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Original article

Structural analysis of Ceramide Aminoethyl Phosphonate Derived from Pacific Oyster (*Crassostrea gigas*) By-product

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Summary

Oysters contain various nutritional and functional components, such as zinc, glycogen, and taurine. Therefore, oyster extract containing extracted and concentrated nutritional and functional components are marketed. However, the effective use of oyster extract by-products (OEBP) needs to be realized. A previous study revealed that several types of mollusc bivalves characteristically contain ceramide aminoethyl phosphonate (CAEP), a sphingophosphonolipid, that improves skin barrier function. In this study, we analyzed the chemical structure of CAEP in OEBP to differentiate it from other marine CAEP. CAEP was purified by alkali and acid treatments, followed by three types of column chromatography for Pacific oyster by-products. To analyze the chemical structure of CAEP, fatty acid composition analysis via methyl esterification and long-chain base composition analysis via trimethylsilyl derivatization were performed. The results showed that 26% of OEBP-derived phospholipids were CAEP. Chemical structural analysis indicated that the fatty acid composition of the OEBP-derived CAEP was C16:0 (68%), C17:0 (5%), and C18:0 (27%). Furthermore, the long-chain base composition of OEBP-derived CAEP was d16:1 (24%), d18:1 (13%), d18:2 (39%), d20:1 (4%), and d20:2 (12%). These results indicate that most of the long-chain bases of marine CAEP reported to date have carbon numbers of ≤19, whereas Pacific oyster-derived CAEP characteristically contains long-chain bases with carbon numbers of 20.

Introduction

Oysters are known as a “milk of the sea” because they contain various nutritional and functional components, such as zinc, glycogen, and taurine. Oyster extract containing various (extracted and concentrated) nutritional and functional components has been marketed to derive optimal benefits of these components. It has been reported that the oyster-extract has the food functions related platelet aggregation inhibition and hepatoprotective effect[1,2]. In addition, our research group has reported the effects of oyster extract on colorectal precancerous lesions and renal injury in animal studies[3,4]. However, the oyster extract by-product (OEBP) is discarded, although it contains high concentrations of lipids and proteins, which are the main components of oyster meat. The effective utilization of OEBP is desired from the perspective of environmental issues. Previous studies have found that the triacylglycerol in OEBP contains approximately 10% *n*-3 polyunsaturated fatty acids, such as eicosapentaenoic acid and docosahexaenoic acid[5]. In addition, OEBP is expected to contain other functional components.

Scallops (*Patinopecten yessoensis*) and pearl oysters (*Pinctada martensii*), which are molluscan bivalves as well as oysters, characteristically contain ceramide aminoethyl phosphonate (CAEP), a sphingophosphonolipid[6,7]. Moreover, some bivalve-derived CAEP are composed of components such as hydroxy fatty acids and sphingatrienine, which are not found in common sphingolipids. Marine-derived CAEP improves skin barrier function[6]. In addition, the health-promoting functions of sphingolipids are dependent on their chemical structure[8]. Therefore, we

analyzed the chemical structure of CAEP in OEBP with the aim of making effective use of OEBP in the future.

Materials and Methods

Materials

OEBP derived from the Pacific oyster (*C. gigas*) was provided by Japan Clinic Co., Ltd. (Kyoto, Japan); it was composed of 63.7 wt% moisture, 22.6 wt% crude protein, 9.6 wt% crude fat, and 3.3 wt% crude ash. OEBP used in the experiments were boiled and dehydrated in acetone. During this process, the acetone layer was extracted to the extent that no coloration was observed; thus, acetone-soluble fats and pigments were also removed.

