Anticoagulant Activity of Sulfoalkyl Derivatives of Curdlan

Kyung Bok Lee[1], Jong Hwan Bae[1], Jong Seung Kim[2], Yung Choon Yoo[3], Beom Soo Kim[4], Sang Tae Kwak[1], and Yeong Shik Kim[5]

[1]Department of Biochemistry, College of Medicine, [2]Department of Chemistry, [3]Department of Microbiology, College of Medicine, [4]Department of Chemical Engineering, Konyang University, Nonsan, Chungnam 320-711, Korea, and [5]Natural Products Research Institute, Seoul National University, Seoul 110-460, Korea

(Received February 5, 2001)

Curdlan is a natural β-1,3-glucan produced by *Agrobacterium biovar* 1. In this study, the anticoagulant activity of sulfoalkyl derivatives of curdlan was investigated by carrying out activated partial thromboplastin time (APTT) assay and compared with that of *o*-sulfonated curdlan. Approximately 100-fold higher concentration of *o*-sulfonated curdlan than heparin was required to obtain the same level of the clotting time. Anticoagulant activity of curdlan derivatives was dependent on the degree of sulfation in prolonging the clotting time. However, the chain length of the substituent did not play a role in prolonging the clotting time. The curdlan derivatives enhanced thrombin inhibition by mediating through antithrombin III. The inhibition of thrombin by *o*-sulfonated curdlan was found to be approximately 10-fold weaker than that by heparin.

Key words: Anticoagulant activity, Sulfoalkyl derivatives, Curdlan, Activated partial thromboplastin time

INTRODUCTION

Curdlan is a totally linear β-1,3-glucan with no branching (Fig. 1) (Kim *et al.*, 2000). It was discovered by Harada *et al.* (Harada *et al.*, 1968). Although curdlan is mainly used in the food industry, many chemical modifications have been tried to obtain various biological activities including antitumor activity (Demleitner *et al.*, 1992; Kurachi *et al.*, 1990; Usui *et al.*, 1997; Zhang *et al.*, 2000), anti AIDS virus activity (Gao *et al.*, 1997; Yoshida *et al.*, 1995) and anticoagulant activity (Alban *et al.*, 1995). Anticoagulant and antithrombotic activities are the most widely studied biological activities of sulfated polysaccharides (Wu *et al.* 1998). Many sulfated polysaccharides, either naturally-occurring polysaccharides (Farias *et al.*, 2000) or chemically-sulfated polysaccharides, have been described as anticoagulants. Many studies on the anticoagulant activity of sulfated curdlan derivatives have been carried out and the relationship between sulfation pattern of curdlan sulfates and their anticoagulant activity was investigated (Franz and Alban, 1995). In an effort to develop better anticoagulant drugs, many attention has been focused on sulfated polysaccharides (Alban *et al.*, 1995; Kim *et al.*, 2000). However, no studies have been carried out on anticoagulant activity of sulfoalkyl derivatives of curdlan. It was of interest to investigate whether these types of derivatives show improved anticoagulant activity. In this study, 2-sulfoethyl, 3-sulfopropyl, and 4-sulfobutyl derivatives of curdlan and sulfonated curdlan have been synthesized (Demleitner *et al.*, 1992) and the anticoagulant action of these derivatives is presented.

Correspondence to: Kyung Bok Lee, Department of Biochemistry, College of Medicine, Konyang University, Nonsan, Chungnam 320-711, Korea
E-mail: kyunglee@kytis.konyang.ac.kr

MATERIALS AND METHODS

Materials and Apparatus

IR spectra obtained with a Bio-Rad Series FT-IR on deposited KBr window in the case of gel, respectively, were recorded in reciprocal centimeters. ^{1}H and ^{13}C NMR spectra were recorded as chemical shifts (δ) downfield from the internal standard, DSS using a 400 MHz (Bruker ARX-400) and an 100 MHz spectrometer, respectively. Approximately 20 mg of each sample was dissolved in D_2O and spectra were obatined. Elemental analysis was performed by Elemental Analyzer (Vario EL) in Korea Basic Science Institute in Seoul. Curdlan (M.W. 89,000) was obtained from Wako Pure Chemical Indu-

stries (Tokyo, Japan). The activated partial thromboplastin time (APTT) reagent, thrombin, antithrombin and *N*-*p*-tosyl-Gly-Pro-Arg-*p*-nitroanilide, were purchased from Sigma (St. Louis, MO). Unless specified otherwise, reagent-grade reagents and solvents were obtained from chemical suppliers and used without further purification.

