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Glycosylceramides Obtain from the Starfish *Asterias amurensis* Lütken

A.K.M. Azad Shah[1], Mikio Kinoshita[2], Hideyuki Kurihara[1], Masao Ohnishi[2] and Koretaro Takahashi[1]*

[1] *Division of Marine Life Science, Graduate School of Fisheries Sciences, Hokkaido University (Hakodate 041-8611, JAPAN)*
[2] *Department of Agricultural and Life Science, Obihiro University of Agriculture and Veterinary Medicine (Obihiro 080-8555, JAPAN)*

Abstract: Complex lipids in the starfish *Asterias amurensis* were characterized and the influence of sphingoid bases on human colon carcinoma Caco-2 cells was also investigated. Lipid content of gonad and viscera were 3.3% and 6.8%, respectively, in wet basis. The main lipid class in gonad was ceramide monohexoside (CMH) while triglyceride (TG) was predominant in the viscera. The most abundant fatty acid in the polar lipid was eicosapentaenoic acid (EPA, C20:5n-3), with the gonad and viscera samples having the highest proportion of 41.5% and 32.7%, respectively, of total fatty acids. Starfish internal organ contained enormous amount (0.7% in wet base) of glycosylceramide. Sphingoid bases of the glycosylceramide were mainly consisted of d22:2, d22:1 and d18:3. This sphingoid base exerted an apoptotic activity on Caco-2 cells. Thus, starfish could be used as a potential source of precious and useful complex lipids.

Key words: *Asterias amurensis*, glycosylceramide, sphingoid base

1 INTRODUCTION

The starfish *Asterias amurensis* Lütken is widely distributed in the North Pacific. Asteroids in the genus *Asterias* are notoriously efficient predators, attacking mollusks and other echinoderms[1]. Any outbreak of this starfish causes severe damage to the fishery and aquacultural grounds for benthic shellfish[2,3]. Starfish, very often, eats away scallops and seriously damages fishing gears. For this reason, elimination has been done continuously and it has been a burden to fisherman. In the Nemuro Bay area (Hokkaido) of northern Japan, physical removal of *A. amurensis* is routinely practiced in the scallop culture ground[4]. As a result, a huge number of starfish are collected and accumulate as waste. In Hokkaido, the amount of starfish waste is estimated to be about 15,000 tons per year. Although such starfish are a nuisance to fishermen, some trials have attempted to utilize starfish waste as fertilizer and plant growth regulators[5,6]. It has been reported that *A. amurensis* powder is rich in EPA bound phosphatidylcholine[7]. Moreover, in starfish, several unique glycosphingolipids (GSLs) such as gangliosides having a sialic acid in the middle of sugar chains[8,9], those with a sialic acid bound to the glycolyl group of the penultimate sialic acid[10], and neutral GSLs with d22:2 long chain bases[11,12] have been reported. In addition, steroidal glycosides and related compounds are predominant metabolites in starfish and have a broad variety of biological activities[13,14].

Sphingolipid hydrolysates, such as sphingosine, inhibit protein kinase C (PKC), a pivotal enzyme in cell regulation and signal transduction. Sphingolipids affect significant cellular responses and exhibit antitumor promoter activities in various mammalian cells. These molecules may function as endogenous modulators of cell function and possibly as second messengers[15]. It has been reported that ceramide; sphingosine and sphinganine induce apoptosis in HT-29 and HCT-116 human colon cancer cell[16]. Although various sphingoid bases were identified in the spermatozoa of *A. amurensis*[17], the existence of sphingoid bases in the internal organs including gonad and their biological activity is poorly understood. Therefore, the present study was conducted to investigate the variation of total lipid content, lipid class composition and fatty acid composition in the internal organs of starfish. The composition of sphingoid bases in starfish cerebrosides and their effect on

*Correspondence to: Koretaro Takahashi, Division of Marine Life Science, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, JAPAN
E-mail: kore@fish.hokudai.ac.jp
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human colon carcinoma Caco-2 cells were also investigated.

2 EXPERIMENTAL

2.1 Materials

Asterias amurensis was collected at the coast of Kushiro city, Hokkaido, Japan, during the month of June, 2004. Starfish samples were pooled into six groups. Gonad and viscera were carefully removed from starfish, and stored at −40℃ until use.

