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Regulation of the Activities of Thrombin and Plasmin by Cholesterol Sulfate as a Physiological Inhibitor in Human Plasma[1]

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Thrombin and plasmin, both of which are serine proteases in the plasma of vertebrates, play essential roles in blood clotting and fibrinolysis, respectively, and regulation of their activities is important to suppress the excessive reactions within the vascular network and to prevent tissue injury. Along with the peptidic inhibitors belonging to the serpin family, we found that cholesterol sulfate (CS), which is present at the concentration of 2.0±1.2 nmol/ml in human plasma, was a potent inhibitor of both plasma thrombin and plasmin. Thrombin, as determined both using a chromogenic substrate and the natural substrate, fibrinogen, was inactivated upon reaction with CS in a dose-dependent manner, but not in the presence of the structually related steroid sulfates, I^3SO_3-GalCer and $II^3NA\alpha$-LacCer, suggesting that both the sulfate group and the hydrophobic side chain of CS are necessary for the inhibitory activity of CS. Preincubation of thrombin with CS at 37°C for 10 min was required to achieve maximum inhibition, and virtually complete inhibition was achieved at a molar ratio of CS to thrombin of 18:1. CS-treated thrombin had the same K_m and a lower V_{max} than the original enzyme, and a higher molecular weight. The molecular weight and activity of the original enzyme were not observed on the attempted separation of the CS-treated enzyme by gel permeation chromatography and native PAGE, indicating that the inactivation of thrombin by CS is irreversible. In contrast, CS was readily liberated from the enzyme by SDS-PAGE, suggesting that hydrophobic interactions are involved in the CS-mediated inactivation of thrombin. When acidic lipids were reacted with thrombin after dissolving them in DMSO, I^3SO_3-GalCer, steroid sulfates and $II^3NA\alpha$-LacCer, as well as CS, but not SDS and sodium taurocholate, exhibited inhibitory activity, probably due to micellar formation facilitating interaction between thrombin and negatively charged lipids. On the other hand, plasmin, as determined using a chromogenic substrate, was more susceptible to acidic lipids than thrombin. CS, I^3SO_3-GalCer and $II^3NA\alpha$-LacCer, all of which are present in serum, inhibited the activity of plasmin in aqueous media, as well as in DMSO-mediated lipid solutions. Thus, acidic lipids in plasma were demonstrated to possess regulatory activity as endogenous detergents toward both enzymes for blood clotting and fibrinolysis.

Key words: fibrinolysis, fibrinogen, inhibition, serine protease, thrombogenesis.

Thrombin [EC 3.4.21.5] and plasmin [EC 3.4.21.7] are serine proteases in the plasma of vertebrates and are primarily involved in blood clotting and fibrinolysis, respectively (*1, 2*). The physiological substrate for thrombin is fibrinogen, which is converted to fibrin by its partial proteolysis by thrombin, followed by polymerization of the fibrin monomers by end-to-end and side-to-side aggregation to form a gel or a tough insoluble clot composed of fibrin strands and blood cells, while plasmin can cleave fibrin in the process of thrombolysis. To maintain the homeostasis of the blood clotting system within the vascular network, the levels of these enzymes in plasma are strictly regulated by activation of their inactive zymogens, namely, prothrombin and plasminogen, as well as by inhibition of the active enzymes by their specific inhibitors belonging to the serpin family. In plasma, nearly 10% of the total protein consists of proteinase inhibitors of the serpin family, and an imbalance in the ratio of proteinase to its inhibitor causes

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Abbreviations: AMC, 7-amino-4-methyl coumarin; Boc-Asp(OBzl)-Pro-Arg-MCA, *t*-butyloxycarbonyl β-benzyl-L-aspartyl-L-prolyl-L-arginine 4-methylcoumaryl-7-amide; Boc-Val-Leu-Lys-MCA, *t*-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine 4-methylcoumaryl-7-amide; CS, cholesterol sulfate; DMSO, dimethylsulfoxide; HPLC, high-performance liquid chromatography; NAα, sialic acid; $II^3NA\alpha$-LacCer, NAα2-3Galβ1-4Glcβ1-1′ ceramide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; I^3SO_3-GalCer, SO_3-3Galβ1-1′ceramide.

