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Occurrence of gangliosides in the common squid and pacific octopus among protostomia

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Abstract

Acidic lipids from tissues of the common squid *Todarodes pacificus* and the pacific octopus *Octopus vulgaris* were characterized. Hepatopancreatic tissues of both animals had complex compositions of resorcinol-positive acidic lipids, many of which became reactive with cholera toxin B subunit and anti-G_{M1} antibody after in situ treatment with sialidase on TLC. One of the major acidic lipids in squid tissue was isolated and examined for its structure. This acidic lipid was identified to be the ganglioside G_{D1a} based upon the susceptibility to sialidases of different substrate specificity, characterization of reaction products, and electrospray ionization-mass spectrometry of the lipid. Hepatopancreatic tissues of squid and octopus also contained acidic lipids that reacted with A2B5, a monoclonal antibody specific to c-series gangliosides. Cerebral ganglia of both animals expressed resorcinol-positive acidic lipids, though their compositional patterns differed from the hepatopancreatic tissues. *N*-Acetylneuraminic acid was identified as the main species in lipid-bound sialic acid in both tissues. The contents of lipid-bound sialic acid in cerebral ganglia were significantly lower than those of hepatopancreatic tissues in both animals. The present study presents the first evidence for the occurrence of gangliosides in protostomia.

Keywords: Ganglioside; c-Series ganglioside; Sialic acid; A2B5; Mollusk; Protostomia

1. Introduction

Gangliosides are a family of glycosphingolipids that contain one or more sialic acid residues. They are localized mainly on the surface of plasma membranes and play important roles in diverse cellular functions [1–3]. It is generally accepted that gangliosides exist only in echinoderms (e.g., starfish and sea urchin) or higher animal phyla of deuterostomia (e.g., fish, reptile, amphibian, avian, and mammalian) [4,5]. In vertebrates, gangliosides are enriched in brain tissues, in which gangliotetraose gangliosides constitute the major ganglioside species [6,7]. Phylogenetically, echinoderm has been considered to be the lowest animal phylum that expresses gangliosides. Their gangliosides are distinct from those of vertebrates and possess unique structures; they include those with sialic acids linked to glucosyl ceramide at the C6 position of the glucose moiety [8–10] or those with sialic acids interposed in their oligosaccharide chains [9–14]. As for the expression of gangliosides in lower animal phyla among protostomia, no evidence has been provided to date [4,5]. In the

Abbreviations: Cer, ceramide; GlcCer, Glc1-1′Cer; LacCer, Galβ1-4Glc1-1′Cer; GgOse3Cer, GalNAcβ1-4Galβ1-4Glc1-1′Cer; GgOse4Cer, Galβ1-3GalNAcβ1-4Galβ1-4Glc1-1′Cer

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present study, we were able to obtain the first evidence for the occurrence of gangliosides in some mollusks among protostomia, i.e., the common squid *Todarodes pacificus* and the pacific octopus *Octopus vulgaris*.

2. Materials and methods

2.1. Materials

Fresh common squid *T. pacificus* and pacific octopus *O. vulgaris* were obtained from local fish markets. A preparation of c-series ganglioside-specific monoclonal antibody A2B5 was obtained as follows. A2B5-producing hybridomas (CRL 1520, American Type Culture Collection, USA) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The culture medium containing the antibody (IgM-type) was used for experiments [15]. A polyclonal antibody against G_{M1} or asialo G_{M1} (IgG-type for both) was prepared by immunizing rabbits with purified glycolipid [16]. GlcCer, LacCer, GgOse3Cer, and GgOse4Cer were purified from mild acid hydrolysates of rat brain gangliosides in our laboratory [17]. Other chemicals and reagents were obtained from the following companies: *Clostridium perfringens* sialidase, *Arthrobacter ureafaciens* sialidase, goat peroxidase-conjugated antibodies against mouse IgM or rabbit IgG, peroxidase-conjugated cholera toxin B subunit, *N*-acetylneuraminic acid, and *N*-glycolylneuraminic acid (Sigma, USA), *Salmonella typhimurium* sialidase (α2,3-specific, cloned from *S. typhimurium* LT2 and expressed in *Escherichia coli*) (Takara Shuzo, Japan), high performance thin-layer chromatographic (TLC) plates (nanoplates, E. Merck, Germany), and enhanced chemiluminescence (ECL) Western blotting detection kits (Amersham, USA).