Caco-2 cell was obtained from RIKEN Gene Bank, Tsukuba, Japan. Dulbecco's Modified Eagle's Medium (DMEM), penicillin and streptomycin were obtained from GIBCO (Grand Island, NY, USA). Fetal calf serum (FCS) and L-glutamine were obtained from ICN Biomedicals, Inc. (Costa Mesa, CA, USA). Sodium periodate and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemical solvents were of analytical grade.

2.2 Lipid extraction

The lipids were extracted from gonad and viscera of starfish samples with a solvent combination of chloroform: methanol: distilled water according to the method of Bligh and Dyer[18] with slight modification making those final ratios 10:5:3, v/v/v. The extracted lipid was redissolved in chloroform and stored under argon gas in dark at −20℃ till further analysis.

2.3 Fractionation of total lipids

Polar lipid (PL) and non-polar lipid (NL) were separated from the total lipid by using Sep-Pak Vac 12cc silica cartridges (Waters Corporation, Milford, MA, USA) as described by Juaneda and Rocquelin[19]. The lipid samples (200 mg of total lipid) were loaded on the top of the cartridges. Then non-polar and polar lipids were eluted with chloroform and methanol respectively, in sequential order.

2.4 Lipid class composition analysis

The lipid class compositions of non-polar lipid were determined by using a commercial silica gel $60F_{254}$ (Merck KGaA, Darmstadt, Germany) thin-layer chromatograph (TLC) plate using a single step development system consisting of *n*-hexane: diethyl ether: acetic acid (80:20:1, v/v/v). The plate was sprayed with 50% H_2SO_4 and heated at 150-160℃ for 15 min. Each spot was identified by authentic lipid standards and then lipid compositions were analyzed by using scanner and Scion Image software (Scion Corporation, Frederick, Maryland, USA). Monoglycerides (1-MG and 2-MG) were determined by TLC using *n*-hexane: diethyl ether: acetic acid (50:50:1, v/v/v) as the developing solvent and 50% H_2SO_4 as a detection reagent. Lipid composition of polar lipid was determined by TLC with the solvent system of chloroform: methanol: water (65:25:4, v/v/v) and 50% H_2SO_4 as a detection reagent. Phosphatidylserine (PS) and lysophosphatidylethanolamine (LPE) were determined by using the solvent system of chloroform: methanol: 25% ammonia (65:25:5, v/v/v) and ninhydrin as a detection reagent.

2.5 Fatty acid composition of polar lipid

Fatty acid methyl esters were derived from the polar lipid of gonad and viscera samples following the method described by Prevot and Mordret[20]. In brief, dried lipid samples were dissolved in 1 mL *n*-hexane, and 0.2 mL of methanolic 2N-NaOH solution was added. The mixture was shaken and kept at 50℃ for 20 seconds and then 0.2 mL of methanolic 2N-HCl solution was added. The *n*-hexane layer was collected, concentrated and subjected to gas chromatographic analysis with a Hitachi 163 Gas Chromatograph (Hitachi, Ltd. Tokyo, Japan) connected with a PEG-20M liquid phase-coated G-300 column (1.2 mm i.d. × 40 m, 0.5 μm; Chemicals Evaluation and Research Institute, Saitama, Japan) with flame ionization detection. The temperature of the column, detector and injector were 170, 250 and 240℃, respectively. The fatty acids were identified by comparing the peak retention times with authentic standards (GL Sciences Inc. Tokyo, Japan) and by following the theory of linear relationship between the carbon number unit or the number of double bonds of fatty acid and logarithm of the corresponding retention times.