uncontrolled proteolysis, resulting in tissue injury, such as is observed in familial emphysema due to abnormally low concentrations of a proteinase inhibitor in plasma (*3, 4*). The serpins antithrombin-III and α_2-antiplasmin of thrombin and plasmin, respectively, are both well characterized, but, in addition to these inhibitors, thrombin and plasmin activities are also regulated by other inhibitors, such as α_1-antitrypsin, C1-inhibitor, α_2-macroglobulin, and heparin cofactor II (*5-8*). Thrombomodulin and fibrin also act to fix thrombin within a restricted area to avoid its diffusion (*9*). Thus, although the expression of activities of both enzymes is essential for thrombogenesis and thrombolysis, regulation of their strong degradative activities, particularly by inhibitors, is important in order to prevent injury of tissues and the vascular network. Recently, we found that sulfated lipids in the epithelial cells of the duodenum, jejunum, and ileum, which were exposed to pancreatic juice containing several digestive enzymes, possessed inhibitory activities toward pancreatic serine proteases, trypsin, chymotrypsin, and elastase (*10, 11*). Since the same sulfated lipids as those in the gastrointestinal tract were shown to be present in plasma (*12*), we speculated that thrombin and plasmin in plasma are also regulated by a similar mechanism, mediated by lipid inhibitors, to those in the gastrointestinal tract.

MATERIALS AND METHODS

Materials—Thrombin from bovine plasma and plasmin from human plasma were purchased from Wako Chemicals (Tokyo) and Sigma (St. Louis, MO, USA), respectively, and were used without further purification. *t*-Butyloxycarbonyl β-benzyl-L-prolyl-L-aspartyl-L-arginine 4-methyl coumaryl-7-amide [Boc-Asp(OBzl)-Pro-Arg-MCA], the substrate for thrombin, *t*-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine 4-methyl coumaryl-7-amide (Boc-Val-Leu-Lys-MCA), the substrate for plasmin, and 7-amino-4-methyl coumarin (AMC) were purchased from Peptide Institute (Osaka) (*13, 14*). Fibrinogen from bovine plasma, sodium dodecyl sulfate, sodium taurocholate, dehydroepiandrosterone sulfate, pregnenolone sulfate, and estrone sulfate were obtained from Sigma. Cholesterol sulfate (CS) was synthesized by sulfation of the 3β-hydroxyl group of cholesterol (Wako Chemicals) with pyridinium sulfate and purified by Iatrobeads (Iatron, Tokyo) column chromatography. I^3SO_3-GalCer and $II^3NA\alpha$-LacCer were prepared from human brain and placenta, respectively, in our laboratory.

Determination of Acidic Lipids in Human Plasma and Hemocytes—Human whole blood (male, 30-35 years old) and platelets were supplied by the Japanese Red Cross Society (Tokyo). Plasma was obtained by centrifugation of blood at $400 \times g$ for 20 min, and the hemocytes, namely, erythrocytes, neutrophils, and lymphocytes plus monocytes, were prepared using Ficoll Hypaque solution (Monopoly Separation Medium, Dainippon Pharmaceutical, Osaka) according to the manufacturer's instructions. After examination of the purity and number of cells, they were lyophilized and the acidic lipids in the cells were determined as follows (*12*). The total lipids were extracted from the lyophilized cells with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1, by volume), and the combined extracts were applied onto a column packed with DEAE-Sephadex A-25 (acetate form; Pharmacia, Uppsala, Sweden). After elution of the unabsorbed lipids with 3 volumes of chloroform/methanol (1:1, by volume) and 1 volume of methanol, the acidic lipids absorbed on the column were eluted with 10 volumes of 0.3 M sodium acetate in methanol, then saponified with 0.5 M sodium hydroxide in methanol at 40°C for 30 min to cleave the ester-containing acidic phospholipids. The acidic lipids thus obtained were chromatographed on a TLC plate (E. Merck, Darmstadt, Germany) using the solvent system of chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1, by volume) and detected with cupric acetate-phosphoric acid for organic compounds, ferric chloride-sulfuric acid for steroids, and orcinol-sulfuric acid for glycolipids. Known amounts of CS, I^3SO_3-GalCer and $II^3NA\alpha$-LacCer were spotted on the same plate, and the density of the spots was determined at a sample wavelength of 420 nm and a control wavelength of 700 nm with a TLC-densitometer (CS-9000; Shimadzu, Kyoto). The lower limit for detection was 0.05 μg and the standard curves were linear up to 1.5 μg. CS was identified by fast-atom bombardment mass spectrometry after purification by Iatrobeads column chromatography as described previously (*15*). I^3SO_3-GalCer and $II^3NA\alpha$-LacCer in the acidic lipid fraction were identified by TLC-immunostaining with monoclonal anti-I^3SO_3-GalCer (TCS-I) and anti-$II^3NA\alpha$-LacCer (M2590) antibodies (*12, 16*).