2.2. Isolation of acidic lipids

Total lipids were extracted from tissues with 20 vols. of chloroform/methanol (1:1) and separated into neutral and acidic lipids by DEAE-Sephadex column chromatography. Acidic lipids were incubated in 0.2 M methanolic NaOH at 37°C for 1 h, then neutralized with acetic acid. Acidic lipids were obtained after desalting the neutralized mixture by Sephadex LH-20 column chromatography.

2.3. Overlay analysis

Overlay analysis of acidic lipids with glycolipid-specific ligands was carried out based upon a method reported previously [18]. Briefly, acidic lipids were developed on a TLC plate. After coating with a 0.4% polyisobutylmethacrylate solution, the plate was overlaid consecutively by an anti-glycolipid antibody and peroxidase-conjugated second antibody at room temperature for 1.5 h. In the case of cholera toxin B subunit, a peroxidase-conjugated toxin preparation was used [19]. The reacted band(s) were detected on an X-ray film by the ECL method. Acidic lipids on the plate were then visualized with resorcinol-HCl reagent [20]. Densitometric analysis of acidic lipids was carried out using a densitometric image analyzer (Atto Densitograph AE-6920M, Atto, Tokyo, Japan).

Structural analysis of acidic lipids by an overlay technique was carried out as follows [21]. Acidic lipids were developed on TLC and treated in situ with *Cl. perfringens* sialidase (250 mU/ml of 0.1 M sodium acetate buffer, pH 4.8) at room temperature for 1.5 h. The modified lipid structures were examined using glycolipid-specific ligands as described above.

2.4. Characterization of acidic lipid using different sialidases

An acidic lipid in hepatopancreatic tissue of squid was isolated by preparative TLC, followed by purification on high performance liquid chromatography (HPLC) with a size exclusion chromatographic column (TSKgel α-2500, Tosoh, Japan). The lipid was treated with one of three different sialidases, i.e., the enzyme derived from *Cl. perfringens*, *S. typhimurium*, or *A. ureafaciens*. In the case of *Cl. perfringens* sialidase, the reaction mixture consisted of 100 mM sodium acetate (pH 4.8), acidic lipid, and 250 mU/ml of the enzyme in a final volume of 0.1 ml. The mixture was incubated at 37°C for 45 min. The reaction with *S. typhimurium* sialidase was carried out in a similar manner, except that the pH was 5.5. As for the *A. ureafaciens* enzyme, the lipid was treated with the enzyme (250 mU/ml, pH 4.8) in the presence of

sodium deoxycholate (0.5 mg/ml) at 37°C for 2 h. The ganglioside G_{D1b} (used as reference) was also treated in similar manners. After reaction, the reaction product(s) were desalted by LH-20 column chromatography, developed on TLC with a solvent system of chloroform/methanol/0.2% $CaCl_2 \cdot 2H_2O$ (50:45:10), and characterized by overlay analysis with specific ligands. Visualization of resorcinol-negative acidic lipids was carried out using orcinol-H_2SO_4 reagent [22].

2.5. Negative ion electrospray ionization (ESI)-mass spectrometry (MS) of acidic lipid

The structure of acidic lipid was analyzed using an LCQ ion trap mass spectrometer equipped with an ESI source (Finnigan MAT, USA). The purified acidic lipid was dissolved in methanol at a concentration of 10 pmol/μl and introduced into the electrospray needle by mechanical infusion at a flow rate of 3 μl/min. The ESI capillary was kept at a voltage of −4 V at 200°C. The tube lens offset was set at 30 V. The collision-induced dissociation (CID)-MS^2 and MS^3 spectra were taken using helium as the collision gas. The relative collision energy scale was set at 2.5 eV. Mass spectra were averaged over ten scans.

2.6. Fluorometric HPLC analysis of sialic acids

Lipid-bound sialic acids were analyzed using a fluorometric HPLC method [23]. Acidic lipids were hydrolyzed by incubation in 25 mM sulfuric acid at 80°C for 1 h. The hydrolysates were incubated with a 1,2-diamino-4,5-methylenedioxybenzene (DMB) reagent at 60°C for 2.5 h. The fluorescent product(s) were analyzed by HPLC with an ODS column and fluorescence detector. The fluorescence excitation and emission wave lengths were at 373 and 448 nm, respectively. Quantitation of sialic acids was carried out based upon the standard curves obtained with the *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid solutions of different concentrations.