2.6 Determination of sphingoid bases from starfish cerebroside

Cerebrosides were separated from polar lipid fraction of internal organs by using TLC developed in chloroform: methanol: water (65:25:4, v/v/v). The lipid band corresponding to cerebrosides were visualized under UV light irradiation in a dark room and compared with an authentic standard. The cerebroside-containing band was scraped off and immediately eluted with methanol. Methanol elutes were evaporated, dissolved in a chloroform: methanol: water (10:5:3, v/v/v) mixture and placed in a separatory funnel to remove silica gel. The separatory funnel was kept at 4-5℃ overnight, and then the chloroform layer was collected and evaporated to obtain the cerebrosides. To isolate the sphingoid bases, the prepared cerebrosides were subjected to strong alkaline hydrolysis (10% aqueous $Ba(OH)_2$/dioxane, 1:1, 24 h at 110℃)[21]. The liberated sphingoid bases were then extracted with diethyl ether and purified by silica TLC developed in chloroform: methanol: ammonia (40:10:1, v/v/v). The composition of the sphingoid bases was determined by oxidation with sodium periodate and subsequent GC-MS of the resulting fatty aldehyde[22]. Sphinganine (d18:0) was added as an internal standard. The GC-MS analysis was performed with a QP5050A instru-

ment (Shimadzu, Kyoto, Japan) equipped with a CP-Sil 88 column (0.25 mm i.d. × 50 m, 0.2 μm; Chrompak, Middelburg, The Netherlands). The column temperature was programmed from 80℃ to 160℃ at 10℃/min and then to 200℃ at 2℃/min. The injector and detector were held at 250℃. The mass spectra were identified by referring to the data reported by Karlsson *et al*.[23]. The sphingoid bases were also analyzed by GC-MS in the DB-1 capillary column at 220℃ after converting to *N*-acetylated-*O*-trimethylsilylated derivatives[24].

2.7 Cell culture

Caco-2 cells were cultured in DMEM containing 10% FCS and supplemented with antibiotics (100 IU/ml of penicillin and 100 μg/ml of streptomycin), 2 mM glutamine, and 0.1 mM non-essential amino acids. The cells were kept at 37℃ in a humidified atmosphere containing 5% CO_2.

2.8 Microscopic assay

The apoptotic cells were evaluated by their morphological changes, the condensed chromatin fragments being visualized under a fluorescence microscope after being stained with 4′,6-diamidino-2-phenylindole (DAPI). Caco-2 cells were seeded at 5 × 10^4 cells/well in 8-well chamber slides (Nunc, Naperville, IL, USA) and cultured for 24 h, before changing the medium which containing 20 μM sphingoid bases and culturing for a further 24 h, and then the cells were stained with 0.05% DAPI[25].

2.9 Statistical analysis

The Q value examination proposed by Dean and Dixon[26] was used to minimize the error. Student's *t*-test was used to determine significant differences between samples. Trends were considered significant when the mean of compared sets differed at $p<0.05$.

3 RESULTS AND DISCUSSION

3.1 Lipid content and lipid class composition

Morphological parameters and lipid content of internal organs in starfish are presented in Table 1. The mean lipid content of gonad and viscera was 3.3% and 6.6%, respectively, in wet weight basis, which was comparatively higher than that of whole starfish (1.8%) (data not shown). Lipid content in the internal organs of starfish was found to be significantly higher in viscera than in gonad. The polar lipid proportion in gonad total lipids was rather different to that found in viscera (59.9% and 29.2% of total lipid) (Table 2). The major lipid class of internal organs was triglyceride (TG), diglyceride (DG), phosphatidylcholine (PC), and ceramide monohexoside (CMH). Significantly highest amount of PC and CMH was found in the gonad, on the other hand TG and DG were found to be significantly highest in the viscera. Internal organs of starfish contained enormous amount (0.7% in wet base) of cerebrosides, which was much higher than that reported by Sugawara *et al*.[27]. They found that sea cucumber contained about 200 mg cerebrosides per 100 g of dry powder. In another study, very small amount (1-40 mg/100 g dry weight) of cerebrosides was found in some plant sources[28]. Cerebrosides are used as food and cosmetic ingredients; therefore, cerebrosides from starfish internal organs may be used for industrial purposes.

Table 1 Morphological Parameters and Lipid Content of Starfish (*Asterias amurensis*).

Morphological parameters[1]	
Body length (cm)	13.10
Arm length (cm)	4.77
Body weight (g)	296.43
Gonad weight (g)	38.27
Viscera weight (g)	47.30
Gonad Index[2]	12.90
Lipid content (% wet weight basis)	
Gonad	3.33 ± 0.13*
Viscera	6.68 ± 1.08*

[1] Each value is expressed as mean (n = 55).

