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Original article

Chemical structure analysis of mussels (*Mytilus galloprovincialis*)-derived ceramide 2-aminoethylphosphonate

Koki Sugimoto[1)†], Nao Onuma[1)], Ayano Kosaka[2)], Ryota Hosomi[2)], and Kenji Fukunaga[2)]

[1)]*Faculty of Food and Nutritional Sciences, Toyo University**
[2)]*Faculty of Chemistry, Materials, and Bioengineering, Kansai University***
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Summary

Although mussels (*Mytilus galloprovincialis*) are a popular seafood, their lipid composition remains inadequately characterized. According to previous studies, several bivalve species contain ceramide 2-aminoethylphosphonate (CAEP), a type of sphingophosphonolipid. CAEP is recognized for its health-promoting properties, including its ability to lower serum and liver cholesterol levels and enhance skin barrier function. This study aimed to investigate the lipid composition of mussels with a particular focus on CAEP, to facilitate their effective utilization as a CAEP source. Moreover, chemical structural analysis was performed to elucidate the health-promoting functions of CAEP. The results showed that approximately 60 wt% of phospholipids fraction in mussels are composed of CAEP. Furthermore, chemical structural analysis revealed that CAEP is characterized by short fatty acids with carbon chain lengths of 14 or 15. By contrast, the long-chain base present in mussels-derived CAEP was predominantly composed of d16:1, d18:0, and d18:1, which are commonly found in CAEP derived from bivalves. These findings are expected to provide valuable insights for the future utilization of mussels as a CAEP source.

Introduction

The lipid composition of shellfish is characterized by various complex lipids that are bound to sugar chains and phosphorus. Notably, several bivalve species contain sphingophosphonolipids (sphingolipids that feature carbon–phosphorus bonds that are rarely found in other higher animals). According to previous studies, bivalves, including pearl oysters (*Pinctada martensii*) and scallops (*Patinopecten yessoensis*), contain ceramide 2-aminoethylphosphonate (CAEP), a specific type of sphingophosphonolipid[1,2)]. Furthermore, CAEP has been reported to exhibit health-promoting properties, including its ability to reduce serum and liver cholesterol levels and enhance skin barrier function[3,4)]. However, the chemical structure of CAEP varies among species[1)], causing differences in these health-promoting effects. Therefore, a thorough understanding of the chemical structure of CAEP is essential for its utilization as a functional component.

Mussels (*Mytilus galloprovincialis*), a bivalve species that inhabits the intertidal zone of marine environments, are commonly found in the Kanto region of Japan and considered an invasive species originating from the Mediterranean Sea. Other major mussels-producing countries include Chile, China, and Spain. However, despite the widespread consumption of mussels, their lipid composition remains poorly understood. Considering mussels are bivalves similar to pearl oysters and scallops, they may likely contain CAEP.

In this study, we aimed to investigate the lipid composition of mussels, with a particular focus on the presence of CAEP. In addition, CAEP derived from bivalves comprises hydroxy fatty acids and sphingatrienine, which are not typically found in general sphingolipids[5)]. The health-promoting functions of sphingolipids are known to depend on their chemical structure[6)]. Therefore, we conducted a chemical structural analysis of sphingophosphonolipids present in mussels to elucidate their health-promoting functions.

† Corresponding author: Koki Sugimoto, E-mail: sugimoto080@toyo.jp
* Address: 48-1, Oka, Asaka-shi, Saitama 351-8510, Japan
** Address: 3-3-35, Yamate-cho, Suita, Osaka 564-8680, Japan

Materials

Commercially available Chilean mussels (*M. galloprovincialis*) that were harvested in 2023 were purchased. The mussels used in the experiments were boiled and dehydrated using acetone. During this process, the acetone layer was extracted to ensure that no coloration was observed. Subsequently, acetone-soluble fats and pigments were removed. The wet weight of the mussels before these processes was 862 g, and the dry weight after the processes was 214 g.

The standard CAEP used in this study was derived from clam (*Ruditapes philippinarum*), which was independently prepared by our laboratory. In addition, the standard ceramide phosphoethanolamine (CPE) derived from fruit fly (*Drosophilidae*) was provided by the chemistry laboratory of Shiga University. Other chemicals were procured from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); Nacalai Tesque, Inc. (Kyoto, Japan); and Merck KGaA (Darmstadt, Germany).

