Glycoconjugate Journal 17, 787–793, 2000

Inhibitory activity of sulphoglycolipid derivatives towards pancreatic trypsin

Hiroko Suzuki[1], Makoto Ito[2], Takayoshi Kimura[3], Yuriko Iwamori[1] and Masao Iwamori[1,*]

[1]*Department of Biochemistry, Faculty of Science and Technology, Kinki University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502,* [2]*Department of Bioscience and Biotechnology, Division of Bioresource and Bioenviromental Science, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 814-8581, Japan,* [3]*Department of Physical Chemistry, Faculty of Science and Technology, Kinki University, 3-4-1 Kowakae, Higashiosaka, Osaka 577-8502*

Amphipathic sulpholipids have been shown to inhibit pancreatic serine proteases due to their detergent-like properties. To evaluate the structural requirement for this inhibitory activity, we examined the effects of various derivatives of sulphoglycolipids, some of which were prepared by deacylation with sphingolipid ceramide *N*-deacylase, followed by acylation with acyl chloride, on the activity of pancreatic trypsin. Both deacylated sulphatides and seminolipids exhibited inhibitory activity towards trypsin without any requirement for solubilisation and preincubation. On the other hand, stronger inhibition was observed for acylated sulphatides than for deacylated ones, but increasing the chain length of the fatty acid moiety resulted in the need for a solubilisation agent and preincubation in order to achieve maximal inhibitory activity. The structural isomers of sulphoglycolipids, such as I^6SO_3-GalCer, and phytosphingosine- and diglyceride-containing sulphoglycolipids, showed similar inhibitory activity, indicating the involvement of sulphate and hydrophobic groups, irrespective of the fine structure, in the inhibition. Among the sulphoglycolipids examined, II^3SO_3-LacCer was found to exhibit the highest inhibitory activity.

Keywords: **amphipathic lipid, micelle, seminolipid, sphingolipid, sulphatide**

Introduction

Two major types of sulphoglycolipids, sulphatides and seminolipids, whose core structures are those of sphingo- and glyceroglycolipids, respectively, exhibit restricted distributions in mammalian tissues. The former has been detected in the myelin sheath, the epithelia of the gastrointestinal tract and the kidneys, while the latter has been exclusively found in the testis [1,2]. The syntheses of sulphatides in myelin and seminolipids in testis are triggered at the stages of myelination and spermatogenesis, respectively. Disruption of the gene encoding the biosynthetic enzyme for the precursor glycolipids of sulphatides has been found to result in an electrophysiological deficit, showing the significance of ensuring a proper axo-oligodendrocyte interaction [3,4]. In the gastrointestinal tract, sulphatides are localized in the epithelial lining of the mucosa [5]. Those on the basolateral side bind to laminin in the basement membrane to form epithelial glandular structures [6], whereas those on the apical side endow the gastric epithelial cells with acid-resistant properties [7], and act as receptors for *Helicobactor pylori* [8] and selectins [9]. In addition, sulphatides have been detected in gastric juices [10], and found to inhibit pancreatic proteases, suggesting that they play a role in protection against the actions of endogenous and exogenous proteases [11–13]. However, the same inhibitory activity towards proteases was observed with cholesterol sulphate (CS) in the gastrointestinal epithelia, but not with steroid sulphates without the hydrophobic side chain of CS, indicating that both the sulphate group and proper hydrophobicity are requisite for sulpholipids with inhibitory activity towards proteases [11–13]. The interaction of sulpholipids with bioactive proteins was thought to be due to their detergent properties, but some proteases, particularly those of bacterial origin, were not inhibited by sulphatide or CS at all [11,12], suggesting that the structure of enzyme proteins is also involved in the susceptibility to sulpholipids. Thus, the relationship between the structure of sulpholipids and their inhibitory activity towards proteases is an important subject, not only for clarifying the functional significance of natural detergents, but also for applying them to regulation of the protease activity as anti-ulcer agents. In this study, we prepared various sulphoglycolipids, and compared their inhibitory activities towards pancreatic trypsin.

*To whom correspondence should be addressed: Masao Iwamori.
Tel.: +81-6-6721-2332; Fax: +81-6-6723-2721;
E-mail: iwamori@chem.kindai.ac.jp

Materials and methods

Materials

A sulphatide, 3′-sulpho-*β*-D-galactopyranosyl ceramide, and a seminolipid, 1-*O*-hexadecyl-2-*O*-acyl-3-*O*-(3′-sulpho-*β*-D-galactopyranosyl)-*sn*-glycerol, were purified in our laboratory from bovine brain and boar testis, respectively. A lyso-seminolipid, 1-*O*-hexadecyl-2-hydroxy-3-*O*-(3′-sulpho-*β*-D-galactopyranosyl)-*sn*-glycerol, was prepared from the seminolipid by saponification with 0.5 M NaOH in methanol at 40°C for 30 min and then purified by silica gel column chromatography (6RS8010, Iatrobeads, Iatron, Tokyo) [14]. 6′-Sulpho-*β*-D-galactopyranosyl ceramide (I^6SO_3-GalCer) was kindly donated by Dr. K Akiyama, Tokyo Metropolitan Institute of Gerontology, Tokyo.

