot water, fibroin was dried under reduced pressure.

Sulfation of fibroin

One gram of dried fibroin was suspended in 50 mL of MilliQ water and warmed to 70°C. Sulfuric acid in the amount of 39.4 mL (Wako Chemical Co., Ltd., Tokyo) was added slowly to the suspension. Final sulfuric acid concentration became 80 wt %. Sulfation proceeded at 70°C for a predetermined time with stirring. After reaction, the solution was neutralized by equivalent molar sodium hydroxide solution and dialyzed against water due to desalting. Sulfated fibroin was stored until use in a dessicator after freeze drying. To determine the sulfated fibroin yield, reactions were repeated at least three times and weights after freeze drying were recorded.

Analysis of sulfated fibroin

Infrared spectra for sulfated fibroin analysis obtained using KBr pellets were recorded by FT/IR-350 (Jasco Co., Tokyo, Japan). Amino acid analyses were performed using a high speed amino acid analyzer, Model L-8500 (Hitachi Co., Tokyo, Japan) after hydrolysis with 6*M* hydroxyl chloride. A gel filtration chromatograph was performed using G3000sw column (Toso Co., Tokyo Japan) by high performance liquid chromatograph system (Shimazu Co., Kyoto, Japan). Elution buffer used 0.1*M* phosphate buffer (pH 7.0) and the flow rate was 1 mL/min. Reverse phase chromatography was carried out using C18 column (Toso Co., Tokyo, Japan) and acetonitrile–water solvent system was used for elution.

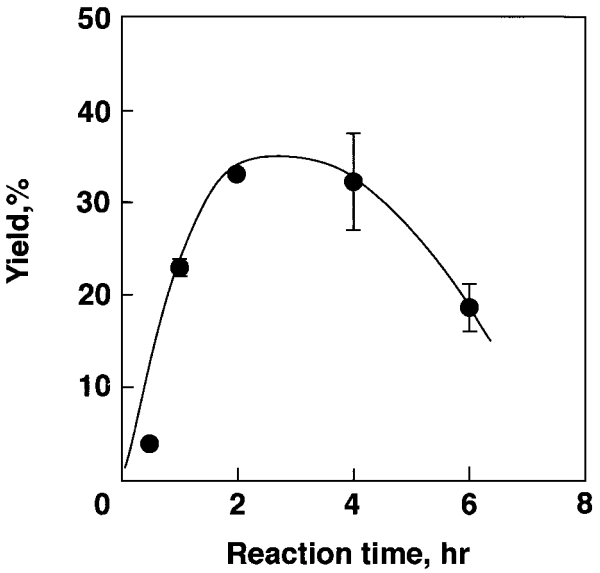

Figure 1 Yield of sulfated fibroin with reaction time. Sulfuric acid concentration was 80 wt % and reaction temperature was 7°C. The water-soluble fraction was collected and weighed.

Content of sulfate groups was determined by means of acidimetric titration. Sulfated fibroins were dissolved in distilled water and passed through ion exchange resins (Amberlite 118H, Organo, Tokyo) in order to change sodium sulfate in the sulfated fibroin to the sulfatic acid form; the solution was then titrated with 0.1*M* sodium hydroxide. Three samples were used for determining sulfate group content.

Anticoagulant activity of sulfated fibroins

Anticoagulant activity of sulfated fibroins was determined as prolongation of whole blood clotting time by recalcification time. Citrated human blood (0.9 mL) was added to glass test tubes containing 0.1 mL of sulfated fibroin solution of various concentrations and incubated for 3 min. The 4 h reaction sample was used. As a trigger for blood coagulation, 1 wt % calcium chloride solution (0.1 mL) was added to blood solutions and the solution was let to stand in a 37°C water bath for a predetermined time. Clotting time was defined as the time when no solution flow was observed upon inverting the tube. This test was repeated four times using two different blood samples.