CAEP purification

CAEP derived from mussels was purified in accordance with the method by Sugita *et al.*,[7] with some modifications (Fig. 1). Briefly, the crude complex lipid fraction was extracted from mussels using a chloroform–methanol mixture. The fraction was stirred in acetone, and the insoluble powder was collected as the phospholipids fraction. Then, alkaline saponification and acid treatment were performed to decompose glycerophospholipids. Moreover, acetone was added to obtain an acetone-insoluble powder as a sphingolipids fraction. The sphingolipids fraction was fractionated into a chloroform/methanol/water fraction and an ammonium acetate fraction by QAE-Sephadex (OH^-) column (Tosoh Co., Tokyo, Japan) chromatography. The elution from the column was performed using chloroform/methanol/water (30:60:8), 0.05 M ammonium acetate–methanol solution, and 0.45 M ammonium acetate–methanol solution. The elution of 0.05 M ammonium acetate fraction was dialyzed in running water and then subjected to DEAE-Sephadex (COO^-) column (Tosoh Co.) chromatography. The fraction that passed through the column in chloroform/methanol/water (30:60:8) was collected and subjected to silica gel column chromatography. Fractions were collected over time while chloroform/methanol (1:1) was flowing through the column. Moreover, fractions containing the same Rf values as the standard CAEP by thin-layer chromatography (TLC) were concentrated to purify the mussels-derived CAEP.

Phospholipids fraction analysis

The phospholipid class was confirmed by TLC. Silica gel 60 (Merck KGaA) was employed as the TLC plate, and chloroform/methanol/water (60:30:5) was used as the developing solvent. Spots were detected with a 50% (v/v) sulfuric acid solution, ninhydrin solution, and molybdic acid solution. This was followed by the use of standard phosphonolipids and phospholipids (CAEP, CPE, phosphatidylcholine [PC], and phosphatidylethanolamine [PE]) for identification. The phospholipid class composition was calculated based on spot intensity using the JustTLC software (version 4.0.3, Lund, Sweden).

Fatty acid composition analysis

CAEP was reacted with 1 M methanolic hydrochloride and heated at 100℃ for 3 h. The fatty acid methyl esters were extracted using *n*-hexane and analyzed with a gas chromatography (GC) system (GC-2014; Shimazu Co.) and

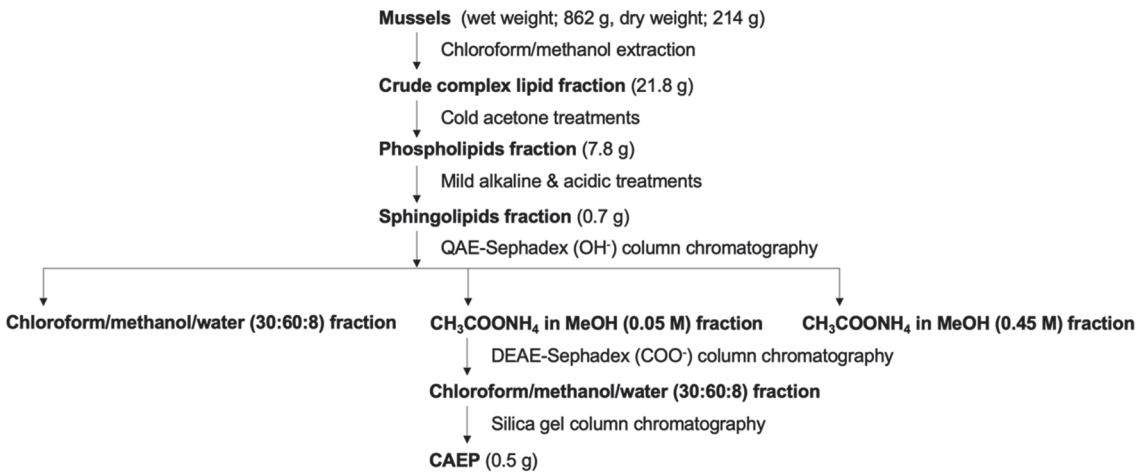

Fig. 1. Scheme for the preparation and fractionation of ceramide 2-aminoethylphosphonate from mussels
CAEP, ceramide 2-aminoethylphosphonate.