Preparation of sulphatides with various chain lengths of fatty acids

Deacylation of sulphatides was performed by incubation with sphingolipid ceramide *N*-deacylase (*Pseudomonas sp*. TK4) in 20 mM acetate buffer (pH 6.0) containing 0.8% (w/v) Triton X-100 at 37°C for 18 h [15], and the product, lyso-sulphatide, was purified by silica gel column chromatography (6RS8010; Iatrobeads) with a gradient of chloroform/methanol/water (70 : 30 : 0.5 and 70 : 30 : 5, by vol.). Then, the lyso-sulphatide (10 μmol) was acylated with 10 μmol of acyl chloride in 0.4 ml of tetrahydrofuran and 0.5 ml of sodium acetate (50% in water) for 90 min, and the products were purified by solvent partitioning [16], followed by silica gel column chromatography as above. The structures of the products were confirmed by negative ion fast atom bombardment mass spectrometry (FABMS) as follows [17]. About 5 μg of glycolipid in chloroform/methanol (2 : 1, by vol.) was mixed with 2 μl of triethanolamine, followed by analysis through collision with a neutral xenon beam with a kinetic energy of 4 keV and detection of negative ions with a mass spectrometer (HX-110; JEOL, Tokyo). Perfluoroalkyl phosphazine was used for assignment of the mass numbers. In addition, phytosphingosine and monoalkylglycerol were analysed by gas liquid chromatography-mass spectrometry (GC-MS) as their *N*-acetyl-*O*-trimethylsilyl and *O*-trimethylsilyl derivatives, respectively [14].

Determination of trypsin activity

Trypsin (EC 3.4.21.4, porcine pancreas; Wako Chemicals, Tokyo) was measured with either casein or *N*-α-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) as the substrate [11]. Prior to the reaction, trypsin (20 μg) in 350 μl of 100 mM Tris-HCl (pH 8.0) was incubated with a glycolipid or CS in 50 μl of either the same buffer containing dimethylsulphoxide (DMSO), or the buffer containing 10 μg of bile acids at 37°C for various times. The enzyme activity was measured with 450 μg of casein in 650 μl of the buffer, or 250 μg of BAPNA in 50 μl of DMSO and 350 μl of the buffer at 30°C for 20 min. The reaction with casein was terminated by the addition of 150 μl of 15% of trichloroacetic acid, and then the optical density at 280 nm was measured after centrifugation at 15000 rpm for 10 min. While the reaction with BAPNA was terminated by the addition of 200 μl of 45% acetic acid, followed by measurement of the optical density at 405 nm with *p*-nitroaniline as the product.

Results

Sulphoglycolipid derivatives

The structures of the major sulphoglycolipids used in this experiment are listed in Figure 1. On GC-MS, 2-D-amino-octadec-4-ene-1,3-D-diol (4-sphingenine) was found to com-

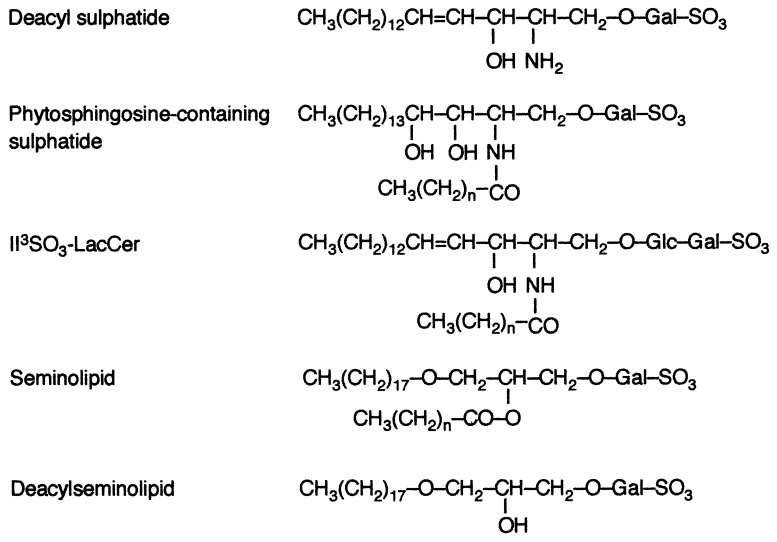

Figure 1. Structures of glycolipids.