Determination of Thrombin Activity—Thrombin activity was measured with either Boc-Asp(OBzl)-Pro-Arg-MCA or fibrinogen as the substrate (*13*). In the former case, 1 μg of thrombin in 20 μl of 50 mM Tris-HCl (pH 8.0) was preincubated with lipids either suspended in 230 μl of the same buffer by sonication or dissolved in 20 μl of DMSO, followed by dilution with 210 μl of the buffer, at 37°C for 10 min. The proteolytic reaction was started by addition of 50 μl of 0.2 mM Boc-Asp(OBzl)-Pro-Arg-MCA in 2% (v/v) DMSO in the buffer and 500 μl of the buffer, allowed to proceed at 37°C for 20 min, and terminated by the addition of 200 μl of 20% acetic acid. The amount of liberated AMC was determined by measuring its fluorescence intensity at an excitation wavelength of 380 nm and an emission wavelength of 460 nm with a fluorescence spectrophotometer (Hitachi, Tokyo), and by comparing this with a calibration curve obtained with AMC. When fibrinogen was used as the substrate, the effect of various lipids on the activity of thrombin was measured by SDS-PAGE. In brief, thrombin (10 μg) in 10 μl of 50 mM Tris-HCl (pH 8.0) was incubated with 10 μg of lipids in 1 μl DMSO at 37°C for 10 min, followed by incubation with 10 μg of fibrinogen in 10 μl of the same buffer at 37°C for 1 h. The solution was diluted with 20 μl of the sample buffer (10% glycerol, 2% SDS, 0.05% β-mercaptoethanol, and 62.5 mM Tris-HCl, pH 6.8) and boiled for 4 min. The samples thus prepared were electrophoresed on a 12% acrylamide gel, and the gel was stained with Coomassie Brilliant Blue (*17*).

Determination of Plasmin Activity—Plasmin was measured using Boc-Val-Leu-Lys-MCA as the substrate (*14*) by the same procedure as detailed above. Lipids were evaporated to dryness in a test tube, then either dissolved in 20 μl DMSO, followed by dilution with 720 μl of 50 mM Tris-HCl (pH 8.0), or suspended in 740 μl of the buffer by sonication. To the lipids were added 10 μg of plasmin in 10 μl of the same buffer and 50 μl of 0.2 mM substrate in the buffer, and the mixture was incubated at 37°C for 1 h. The reaction was terminated by the addition of 200 μl of 20%

acetic acid, and the AMC released was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm with a fluorescence spectrophotometer.

Analysis of Thrombin and CS-Treated Thrombin by HPLC, Native and SDS-PAGE—To assess the molecular mechanism of its inhibition by CS, thrombin (10 μg) either treated or not treated with 10 μg of CS as described above was injected into a HPLC equipped with a gel permeation column (TSK gel G3000, 7 mmϕ × 60 cm, Toso, Tokyo), and the eluates were monitored at 280 nm and by measuring the specific activity and concentration of CS in each fraction tube. The eluates were also analyzed by native and SDS-PAGE on 12% polyacrylamide gels (*17*).

RESULTS

Acidic Lipids in Human Plasma and Hemocytes—CS, I^{3}SO$_3$-GalCer, and II^{3}NAα-LacCer were contained in the acidic lipid fractions of human plasma and hemocytes. The structures of I^{3}SO$_3$-GalCer and II^{3}NAα-LacCer were identified by comparing their mobilities on a TLC plate with those of standard glycolipids and from their reactions with monoclonal anti-I^{3}SO$_3$-GalCer (TCS-1) and anti-II^{3}NAα-LacCer (M2590) antibodies. CS was also characterized by analysis of the purified material by fast-atom bombardment mass spectrometry, which gave an intense molecular ion, $[M-H]^-$, at m/z 465 and fragment ion, $[HSO_4]^-$, at m/z 97. CS was shown to be ubiquitously distributed in the human plasma and hemocytes, and the highest concentration was noted in the lymphocytes plus monocytes fraction. The concentrations of CS, I^{3}SO$_3$-GalCer, and II^{3}NAα-LacCer in the plasma were 2.0±1.2, 0.6±0.3, and 6.3±0.9 nmol per ml, respectively (Table I). The concentrations of CS in erythrocytes and platelets were 0.6×10^{-18} and 1.7×10^{-18} mol per cell, respectively.

TABLE I. **Concentrations of acidic lipids in human plasma and hemocytes.**

	CS	I^{3}SO$_3$-GalCer	II^{3}NAα-LacCer
		(nmol/ml)	
Plasma	2.0±1.2	0.6±0.3	6.3±0.9
		(nmol/g dry mass)	
Erythrocytes	19.1±1.0	8.2±1.1	24.7±2.5
Platelets	22.0±2.7	15.2±2.6	35.5±2.0
Neutrophils	26.3±1.9	2.0±1.8	14.8±2.3
Lymphocytes plus monocytes	51.5±3.3	7.1±1.5	34.4±2.1

Regulation of the Activity of Thrombin by Sulfated Lipids—Based on our previous observations (*10*), the effects of sulfated lipids and their desulfated counterparts on the activity of thrombin were measured using the chromogenic substrate Boc-Asp(OBzl)-Pro-Arg-MCA. As shown in Fig. 1, thrombin activity was inhibited by sulfated lipids in a dose-dependent manner, but not at all by their desulfated counterparts. However, while CS inhibited thrombin to a similar extent in solution both with and without DMSO, the inhibitory activity of I^{3}SO$_3$-GalCer was not seen in solution without DMSO. Although mixing of thrombin (1 μg) with 1 μg of CS in the buffer alone resulted in a 30% reduction of the activity, further incubation of the mixture at 37°C for 10 min was necessary to attain the maximal level of inhibition (Fig. 2).