GC-mass spectrometry (MS) system (GCMS-QP2010/PARVUM2; Shimadzu Co.), equipped with the Omegawax® Capillary GC Column (cat no. 24152, Merck KGaA). The parameters were set as follows: injector and interface temperatures of 250℃ and a column temperature of 120℃, followed by a gradual increase to 240℃ at a rate of 2℃/min and held at 240℃ for 10 min, with helium as the carrier gas at a flow rate of 1 mL/min. The parameters for the MS system were as follows: ion source temperature, 200℃; electronic ionization (EI), 70 eV; and full scan range, *m/z* 45–350 amu. The fatty acid composition was determined by GC analysis, and peak compounds were identified by GC–MS analysis.

Long-chain base composition analysis

CAEP was added to 8.6% aqueous methanol hydrochloric acid and heated at 70℃ for 18 h[8]. Subsequently, the fatty acid methyl esters were extracted using *n*-hexane, after which, a mixture of 1 M sodium hydroxide solution in methanol and chloroform was added. The solution was then centrifuged to achieve phase separation. The chloroform layer was concentrated under a nitrogen stream to prepare the long-chain base fraction. Trimethylsilyldiazomethane was introduced to the resulting residue and heated at 60℃ for 30 min. GC and GC–MS were used to analyze this trimethylsilyl derivative, determine the long-chain base composition, and identify peak compounds, respectively. A nonpolar 5% phenylmethyl silicon chemically bonded silica capillary column (Shimadzu HiCap-CBP 5) with a film thickness of 0.25 μm was used as the analytical column. The parameters for the GC system were as follows: injector and interface temperatures of 250℃, column temperature maintained at 170℃, followed by a gradual increase to 240℃ at a rate of 4℃/min and held at 240℃ for 20 min, with helium as the carrier gas at a flow rate of 1 mL/min. Meanwhile, the parameters for the MS system were as follows: ion source temperature, 200℃, EI, 70 eV; and full scan range, *m/z* 45–500 amu.

Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) analysis

CAEP was dissolved in methanol and then subjected to *N*-acetylation using pyridine and acetic anhydride for 30 min at room temperature. The acetylated CAEP and matrix (*α*-cyano-4-hydroxycinnamic acid) were dried on glass slides and analyzed using MALDI-TOF MS (AXIMA® Confidence™, Shimadzu Co.). A nitrogen laser (laser wavelength, 377 nm) was used as the ion source, and the measurements were conducted in the negative-ion mode.

Results and Discussion

CAEP purification

The mussels used in this study were pretreated by heating and subsequently treated with acetone, which effectively removed neutral lipids and pigments while dehydrating the tissue. Consequently, the dry tissue weight reported herein reflects the weight after the acetone washing process. Fig. 1 shows that 21.8 g of the crude complex lipid fraction and 7.8 g of the phospholipids fraction were obtained from 862 g of mussels. The TLC results of the phospholipids fraction are shown in Fig. 2. This fraction comprised 61.9 wt% CAEP, 22.8 wt% PC, 12.5 wt% CPE, and 1.5 wt% PE (Table 1). In our analyses of other bivalves, the phospholipids fraction of pacific oysters (*Crassostrea gigas*) was composed of 26.0 wt% CAEP,

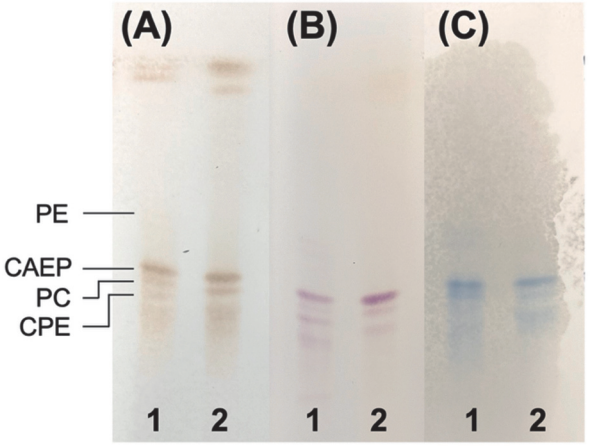

Fig. 2. Thin-layer chromatogram of the phospholipids and sphingolipids fractions from mussels
Lane 1, phospholipids fraction prepared from mussels; lane 2, sphingolipids fraction prepared from mussels. The plate was developed in chloroform/methanol/water (60:30:5, v/v/v) and the spots were visualized with 50% (v/v) sulfuric acid solution (A), ninhydrin solution (B), and molybdic acid solution (C).
CAEP, ceramide 2-aminoethylphosphonate; CPE, ceramide phosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Table 1. Phospholipid class of mussels-derived phospholipids fraction