RESULTS AND DISCUSSION

Sulfation of fibroin

Figure 1 shows the yield change of sulfated fibroin as a function of reaction time with 80 % sulfuric acid at 70°C. The yield was very low at 0.5 h reaction time and subsequently increased with reaction time. Maximum

TABLE I
Influence of Reaction Time on Amino Acid Composition of Sulfated Fibroin

Amino acid	Reaction time (h)				
	0	0.5	1	4	6
Gly	44.26	26.02	24.13	23.63	21.12
Ala	30.15	19.34	18.14	17.55	15.80
Ser	10.84	7.13	6.55	6.48	9.93
Tyr	5.19	7.79	7.60	7.46	7.90
Val	2.41	6.97	7.60	9.63	8.95
Asp	1.28	1.12	1.22	1.34	1.40
Glu	1.22	7.58	8.07	7.90	8.74
Thr	0.90	3.13	3.30	2.73	3.81
Ile	0.51	4.37	4.78	4.50	4.98
Leu	0.37	3.34	3.57	3.45	3.35
Phe	0.61	2.55	2.68	2.73	2.63
Pro	0.00	0.00	0.00	0.00	0.00
Met	0.18	0.88	1.25	1.68	0.94
Cys	0.00	0.40	0.50	0.67	0.84
Lys	0.26	2.35	2.55	2.52	2.68
His	0.92	5.03	4.88	4.70	3.17
Arg	0.46	2.90	3.20	3.12	3.31

yield was obtained at around 2–4 h; thereafter, it decreased at 6 h and more. Although the reaction solution was turbid with suspended fibroin, the solution clarified during the reaction and the silk finally dissolved in the reaction solution after about 2 h reaction. This result indicates that water-insoluble fibroin changed to a water soluble form due to hydrolysis and addition of sulfate groups up to 4–6 h sulfation; also, more sulfation results in further molecular degradation of water-soluble sulfated fibroin and its subsequent loss during dialysis.

Amino acid analysis of sulfated fibroin

Amino acid analysis results of sulfated fibroin reacted for various times are summarized in Table I. Composition of major amino acid residues of fibroin, which are glycine, alanine, and serine, decreased with reaction time, while composition of minor amino acid residues increased relatively. The ratio of major amino acid composition gives information of primary structure change of fibroin by reaction. Since the abundant sequence in fibroin molecules was glycine–alanine–glycine–alanine–glycine–serine (Gly–Ala–Gly–Ala–Gly–Ser), which compose the crystal region in H-chain of fibroin molecule,[31] the ratio of Gly, Ala, and Ser residues was theoretically calculated as 3 : 2 : 1. The actual ratio of nonreacted fibroin differed from the theoretical one because amorphous regions exist in the H-chain molecules of fibroin, and because other polypeptides such as L chains and p25 molecules are present in the original silk.[32] The ratio was almost unchanged during the reaction until 4 h, but changed drastically by the 6 h reaction point. This result suggests that fibroin molecule crystal regions did not break down significantly by sulfation until 4 h reaction; consequently, longer reaction times affected crystal regions.

FTIR measurement

Sulfated fibroin FTIR spectra show a strong absorption band at around 1200 cm^{-1} that was attributable to stretching vibrations of the SO_2 group. Although two strong absorption bands at around 1400 and 1200 cm^{-1} were observed and attributed to asymmetric and symmetric stretching vibration of SO_2 group, respectively, sulfated fibroin spectra show a strong wide absorption band due to overlapping with the fibroin band. This result indicates that reaction with sulfuric acid succeeded in incorporating sulfated groups in fibroin molecules. (See Fig. 2.)

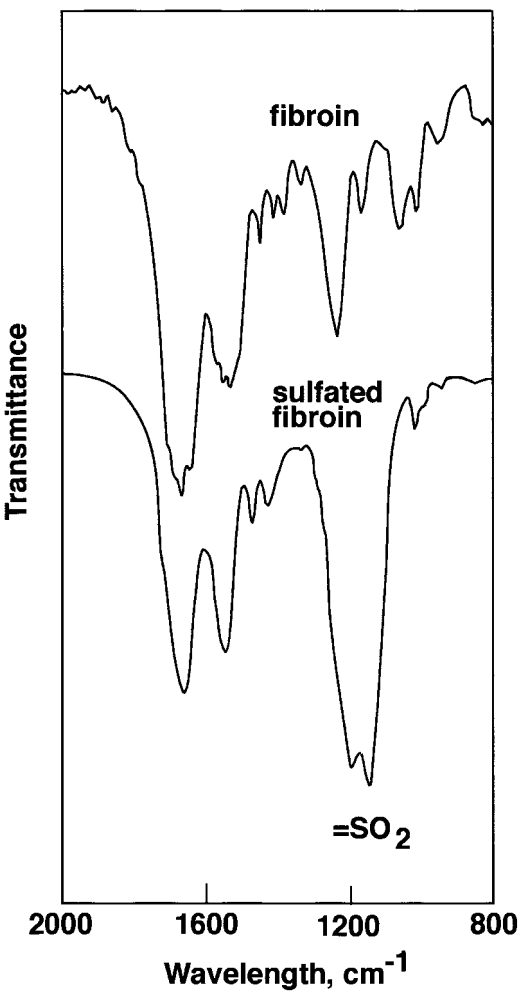

Figure 2 FTIR spectra of fibroin and sulfated fibroin. The sulfated fibroin was prepared by reaction for 4 h at 70°C using 80 wt % of sulfuric acid.