2.7. Determination of sialic acids by gas-liquid column chromatography (GLC)-MS

Lipid-bound sialic acids were identified by GLC-MS. Total acidic lipids from squid hepatopancreatic tissue were treated in 0.1 M HCl at 100°C for 30 min. The hydrolysates were incubated in 5% HCl in methanol at 80°C for 1 h, followed by trimethylsilylation using a TMS-HT kit (Tokyo Kasei, Japan). Authentic *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid were treated in similar manners. The presence of per-*O*-trimethylsilyl derivatives of sialic acid methyl esters was examined by GLC-MS [24].

3. Results

3.1. TLC and overlay analysis of acidic lipids from hepatopancreatic tissues of squid and octopus

Acidic lipids were isolated from hepatopancreatic tissues of the common squid and pacific octopus, developed on TLC, and visualized with resorcinol-HCl reagent. As shown in Fig. 1A, the hepatopancreatic tissues of both animals had complex compositions of resorcinol-positive acidic lipids. These acidic lipids were analyzed using the overlay method with ganglioside-specific ligands. First, the acidic lipids were treated with cholera toxin B subunit on TLC plates (Fig. 1B, left panel). Among rat liver gangliosides (used as reference), G_{M1} and G_{D1b} were detected by the toxin. In both cases of hepatopancreatic tissues from squid and octopus, a positive band was observed at the same position as G_{M1} on TLC. The toxin also detected a few minor lipids in squid tissue. Second, the acidic lipids were treated in situ with *Cl. perfringens* sialidase on TLC, followed by reaction with cholera toxin B subunit (Fig. 1B, right panel). In the case of rat liver, gangliosides including G_{D1a}, G_{T1b}, and G_{Q1b} were newly detected after enzyme treatment. Similarly, many acidic lipids of hepatopancreatic tissues became reactive with the toxin. These acidic lipids included those having the same chromatographic mobility as G_{D1a}, G_{T1b}, or G_{Q1b}. An acidic lipid corresponding to G_{D1b} was observed only in squid tissue. Analysis of acidic lipids with an anti-G_{M1} antibody produced similar chromatographic patterns as observed with cholera toxin B subunit (Fig. 1C). Some acidic lipids from hepatopancreatic tissues reacted with a c-series ganglioside-specific monoclonal antibody A2B5 (Fig. 1D). They included those migrating above G_{D1b} (lip-

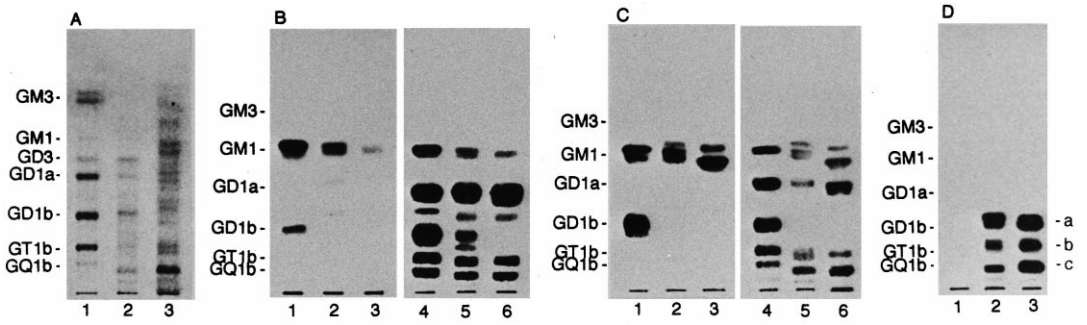

Fig. 1. TLC and overlay analysis of acidic lipids from hepatopancreatic tissues of the common squid and pacificus octopus. Acidic lipids were isolated from tissues and developed on TLC with a solvent system of chloroform/methanol/0.2% $CaCl_2 \cdot 2H_2O$ (45:40:10). (A) Acidic lipids were visualized by resorcinol-HCl reagent. (B) Acidic lipids were reacted with peroxidase-conjugated cholera toxin B subunit (lanes 1–3) or reacted with the toxin after in situ treatment with *Cl. perfringens* sialidase (lanes 4–6). (C) Acidic lipids were reacted with anti-G_{M1} antibody (lanes 1–3) or reacted with the antibody after sialidase treatment (lanes 4–6). (D) Acidic lipids were reacted with A2B5. (A,D) Lanes: 1, rat liver gangliosides (used as reference); 2 and 3, acidic lipids from squid and octopus tissues. (B,C) Lanes: 1 and 4, rat liver gangliosides; 2 and 5, acidic lipids from squid tissue; 3 and 6, acidic lipids from octopus tissue.

id a), between G_{D1b} and G_{T1b} (lipid b), and below G_{Q1b} (lipid c).