[2] Gonad Index was calculated as gonad weight / total body weight × 100.

* Lipid content of gonad and viscera are significantly different ($P < 0.05$). Results represent mean ± SD; n = 6 as samples were pooled into six groups

3.2 Fatty acid composition of polar lipid

The fatty acid compositions of polar lipid in starfish internal organs are presented in Table 3. With some exceptions, the same patterns of fatty acids were found in the gonad and viscera. The major fatty acids were C18:0, C20:1n-9, C20:4n-6 and C20:5n-3, and they comprised more than 73% in gonad and 67% in viscera of the total fatty acid composition in the polar lipid. Among monounsaturated fatty acids, C20:1n-9 was most dominant fatty acids in the gonad and viscera polar lipids. Significantly higher amount of polyunsaturated fatty acids (PUFAs) was found in gonad (57.90%) compared to viscera (45.62%). Within this group, the major fatty acids were C20:4n-6 and C20:5n-3. Eicosapentaenoic acid (EPA, C20:5n-3) was found in more significant amount than docosahexaenoic acid (DHA, C22:6n-3) in the gonad and viscera. However, the total amount of EPA and DHA in the gonad and viscera were 46.46% and 36.74%, respectively. Significantly higher amount of EPA was observed in gonad compared to viscera.

Diversity of fatty acids in terms of chain length, degree of unsaturation and position of the double bonds is respon-

Table 2 Lipid Class Composition (% of total lipid) of Internal Organs in Starfish[1].

Lipid component	Gonad	Viscera
Non-polar lipid		
1-monoglyceride	0.27 ± 0.06	0.36 ± 0.09
2-monoglyceride	0.14 ± 0.04	0.42 ± 0.13*
1,2-diglyceride	0.75 ± 0.12	1.28 ± 0.16*
Sterols	3.76 ± 0.49	5.50 ± 0.71*
1,3-diglyceride	0.58 ± 0.16	0.68 ± 0.09
Free fatty acid	2.97 ± 0.94	5.12 ± 1.79*
Triglyceride	21.60 ± 1.65	43.76 ± 5.12*
Diglyceride	6.92 ± 1.39	13.62 ± 1.18*
Hydrocarbon	3.16 ± 1.37	tr[+]
Polar lipid		
Lysophosphatidylcholine	3.64 ± 1.40	1.51 ± 0.59*
Phosphatidylserine	2.10 ± 0.54	1.45 ± 0.14*
Lysophosphatidylethanolamine	1.96 ± 0.43	1.47 ± 0.39*
Phosphatidylcholine	15.44 ± 1.38	6.46 ± 0.58*
Phosphatidylethanolamine	8.19 ± 1.63	2.45 ± 0.38*
Ceramide monohexoside	22.71 ± 2.54	10.91 ± 1.28*
PL others	5.83 ± 1.71	4.97 ± 1.28

[1] Pairs of mean corresponding to gonad and viscera of starfish were compared and those that were significantly different ($P < 0.05$) are indicated by (*). Results represent mean ± SD (n = 6); [+]trace (less than 0.05%)

sible for the ultimate characteristics of lipids among different organisms. Polyunsaturated fatty acids (PUFA) are considered essential fatty acids for marine invertebrates because of their inability to synthesize them endogenously[29,30]. In the present study, we found that starfish internal organs contained high proportion of polyunsaturated fatty acids (PUFAs) and the most predominant fatty acid was eicosapentaenoic acid (EPA). Eicosapentaenoic acid is shown to be characteristic fatty acid of most of the Japan sea invertebrates and high level of EPA was observed in echinoderms, in two species of holothurians: *Cucumaria fraudatrix* and *Cucumaria japonica*[31]. Romashina[32] also reported that the main fatty acid of the majority of 23 Japan sea invertebrates was EPA.