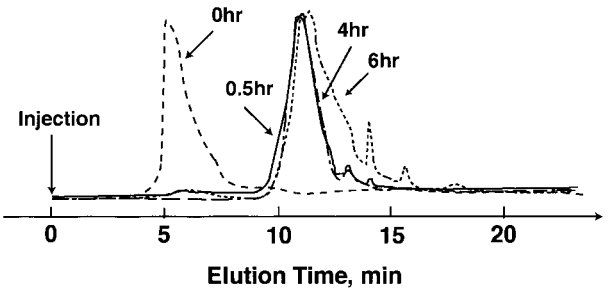

Figure 3 Gel filtration chromatography profile of sulfated fibroin with reaction time. Sulfuric acid concentration was 80 wt % and reaction temperature was 70°C.

Gel filtration chromatograph analysis

Gel filtration profiles of sulfated fibroin prepared at various reaction times are shown in Figure 3. Since water-insoluble portions were present in reaction solution at 0.5 h, water-soluble parts of reactants were analyzed. Fibroin molecule size drastically decreased through reaction with sulfuric acid due to hydrolysis. Profiles of 0.5 and 4 h reaction samples were almost identical except for the small peak shoulder attribute from the large molecular size in 0.5 h reaction sample, which was diminished in the 4 h reaction sample. Still, a significant difference in the 6 h reaction sample profile was observed. Compared with a 4 h reaction sample, the main peak slightly shifted to low molecular size and a large shoulder at the lower molecular size side of the peak appeared in the 6 h reaction sample. Both chromatograph and amino acid analysis indicate that fibroin degradation occurred gradually by sulfation, and that the molecular size and amino acid composition of produced peptides remained almost unchanged until the 4 h reaction point. Still, longer sulfation results in further peptide degradation. The main peak molecular weight was estimated at around 10,000 by globular protein standards.

Reverse phase chromatograph analysis

Figure 4 shows elution profiles of sulfated fibroins from a C18 column with the acetonitrile–water solvent system. Reaction samples from 4 and 6 h showed almost identical profiles, while molecules with longer retention time were observed in a 0.5 h reaction sample profile. This result suggests that more kinds of peptides produced by sulfation from fibroin are present at the first stage of reaction than at longer reaction times.

Sulfate group contents

The sulfate group contents of sulfated fibroin reacted for 4 h was 0.3 mmol/g (Table II). Theoretical amounts

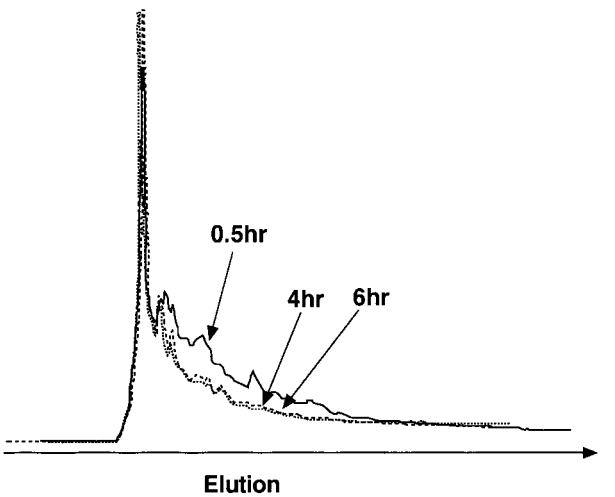

Figure 4 Reverse phase chromatography profile of sulfated fibroin.

of sulfate groups incorporated in original fibroin and sulfated fibroin molecules can be calculated from each amino acid composition (Table I). Possible amino acids reacted by sulfuric acid are mainly serine, tyrosine, lysine, and arginine in the fibroin molecule. When total mol % of these amino acids in the molecule and average amino acid molecular weight are represented as rAA and aveAA, respectively, the theoretical amount of sulfate group content per gram of sample can be presented as follows:

$$\text{Sulfate group content (mol/g)} = \frac{\text{rAA (mol \%)}}{100 \times \text{aveAA}}$$

Since rAA and aveAA of fibroin and sulfated fibroin (4 h) calculated from Table I were 17.65, 22.31, and 93.6, 116, respectively, the sulfate group contents were estimated at 1.90 and 1.92, as shown in Table II. Therefore, reaction efficiency is calculated as 15.7%.