Sulfation

A mixture of 20 g of triethylamine-sulfur trioxide and 250 ml of dimethylformamide was cooled to 0°C. Curdlan (5.0 g) was added and reacted for 24 h at 0°C with constant stirring. The material was dialyzed for 24 h against a solution of 10% sodium hydrogen carbonate and subsequently dialyzed for 48 h against distilled water. The product was collected on a Bucher funnel, washed with 95% ethanol, and dried over calcium chloride under reduced pressure. The colorless gel was obtained with a quantitative yield. IR (KBr window, cm^{-1}) 1070 (SO_2).

Sulfoethylation

The curdlan (1.0 g) was suspended in 2-propanol (10 ml). To this suspension, a solution of NaOH (0.66 g) and sodium 2-chloroethanesulfonate (2.04 g) in water (2 ml) was added in two portions with an interval of 1 h and the reaction mixture was refluxed for 3 h at 85°C. A solution of the derivative was neutralized with glacial acetic acid and dialyzed several times. The colorless gel was obtained with a quantitative yield. IR (KBr window, cm^{-1}) 1328 (SO_2), 1070 (SO_2). ^{1}H NMR (D_2O): δ 5.16 (m, HOC-*H*), 4.57 (m, HOC -*H*), 3.87-3.64 (m, HOC), 3.46-3.55 (m, HOC), 3.15 (t, -OCH_2C$H_2$$SO_3$Na), 2.83 (m, HOC).

Sulfopropylation

The curdlan (1.0 g) was suspended in 15 ml of 2-propanol-water (25:3). To this suspension, a solution of NaOH (1.2 g) was added and the resulting suspension was stirred at 50°C for 1 h. 1,3-Propane sultone (4 ml) in acetone (1 ml) was added and the mixture was stirred for 6 h at 50°C. A solution of the derivative in water was neutralized with glacial acetic acid and dialyzed. IR (KBr window, cm^{-1}) 1312 (SO_2), 1034 (SO_2). ^{1}H NMR (D_2O): δ 5.36 (m, HOC -*H*), 4.56 (m, HOC -*H*), 3.84-3.63 (m, HOC), 3.63-3.36 (m, HOC), 2.96 (t, -OCH_2CH_2C$H_2$$SO_3$Na), 1.98 (m, -OC$H_2CH_2CH_2$$SO_3$Na). ^{13}NMR ($D_2O$): 76.4, 72.1, 70.0, 48.5, 25.5, 24.7.

Sulfobutylation

The curdlan (1 g) was suspended in 15 ml of 2-propanol-water (25:3). To a suspension, NaOH (0.8 g) was added and the suspension was stirred at 60°C for 1 h. 1,4-butane sultone (2.5 ml) was added and the mixture was stirred for 6 h at 60°C. The reaction mixture was neutralized with glacial acetic acid and dialyzed. The colorless gel was obtained with a quantitative yield. IR (KBr window, cm^{-1}) 1312 (SO_2), 1034 (SO_2). ^{1}H NMR (D_2O): δ 5.16 (m, HOC -*H*), 4.56 (m, HOC -*H*), 3.84-3.63 (m, HOC), 3.63-3.36 (m, HOC), 2.88 (t, -OCH_2CH_2CH_2C$H_2$$SO_3$Na), 1.92 (m, -OC$H_2CH_2CH_2CH_2$$SO_3$Na).