3.3 Composition of sphingoid bases

The sphingoid bases were analyzed by GC-MS as aldehyde derivatives (Fig. 1). The numbered peaks representative mass spectra are shown in Fig. 2. Some of the bases showed same mass spectrum by GC-MS of the *N*-acetylated-*O*-TMS derivatives, e.g. Peak 3 and 4 had the same mass spectra patterns. The fragment ion correspondent to $[M-18]^+$ were observed in the mass spectra Peak 1 (m/z 276; Fig. 2A), Peak 2 (m/z 205; Fig. 2B) and Peaks 3 and 4 (m/z 274; Fig. 2C). Therefore, the peaks were determined to be the *N*-acetylated-*O*-TMS derivatives of sphingoid bases as follows: Peak 1, d22:1 (M = 294); Peak 2, d18:3 (M = 234); Peak 3 and 4, d22:2 (M = 292). The sphingoid base compositions of cerebrosides in starfish are depicted in Table 4.

The most common sphingoid base of mammalian sphingolipids are sphingosine (*trans*-4-sphingenine, d18:1[4t]) and smaller amounts of others such as sphinganine (dihydrosphingosine, d18:0) and phytosphingosine (4-hydroxysphinganine, t18:0) are frequently present[33]. The main components of sphingoid bases from starfish cerebrosides were d22:2, d22:1 and d18:3 which suggested that the sphingoid base components of starfish cerebrosides are quite different from that of mammalian sphingolipids. In addition, the long chain bases (LCBs) of the glycolipid consisted of dihydroxy (d18:2, d18:3, d19:3 and d22:2) and trihydoxy (t22:1) types were identified in the spermatozoa of *A. amurensis*[17]. Previously d22:2, d18:3 and d19:3 LCBs have been reported in marine invertebrates: d22:2 in *Asterias rubens*[34]; d18:3 and d19:3 in the ascidians[35], and in the gonad and body walls of the Patagonian starfish *Allostichaster inaequalis*[36].

3.4 Apoptotic activity on Caco-2 cells

Marine-derived cerebrosides and gangliosides have been

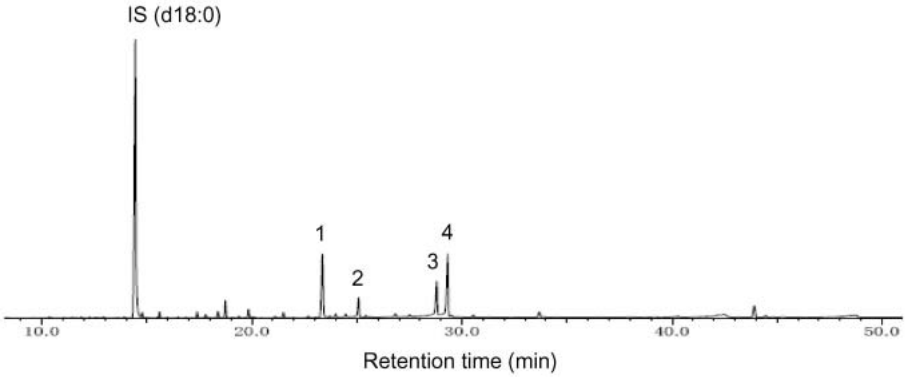

Fig. 1 Total Ion Chromatogram of Aldehydes Derived from the Sphingoid Bases in Starfish Cerebroside.
IS = Internal standard.