Mode of Inhibition of Thrombin by Sulfated Lipids—As

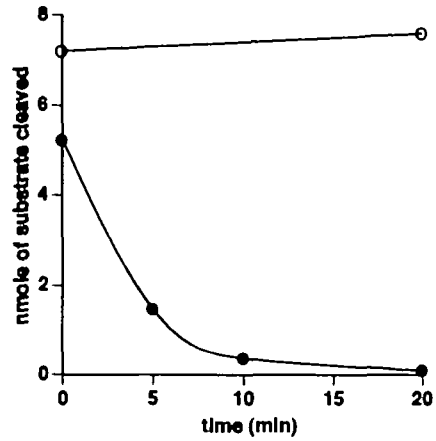

Fig. 2. **Effect of preincubation of thrombin with 1 μg of CS (●) and cholesterol (○) on its activity.** Thrombin activity was measured as described in "MATERIALS AND METHODS," with the indicated preincubation times. Data obtained with and without DMSO were essentially similar.

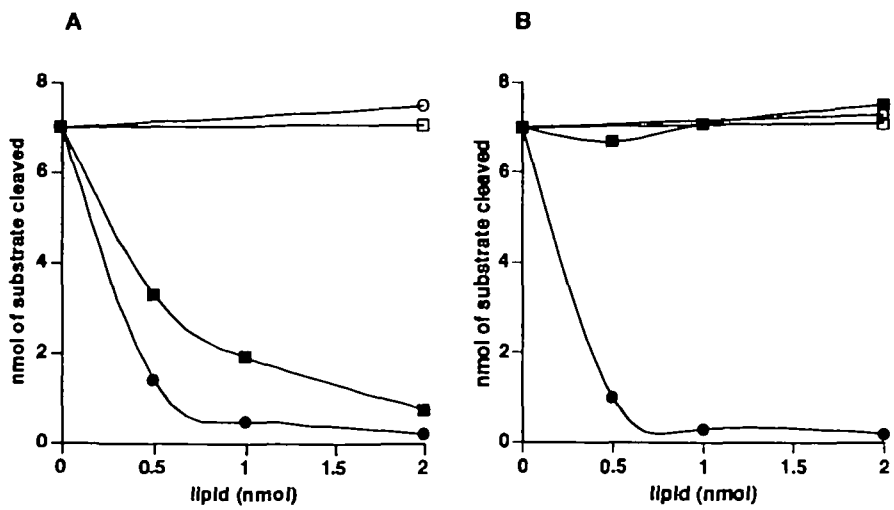

Fig. 1. **Effects of cholesterol (○), GalCer (□), CS (●), and I^{3}SO$_3$-GalCer (■) on the activity of thrombin.** Thrombin activity was measured with Boc-Asp(OBzl)-Pro-Arg-MCA as substrate as described in "MATERIALS AND METHODS," except that preincubation was for 30 min. Dried lipids were dissolved in 20 μl of DMSO and diluted with 210 μl of 50 mM Tris-HCl (pH 8.0) (A) or suspended in 230 μl of the buffer by sonication (B).

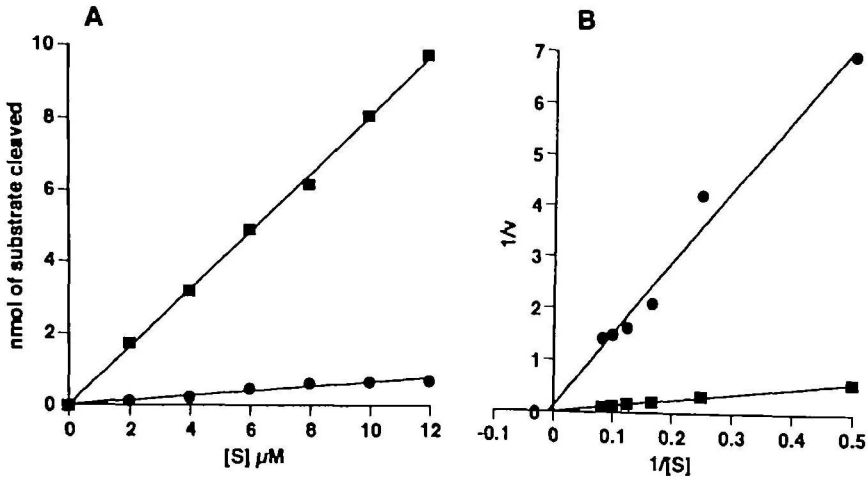

Fig. 3. **Activities of thrombin (1 μg) treated (●) or not treated (■) with CS (0.5 nmol), as a function of substrate concentration (A), and as a double reciprocal plot (B).**