prise more than 97% of the long chain bases of sulphatides (I^3SO_3-GalCer) on the synthesis of the sulphated derivatives with different fatty acids, and 4-sphingenine and 2-D-aminooctadecane-1,3,4-triol (phytosphingosine) were the major long chain bases of II^3SO_3-LacCer (89%) and phytosphingosine-containing sulphatide (93%), respectively. Also, batyl alcohol was exclusively present in the monoalkylglycerol (90%) of the seminolipid. The purity of the glycolipids was sufficient, as determined by TLC (Figure 2) and negative ion FABMS (Figure 3). The molecular ions at m/z 540, m/z 582 and m/z 694 were in accord with deacylated sulphatide, *N*-acetyl sulphatide and *N*-caproyl sulphatide, respectively. Sulphatides with nervonic acid (24 : 1) increased on the purification of brain sulphatides by silica gel column chromatography, while the sulphatides giving the molecular ion at m/z 906 in spectra 5 and 6 in Figure 3 were found to have *N*-cerebronoyl 4-sphingenine and *N*-nervonoyl phytosphingosine as their ceramide moieties, respectively. Similarly, *N*-cerebronoyl 4-sphingenine was the major ceramide of I^6SO_3-GalCer (spectrum 7 in Figure 3). On the other hand, the long chain base and fatty acid compositions of II^3SO_3-LacCer comprised 4-sphingenine and palmitic to lignoceric acids, respectively, yielding the molecular ions between m/z 940 to m/z 1052 (spectrum 8 in Figure 3).

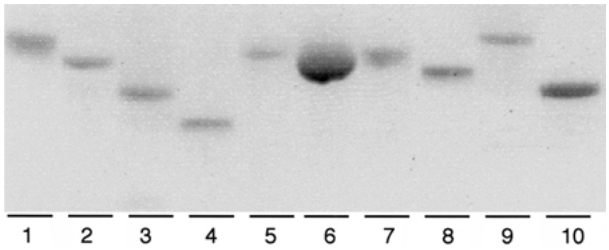

Figure 2. TLC of various derivatives of sulphatides and seminolipids. Glycolipids were subjected to TLC with chloroform/methanol/water (65 : 35 : 8, by vol.), and the spots were visualised with orcinol-H_2SO_4 reagent. 1, sulphatides from bovine brain; 2, sulphatide with capric acid; 3, sulphatide with acetic acid; 4, deacylated sulphatide; 5, sulphatides with 2-hydroxy fatty acids from bovine brain; 6, sulphatide with phytosphingosine from human meconium; 7, I^6SO_3-GalCer; 8, II^3SO_3-LacCer; 9, seminolipid from boar testis; 10, deacyl seminolipid.

Effects of sulphoglycolipid derivatives on trypsin

As reported previously [10–13], although CS exhibits inhibitory activity towards several proteases including trypsin, steroid sulphates without the hydrophobic side chain of CS did not inhibit proteases at all, indicating that both the sulphate group and appropriate hydrophobicity are required for the inhibitory activity of sulpholipids. Accordingly, we first examined the inhibitory activity of deacylated derivatives of the sulphatide and seminolipid towards pancreatic trypsin, as shown in Figure 4. The deacylated seminolipid inhibited the activity of trypsin in a dose-dependent manner, and its