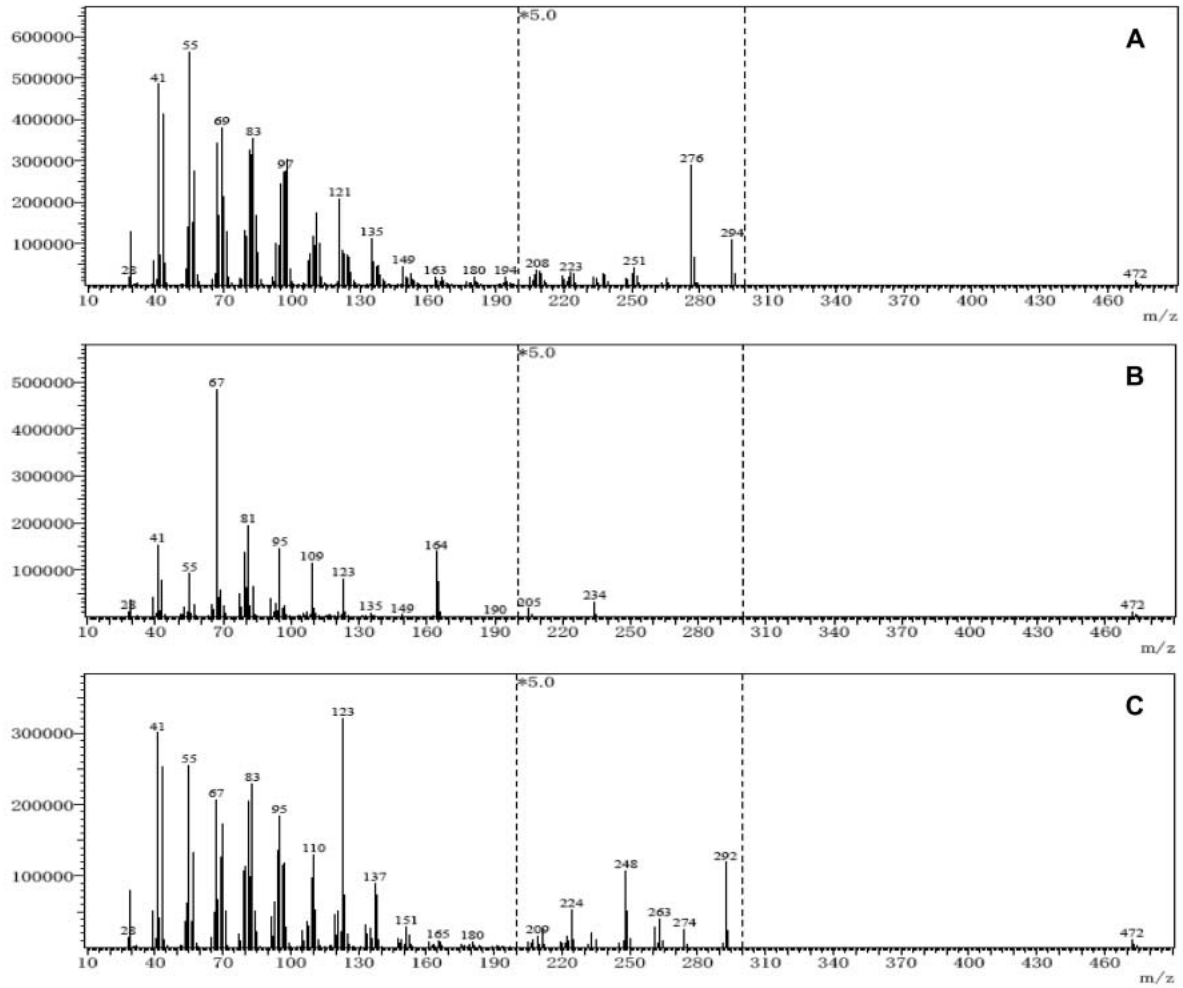

Fig. 2 Mass Spectra (EI mode) of Peak 1 (A), Peak 2 (B) and Peak 3 (C) in **Fig. 1**.

Table 3 Fatty Acid Composition (% of total fatty acids) of Polar Lipids in Starfish Internal Organs[1].

Fatty acid	Gonad	Viscera
C16:0	1.62 ± 0.31	2.44 ± 0.34*
C18:0	6.18 ± 0.49	8.32 ± 0.73*
Others	−	1.34 ± 1.22
Σ saturated	7.80 ± 0.79	12.10 ± 1.96*
C16:1n-7	1.32 ± 0.19	1.58 ± 0.25
C17:1n-9	2.84 ± 0.43	2.70 ± 0.89
C18:1n-9	4.36 ± 0.43	5.08 ± 0.83*
C20:1n-9	17.58 ± 1.55	19.42 ± 1.75
Others	0.22 ± 0.05	1.18 ± 0.82*
Σ monounsaturated	26.32 ± 1.05	29.96 ± 2.89*
C15:2	0.72 ± 0.67	1.22 ± 0.76
C20:4n-6	8.08 ± 0.65	7.36 ± 0.44*
C20:5n-3	41.52 ± 1.79	32.76 ± 1.69*
C22:5n-6	0.94 ± 0.53	−
C22:6n-3	4.94 ± 0.74	3.98 ± 1.44
Others	1.70 ± 0.60	0.30 ± 0.21*
Σ polyunsaturated	57.90 ± 2.42	45.62 ± 3.04*
Others	7.98 ± 1.74	12.32 ± 1.59*

