Analyses of Glycolipids from Fish, Shellfish, and Sea Snake Lipids by High-Performance Liquid Chromatography

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To determine the existence of glycolipids (neutral glycosphingolipid and glycoglycerolipid) in sea snake, round frigate mackerel, sardine, sea urchin, and abalone, we performed silica gel chromatography and high-performance liquid chromatography (HPLC) using an Aquasil-SS column and a C_8-reversed phase silica gel column. HPLC with a UV absorption detector was used to analyze neutral glycosphingolipid. These chromatograms showed typical peaks in round frigate mackerel lipid, in sea snake crude fat, in abalone intestine lipid, and in sea urchin intestine lipid. UV-HPLC was also used to analyze glycoglycerolipid. These chromatograms indicated a large peak in round frigate mackerel lipid and a small peak in purified sardine oil. In addition, we observed the same peaks in the glycolipid fraction of round frigate mackerel muscle lipids and sea snake crude fat using a differential refractometer detector. The results of this study suggest that the peaks are neutral glycosphingolipid or glycoglycerolipid and that neutral glycosphingolipid and glycoglycerolipid may have specific physiological functions in each living creature.

Keywords: *Glycolipid; sea snake; round frigate mackerel; sardine; sea urchin; abalone; HPLC*

INTRODUCTION

Glycosphingolipid, a class of glycolipids, is composed of hydrophobic ceramide (sphingosine and fatty acids) and hydrophilic oligosaccharide chains. Glycolipid is one of the components of cell plasma membrane. Several functional studies of glycosphingolipid in the fields of cell biology and hematology have recently been reported (Nakamura, 1997). It contributes to the function of the carbohydrate-rich glycocalyx and thus affects the surface properties of cells. Recent growing interest in the physiological roles of glycosphingolipid has generated a need to separate glycolipid from a variety of species and tissues. High-performance liquid chromatography (HPLC) is a rapid and sensitive analytical method commonly used to separate glycolipids including glycosphingolipid (Ullman and McCluer, 1987; McCluer and Gross, 1985; Kaye and Ullman, 1984).

In oriental societies, sea snake (*Laticauda semifasciata*) has long been known to relieve general tiredness, muscle stiffness of the shoulder, dysesthesia in hands and legs, decreased body stamina, and constipation. It has also been established that fish oil containing *n*-3 polyunsaturated fatty acids, such as eicosapentaenoic and docosahexaenoic acids, prevents coronary artery disease (Conner and Conner, 1997) and cancer (Singh et al., 1997; Jiang et al., 1997; Kato et al., 1997) and increases learning and memory ability (Salem et al., 1986; Neuringer et al., 1988; Suzuki et al., 1998). Furthermore, fish oil is known to alleviate the symptoms of inflammatory disorders such as rheumatoid arthritis (Kremer et al., 1995; Endres et al., 1995) and atopic dermatitis (Ziboh, 1996). Many studies on the isolation of lipid components such as neutral lipids and phospholipids extracted from fish have been conducted. However, there have been few reports on the glycolipid content of different fish, shellfish, and sea snake.

In the present study, we extracted glycolipids from different fish, shellfish, and sea snake by silica gel chromatography. We then analyzed glycolipid classes (neutral glycosphingolipid and glycoglycerolipid) by HPLC with ultraviolet (UV) absorption and differential refractometer (RI) detectors to determine the existence of glycolipids and to separate different types of glycolipids.

MATERIALS AND METHODS

Reagents. Chloroform, acetone, methanol, 2-propanol, *n*-hexane, and distilled water of HPLC grade were obtained from Wako Pure Chemicals (Osaka, Japan) and were used without further purification. Sodium sulfate (Na_2SO_4) and silica gel (45−75 μm, for silica gel column chromatography) were also purchased from Wako Pure Chemicals.