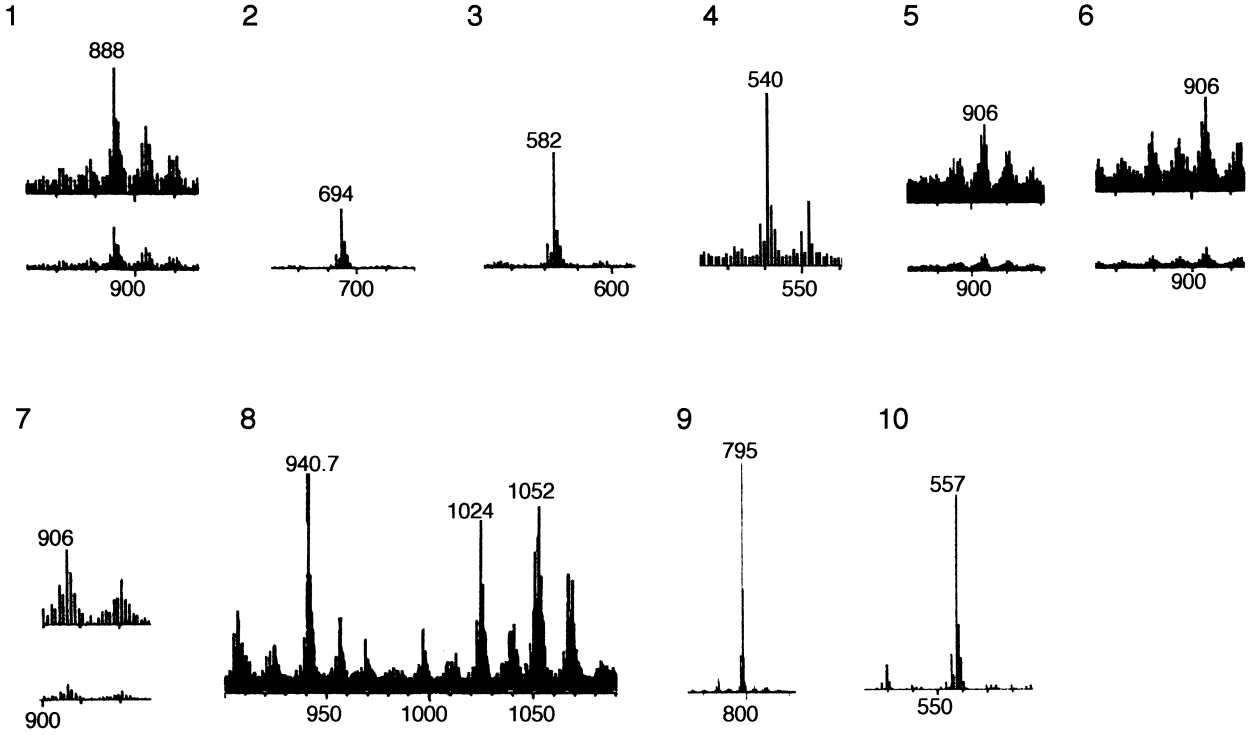

Figure 3. Molecular ions of various derivatives of sulphatides and the seminolipid on negative ion FABMS. The numbers of the spectra are same as those in the legend to Figure 2.

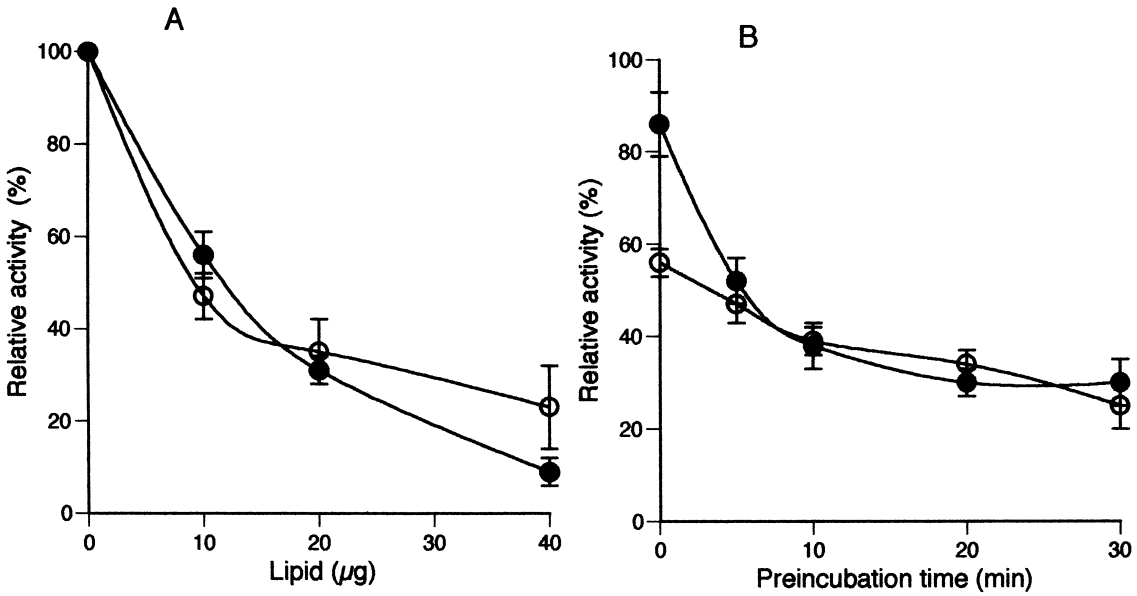

Figure 4. Effects of cholesterol sulphate (●) and the deacylated seminolipid (○) on the activity of pancreatic trypsin as functions of the amount of sulpholipids (A) and the preincubation time (B). Trypsin (40 μg) was preincubated with various amounts of sulpholipids in DMSO (50 μl) for A, and with 20 μg of sulpholipids in DMSO (50 μl) for B, and then the activity was measured as described in the text.