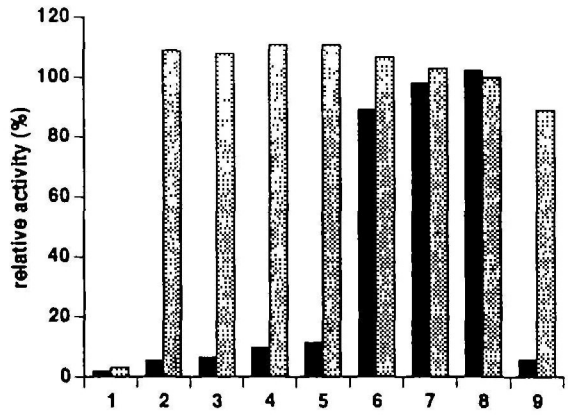

Fig. 4. **Effects of sulfated lipids and II³NAα-LacCer on the activity of thrombin.** Thrombin activity was measured with Boc-Asp(OBzl)-Pro-Arg-MCA as substrate as described in "MATERIALS AND METHODS." Closed column: 20 nmol of lipids were dissolved in 20 μl of DMSO and the solution was diluted with 210 μl of the buffer by sonication; dotted column: 20 nmol of lipids were dissolved and suspended in 230 μl of the buffer. 1, CS; 2, I³SO_3-GalCer; 3, dehydroepiandrosterone sulfate; 4, pregnenolone sulfate; 5, estrone sulfate; 6, SDS; 7, sodium taurocholate; 8, sodium sulfate; 9, II³NAα-LacCer.

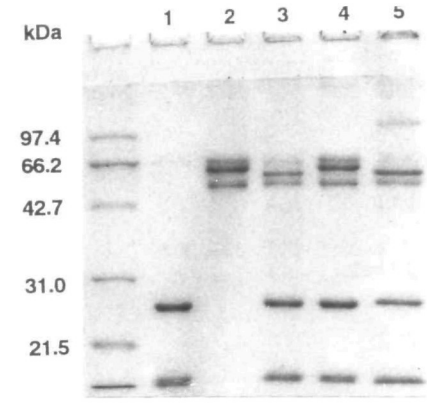

Fig. 5. **SDS-PAGE of thrombin (lane 1), fibrinogen (lane 2), and products of cleavage of fibrinogen by thrombin (lane 3), CS-treated thrombin (lane 4), and cholesterol-treated thrombin (lane 5).** Analysis was performed as described in the text.

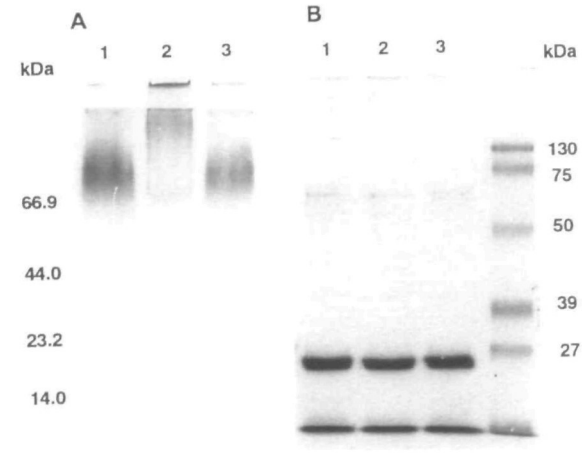

Fig. 6. **Native (A) and SDS-PAGE (B) of thrombin (1), CS-treated thrombin (2), and cholesterol-treated thrombin (3).**

shown in Fig. 1, about 85% of the activity of thrombin (1 μg) was lost by treatment with 0.5 nmol of CS, corresponding to a molar ratio of CS to thrombin of 18:1. This indicates that the concentration of CS in human plasma (Table I) is sufficient for the inhibition of thrombin. As in the case of trypsin (*10*), treatment with CS greatly reduced the V_{max} of thrombin (25.6 nmol/μg protein for original enzyme and 6.4 nmol/μg protein for CS-treated enzyme), but did not affect the value of K_m (1.3×10^{-4} M), suggesting that CS modifies thrombin in a region other than the active site in the mode of noncompetitive inhibition (Fig. 3). The inhibitory effects of excess concentrations (20 nmol) of various lipids including steroid sulfates structurally related to CS were then compared, as shown in Fig. 4. When lipid solutions were prepared by dissolving lipids in 20 μl of DMSO, followed by dilution with 210 μl of 50 mM tris-HCl (pH 8.0), thrombin activity was inhibited not only by CS but also by I³SO_3-GalCer, dehydroepiandrosterone sulfate, pregnenolone sulfate, estrone sulfate, and II³NAα-LacCer. The concentrations of I³SO_3-GalCer, steroid sulfates and II³NAα-LacCer required for 50% inhibition of the total