3.2. Characterization of acidic lipid in hepatopancreatic tissue using different sialidases

As shown in Fig. 1A, the hepatopancreatic tissue of squid contained a major acidic lipid that migrated to the same position as G_{D1a}. This acidic lipid (designated as lipid X) was isolated and characterized using sialidases with different substrate specificity. Lipid X was partially hydrolyzed by *Cl. perfringens* sialidase or *S. typhimurium* sialidase, generating a resorcinol-positive reaction product that had the same chromatographic mobility as G_{M1} on TLC (Fig. 2A, lanes 2, 4, 6). This degradation product reacted with cholera toxin B subunit (Fig. 2B, lane 4) and anti-G_{M1} antibody (data not shown). G_{D1b}

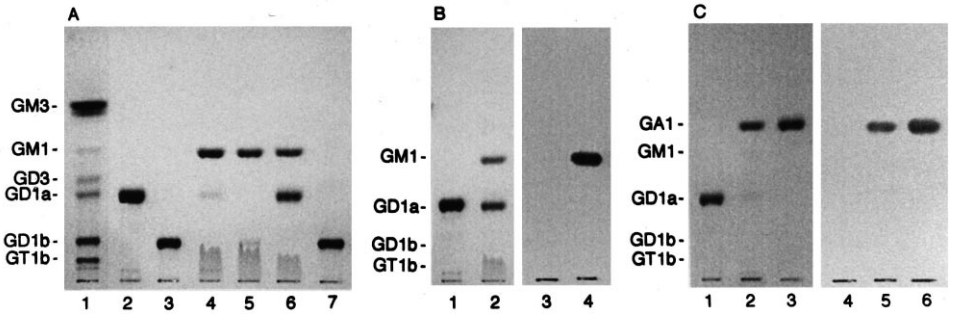

Fig. 2. Characterization of acidic lipid in squid hepatopancreatic tissue using different sialidases. One of the major acidic lipids in squid hepatopancreatic tissue (designated as lipid X) was treated in tube reaction with one of the sialidases having different substrate specificity, and the reaction products were developed on TLC with a solvent system of chloroform/methanol/0.2% $CaCl_2 \cdot 2H_2O$ (50:45:10). (A) The reaction products from lipid X by treatment with *Cl. perfringens* sialidase and *S. typhimurium* sialidase were visualized with resorcinol-HCl reagent. G_{D1b} was used as reference. Lanes: 1, rat liver gangliosides; 2, lipid X; 3, GD1b; 4 and 6, reaction product from lipid X by treatment with *Cl. perfringens* and *S. typhimurium* sialidase, respectively; 5 and 7, reaction product from G_{D1b} by treatment with *Cl. perfringens* and *S. typhimurium* sialidase, respectively. (B) The main reaction product from lipid X by treatment with *S. typhimurium* sialidase was analyzed using cholera toxin B subunit. Lanes: 1 and 2, visualized with resorcinol-HCl reagent; 3 and 4, overlay analysis with cholera toxin B subunit; 1 and 3, lipid X; 2 and 4, reaction product from lipid X by treatment with *S. typhimurium*. Bands with tailing above the origin were free sialic acids (A, lanes 4–6; B, lane 2). (C) The main reaction product from lipid X by treatment with *A. ureafaciens* was analyzed with anti-asialo G_{M1} antibody. Lanes: 1–3, visualized with orcinol-H_2SO_4 reagent; 4–6; immunostained with an anti-asialo G_{M1} antibody; 1 and 4, lipid X; 2 and 5, reaction product from lipid X by treatment with *A. ureafaciens* sialidase; 3 and 6, authentic asialo G_{M1} (indicated as GA1 in the figure).