[1] Pairs of mean corresponding to gonad and viscera of starfish were compared and those that were significantly different ($P < 0.05$) are indicated by (*). Results represent mean ± S.D. (n = 6), (−) not detected.

Table 4 Sphingoid Base Composition of Cerebrosides in Starfish.

Peak no	M^+	Aldehyde[1]	Parent base[1]	%
1	294	20:1	d22:1	32
2	234	16:3	d18:3	11
3, 4	292	20:2	d22:2	49
Others				8

[1] The aldehyde and parent bases are designated by chain length, while the second represents the number of double bonds, and d means dihydroxy base.

reported to exhibit various pharmacological effects including antitumor and neuritogenic activities[37,38]. Furthermore, ceramide and sphingosine, the hydrolyzed products from sphingolipids, have induced apoptosis in several cancer cells[16,39]. Apoptotic cells with characteristic nuclear condensation and fragmented nuclei were observed by a fluorescence microscope after being treated with 20 μM starfish sphingoid bases for 24 h (Fig. 3). This result suggested that sphingoid bases derived from starfish cerebroside in the internal organs induce apoptosis on human colon carcinoma Caco-2 cells. Similarly, Aida *et al.*[40] found that 20 μM maize sphingoid bases induce apoptosis on Caco-2 cells. They also reported that the optimum dose and incubation time for the apoptosis inducement of sphingoid bases are 20 μM and 24 h, respectively, because higher doses of sphingoid bases (50 μM) caused necrosis-like cell death. It has also been reported that sphingoid bases prepared from wheat flour consisted of mainly 8-sphingenine induced apoptosis in DLD-1 colon cancer cells[41], and sea

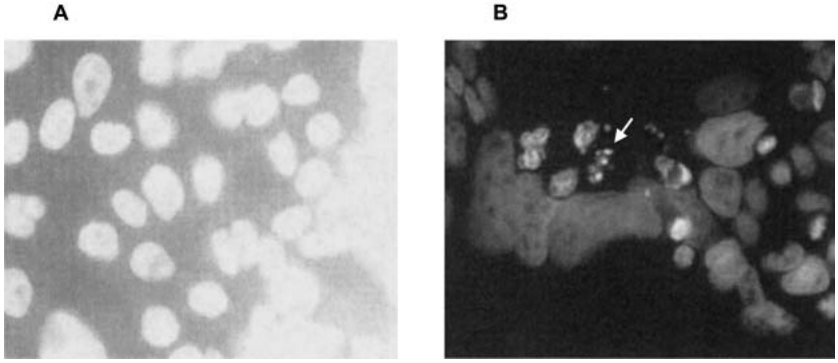

Fig. 3 Nuclear Morphology of Caco-2 Cells Added with Sphingoid Bases Derived from Starfish Cerebroside.
The cells were incubated for 24 h in the presence or absence of starfish sphingoid bases (20 μM), and then stained with DAPI. The nuclear morphology was assessed by fluorescence microscopy (magnification, × 400). A, cells without sphingoid bases: B, cells cultured with sphingoid bases. Under going of apoptosis are clearly seen in the arrow portion.

cucumber sphingoid bases also induced apoptosis in several colon cancer cell lines[27].

4 CONCLUSIONS

Results of the present study demonstrated that internal organs of starfish contained high proportion of PUFA (45.6%-57.9%) in the polar lipid fraction and amount of EPA was more than 40% of total fatty acids in the gonad lipid. Internal organs of starfish contained approximately 0.7% cerebrosides in wet base. Our results also confirmed that sphingoid bases of starfish cerebroside induce apoptosis in human colon carcinoma Caco-2 cells. Thus, starfish could be used as a potential source of precious and useful complex lipids.

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