Materials. Crude fat of sea snake (*L. semifasciata*) extracted with *n*-hexane was obtained from Fuji Pharmaceutical Co., Ltd. (Tokyo, Japan). Sardine (*Sardinella brasiliensis*) oil was provided by NOF Corp. (Tokyo, Japan) and was purified by centrifuging, degassing, decoloring, and deodoring after it was heated and pressed (Kinsella, 1987). Round frigate mackerel (*Auxis rochei risso*) was obtained from Kouchi Prefectural Industrial Technology Center (Kouchi, Japan), and sea urchin (*Echinoidea*) intestine was provided by Kuji (Iwate, Japan). Frozen abalone (*Sulculus diversicolor*) harvested from the coast of Australia was purchased. All of the materials were kept at −25 °C and used within 1 month.

Sample Preparations. Lipids were extracted from the muscle of round frigate mackerel and the intestine of abalone and sea urchin according to the method of Bligh and Dyer (1959). Each sample (30 g) was combined with 60 mL of chloroform and 120 mL of methanol and homogenized for 2 min in a Waring blender (Nihon Seiki Seisakusho Co., Tokyo,

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Japan). Sixty milliliters of chloroform and 60 mL of distilled water were added to each extract, and each resulting mixture was stirred for 30 s. The extract was then filtered through a Whatman No. 2 filter paper using a Büchner funnel into a 500-mL sidearm flask under vacuum. The filtrate was transferred to a 250-mL separatory funnel and partitioned into organic solvents by vigorously shaking the solution for 20−30 s. Sufficient time was allowed for the layers to separate, and the chloroform layer was drained into a flask over anhydrous Na_2SO_4. The chloroform layer was transferred to a round-bottom flask after standing for 10 min at room temperature. The extract was evaporated to dryness using a rotary vacuum evaporator (Tokyo Rikakikai, Co., Tokyo, Japan) with the water bath heated at 40 °C.

Fractionation by Silica Gel Chromatography. Silica gel chromatography was performed according to the method of Privett et al. (1973). Each crude fat and lipid extract (10 g) was applied to a column (25 × 300 mm) packed with silica gel in chloroform. The glycolipid fraction was eluted with 250 mL of acetone after the triglyceride fraction was removed by eluting with 300 mL of chloroform. The phospholipid fraction was eluted with 300 mL of methanol. In this study, the crude lipid fraction was obtained by using 250−300 mL of solvent. After the solvent was reduced by evaporation, the weight of each fraction was measured, and the glycolipid fraction was subjected to HPLC.

HPLC Analyses. HPLC analyses were conducted using a model 880 HPLC pump, a model 860-CO column oven, and a syringe-loading sample injector with a 20-μL loop (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Detection was made with a model 870 UV detector (Japan Spectroscopic Co., Ltd.) operated at a UV absorption wavelength of 208 nm and with a differential refractometer (Japan Spectroscopic Co., Ltd.). Neutral glycosphingolipids in the samples were separated on an Aquasil-SS column (200 mm × 6 mm i.d., particle size 50 Å; Senshu, Co., Tokyo, Japan) using a solvent system of 2-propanol/*n*-hexane/water. This composition was eluted with 20:80:0 for 20 min and then eluted with 55:40:5 for 10 min, at a flow rate of 1.0 mL/min and a column temperature of 40 °C (Suzuki et al., 1990). The injection volume was 20 μL. Furthermore, we performed differential RI-HPLC analyses to detect sugars of neutral glycosphingolipids in the glycolipid fraction of round frigate mackerel and sea snake. The glycolipid fraction was eluted with 2-propanol/*n*-hexane/water (48:48:4) for 20 min (flow rate = 1.0 mL/min; column temperature = 40 °C; injection volume = 20 μL). Glycoglycerolipids in the samples were measured using a C_8-reversed phase silica gel column (250 mm × 4.6 mm i.d., particle size 120 Å; Senshu, Co.) according to the method of Smith et al. (1985). The glycoglycerolipids were separated with methanol/water (96:4) and detected by UV and RI in the same conditions as before (flow rate = 1.0 mL/min; column temperature = 40 °C; injection volume = 20 μL).