Phospholipid class (wt%)	
CAEP	61.9
PC	22.8
CPE	12.5
PE	1.5
Others	1.4

CAEP, ceramide 2-aminoethylphosphonate; CPE, ceramide phosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

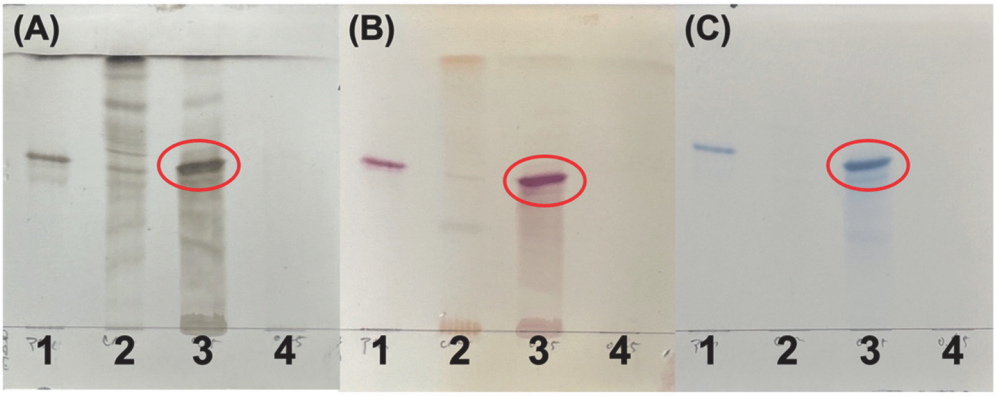

Fig. 3. Thin-layer chromatogram of QAE column chromatography fractions
Lane 1, standard ceramide 2-aminoethylphosphonate; lane 2, chloroform/methanol/water fraction; lane 3, CH_3COONH_4 in MeOH (0.05M) fraction; lane 4, CH_3COONH_4 in MeOH (0.45M) fraction. The plate was developed in chloroform/methanol/water (60:30:5, v/v/v) and the spots were visualized with 50% (v/v) sulfuric acid solution (A), ninhydrin solution (B), and molybdic acid solution (C). The spots circled indicate ceramide aminoethylphosphonate spots.

8.5 wt% PC, and 30.7 wt% PE[2]. In contrast, the phospholipids fraction of scallops (*Patinopecten yessoensis*) consisted of 8.7 wt% CAEP, 63.4 wt% PC, and 21.1 wt% PE, whereas that of clams (*Ruditapes philippinarum*) comprised 39.0 wt% CAEP, 20.0 wt% PC, and 38.8 wt% PE[9]. Compared with these species, mussels exhibited a lower PE content and higher CAEP content, indicating that they represent a superior source of CAEP relative to other bivalves.

Subsequently, QAE-Sephadex (OH^-) column chromatography was conducted. The elution solvent used for the column comprised chloroform/methanol/water (30:60:8) as a neutral solvent, alongside an ammonium acetate–methanol solution as polar solvents. The results of TLC analysis for each fraction are shown in Fig. 3. In the chloroform/methanol/water fraction (which is non-adsorbent to the carrier), various substances were eluted. Bivalves contain sphingoglycolipids with varying numbers of sugar chains, which appear as spots with different Rf values under specified TLC conditions[10]. Notably, bivalves possess high concentrations of neutral glycolipids lacking polarity, which likely eluted without adhering to the support. In contrast, CAEP was confirmed to be eluted in the 0.05 M ammonium acetate fraction.

Fig. 4. Thin-layer chromatogram of mussels-derived ceramide 2-aminoethylphosphonate
Lane 1, mussels-derived ceramide 2-aminoethylphosphonate; lane 2, standard ceramide aminoethylphosphonate. The plate was developed in chloroform/methanol/water (60:30:5, v/v/v) and the spots were visualized with 50% (v/v) sulfuric acid solution.