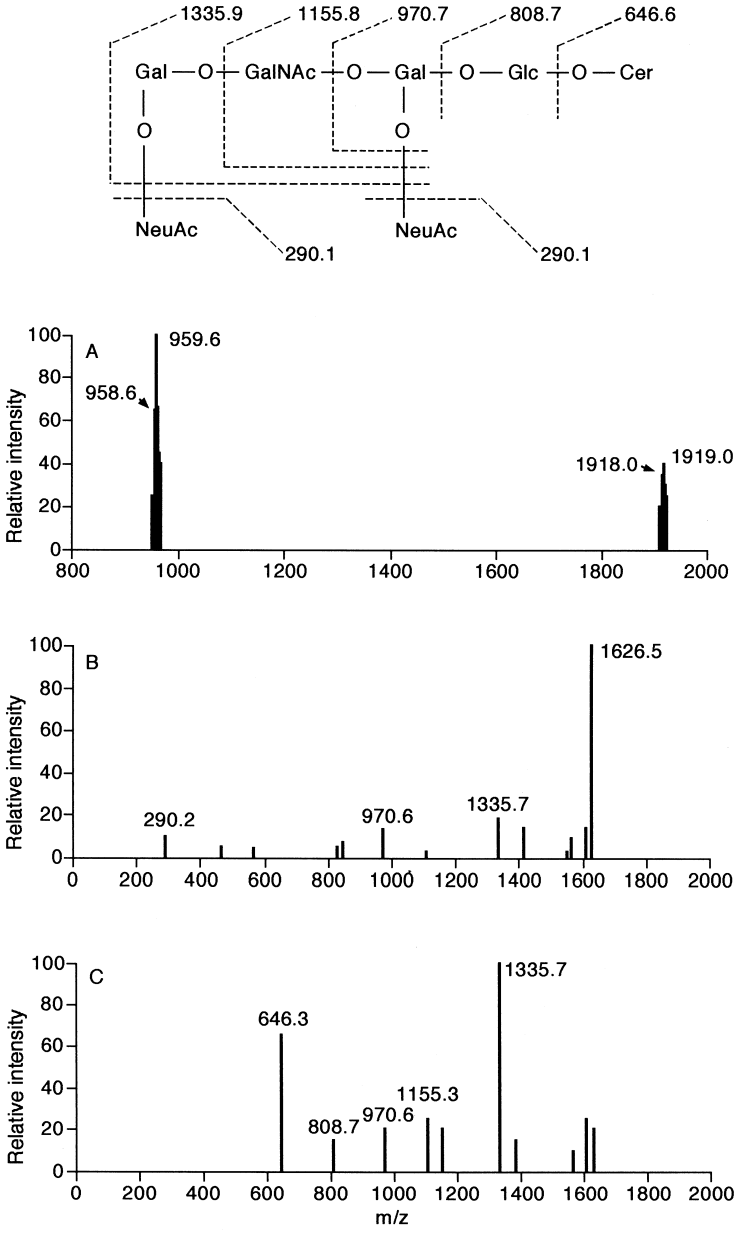

Fig. 3. Negative ion electrospray ionization-mass spectrometry of acidic lipid in squid hepatopancreatic tissue. (A) An ESI-MS spectrum of lipid X. (B,C) CID-MS^2 and CID-MS^3 spectra of the ions at *m/z* 958.6 (A) and 1626.5 (B), respectively.

(used as reference) was hydrolyzed to G_{M1} by *Cl. perfringens* sialidase, but was totally resistant to the action of *S. typhimurium* enzyme (Fig. 2A, lanes 3, 5, 7). The treatment of lipid X with *A. ureafaciens* sialidase generated a major lipid product (Fig. 2C, lane 2). This lipid was resorcinol-negative and had the same chromatographic mobility as asialo G_{M1} on TLC. This lipid was detected specifically with anti-asialo G_{M1} antibody (Fig. 2C, lane 5). These findings suggested that lipid X may be the ganglioside G_{D1a}.

3.3. ESI-MS of acidic lipid in hepatopancreatic tissue

The possibility that lipid X is G_{D1a} was examined by negative ion ESI-MS of the lipid (Fig. 3). The

negative ion ESI mass spectrum of lipid X showed a double-charged ion of $(M\text{-}2H)^{2-}$ at *m/z* 958.6 and a single-charged ion of $(M\text{-}H)^{-}$ at *m/z* 1918.0. The negative ion CID-MS^2 spectrum of the double-charged ion produced single-charged ions at *m/z* 290.2, 970.6, 1335.7, and 1626.5; they corresponded with NeuAc, Gal-Glc-Cer, Gal-GalNAc-Gal-Glc-Cer, and M-NeuAc in the structure of G_{D1a}, respectively. In the negative ion CID-MS^3 spectrum of the ion at *m/z* 1626.5, the fragment ions were observed corresponding to ceramide (*m/z* 646.3), Glc-Cer (*m/z* 808.7), and GalNAc-Gal-Glc-Cer (*m/z* 1155.3). The molecular mass of lipid X was calculated to be 1919.1. These results demonstrated that lipid X is G_{D1a}.