inhibitory activity did not require a solubilising agent, such as DMSO and sodium deoxycholate (10 μg), indicating that its solubility in buffer alone was sufficient for the inhibitory activity due to its relative hydrophilicity. This was in contrast to in the case of CS, which has to be dissolved in DMSO or detergent-containing buffer for maximal inhibition, CS inhibits trypsin by up to 35% when dissolved in buffer; this figure rising to 95% for CS dissolved in DMSO [10]. Exposure of trypsin to the deacylated seminolipid without preincubation resulted in an inhibition of the activity to a level higher than that in the case of CS, showing that the deacylated seminolipid readily reacts with trypsin in comparison to CS (Figure 4B). Also, the deacylated sulphatide showed the same inhibitory activity as above, while no inhibition was observed with sodium dodecyl sulphate, sodium cholate, sodium deoxycholate or sodium taurocholate under the same conditions, probably due to their insufficient hydrophobicity compared with that of the deacylated seminolipid and sulphatide. Inhibition by the deacylated seminolipid occurred in a noncompetitive manner, and the activity of trypsin treated with the deacylated seminolipid could not be restored by gel permeation chromatography on TSK-3000 gel (Toso, Tokyo), indicating the irreversible inhibition by sulpholipids. Also, the molar ratio of deacylated seminolipid to trypsin for 50% inhibition was 18 : 1. The results obtained using BAPNA as described above were identical with those obtained using casein as the substrate. Then, the effects of various derivatives of sulphoglycolipids on trypsin were determined, as shown in Figures 5 and 6. Sulphatides with longer chain fatty acids exhibited a stronger inhibitory activity than those with shorter ones, when the activity was compared with the same amount of sulphatides. Also, structural isomers, such as I^6SO_3-GalCer, and phytosphingosine- and diglyceride-containing sulphoglycolipids, showed similar inhibitory activity, indicating the involvement of the sulphate and hydrophobic groups, irrespective of the fine structure, in the inhibition. Among the sulpholipids examined, II^3SO_3-LacCer was found to exhibit the highest inhibitory activity.

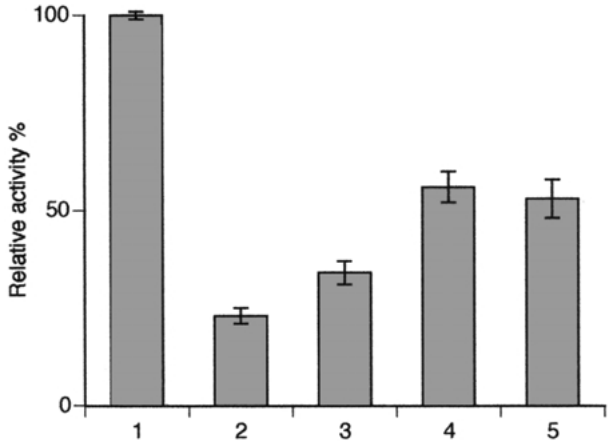

Figure 5. Effects of sulphatides with various chain lengths of fatty acids on the activity of trypsin. Trypsin (40 μg) was preincubated with 10 nmoles of sulphatide in DMSO (50 μl), and then the activity was measured as described in the text. 1, control (DMSO alone as a solubilising agent); 2, brain sulphatides with nervonic acid as the major fatty acid; 3, sulphatide with capric acid; 4, sulphatide with acetic acid; 5, deacylated sulphatide.

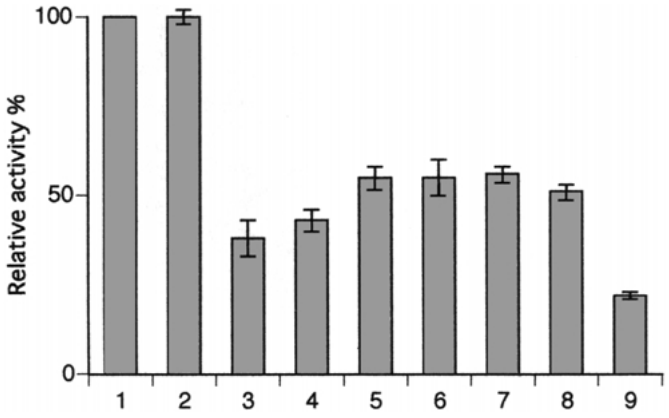

Figure 6. Effects of various sulpholipids on the activity of trypsin. Trypsin (40 μg) was preincubated with 10 nmoles of sulpholipids in DMSO (50 μl), and then the activity was measured as described in the text. 1, control (DMSO alone as a solubilising agent); 2, sodium dodecyl sulphate; 3, cholesterol sulphate; 4, seminolipid; 5, deacylated seminolipid; 6, brain sulphatides with cerebronic acid as the major fatty acid; 7, phytosphingosine-containing sulphatide; 8, I^6SO_3-GalCer; 9, II^3SO_3-LacCer.