Agarose gel electrophoresis

Curdlan derivatives were analyzed by agarose gel electrophoresis, as described previously (Wu *et al.*, 2000). Briefly, 100 μg of samples were applied to a 1% gel in TBE buffer (0.045 M Tris-borate, 0.001 M EDTA) and run at 80 V for 1 h. The gel was visualized with 0.5% Azure A in 1% acetic acid.

Determination of the molecular weight

The molecular weight of curdlan derivatives was determined by HPLC on a GPC column of TSK G3000SW (Uppsala, Sweden) equilibrated with 100 mM NaCl. Chondroitin sulfate (M.W. 15,000), heparin (M.W. 12,000) and dextran sulfate (M.W. 10,000) were used as standards for the calibration of the column.

Anticoagulant activity

The anticoagulant activity was evaluated by measuring the activated partial thromboplastin time (APTT). APTT test was performed on a Amelung KC 1A micro coagulation analyzer (St. Louis, MO, USA). $CaCl_2$ solution (20 mM) and APTT reagent were preincubated for 30 min at 37°C. Sample (50 μl) and human plasma (100 μl) were mixed well and 50 μl of the above mixture was taken into the bottom of the cuvette and incubated for 2 min. APTT reagent (50 μl) was added and incubated for 3 min. And thereafter 50 μl of 20 mM $CaCl_2$ was added and the clotting time was measured (Lee *et al.*, 2000).

Anti IIa activity

The anti IIa activity of curdlan derivatives was measured according to a modification of published method (Farias *et al.*, 2000). Sample solution (10 μl) and 5 μl of 1 unit/ml antithrombin III were mixed with 19 μl of 10 units/ml thrombin in 66 μl of 0.015 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 1 mg/ml polyethylene glycol. After 1-min incubation, 500 μl of 0.24 mM N-*p*-tosyl-Gly-Pro-Arg-p-nitroanilide was added and the residual thrombin activity was recorded for 2 min at 405 nm. Measurements were performed on a Beckman DU 650 spectrophotometer.

RESULTS AND DISCUSSION

Synthesis and structural characterization

Sulfation and *n*-ethyl-, *n*-propyl-, *n*-butyl-sulfonation of curdlan were carried out with some modifications of the