3.4. Identification and quantitation of lipid-bound sialic acids in hepatopancreatic tissues

The presence and molecular species of sialic acids in lipid X were examined using the fluorometric HPLC method. A peak corresponding to *N*-acetylneuraminic acid, but not to *N*-glycolylneuraminic acid, was observed (Fig. 4). Similar results were obtained for total acidic lipids from hepatopancreatic tissues of squid and octopus (data not shown). The presence of *N*-acetylneuraminic acid was confirmed by GLC-MS of mild acid hydrolysates of total acidic lipids from squid hepatopancreatic tissue. The electron impact mass spectrum was identical to that of authentic *N*-acetylneuraminic acid (Fig. 5). The fragment ions included those at *m/z* 173, 205, 298, 317, 478, 624, and 668, all of which are characteristic to *N*-acetylneuraminic acid [24]. *N*-Glycolylneuraminic acid was not detected by GLC-MS.

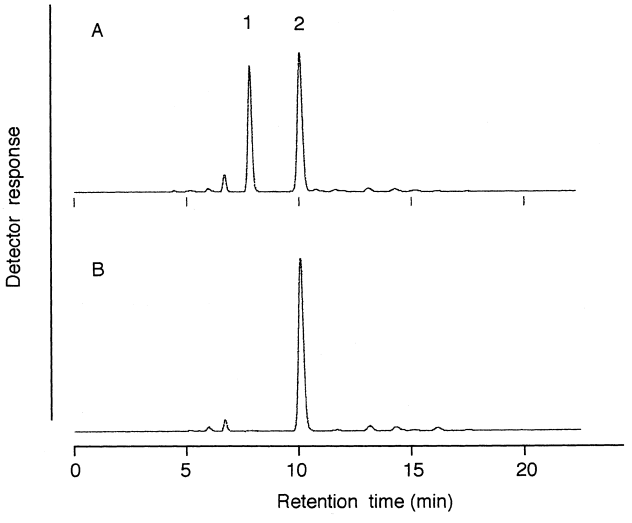

Fig. 4. Detection of sialic acids using a fluorometric HPLC method. Lipid X in squid hepatopancreatic tissue was hydrolyzed in 25 mM sulfuric acid at 80°C for 1 h. The hydrolysate was incubated with a DMB reagent at 60°C for 2.5 h, and the fluorescent product(s) were analyzed by HPLC. (A) The peaks for authentic *N*-glycolylneuraminic acid (peak 1) and *N*-acetylneuraminic acid (peak 2) were observed at a retention time of 7.83 and 10.05 min, respectively. (B) A fluorescent peak, which had the same retention time of *N*-acetylneuraminic acid, was detected for the hydrolysate of lipid X in squid tissue.

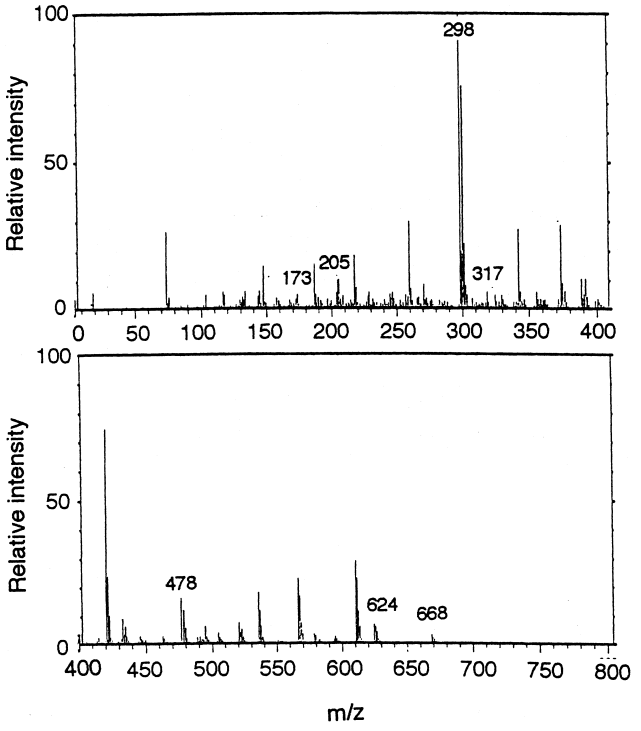

Fig. 5. Electron impact mass spectrum (70 eV) by GLC-MS of mild acid hydrolysate of total acidic lipids from squid hepatopancreatic tissue. Total acidic lipids of squid tissue were treated in 0.1 M HCl at 100°C for 30 min. The hydrolysate was incubated in 5% HCl in methanol at 80°C for 1 h, followed by trimethylsilylation using a TMS kit.