Statistics. The weight percentages of each lipid fraction are expressed as means of five replicates ± SD. The significant difference in comparing each lipid fraction between samples was analyzed by one-way analysis of variance Duncan's pairwise comparisons using the SIGMASTAT statistical program package (Jandel Corp., Erkrath, Germany).

RESULTS AND DISCUSSION

Percentages of Glycolipid Fraction in Fish, Shellfish, and Sea Snake Lipids. A comparative study was conducted to determine the differences in the amounts of triglyceride, glycolipid, and phospholipid fractions contained in different fish, shellfish, and sea snake lipids. The results are shown in Table 1. As can be seen, sea snake crude fat and purified sardine oil were richest in triglyceride fraction (89.8 and 92.6%, respectively) among the samples, sea urchin and abalone intestine lipids had an intermediate percentage (56.7 and 62.5%, respectively), and a low percentage (35.8%) of triglyceride fraction was obtained in round frigate mackerel lipid. In the glycolipid fraction, round frigate mackerel muscle lipid (10.1%) was rich compared to other samples. Sea urchin and abalone intestine lipids contained 7.1 and 5.2% glycolipid fraction, respectively, whereas sea snake crude fat and purified sardine oil contained <5% glycolipid fraction. The mean percentage of phospholipid fraction (31.9%) was high in round frigate mackerel muscle lipid, and 16.6 and 13.7% of phospholipid fraction were obtained in sea urchin and abalone intestine lipids, respectively. Sea snake crude fat and purified sardine oil contained <2% phospholipid fraction.

Table 1. Weight Percentages of Triglyceride, Glycolipid, and Phospholipid Fractions Separated from Different Fish, Shellfish, and Sea Snake by Silica Gel Chromatography[a]

sample	triglyceride fraction (%)	glycolipid fraction (%)	phospholipid fraction (%)
round frigate mackerel muscle lipid	35.8 ± 2.5[a]	10.1 ± 1.5[a]	31.9 ± 2.7[a]
sea urchin intestine lipid	56.7 ± 2.8[b]	7.1 ± 1.1[b]	16.6 ± 1.7[b]
abalone intestine lipid	62.5 ± 3.2[c]	5.2 ± 1.7[c]	13.7 ± 1.7[c]
sea snake crude fat	89.8 ± 0.9[d]	3.3 ± 0.4[d]	0.9 ± 0.1[d]
purified sardine oil	92.6 ± 0.7[de]	1.1 ± 0.2[e]	1.1 ± 0.2[de]

[a] The values are means of five replicates ± SD. Values for each sample with different superscript roman letters in the same fraction are significantly different at $p < 0.05$ by Duncan's pairwise comparisons.

Round frigate mackerel muscle lipid, sea urchin, and abalone intestine lipid were extracted with chloroform/methanol. They may not have been purified and they may have contained other lipid classes in each fraction. The most dominant fraction in sea snake crude fat and purified sardine oil was triglycerides because they were extracted with hexane and purified. The sum of percentages was 77.8 for round frigate mackerel muscle lipid, 80.4 for sea urchin intestine lipid, 81.4 for abalone intestine lipid, 94.0 for sea snake crude fat, and 94.8 for purified sardine oil. Differences among samples can be explained by the fact that round frigate mackerel muscle lipid, sea urchin intestine lipid, and abalone intestine lipid contain other components such as lipoprotein, so it is difficult to elute them completely using methanol.