Furthermore, the 0.05 M ammonium acetate fraction, which contains phospholipids, was recovered via DEAE-Sephadex (COO^-) column chromatography and subsequently purified using a silica gel column, resulting in the extraction of 0.5 g of CAEP. The TLC results for CAEP derived from mussels are presented in Fig. 4. It was compared to the Rf value of standard CAEP, indicating that CAEP was purified from mussels. Similar to mussels, scallops, which are also bivalves, show two spots of CAEP with different Rf values on TLC[5]. This finding is thought to be because of the presence of highly polar fatty acids (e.g., hydroxy fatty acids) and long-chain bases as components of ceramide. However, since mussels-derived CAEP was detected in only one spot, it was predicted that ceramide components with significantly different polarities were not included.

Ceramide components of CAEP

The identification of fatty acids and long-chain bases comprising the ceramide derived from mussels in CAEP was accomplished through GC and GC–MS analyses. The compositions of the fatty acids and long-chain bases are presented in Tables 2 and 3, respectively. In addition, their chromatograms are shown in Fig. 5. The MS spectrum of fatty acid methyl esters was characterized by the pres-

Table 2. Fatty acid composition of mussels-derived ceramide 2-aminoethylphosphonate

Peak	Fatty acid (wt%)	
a	C14:0	4.1
b	C15:0	2.4
c	C16:0	62.9
d	C16:1	3.7
e	C17:0	1.5
f	C18:0	8.6
g	C18:1	9.5
h, i	Others	7.4

Peaks a-i in Table 2 are corresponding to peaks in Fig. 5 A.

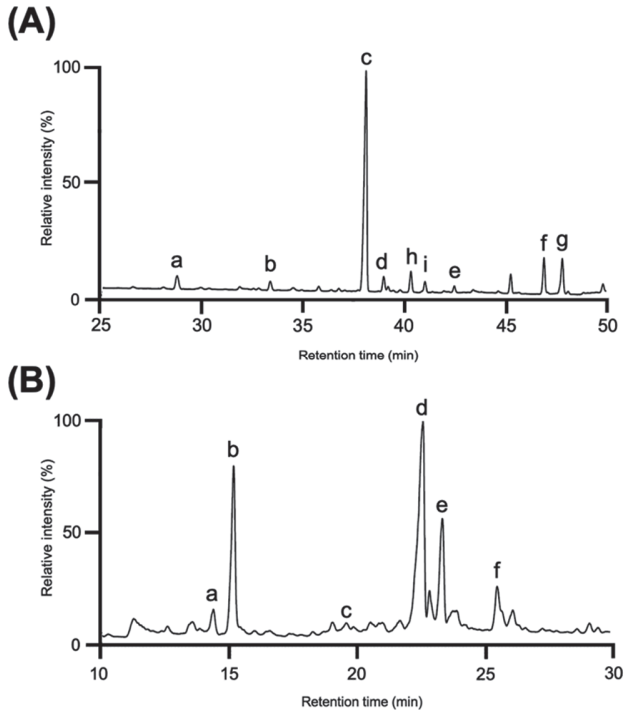

Fig. 5. Gas chromatograms of fatty acids (A) and long-chain bases (B) of mussels-derived ceramide 2-aminoethylphosphonate
A: Fatty acid methyl esters of mussels-derived ceramide 2-aminoethylphosphonate; a, C14:0; b, C15:0; c, C16:0; d, C16:1; e, C17:0; f, C18:0; g, C18:1; h and i, unknown.
B: Trimethylsilylated long-chain bases of mussels-derived ceramide 2-aminoethylphosphonate; a, d16:0; b, d16:1; c, d17:1; d, d18:0; e, d18:1; f, unknown.

ence of molecular ion peaks, *m/z* 74, and molecular ion peaks −31 and −43[11]. Based on this information, the fatty acid composition of mussels-derived CAEP was identified as follows: C14:0 (4.1%), C15:0 (2.4%), C16:0 (62.9%), C16:1 (3.7%), C17:0 (1.5%), C18:0 (8.6%), and C18:1 (9.5%) (MS spectra were not shown). The long-chain bases were identified from GC and GC–MS data as trimethylsilyl derivative. The MS spectrum of trimethylsilylated long-chain bases was characterized by molecular ion peaks, *m/z* 132, and molecular ion peaks −105 and −132. The composition of the long-chain bases in the mussels-derived CAEP was determined to be d16:0 (3.9%), d16:1 (27.8%), d17:1 (2.2%),

Table 3. Long-chain base composition of mussels-derived ceramide 2-aminoethylphosphonate

Peak	Long-chain base (wt%)	
a	d16:0	3.9
b	d16:1	27.8
c	d17:1	2.2
d	d18:0	15.9
e	d18:1	41.0
f	Others	9.0

Peaks a-f in Table 3 are corresponding to peaks in Fig. 5B.

d18:0 (15.9%), and d18:1 (41.0%) (Table 3). In Fig. 5, peak (f) shows the MS spectrum characteristic of the long-chain base. However, the chemical structure of this compound could not be determined.