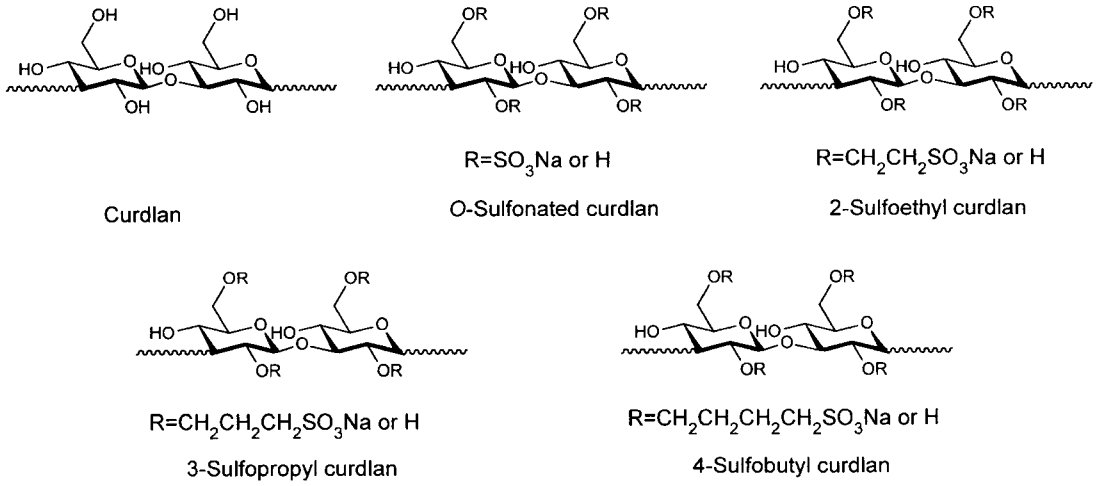

Fig. 1. Chemical structures of curdlan and its derivatives

reported method (Demleitner *et al.*, 1992). Each of final product was identified by NMR and IR spectrometer. Two characteristic IR bands appeared at ~1300 and ~1060 cm^{-1} indicate sulfonyl (O=S=O) double bond in the product. In NMR spectra, the alkyl chains connecting between primary alcohols and sulfonyl groups were shown in the range of 3.3-1.1 ppm. Especially, in the cases of 3-sulfopropylation and 4-sulfobutylation, aliphatic -CH_2- group (-$OCH_2CH_2CH_2SO_3Na$ and -$OCH_2CH_2CH_2CH_2SO_3Na$) appeared at a higher field than 2.0 ppm as well as the sulfur containing microchemical analysis results imply that the corresponding sulfoalkylated cudlan was successfully synthesized. However, the predominant position for sulfoalkylation in parent curdlan could not be determined because of complicated IR and NMR splitting patterns.

Agarose gel electrophoresis and determination of molecular weight

The sulfonation introduced the fixed charges into a neutral polysaccharide, curdlan. Therefore, agarose gel electrophoresis can be performed to determine the molecular weight and polydisperisty of the derivatives (Fig. 2). Agarose gel electrophoresis analysis revealed that curdlan was degraded during the derivatization. The molecular weight of *o*-sulfonated curdlan was similar to that of 2-sulfoethyl curdlan as shown in lane 2 and lane 3 in Fig. 2. The molecular weights of 3-sulfopropyl, and 4-sulfobutyl derivatives of curdlan were lower than that of *o*-sulfonated curdlan as shown in lane 4 and lane 5 in Fig. 2. It was reported that anticoagulant activity was dependent on the molecular weight (Franz and Alban, 1995). Therefore, the average molecular weights of derivatives were determined by high performance liquid chromatography (HPLC) on gel permeation column, TSK G3000SW. The average molecular weights of *o*-sulfonated curdlan, 2-sulfoethyl, 3-sulfopropyl, and 4-sulfobutyl derivatives of curdlan were 16×10^3, 15.5×10^3, 13×10^3, and 14.5×10^3, respectively. In order to investigate relationship between the degree of sulfation and anticoagulant activity, the degree of sulfonation was estimated by elemental analysis and results are summarized in Table I.

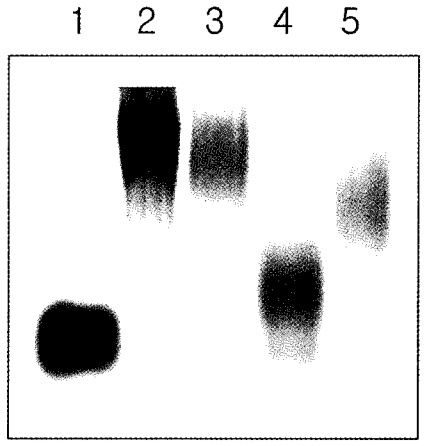

Fig. 2. Agarose gel electrophoresis of heparin and curdlan derivatives. Lane 1, heparin; lane 2, *o*-sulfonated curdlan; lane 3, 2-sulfoethyl curdlan; lane 4, 3-sulfopropyl curdlan; lane 5, 4-sulfobutyl curdlan

Determination of anticoagulant activity

Anticoagulant activity was determined using heparin as reference by performing APTT assay. Fig. 3 illustrates the anticoagulant activity of curdlan derivatives. Curdlan itself did not show any anticoagualnt activity. Heparin showed a more potent anticoagulant activity than any other curdlan derivatives. We investigated the relationship between the chain length of the substituent and

Table I. Sulfoalkylation of curdlan

Derivative	Elemental analysis (%)			Degree of substitution[a]	M_r[b]
	C	H	S		
O-sulfonated curdlan	28	4.4	9.8	0.86	16,000
2-sulfoethyl curdlan	35.2	5.5	4.3	0.41	15,500
3-sulfopropyl curdlan	33.1	5.4	8.3	0.84	13,000
4-sulfobutyl curdlan	35.7	5.9	5.3	0.56	14,500