The standard CAEP was a scallop-derived substance prepared independently in our laboratory. Other chemicals were purchased from Nacalai Tesque, Inc. (Kyoto, Japan); Merck KGaA (Darmstadt, Germany); and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

CAEP purification

CAEP was purified according to the method described by Sugita *et al.*[6], with some modifications (Fig. 1). In brief, the crude complex lipid fraction was extracted from OEBP using a chloroform–methanol (C/M; 2:1) mixture, and the solvent was eluted under reduced pressure (C/M extract in Fig. 1). The C/M extract was stirred in acetone, and the insoluble powder was collected as the phospholipid fraction. To decompose and remove glycerophospholipids, alkaline saponification and acid treatment (pH = 1) were performed, and the solution was dialyzed under running water for 2 days. The solution in the dialysis membrane was concentrated and acetone was added to prepare an acetone-insoluble powder (sphingolipid fraction). The sphingolipid fraction was fractionated into chloroform/methanol/water (C/M/W) and ammonium acetate fractions using TOYOPEAL QAE-550 (Tosoh Co., Tokyo, Japan) column chromatography. Elution from the column was performed using 5-volume of C/M/W (30:60:8) and equal-volume of methanol as the neutral solvent and 5-volume of 0.05 M ammonium acetate–methanol solution as the polar solvent. Elution of 0.05 M ammonium acetate fraction was concentrated and dialyzed in running water for 2 days, followed by TOYOPEAL DEAE-650 (Tosoh Co.) column chromatography. The fractions that passed through the column in 5-volume of C/M/W (30:60:8) were collected, concentrated, and subjected to silica gel column chromatography. Fractions were collected over-time while C/M (1:1) was flowed through the column, and fractions containing with the same Rf values as the standard CAEP by thin-layer chromatography (TLC) were concentrated to purify the OEBP-derived CAEP.

Phospholipid class analysis

TLC and infrared absorption (IR) spectral analyses were performed to confirm the phospholipid class. The TLC plate used was silica gel 60 (Merck KGaA), and C/M/W (60:30:5) was used as the developing solvent. Spots were detected with 50% (v/v) sulfuric acid and ninhydrin solutions and identified using standard phospholipids [CAEP, phosphatidylcholine (PC), and phosphatidylethanolamine (PE)]. The phospholipid class composition was calculated based on spot intensity using JustTLC software (version 4.0.3; Lund, Sweden). IR spectra of the OEBP-derived CAEP were measured using a Shimadzu Fourier-transform infrared spectrophotometer FTIR-8400 S (Shimadzu Co., Kyoto, Japan).