The concentrations of gangliosides in hepatopancreatic tissues of both animals were quantitated using the fluorometric HPLC method. The tissues of common squid ($n=5$) and pacific octopus ($n=4$) contained 2.49 ± 1.44 and 0.93 ± 0.93 μg of *N*-acetylneuraminic acid per gram of wet tissue, respectively.

Densitometric analysis of squid hepatopancreatic

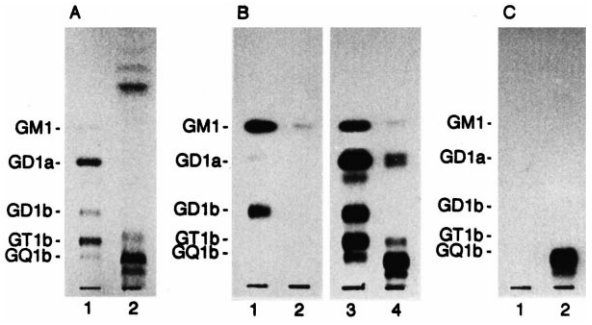

Fig. 6. TLC and overlay analysis of acidic lipids from cerebral ganglion of the common squid. Acidic lipids were analyzed using similar methods as described in Fig. 1. Acidic lipids were visualized with resorcinol-HCl reagent (A), reacted with cholera toxin B subunit (B, left panel), reacted with the toxin after sialidase treatment (B, right panel), or reacted with A2B5 (C). (A,C) Lanes 1 and 2, rat brain gangliosides and acidic lipids from cerebral ganglion of common squid, respectively. (B) Lanes: 1 and 3, rat brain gangliosides; 2 and 4, acidic lipids from cerebral ganglion of common squid.

gangliosides demonstrated that G_{D1a} amounted to 16.7% of total gangliosides.

3.5. Characterization of acidic lipids from cerebral ganglia of squid and octopus

Acidic lipids in cerebral ganglia of common squid and pacific octopus were analyzed. The cerebral ganglion of squid contained resorcinol-positive acidic lipids, though the compositional pattern was simpler than that of hepatopancreatic tissue (Fig. 6A). The composition of cholera toxin-reactive lipids also differed from that of hepatopancreatic tissue; acidic lipids corresponding to G_{D1b} and some other acidic lipids were not detected in the cerebral ganglion (Fig. 6B). Overlay analysis with anti-G_{M1} antibody gave similar chromatographic patterns as observed with cholera toxin B subunit (data not shown). Squid ganglion contained at least two A2B5-reactive lipids, both of which migrated below G_{Q1b} on TLC (Fig. 6C). *N*-Acetylneuraminic acid was shown to be the sole sialic acid species using the fluorometric HPLC assay (data not shown). The concentrations of gangliosides in cerebral ganglia of *T. pacificus* and *O. vulgaris* were 0.069 ± 0.030 and 0.084 ± 0.060 μg of *N*-acetylneuraminic acid per gram of wet tissue, respectively ($n = 5$ for both).

4. Discussion

In the present study, we characterized acidic lipids from hepatopancreatic tissues and cerebral ganglia of the common squid and pacific octopus. The stability of acidic lipids during mild base treatment and specific coloration with resorcinol-HCl reagent suggested that these acidic lipids may be gangliosides. This possibility was supported by their reactivity with anti-ganglioside ligands and susceptibility to sialidases and the presence of lipid-bound sialic acids. The expression of gangliosides in hepatopancreatic tissues was confirmed by identifying one of the major acidic lipids (lipid X) to be the ganglioside G_{D1a}. Overlay analysis with specific ligands revealed that lipid X contains the same oligosaccharide structure as G_{M1}. The structure of lipid X was further analyzed using sialidases of different substrate specificity. It is known that the action of *S. typhimurium* sialidase is linkage-specific; it cleaves $\alpha_{2,3}$-sialic acids at much faster rates than other sialic acid residues [25]. Differing from *Cl. perfringens* and *S. typhimurium* enzymes, *A. ureafaciens* sialidase can efficiently hydrolyze the sialic acid residue linked to the inner galactose of the gangliotetraose structure, especially in the presence of detergents [26]. Based upon the susceptibility to individual sialidases and characterization of the reaction products, it was suggested that the lipid X possesses the gangliotetraose oligosaccharide backbone with two sialic acid residues: one connected to the internal galactose moiety and the other linked to the terminal galactose through an α2,3-linkage. Among gangliosides of known structures, the plausible candidate for lipid X was G_{D1a}. This assumption was confirmed by ESI-mass spectrometry of the lipid. While neutral glycosphingolipids are known to be present in different animals among protostomia [27–32], no information about the expression of gangliosides has been provided. This is the first evidence for the occurrence of gangliosides among protostomia.