[a]The number of sulfonate group per sugar unit in curdlan derivatives was determined by elemental analysis.
[b]Determined by gel permeation chromatography as described in Materials and Methods

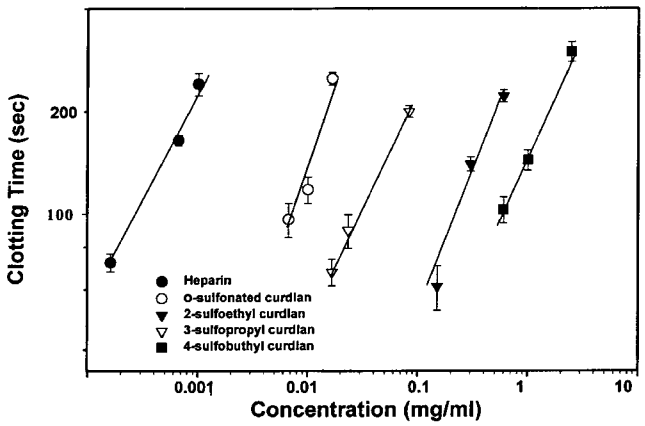

Fig. 3. Comparison of the anticoagulant activity of heparin and curdlan derivatives. The clotting time was measured by the APTT assay as described under Materials and Methods.

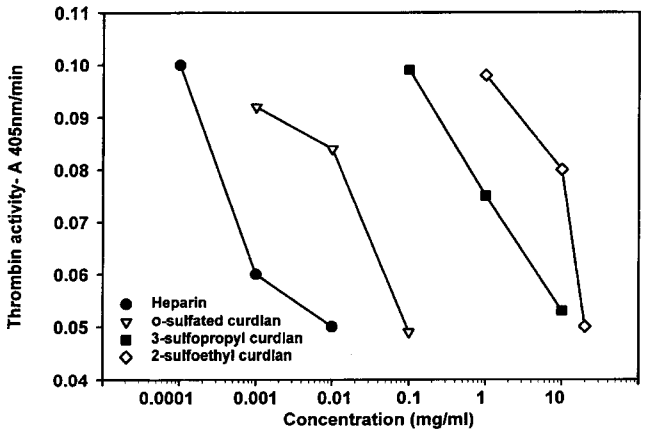

Fig. 4. Comparison of the anti-factor IIa activity of heparin and curdlan derivatives. Antithrombin III was incubated with thrombin in the presence of various concentrations of the curdlan derivatives as described under Materials and Methods.

anticoagulant activity. Unfortunately, the chain length of the substituent did not play a role in prolonging clotting time.

The anticoagulant activity of curdlan derivatives has a tendency to be enhanced with an increasing degree of substitution (Table I). There are many reports that increased sulfation of glycosaminoglycans resulted in their anticoagulant activity (Maaroufi *et al.*, 1990; Casu *et al.*, 1994; Farias *et al.*, 2000). To establish a relationship between the degree of sulfation and anticoagulant activity, a series of curdlan derivatives were synthesized by under the various conditions. In order to obtain the same clotting time as heparin, more than 10-fold higher concentration of *o*-sulfonated curdlan was required.

The inhibition of factor IIa (thrombin) was determined using a chromogenic substrate (Fig. 4). *O*-sulfonated curdlan showed approximately 10-fold lower antifactor IIa activity than that of heparin. Antifactor IIa activities of 2-sulfoethyl and 3-sulfopropyl derivatives of curdlan were approximately 100-fold lower than that of *O*-sulfonated curdlan. *O*-sulfonated curdlan showed maximal inhibiton of factor-IIa at a concentration of approximately 10 μg/ml. It was observed that 4-sulfobutyl derivatives of curdlan were unable to inhibit the amidolytic activity of factor IIa (not shown).

Studies for new biological activities of prepared sulfoalkyl curdlan derivatives including anticancer, antiHIV and immuno-modulating activities are currently being performed.