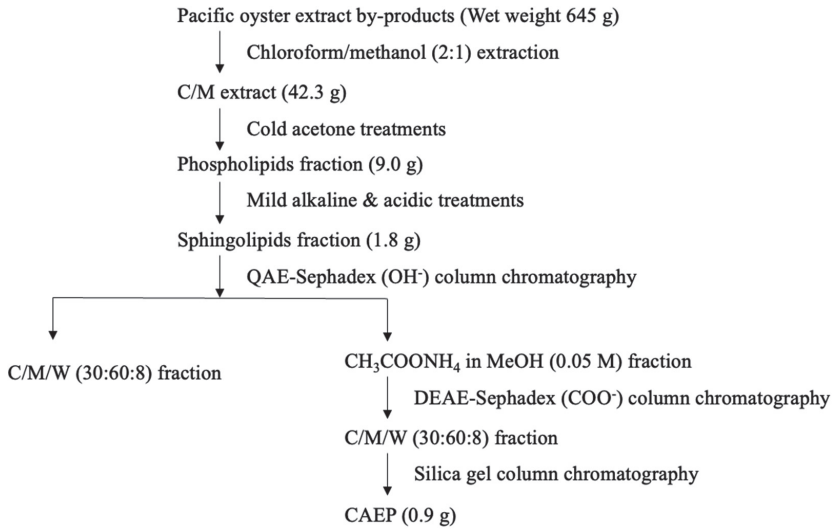

Fig. 1. Scheme for preparation and fractionation of CAEP from OEBP

Fatty acid composition analysis

Two hundred μg of OEBP-derived CAEP was added to 1 M methanol hydrochloride and heated at 100 ℃ for 3 h. After cooling, fatty acid methyl esters were extracted three times with 0.2 mL *n*-hexane and analyzed by gas chromatography (GC) system (GC-2014; Shimadzu Co.) and GC-mass spectrometry (MS) system (GCMS-QP2010/PARVUM2; Shimadzu Co.), equipped with Omegawax® capillary GC column (cat no. 24152, Merck KGaA). The GC system parameters were set as follows: injector and interface temperatures, 250 ℃; column temperature, 120 ℃; gradual heating to 240 ℃ at a rate of 2 ℃/min, held at 240 ℃ for 10 min; and carrier gas, 1 mL/min helium. MS system parameters were set as follows: ion source temperature, 200 ℃; electronic ionization(EI), 70 eV; full scan m/z 45–350 amu. Fatty acid composition was determined by GC analysis, and peak compounds were identified by GC-MS analysis.

Long-chain base composition analysis

Five hundred μg of OEBP-derived CAEP was added to 0.2 mL of 8.6% aqueous methanol hydrochloric acid and heated at 70℃ for 18 h[9]. After cooling, the fatty acid methyl esters were removed three times with 0.2 mL *n*-hexane, and methanol was removed from the remaining solution. Then, 0.6 mL of 1 M sodium hydroxide:methanol (3:4, v/v) and 0.72 mL of chloroform were added, stirred, and centrifuged to separate into two layers. The chloroform layer was washed with 0.4 mL of water/methanol (1:1, v/v) and concentrated under nitrogen to obtain the long-chain base fraction. Trimethylsilyl-diazomethane was added to the resulting residue and heated at 60 ℃ for 30 min, and the trimethylsilyl derivative was subjected to GC and GC-MS analyses to measure long-chain base composition and identify peak compounds, respectively. The analytical column was nonpolar 5% phenylmethyl silicon chemically bonded (film thickness, 0.25 μm) silica capillary (Shimadzu HiCap-CBP 5). The GC system parameters were set as follows: injector and interface temperatures, 250 ℃; column temperature, 170 ℃; gradual heating to 240 ℃ at a rate of 4 ℃/min, held at 240 ℃ for 20 min; and carrier gas, 1 mL/min helium. MS system parameters were set as follows: ion source temperature, 200 ℃; electronic ionization(EI), 70 eV; full scan m/z 45–500 amu.

MALDI-TOF MS analysis

OEBP-derived CAEP was dissolved in 500 μL of methanol, and *n*-acetylation was performed by adding 10 μL of pyridine and 50 μL of acetic anhydride for 30 min at room temperature. The acetylated CAEP and matrix (*α*-cyano-4-hydroxycinnamic acid) were dried on slides and subjected to MALDI-TOF MS (AXIMA® Confidence™; Shimadzu Co.) analysis. A nitrogen laser (laser wavelength: 377 mm) was used as the ion source, and measurements were performed in negative-ion mode.

Results and Discussion

CAEP purification

As shown in Fig. 1, 42.3 g of C/M extract and 9.0 g of phospholipid fraction were obtained from 645 g of OEBP. TLC results for the phospholipid fraction are shown in Fig. 2. This fraction comprised 30.7% PC, 26.0% CAEP, and 8.5% PE (Table 1).

In addition, 0.9 g of CAEP was purified from 9.0 g of phospholipid fraction by this experimental purification method. The TLC results for OEBP-derived CAEP are shown in Fig. 3. CAEP is colored by ninhydrin and sulfuric acid because it contains lipid-containing amino groups. The Rf value of the extract was also compared with that of the standard CAEP, and it was concluded that CAEP was purified from OEBP. The reason for the presence of