The fatty acids present in mussels-derived ceramide are predominantly composed of C16:0, C18:0, and C18:1, which are commonly found in bivalve-derived CAEP. In addition to these, mussels-derived CAEP also contained C14:0 and C15:0 fatty acids. Most fatty acids found in marine-derived CAEP comprise carbon chains with 16 or more carbon atoms, whereas short fatty acids with carbon chain lengths of 14 or 15 are rarely reported. The findings of this study indicate that mussels-derived CAEP uniquely contains fatty acids with 14 or 15 carbon chains. Conversely, the long-chain base of ceramides contained in mussels-derived CAEP are primarily composed of d16:1, d18:0, and d18:1, which are typically found in bivalve-derived CAEP. Further investigation is required to elucidate the factors that contribute to the observed differences in carbon chain length. In addition, the chemical structure of ceramide has been suggested to be similar within the same taxonomic order of animals[1]. Although no other information is currently available with regard to CAEP in mollusks of the *Mytilida* order, CAEP in other bivalves belonging to this order may exhibit a similar ceramide chemical structure to that identified in this study.

MALDI-TOF MS analysis

MALDI-TOF MS operates in two measurement modes: positive-ion and negative-ion modes, which depend on the functional groups present in the analyzed sample. For lipids containing acidic functional groups, the ionization efficiency is lower in the positive-ion mode[1]. In addition, for phospholipids with free amino groups, analyzing them as *N*-acetyl derivatives proves to be effective[7]. Consequently, in the case of mussels-derived CAEP, MALDI-TOF MS analysis was performed in the negative-ion mode following the *N*-acetylation of free amino groups. The MS spectrum is shown in Fig. 6. Forty-two masses (CH_3COO = 42) more spectra based on each molecular species [M-H] were ob-

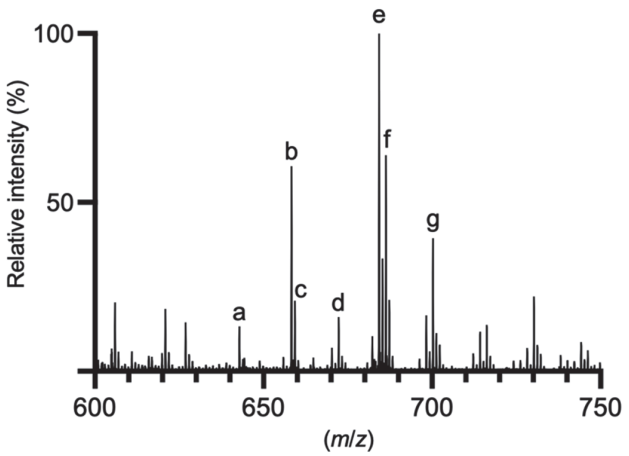

Fig. 6. Negative-ion mode of matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry spectra of *N*-acetylated mussels-derived ceramide 2-aminoethylphosphonate
a, *m/z* 643.98 (C15:0 fatty acid-d16:1 long chain base, calculated value 644.68); b, *m/z* 654.08 (C16:1-d16:1, 654.68); c, *m/z* 656.09 (C16:1-d16:0 and C16:0-d16:1, 656.69); d, *m/z* 666.09 (C16:0-d17:1, 666.69); e, *m/z* 680.16 (C16:0-d18:1 and C16:1-d18:0, 680.72); f, *m/z* 682.27 (C16:0-d18:0 and C18:0-d16:0, 682.73); g, *m/z* 702.12 (C18:1-d18−1, 702.72).