Discussion

Amphipathic lipids carrying both hydrophobic and hydrophilic groups are essentially detergents in nature, and their behavior in aqueous media, such as micellar and bilayer formation, is known to be largely influenced by their structures. The chain lengths of fatty acids and long chain bases in the ceramide moiety, and the types of carbohydrates of the glycosphingolipids determine the relative masses, hydrodynamic radii and Svedberg coefficients of their micelles [18–20], as well as their binding capacity with proteins and cells [21–23].

Due to the diversity in the hydrophilic carbohydrate structures of glycosphingolipids, one can expect that they have a wide range of physicochemical properties. In fact, sialoglycosphingolipids in the phospholipid bilayer are known to form a cluster [24], and some of them have been supposed to play a role in the transfer of lipophilic steroid hormones as mediators [25]. Also, glycosphingolipids with shorter fatty acids in the cell membrane have been found to be shed into the culture medium [26], and tumor cells grown to high density in either culture or host animals have been found to release glycosphingolipids into the cell-conditioned medium or the plasma [27,28]. We also found that sulphatides were present in the plasma and gastric juices [10,28]. When the lipid compositions of the epithelia of the digestive tract and the gastric juices were compared, the relative concentration of sulphatide in the gastric juices was found to be exceedingly high, indicating that sulphatide in the epithelia is released from the epithelia more easily than the other lipids, including phospholipids and neutral glycolipids. Also, sulphatide released into the gastric juices was associated with proteins, probably through their hydrophobic and sulphate groups [10,21]. Since a hydrophobic interaction is the major force maintaining the tertiary structure of globular proteins in water, binding with sulphatides might cause alteration of the structure. Concerning the sulphate group, an anion binding exosite I exhibiting the strong affinity with the sulphate group of globular proteins was shown to be involved in the inhibition by sulphated dextran and hirudin of thrombin [29,30]. Accordingly, sulphoglycolipids with both sulphate and hydrophobic groups have the ability to interact with proteins and to modify their physiological activity, as reported in the case of trypsin in this paper. The reason why no inhibitory activity was observed with sodium dodecyl sulphate, sodium deoxycholate or sodium taurocholate seemed to be due to low hydrophobicity being insufficient for modification of the structure and activity of trypsin.

The deacylated sulphatide, 3′-sulpho-β-D-galactopyranosyl 4-sphingenine, and deacylated seminolipid, 1-*O*-hexadecyl-2-hydroxy-3-*O*-(3′-sulfo-β-D-galactopyranosyl)-*sn*-glycerol, both of which are composed hydrophobic groups with the chain length of C18, had the ability to inhibit trypsin, and increasing chain length of the fatty acids resulted in stronger inhibitory activity. In other words, sulphoglycolipids with lower hydrophobicity are required a greater amounts than ones with higher hydrophobicity to exhibit the same inhibitory activity. In accordance with the general properties of detergents, that is, micellar formation in water, sulphatides with longer chain fatty acids, which are difficult to solubilise in water, required the aid of a solubilising agent, such as DMSO, bile acids and detergents, to express their maximum activity, but deacylated sulphoglycolipids were readily solubilised in water without a solubilising agent and inhibited the activity, indicating that the inhibitory activity is dependent on the form of the micelles of sulphoglycolipids. A difference in the physicochemical properties between native sulphoglycolipids and their deacylated derivatives was also observed in the time of preincubation of trypsin with the lipids at 37°C, the latter inhibiting trypsin to a significant extent without preincubation. Micelles of sodium dodecyl sulphate, bile acids and steroid sulphates were incapable of inhibiting enzymes due to their lower hydrophobicity. In addition to the hydrophobic group, the contribution of the carbohydrate portion to the micellar structure seemed to be significant, since II^3SO_3-LacCer showed the highest inhibitory activity, suggesting that a proper distance of the sulphate group on the micellar surface is involved in the affinity between proteins and micelles. To clarify the effect of the carbohydrate moiety, the preparation of various sulphoglycolipids, including I^6SO_3-LacCer, II^3SO_3-Gg_3Cer, I^6, II^6, $III^6(SO_3)_3$-Gg_3Cer, IV^3SO_3-Gg_4Cer, II^3SO_3-Gg_4Cer and II^3, $IV^3(SO_3)_2$-Gg_4Cer, is now in progress in our laboratory.