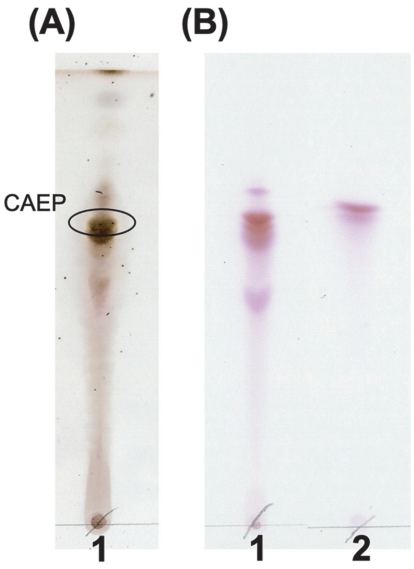

Fig. 2. Thin-layer chromatogram of the phospholipid fraction from the Pacific oyster by-product
Lane 1: phospholipid fraction prepared from OEBP; lane 2: standard CAEP. Plate was developed in chloroform/methanol/water (60:30:5, v/v/v), and the spots were visualized using 50% (v/v) sulfuric acid solution (A) and ninhydrin solution (B).
OEBP, oyster extract by-product.

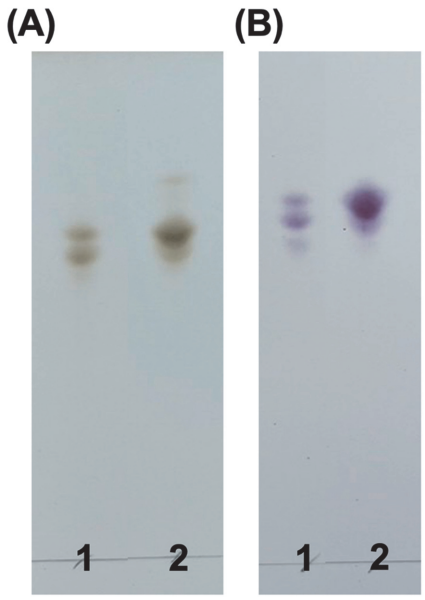

Fig. 3. Thin-layer chromatogram of OEBP-derived CAEP
Lane 1, standard CAEP; lane 2, OEBP-derived CAEP. Plate was developed in chloroform/methanol/water (60:30:5, v/v/v), and the spots were visualized using 50% (v/v) sulfuric acid solution (A) and ninhydrin solution (B). CAEP, ceramide aminoethyl phosphonate; OEBP, oyster extract by-product.

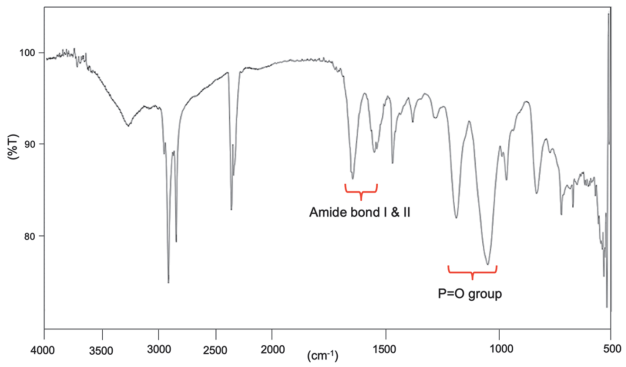

Fig. 4. Infrared spectra of OEBP-derived CAEP

Table 1. Phospholipid class of OEBP-derived phospholipids fraction

Phospholipid class (wt%)	
PE	30.7
CAEP	26.0
PC	8.5
Others	34.8

CAEP, ceramide aminoethyl phosphonate; OEBP, oyster extract by-product; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Table 2. Ceramide composition of OEBP-derived CAEP

Peak	(wt%)	
	Fatty acid	
A	C16:0	67.7
B	C17:0	5.2
C	C18:0	27.2
	Long-chain base	
A	d16:1	25.3
C	d18:1	13.3
B	d18:2	41.2
F	d20:1	4.2
E	d20:2	12.3
D	Unknown	3.6

Peaks a–c (fatty acids) and a–f (long-chain bases) in Table 2 correspond to the peaks shown in Fig. 5 A and 5B, respectively. CAEP, ceramide aminoethyl phosphonate; OEBP, oyster extract by-product.