served than before *N*-acetylation, corresponding to the major components of ceramides detailed in Tables 2 and 3. The primary spectra observed were as follows: a) *m/z* 643.98 (C15:0 fatty acid-d16:1 long-chain base, calculated value 644.68), b) *m/z* 654.08 (C16:1-d16:1, 654.68), c) *m/z* 656.09 (C16:1-d16:0 and C16:0-d16:1, 656.69), d) *m/z* 666.09 (C16:0-d17:1, 666.69), e) *m/z* 680.16 (C16:0-d18:1 and C16:1-d18:0, 680.72), f) *m/z* 682.27 (C16:0-d18:0 and C18:0-d16:0, 682.73), and g) *m/z* 702.12 (C18:1-d18−1, 702.72). These findings confirm the presence of the spectra attributed to the product. The results indicate that the majority of fatty acids listed in Table 1, along with long-chain CAEP molecules, are present. However, there are limitations to the combinations of fatty acids and long-chain bases that constitute ceramides. For example, combinations such as C15:0-d18:1 and C16:1-d17:1 were not detected, suggesting that only specific combinations exist. Further research is needed to elucidate the factors that influence these ceramide molecule combinations.

In this study, we identified the presence of CAEP in mussels. CAEP constitutes over 60 wt% of the total phospholipids fraction, indicating that mussels are a significant source of CAEP compared with other bivalves. Furthermore, we elucidated the chemical structure of CAEP, which is characterized by short fatty acids with carbon chain lengths of 14 or 15. These findings are anticipated to provide essential information for the future utilization of mussels as a CAEP source.

Acknowledgments

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References

1) Itonori S, Kitamura T, Tanaka R, Miyagaki N, Saito H, Sugita M (2006) Aliphatic components of phosphonolipids (ceramide 2-aminoethylphosphonates) from the pearl oyster, *pinctada martensii*. Mem. Fac. Educ., Shiga Univ., Nat. Sci. 56: 51–62.
2) Sugimoto K, Hosomi R, Yoshida M, Fukunaga K (2021) Dietary phospholipids prepared from scallop internal organs attenuate the serum and liver cholesterol contents by enhancing the expression of cholesterol hydroxylase in the liver of mice. Front. Nutr. 8: 761928.
3) Sugimoto K, Kosaka A, Hosomi R, Itonori S, Yoshida M, Fukunaga K (2024) Scallop ceramide 2-aminoethylphosphonate is more effective than egg yolk sphingomyelin in improving cholesterol metabolism in obese/type II diabetic KK-A^y mice. J. Func. Foods 123: 106569.
4) Tomonaga N, Manabe Y, Aida K, Sugawara T (2020) Dietary ceramide 2-aminoethylphosphonate, a marine sphingophosphonolipid, improves skin barrier function in hairless mice. Sci. Rep. 10(1): 13891.
5) Sugita M, Itonori Saki, Makita A, Higashino A, Nozaki H, Kojima H (2008) Structural analysis of phosphonolipids of the giant ezo scallop *patinopecten yesseoensis*. Mem. Fac. Educ., Shiga Univ., Nat. Sci. 58: 21–29.
6) Sang KN, Sung IK (2004) Milk sphingomyelin is more effective than egg sphingomyelin in inhibiting intestinal absorption of cholesterol and fat in rats. J. Nutr. 134: 2611–2616.
7) Itonori S, Shimizu T, Yano K, Kitamura T, Kojima H, Matsumuro Y, Ito M, Toyokawa M, Saito H, Sugita M (2006) Isolation and characterization of ceramide 2-aminoethylphosphonate from umbrella portion of jellyfish, stomolophus nomurai (Echizen-kurage). Mem. Fac. Educ., Shiga Univ., Nat. Sci. 56: 87–96.
8) Sweeley CC, Gaver RC (1965) Methods for methanolysis of sphingolipids and direct determination of long-chain bases by gas chromatography. J. Am. Oil

Chem. Soc. 42: 294–298.
9) Sugimoto K, Itonori S, Hosomi R, Ishida T, Matsui H, Yoshida M, Fukunaga K (2023) Structural analysis of ceramide aminoethyl phosphonate derived from pacific oyster (*Crassostrea gigas*) by-product. Trace Nutrients Research 40: 1–6.
10) Matsubara T, Hayashi A (1978) Structural studies on glycolipid of shellfish. Ⅱ. Occurrence of 3-O-methylgalactosamine in oyster glycolipid. J. Biochem. 83(4): 1195–1197.
11) The lipid web. Mass spectrometry of methyl ester derivatives of fatty acids. (2025, July 14) https://www.lipidmaps.org/resources/lipidweb/index.php?page=ms/methylesters.htm