REFERENCES

Alban, S., Jeske, W., Welzel, D., Franz, G. and Fareed J., Antocoagulant and antithombotic actions of a semi-synthetic β1,3-glucan sulfate. *Thromb. Res.*, 78, 201-210 (1995).

Casu B., Grazioli G., Razi N., Guerrini M., Naggi A., Torri G., Oreste P., Tursi F., Zoppetti G. and Lindahl U., Heparin-like compounds prepared by chemical modification of capsular polysaccharide from *E. coli*. K5. *Carbohydr. Res.*, 263, 271-284 (1994).

Demleitner, S., Kraus, J. and Franz, G., Synthesis and antitumor activity of sulfoalkyl derivatives of curdlan and lichenan. *Carbohydr. Res.*, 26, 247-252 (1992).

Farias, W. R. L., Valente, A.P., Pereira, M.S. and Mourao, P. A. S., Structure and anticoagulant activity of sulfated galactans. *J. Biol. Chem.*, 275, 29299-29307 (2000).

Franz, G. and Alban, S., Structure-activity relationship of antithrombotic polysaccharide derivatives. *Int. J. Biol. Macromol.*, 17, 311-314 (1995).

Gao, Y., Fukuda, A., Katsuraya, K., Kaneko, Y., Mimura,

T., Nakashima, H. and Uryu, T., Synthesis of regioselective substituted curdlan sulfates with medium molecular weights and their specific anti HIV-1 activities. *Macromol.*, 30, 3224-3228 (1997).

Harada, T., Misaka, A. and Satio, H., Curdlan: a bacterial gel forming β-1,3-glucan. *Arch. Biochem. Biophys.*, 124, 292-298 (1968).

Kim, B. S., Jung, I. D., Kim, J. S., Lee, J. H., Lee, I. Y. and Lee, K. B., Curdlan gels as protein drug delivery vehicles. *Biotech. Lett.*, 22, 1127-1130 (2000).

Kurachi, K., Ohno, N. and Yadomae, T., Preparation and antitumor activity of hydroxyethylated derivatives of 6-branched (1-3)-β-D-glucan, SSG, obtained from the culture filtrate of *Sclerotinia sclerotiorum* IFO 9395. *Chem. Pharm. Bull.*, 38, 2527-2531 (1990).

Lee, K. B., Kim, J. S., Yoo, Y. C., Kwak, S. T., Song, K. S. and Kim, Y. S., Isolation and characterization of proteoglycan derived from human placenta and its biological activities. *Arch. Pharm. Res.*, 23, 182-186 (2000).

Maaroufi R. M., Tapon-Bretaudiere J., Mardiguian J. and Sternberg C. Dautzenberg M.D.. Fischer A.M. Influence of the oversulfation method and the degree of sulfation on the anticoagulant properties of dermatan sulfate derivatives. *Thromb. Res.*, 59, 749-758 (1990).

Usui, S., Matsunaga, T., Ukai, S. and Kiho, T. Growth suppressing activity for endothelial cells induced from macrophages by carboxymethylated curdlan. *Biosci. Biotech. Biochem.*, 61, 1924-1925 (1997).

Wu, S. J., Chun, M. W., Shin, K. H., Toida, T., Park, Y., Linhardt, R. J., Kim, Y.S., Chemical sulfonation and anticoagulant activity of acharan sulfate. *Thromb. Res.*, 92, 273-281 (1998).

Yoshida, T., Yasuda, Y., Mimura, TY., Kaneko, Y., Nakashima, H., Yamamoto, N. and Uryu, T. Synthesis of curdlan sulfates having inhibitory effects in vitro against AIDS viruses HIV-1 and HIV-2. *Carbohydr. Res.*, 276, 425-436 (1995).

Zhang, L., Zhang, M., Zhou, Q., Chen, J. and Zeng, F. Solution properties of antitumor sulfated derivative of alpha-(1→3)-D-glucan from *Ganoderma lucidum*. *Biosci. Biotechnol. Biochem.*, 64, 2172-8 (2000).