Analyses of Neutral Glycosphingolipid and Glycoglycerolipid by UV-HPLC. HPLC analyses using a UV detector were conducted to separate neutral glycosphingolipids and glycoglycerolipids. The UV absorption detector is clearly the most sensitive detector for compounds that have double bonds (Munk, 1970). HPLC chromatograms of the lipids in the glycolipid fraction obtained from neutral glycosphingolipid analysis are presented in Figure 1. Chromatogram A shows a typical peak in the glycolipid fraction of round frigate mackerel (retention time = 16 min), and chromatogram B shows that of sea snake (retention time = 14 min). Chromatograms C and D show typical peaks in the glycolipid fraction of abalone intestine (retention time = 11 min) and sea urchin intestine (retention time = 14 min), but the peaks at a retention time of 9 min are not typical due to detection in other samples. Chromatogram E shows small peaks in the fraction of purified sardine oil. These results suggest that there may be different types of neutral glycosphingolipids in the fish, shellfish intestine, and sea snake, and that these types may be related to the physiological functions. Figure 2 shows HPLC chromatograms of the lipids

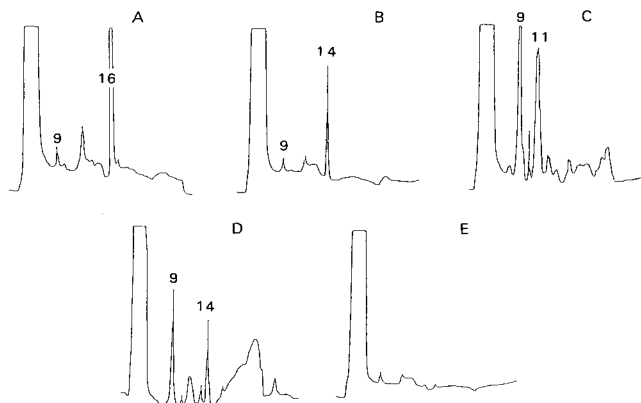

Figure 1. UV-HPLC (208 nm) chromatograms obtained from the glycolipid fraction of (A) round frigate mackerel muscle lipid, (B) sea snake crude fat, (C) abalone intestine lipid, (D) sea urchin intestine lipid, and (E) purified sardine oil under the analytical condition of neutral glycosphingolipid (Suzuki et al., 1990). Retention times are 16, 14, 11, and 9 min.

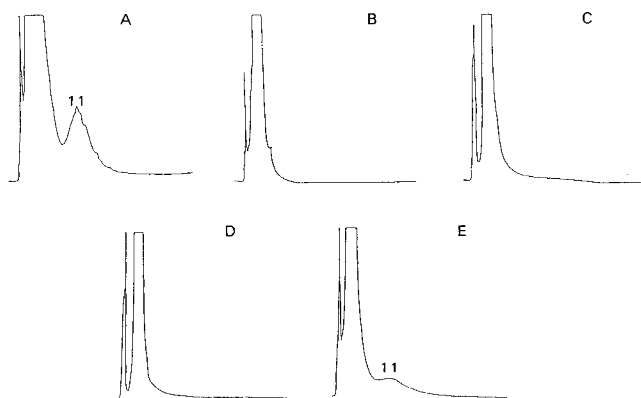

Figure 2. UV-HPLC (208 nm) chromatograms obtained from the glycolipid fraction of (A) round frigate mackerel muscle lipid, (B) sea snake crude fat, (C) abalone intestine lipid, (D) sea urchin intestine lipid, and (E) purified sardine oil under the analytical condition of glycoglycerolipid (Smith et al., 1985). Retention time is 11 min.

in the glycolipid fraction obtained from glycoglycerolipid analysis. Chromatogram A shows a large peak in the glycolipid fraction of round frigate mackerel (retention time = 11 min), and chromatogram E shows a small peak in the faction of purified sardine oil (retention time = 11 min). The results indicate that peaks in HPLC chromatograms of the glycolipid fraction of different fish may be due to the same component. No marked peaks of the glycolipid fraction of sea snake crude fat, sea urchin, and abalone intestine lipids are observed in chromatograms B−D.