activity of thrombin were more than three times higher than that of CS, and CS was shown to exhibit the strongest inhibitory activity toward thrombin among the acidic lipids examined. However, SDS and sodium taurocholate, in which the negatively charged sulfate groups are not directly attached to the steroid or carbohydrate ring, did not exhibit any inhibitory activity. In contrast to the DMSO-containing solutions, the same amounts of the lipids dispersed in the buffer by sonication were inhibitory only in the case of CS, suggesting that CS is a physiological inhibitor of thrombin in human plasma, and that steroid sulfates or sulfated and sialylated glycolipids exhibit inhibitory activity only following suitable micellar formation in the presence of DMSO. To further characterize the inhibitory effect of CS on thrombin, its effect on cleavage of fibrinogen, the physiological substrate of thrombin, was examined, and the results are shown in Fig. 5. The A subunit of fibrinogen with a molecular weight of 65 kDa is normally proteolyzed to smaller peptides by thrombin (lane 3), but following treatment with CS, thrombin was completely unable to cleave fibrinogen (lane 4), while in the presence of cholesterol, it readily cleaved fibrinogen at the same rate as the native enzyme (lane 5). Thus, the inhibition of thrombin by CS can be determined by use of either artificial small peptidic substrates or fibrinogen as a physiological substrate.

Binding of CS to Thrombin—To elucidate the molecular mechanism of the inhibitory effect of CS, thrombin was analyzed directly by native and SDS-PAGE, and HPLC before and after treatment with CS. As shown in Fig. 6, the molecular weight of thrombin after incubation with CS at 37°C for 10 min increased to greater than that of its tetramer, and the bulk of the enzyme was incapable of entering the gel, suggesting formation of large aggregates. However, no change in the molecular weight was observed after treatment with cholesterol. On SDS-PAGE, the aggregate formed by CS dissociated into a peptide with the same molecular weight as native thrombin (Fig. 6); and ^{35}S-CS, which was distributed in the region of the enzyme aggregates on native PAGE, was separated from thrombin and electrophoresed together with bromophenol blue at the gel front. Similar results were observed previously for pancreatic elastase (*11*). In addition, in HPLC on a TSK gel G-3000 column, CS-treated thrombin was coeluted with CS at around the void volume (Fig. 7). Activity and molecular weight of the original enzyme could not be determined by HPLC, either by reducing or increasing the concentration of NaCl, or using a buffer containing EDTA (10 mM) or $CaCl_2$

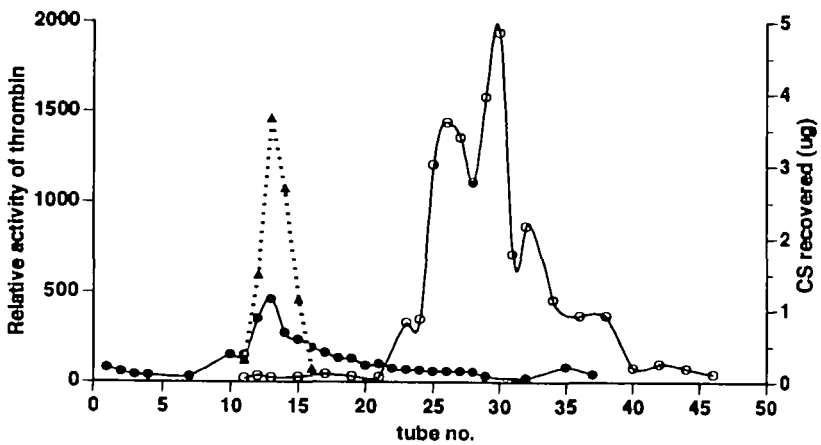

Fig. 7. **HPLC chromatograms of thrombin (○) and CS-treated thrombin (●) as measured by their activities with Boc-Asp(OBzl)-Pro-Arg-MAC (—) and amount of CS recovered from each fraction tube (▲).**