Overlay analysis of acidic lipids suggested that squid and octopus tissues may express gangliosides other than G_{D1a}. In this method, acidic lipids are treated in situ with sialidase on TLC plates; most gangliotetraose gangliosides are hydrolyzed by *Cl. perfringens* sialidase, producing G_{M1}. The generated

G_{M1} is detected with specific ligands. Among these ligands, cholera toxin B subunit specifically reacts with G_{M1} [33–35]. Another specific ligand is an anti-G_{M1} antibody. The preparation of anti-G_{M1} antibody in this study has successfully been employed for detection and identification of this ganglioside [15,36,37]. While these ligands are useful for detection of G_{M1}, they cross-react with other gangliosides that are structurally related with G_{M1}, as shown by the binding of cholera toxin B subunit to fucosyl G_{M1} [38] and of both cholera toxin and anti-G_{M1} antibody to G_{D1b} [39,40]. In the present study, many acidic lipids in squid and octopus tissues reacted with both ligands before or after sialidase treatment. They included acidic lipids having the same chromatographic mobility as G_{M1}, G_{D1b}, G_{T1b}, or G_{Q1b}. Although the structures of these acidic lipids remain to be identified, it was strongly suggested that the common squid and pacific octopus express not only G_{D1a}, but also other gangliosides having the gangliotetraose oligosaccharide structure. There were some differences between the patterns obtained with cholera toxin B subunit and G_{M1} antibody. Though the exact reason for this phenomenon is not known, it may at least partially result from the difference in the reactivity of both ligands. While gangliotetraose gangliosides are a common ganglioside species in vertebrates, they are not found in echinoderms. From the standpoint of ganglioside metabolism, mollusks such as squid and octopus may be closer to vertebrates rather than to echinoderms.

The squid and octopus tissues were also shown to contain A2B5-reactive acidic lipids. The monoclonal antibody A2B5 was originally prepared by immunizing chicken embryonic retinal cells [41]. Although there was some controversy about its specificity, evidence has been accumulated indicating its strict specificity to c-series gangliosides [15,42–45]. Based upon these studies and others, it was suggested that A2B5 specifically reacts with c-series gangliosides including G_{T3}, G_{T2}, G_{T1c}, G_{Q1c}, and G_{P1c} [15]. We have successfully analyzed c-series gangliosides in extraneural tissues using the same A2B5 preparation as in this study [36,37,46–48]. The present study suggested the possible expression of c-series gangliosides in the hepatopancreatic tissues and cerebral ganglia of both animals. A further study will be required for identification of these acidic lipids.

The concentrations of gangliosides in squid and octopus hepatopancreatic tissues are low, but still comparable to those of some extraneural tissues of vertebrates; for example, adult rat liver, pancreas, skeletal muscle, and eye lens contain 22.3 ± 3.0, 12.9 ± 5.0, 3.9 ± 0.3, and 3.6 ± 1.9 μg sialic acid per gram of wet tissue weight, respectively (M. Saito and K. Sugiyama, unpublished data). On the other hand, their concentrations in cerebral ganglia of both animals are extremely low. It is known that the content of gangliosides in brain tissue tends to decrease in lower animal classes in evolutionary terms [49–52]. The low ganglioside concentrations of cerebral ganglia may reflect the evolutionary stages of their neural system, as compared with vertebrates such as fish and higher animals. A distinction between the compositions of resorcinol-positive acidic lipids in hepatopancreatic tissues and cerebral ganglia suggests that gangliosides in these tissues may be expressed in tissue-specific manners, as observed in the higher animals in deuterostomia.

The present study also provided solid evidence that sialic acids exist as normal tissue constituents in some mollusks. The occurrence of sialic acids among protostomia has been reported in few studies. *N*-Acetylneuraminic acid is expressed temporarily at certain developmental stages of *Drosophila melanogaster* [53]. Evidence suggesting the presence of sialic acids in some marine bivalves was also presented [54]. The occurrence of sialic acids in squid and octopus implies that sialic acids may be distributed among protostomia more broadly than believed previously.

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