Anticoagulant activity of sulfated fibroin

Sulfated fibroin prolonged blood coagulation time at about 20 mg/mL of concentration, while unmodified fibroin did not show the effect, as shown in Figure 5. This result indicates that the introduction of sulfate groups results in an increase in the anticoagulant func-

TABLE II
Sulfate Group Content of Sulfated Fibroin After 4 h Sulfation

Theor. Max. (mmol/g)	Experimental (mmol/g)	Exper./Theor.Max. (%)
1.90	0.303 ± 0.094[a]	15.7

[a] Mean ± S.D., n = 3

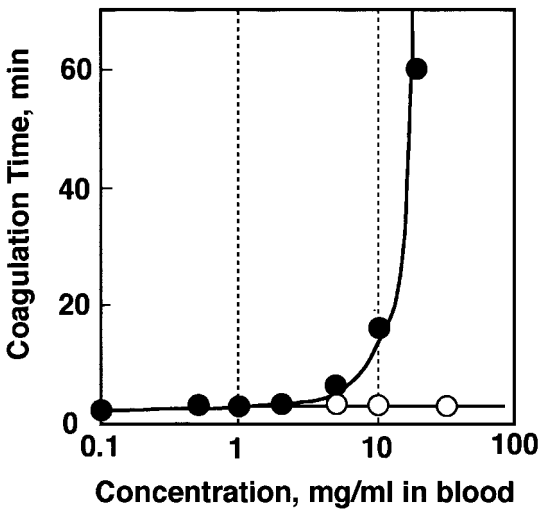

Figure 5 Effect of fibroin (○) and sulfated fibroin (●) concentration on whole blood clotting time. The sulfated fibroin was prepared by reaction for 4 h at 70°C using 80 wt % of sulfuric acid.

tion of fibroin. However, anticoagulant activity was much less than that of heparin, which showed an effective concentration around 0.5 mg/mL in blood (commercial reagent grade heparin, Wako, Co., Tokyo, Japan).

Although sulfation with sulfuric acid makes silk fibroin a novel anticoagulant functional material, the activity was lower than for other sulfated polymers like heparin and sulfonated polyisoprene. It was reported that the molecular size and sulfate group content of sulfated polymers influenced anticoagulant activity; especially a higher sulfate group content was important to achieve higher anticoagulant activity. The sulfate group content of heparin was estimated at 4.5 mmol/g by calculation based on the heparin pentasaccharide analog containing sulfate and sulfoamide.[33] In the case of sulfonated polyisoprene, 2–4 mmol/g of sulfate group was required to show high anticoagulant activity.[27] The sulfate group content of sulfated fibroin in this study is around 0.3 mmol/g, and this would be one reason for lower anticoagulant activity of sulfated fibroin. Introduction of more sulfate group using higher concentrated sulfuric acid or for longer reaction duration breaks the peptide bond of silk fibroin through acidic hydrolysis; the result is decreased molecular size and anticoagulant activity. This indicates that sulfation methods other than a sulfuric acid/aqueous system may be needed to obtain a sulfated fibroin having high anticoagulant activity. At present, further studies with sulfated fibroin will increasingly emphasize anticoagulant activity and determine anticoagulant mechanisms.

Although fibroin molecule degradation occurred through sulfation with sulfuric acid, the crystal region containing Gly–Ala–Gly–Ala–Gly–Ser in the fibroin H-chain molecule[31] could be conserved during sulfation. This result indicates that sulfated fibroin can make a complex easily with fibroin through crystallization without harmful coupling reagents and develop a novel antithrombogenic material based on silk. Sulfated fibroin is a novel type of anticoagulant polymer having a protein backbone; it constitutes an important component for silk use in biomedical applications.

CONCLUSION

Sulfate groups were introduced into silk fibroin molecules by sulfation with sulfuric acid; fibroin molecule degradation occurred simultaneously with sulfation. Sulfated fibroin showed anticoagulant activity, and it is a novel anticoagulant material having a protein backbone. Since the crystal region sequence of fibroin molecule remained after sulfation, sulfated fibroin constitutes an important component for silk use in biomedical applications.

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