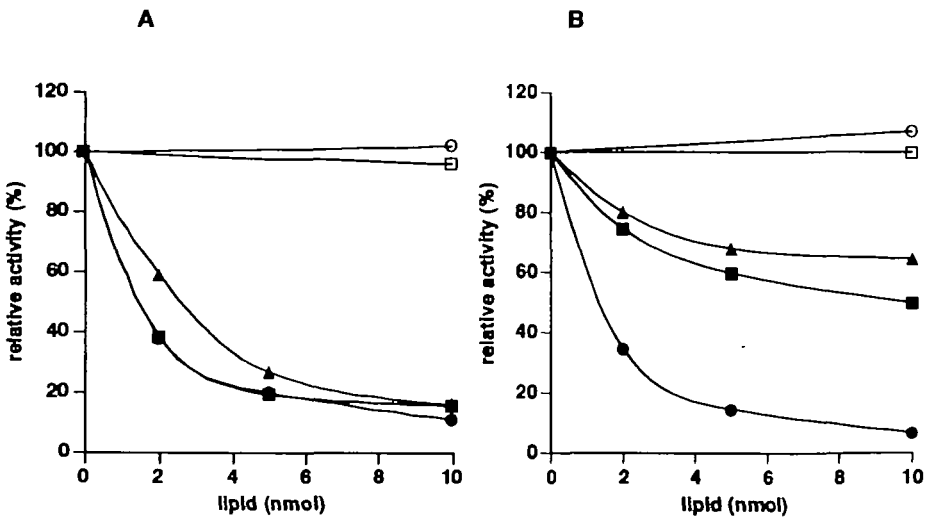

Fig. 8. **Effect of cholesterol (○), GalCer (□), CS (●), I^3SO_3-GalCer (■), and $II^3NA\alpha$-LacCer (▲) on the activity of plasmin.** Dried lipids were dissolved in 20 μl of DMSO and diluted with 720 μl of 50 mM tris-HCl (pH 8.0) (A) or suspended in 740 μl of the buffer by sonication (B). Then 10 μg of plasmin in 10 μl of the buffer was added. The enzyme reaction was started by addition of 50 μl of 0.2 mM Boc-Asp-(OBzl)-Pro-Arg-MCA and 500 μl of the buffer, allowed to proceed at 37°C for 1 h, and terminated by addition of 200 μl of 20% acetic acid. Then AMC liberated was measured with a fluorescence spectrophotometer.

(10 mM), indicating that CS binds strongly to thrombin, leading to irreversible inhibition.

Effect of Acidic Lipids on the Activity of Plasmin—Since plasmin, which is important for thrombolysis, is also a serine protease, the effects of acidic lipids in the human plasma, such as CS, I^3SO_3-GalCer, and $II^3NA\alpha$-LacCer, on the activity of plasmin were examined using similar procedures. All acidic lipids in plasma exhibited inhibitory activity toward plasmin, but their desulfated and desialylated counterparts in solution prepared either with or without DMSO failed to do so (Fig. 8). Although the inhibitory activity of CS as a function of its concentration in the lipid dispersion was identical to that in the DMSO-containing solution, the activities of I^3SO_3-GalCer and $II^3NA\alpha$-LacCer in the buffer were greatly reduced in comparison to those a DMSO-containing solution. In contrast to thrombin, plasmin was inhibited by the acidic lipids even without preincubation of plasmin with the lipid solution. In addition to these acidic lipids, steroid sulfates, dehydroepiandrosterone sulfate, pregnenolone sulfate, and estrone sulfate inhibited plasmin, but 20 nmol of steroid sulfates were required for equivalent inhibitory activity to that observed with 5 nmol of CS. SDS and sodium taurocholate exhibited no inhibitory activity toward plasmin, and the mode of inhibition of plasmin by the various lipid inhibitors was the same as that of thrombin.