However, the inhibition of enzymes by sulphoglycolipid micelles was nonspecific, but the enzymes were not always susceptible to the micelles. For instance, the activities of elastase from *Pseudomonas aeruginosa* and lysyl endopeptidase from *Achromobacter lyticus* were not inhibited even by a higher dose of CS or sulphoglycolipids [11,12], and triggering of the enzyme activity by sulpholipids was observed for the η,

ε and ξ forms of protein kinase C, factor XII and prekallikrein [31–33]. The formation of complexes of proteins with sulphoglycolipid detergents was thought to be important for regulation of the physiological activity. Sulphoglycolipids shed into the gastric juice were present at sufficient concentrations to inhibit the enzyme activity, and might be solubilised in a region close to the duodenal epithelia by bile acids secreted together with pancreatic enzymes for the inhibitory activity. Thus, a novel property of glycolipids as detergents was thought to be involved in various cellular events. Although the research interest in glycolipids so far has been mainly in their carbohydrate portion, the physicochemical properties of glycolipids and their derivatives must be involved in the regulation of bioactive proteins, and some of the bioactivities of glycolipid derivatives reported in the past [34] should be related with their detergent properties.

References

1 Farooqui AA, Metabolism of sulpholipids in mammalian tissues, *Adv Lipid Res* **18**, 159–202 (1981).
2 Ishizuka I, Suzuki A, Yamakawa T, Isolation and characterization of a novel sulphoglycolipid, 'seminolipid,' from boar testis and spermatozoa, *J Biochem* **73**, 77–86 (1973).
3 Coetzee T, Fujita N, Dupree J, Shi R, Blight A, Suzuki K, Suzuki K, Popko B, Myelination in the absence of galactocerebroside and sulphatide: normal structure with abnormal function and regional instability, *Cell* **86**, 209–19 (1996).
4 Dupree J, Coetzee T, Blight A, Suzuki K, Popko B, Myelin galactolipids are essential for proper node of Ranvier formation in the CNS, *J Neurosci* **18**, 1642–9 (1998).
5 Sugano K, Tai T, Kawashima I, Kotani M, Natomi H, Kamisago S, Fukushima Y, Yazaki Y, Iwamori M, Localization of sulphatides in the epithelial lining of gastric mucosa. Studies with a monoclonal antibody to sulphatides, *J Clin Gastroenterol* **21**, 98–103 (1995).
6 Roberts DD, Rao CN, Magnani JL, Spitalnik SL, Liotta LA, Ginsburg V, Laminin binds specifically to sulphated glycolipids, *Proc Natl Acad Sci USA* **82**, 1306–10 (1985).
7 Natomi H, Sugano K, Takaku F, Iwamori M, Glycosphingolipid composition of the gastric mucosa. A role of sulphatides in gastrointestinal mucosal defense, *J Clin Gastroenterol* **12**, 52–7 (1990).
8 Saitoh T, Natomi H, Zhao WL, Okuzumi K, Sugano K, Iwamori M, Nagai Y, Identification of glycolipid receptors for *Helicobacter pylori* by TLC-immunostaining, *FEBS Lett* **282**, 385–7 (1991).
9 Bengtsson T, Grenegard M, Olsson A, Sjogren F, Stendahl O, Zalavary S, Sulphatide-induced L-selectin activation generates intracellular oxygen radicals in human neutrophils: modulation by extracellular adenosine, *Biochim Biophys Acta* **1313**, 119–29 (1996).
10 Iwamori M, Suzuki H, Kimura T, Iwamori Y, Shedding of sulphated lipids into gastric fluid and inhibition of pancreatic DNase I by cholesterol sulphate in concert with bile acids, *Biochim Biophys Acta* **1487**, 268–74 (2000).
11 Iwamori M, Iwamori Y, Ito N, Sulphated lipids as inhibitors of pancreatic trypsin and chymotrypsin in epithelium of the mammalian digestive tract, *Biochem Biophys Res Commun* **237**, 262–5 (1997).
12 Ito N, Iwamori Y, Hanaoka K, Iwamori M, Inhibition of pancreatic elastase by sulphated lipids in the intestinal mucosa, *J Biochem* **123**, 107–14 (1998).
13 Iwamori M, Suzuki H, Iwamori Y, Epithelial differentiation associated expression of cholesterol sulphate and its function as the inhibitor of pancreatic proteases, *Recent Res Dev Lipid Res* **3**, 97–109 (1999).
14 Iwamori M, Sawada K, Hara Y, Nishio M, Fujisawa T, Imura H, Nagai Y, Neutral glycosphingolipids and gangliosides of bovine thyroid, *J Biochem* **91**, 1875–87 (1982).
15 Ito M, Kurita T, Kita K, A novel enzyme that cleaves the *N*-acyl linkage of ceramides in various glycosphingolipids as well as sphingomyelin to produce their lyso forms, *J Biol Chem* **270**, 24 370–4 (1995).
16 Folch J, Lees M, Sloane-Stanley GT, A simple method for the isolation and purification of total lipids from animal tissues, *J Biol Chem* **226**, 497–509 (1957).
17 Iwamori M, Shimomura J, Tsuyuhara S, Nagai Y, Gangliosides of various rat tissues: distribution of ganglio-*N*-tetraose-containing gangliosides and tissue-characteristic composition of gangliosides, *J Biochem* **95**, 761–70 (1984).
18 Yohe HC, Roark DE, Rosenberg A, C20-sphingosine as a determining factor in aggregation of gangliosides, *J Biol Chem* **251**, 7083–7 (1976).
19 Corti M, Degiorgio V, Ghidom R, Sonnino S, Tettamanti G, Laser-light scattering investigation of the micellar properties of gangliosides, *Chem Phys Lipids* **26**, 225–38 (1980).
20 Ulrich-Bott B, Wiegandt H, Micellar properties of glycosphingolipids in aqueous media, *J Lipid Res* **25**, 1233–45 (1984).
21 Hayashi K, Katagiri A, Studies on the interaction between gangliosides, protein and divalent cations, *Biochim Biophys Acta* **337**, 107–17 (1977).
22 Callies R, Schwarzmann G, Radsak K, Siegert R, Wiegandt H, Characterization of the cellular binding of exogenous gangliosides, *Eur J Biochem* **80**, 425–32 (1977).
23 Tomasi M, Roda LG, Ausiello C, D'Agnolo G, Venerando B, Ghidoni R, Sonnino S, Tettamanti G, Interaction of GM1 ganglioside with bovine serum albumin: formation and isolation of multiple complexes, *Eur J Biochem* **111**, 315–24 (1980).
24 Delmelle M, Dufrane SP, Brasseur R, Ruysschaert JM, Clustering of gangliosides in phospholipid bilayers, *FEBS Lett* **121**, 11–14 (1980).
25 Takamiya K, Yamamoto A, Furukawa K, Zhao J, Fukumoto S, Yamashiro S, Okada M, Haraguchi M, Shin M, Kishikawa M, Shiku H, Aizawa S, Furukawa K, Complex gangliosides are essential in spermatogenesis of mice: possible roles in the transport of testosterone, *Proc Natl Acad Sci USA* **95**, 12 147–52 (1998).
26 Young WW Jr, Borgman CA, Wolock DM, Modes of shedding of glycosphingolipids from mouse lymphoma cells, *J Biol Chem* **261**, 2279–83 (1986).
27 Shaposhnikova GI, Prokazova NV, Buznikov GA, Zvezdina ND, Teplitz NA, Bergelson LD, Shedding of gangliosides from tumor cells depends on cell density, *Eur J Biochem* **140**, 567–70 (1984).
28 Cui Y, Iwamori M, Distribution of cholesterol sulphate and its anabolic and catabolic enzymes in various rabbit tissues, *Lipids* **32**, 599–604 (1997).