Detection of Neutral Glycosphingolipid and Glycoglycerolipid by RI-HPLC. We performed HPLC analyses using a RI detector to determine sugars of glycolipids in round frigate mackerel muscle lipids and sea snake crude fat. The RI detector detects the presence of glycolipids on the basis of the difference of the refraction index of the solution of the sample and the solvent (Huber, 1969). A UV detector is not as effective for sugars, which do not possess specific UV-absorbing chromatophores. Although sugars have an appreciable absorbance in the UV region at low wavelengths, the chromatophores are relatively weak due to the poor selectivity over many of the other components encountered in foods (Garcia and Palmer, 1980). The RI detector is the most commonly used instrument in

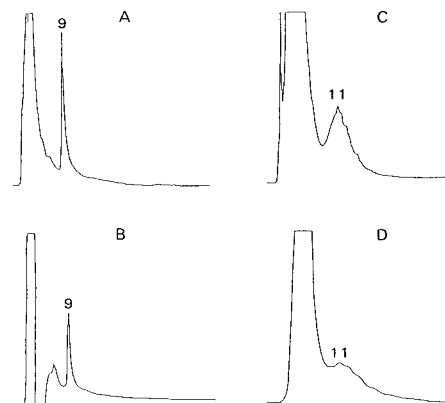

Figure 3. UV-HPLC (A, C) and RI-HPLC (B, D) chromatograms obtained from the glycolipid fraction of round frigate mackerel muscle lipid. Retention times are 9 and 11 min.

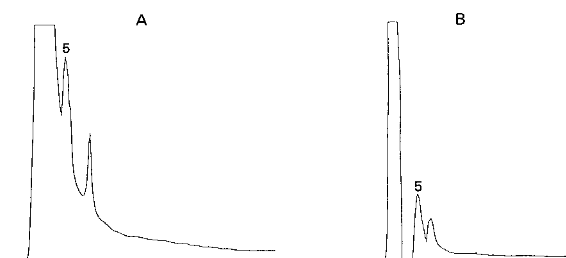

Figure 4. UV-HPLC (A) and RI-HPLC (B) chromatograms obtained from the glycolipid fraction of sea snake crude fat. Retention time is 5 min.

carbohydrate analysis (Macrae, 1985). Figure 3 presents UV-HPLC and RI-HPLC chromatograms of the glycolipid fraction in round frigate mackerel. Chromatograms A (UV) and B (RI) show a typical peak of the same retention time (9 min) under the conditions of neutral glycosphingolipid analysis. Chromatograms C (UV) and D (RI) show a small peak in the same retention time (11 min) under the conditions of glycoglycerolipid analysis. UV- and RI-HPLC chromatograms of the glycolipid fraction in sea snake crude fat under the conditions of neutral glycosphingolipid analysis are shown in Figure 4. Chromatograms A and B indicate a typical peak in the same retention time (5 min). As can be seen in Figures 3 and 4, the peaks of UV-HPLC chromatograms were sharper than those of RI-HPLC chromatograms. This may be because the RI detector has a relatively low sensitivity (Esaiassen et al., 1995). Furthermore, the minimum sample detection limit is 3×10^{-9} g/s, whereas that of the UV detector is 2×10^{-11} g/s (Munk, 1970). Deininger and Halasz (1970) suggested that the sensitivity of the RI detector can be improved by using a heat exchanger through which the mobile phase flows before entering the cell. The above results suggest that these peaks in the glycolipid fraction of round frigate mackerel muscle lipids and sea snake crude fat are neutral glycosphingolipid and glycoglycerolipid.

Conclusions. Our results revealed that the percentage of glycolipid fraction was higher in the lipids of round frigate mackerel than in sea urchin, abalone intestine lipids, and sea snake crude fat. Additionally, we observed typical peaks in the glycolipid fraction of different fish, shellfish, and sea snake by HPLC under the conditions of neutral glycosphingolipid and glycoglycerolipid analyses. Furthermore, we detected typical peaks by a RI detector in the glycolipid fraction of round

frigate mackerel muscle lipids and sea snake crude fat. The present study is only a preliminary experiment on separating glycolipids from fish, shellfish, and sea snake and must be extended by further work to isolate other glycosphingolipid classes and to determine the specific oligosaccharide structure of glycolipids.

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