DISCUSSION

Like the gastric serine proteases, trypsin, chymotrypsin, and elastase, reported previously (*10, 11*), thrombin and plasmin were found here to be inhibited by acidic lipids. The modes of inhibition by CS of the different proteases examined, however, were different. Preincubation of enzyme with CS was required to attain maximum inhibition of elastase and thrombin, while vortex-mixing without preincubation was sufficient for maximum inhibition of trypsin, chymotrypsin and plasmin. In addition, DMSO-mediated dispersion, probably promoting micellar formation in the buffer, and preincubation were necessary for I^3SO_3-GalCer to inhibit the aforementioned proteases. This appears to be attributable to the fact that a hydrophobic group, and the sulfate group are requisite structures for the inhibitory activity of the sulfated lipids. Among the sulfated lipids examined, SDS and sodium taurocholate, in which the sulfate groups are attached to alkyl side chains, were ineffective as inhibitors, indicating that linkage of the sulfate group to a ring structure, such as cyclopentanoperhydrophenanthrene and carbohydrate, is necessary to maintain a proper distance between the sulfate groups on the micellar surface, as it is in the case of polysulfated thrombin inhibitors, carboxymethyl dextran benzoylamide sulfate and fucoidan (*17, 18*). However, since even micromolar concentrations of CS caused inhibition, CS-treated thrombin formed a high-molecular-weight complex, and dissociation of CS from the CS-thrombin complex was achieved by SDS-PAGE, hydrophobic interaction between the hydrophobic region of enzyme and the hydrophobic moiety of sulfated lipids is thought to be involved in the inhibitory mechanism. The differences in the susceptibility of various proteases to sulfated lipids probably depend on the tertiary structure of the individual peptides, which have an appropriate distance between the sulfate and hydrophobic groups. Computer modeling of the protein structures based on the amino acid sequences of CS-sensitive thrombin and plasmin, and CS-resistant elastase (*P. aeruginosa*) and lysyl endopeptidase (*A. lyticus*) (*10, 11*) failed to provide definitive information on the peptides reacting with CS (*19, 20*). For instance, *P. aeruginosa* elastase, which was not inhibited by CS, contains a greater number of hydrophobic clusters than thrombin, and the distribution of these clusters is not related to that of the basic amino acid residues supposed to have an ionic interaction with the sulfate group. Thus the secondary or tertiary structure rather than the primary structure of peptides is suggested to be involved in interaction of the enzyme with CS. To clarify the peptides that bind to CS, analysis of the amino acid sequence after protease digestion of ^{14}C-CS treated thrombin is now in progress in our laboratory (*21*). When compared with antithrombin III (*22*), fibrinopeptide (*23*), and active site-directed inhibitors such as α-azalysine derivatives (*24*), 5,5-*trans*-fused cyclic lactone euphan triterpene (*25*), and serpin- and antistasin-family inhibitors (*26, 27*), the mode of inhibition of thrombin by CS was different in terms of its inhibition kinetics and its broad inhibition specificity. In contrast with the 1:1 ratio of peptidic inhibitors to thrombin, a molar ratio of CS to thrombin of 18:1 was required for 85% inhibition. CS was liberated from the CS-thrombin complex by SDS-PAGE, in contrast to the SDS-stable complex of proteinase inhibitor 8, which was shown to be covalently bound with the arginine residue of proteases (*27*). Regarding the interaction between CS and thrombin, the anion-binding exosite I in thrombin was thought to react with CS, like sulfated dextran and hirudin (*9, 18*), both of which were shown to form complexes with thrombin. This was also the site of interaction with the acidic domain of heparin cofactor II, whose anticoagulant activity was modified by fucoidan (*18*) and dermatan sulfate (*28*). Under physiological conditions, it is clear that antithrombin III, whose activity is modified by heparin, plays a principal role in regulating the activity of thrombin (*29, 30*). Although heparin and dermatan sulfate suppressed the activity of thrombin up to 75%, complete inhibition, in other words, complete inactivation as induced by CS, was not observed (Iwamori *et al.*, unpublished observation). Accordingly, they primarily act on thrombin as modifiers of heparin cofactor II and antithrombin III. Thus, our finding showed that CS was a candidate as an alternative physiological inhibitor of thrombin in plasma.

The concentration of CS in plasma was 2.0 nmol per ml, which was close to our previous observations (*31*) and those of others (*32*), and was sufficient to inhibit thrombin. However, the source of plasma CS is not yet clearly understood. In general, synthesis of CS is mainly observed in epidermal and epithelial tissues (*12*) and is associated with differentiation (*33*). In particular, steroid hormones were shown to affect CS concentration in tissues: for example, activation of cholesterol sulfotransferase and concomitant suppression of CS sulfatase occurred in the uterine epithelia of rabbit after administration of estradiol and chorionic gonadotropin, resulting in sulfation of one-tenth of the cholesterol in the epithelia (*33, 34*). In human placenta, the amount of CS was significantly increased during pregnancy, probably due to hormonal changes, and a simultaneous increase in its concentration was observed in

human plasma during pregnancy (*31*). Since the concentration of only CS among acidic lipids in human plasma was altered, it was strongly suggested that plasma CS was derived from the placenta and that the activities of thrombin and plasmin were kept under control during pregnancy by hormonal control of the level of CS. Since cholesterol sulfotransferase was detected in the lung, kidney, and the endothelium of blood vessels, but not in the erythrocytes or plasma, CS in plasma probably originated from these tissues, and was distributed in the hemocytes by lipid exchange (*35*). In fact, CS in plasma was shown to be readily transferred to erythrocytes in proportion to its concentration in plasma, and to stabilize the erythrocyte membrane against hypotonic hemolysis (*36*).

Regulation of the activities of functional proteins by CS has recently attracted great interest. Enzymes activated by CS include the η, ε, and ζ forms of protein kinase C (*37, 38*), factor XII, and prekallikrein (*39*), and those inhibited by CS include 3-hydroxy-3-methylglutaryl CoA reductase (*40*), phosphatidyl inositol-3-kinase (*41*), trypsin (*10*), chymotrypsin (*10*), pronase (*10*), and elastase (*11*). Thus, amphipathic lipids with sulfate groups were shown to function not only as biomembrane constituents, but also as endogenous detergents regulating the activity of several functional proteins.

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