29 Bock PE, Olson ST, Bjork I, Inactivation of thrombin by antithrombin is accompanied by inactivation of regulatory exosite I, *J Biol Chem* **272**, 19 837–45 (1997).
30 Church FC, Meade JB, Treanor RE, Whinna HC, Antithrombin activity of fucoidan. The interaction of fucoidan with heparin cofactor II, antithrombin III, and thrombin, *J Biol Chem* **264**, 3618–23 (1989).
31 Ikuta T, Chida K, Tajima O, Matsuura Y, Iwamori M, Ueda Y, Mizuno K, Ohno S, Kuroki T, Cholesterol sulphate, a novel activator for the eta isoform of protein kinase C, *Cell Growth Differ* **5**, 943–47 (1994).
32 Denning MF, Kazanietz MG, Blumberg PM, Yuspa SH, Cholesterol sulphate activates multiple protein kinase C isoenzymes and induces granular cell differentiation in cultured murine keratinocytes, *Cell Growth Differ* **6**, 1619–26 (1995).
33 Shimada T, Kato H, Iwanaga S, Iwamori M, Nagai Y, Activation of factor XII and prekallikrein with cholesterol sulphate, *Thromb Res* **38**, 21–31 (1985).
34 Nagai Y, Iwamori M, Biology of gangliosides. In *Biology of Sialic Acids*, edited by Rosenberg A (Plenum, New York, 1995), pp. 197–241.

Received 27 December 2000, revised 1 March 2001, accepted 2 March 2001