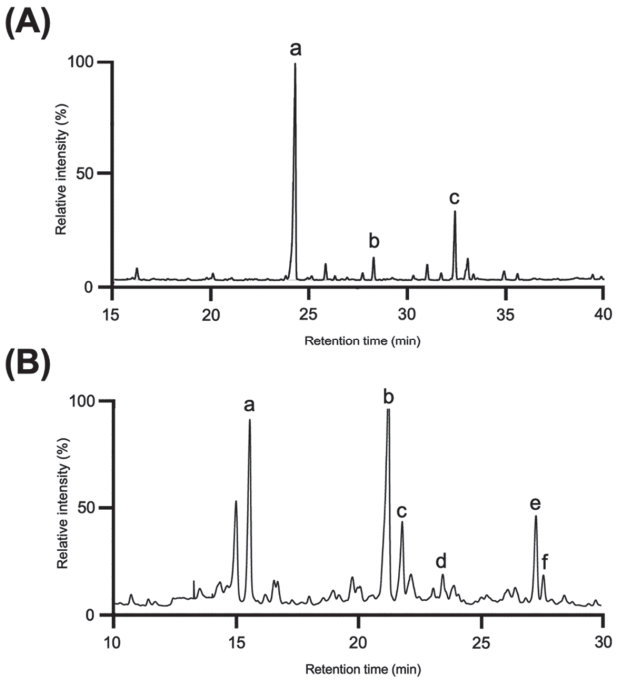

Fig. 5. Gas chromatograms of fatty acid (A) and long-chain base (B) of OEBP-derived CAEP
A: Fatty acid methyl esters of OEBP-derived CAEP; a, C16:0; b, C17:0; c, C18:0.
B: trimethylsilylated long-chain bases of OEBP-derived CAEP; a, d16:1; b, d18:2; c, d18:1; d, unknown; e, d20:2; f, d20:1.
CAEP, ceramide aminoethyl phosphonate; OEBP, oyster extract by-product.

two spots of CAEP with different Rf values on TLC could be the difference in the composition ratio of fatty acids and long-chain bases that compose the ceramides[6].

IR spectra of OEBP-derived CAEP are shown in Fig. 4. The OEBP-derived CAEP showed absorption at 1180 cm^{-1} and 1050 cm^{-1} from the P=O group derived from the phosphate group of the C–P bond and at 1650 cm^{-1} and 1550 cm^{-1} from amide bonds I and II. These results also

confirmed that the substance purified from OEBP was CAEP.

Ceramide components of CAEP

The fatty acid and long-chain base comprising the ceramide of OEBP-derived CAEP were identified using both GC and GC-MS analyses. The compositions and chromatograms of fatty acids and long-chain bases obtained by GC analysis are shown in Table 2 and Fig. 5, respectively. The MS spectrum of fatty acid methyl esters was characterized by 74 and molecular ion peaks −31 and −43[10]. Based on this information, the fatty acid composition of the OEBP-derived CAEP was identified as C16:0 (67.7%), C17:0 (5.2%), and C18:0 (27.2%) (MS spectra not shown). Grand *et al.* reported that the Pacific oyster (*C. gigas*)-derived CAEP contained few fatty acids with 20 or 22-carbon chains, such as C20:5 and C22:6[11]. However, no such fatty acids were detected in the OEBP-derived CAEP. It is unclear whether this is due to differences in the production areas of Pacific oyster or their decomposition during the OEBP purification process, so further research is needed to clarify these.

In addition, the long-chain base composition was identified from the GC and GC-MS data as a trimethylsilyl derivative. In a previous study, it was found that alkaline hydrolysis is necessary when the 9-methyl homologue of the 4, 8, 10-trienine base is present in the base composition[7]. However, OEBP-derived CAEP does not contain a 9-methyl homologue of the 4,8,10-trienine base; therefore, only acid hydrolysis was performed. The MS spectrum of trimethylsilylated long-chain bases was characterized by 73 and 132 and molecular ion peaks −105 and −132. As shown in Table 2, the long-chain base composition of OEBP-derived CAEP was d16:1 (25.3%), d18:1 (13.3%), d18:2 (41.2%), d20:1 (4.2%), and d20:2 (12.3%). Peak (d) in Fig. 5 shows the MS spectrum characteristics of a long-chain base; however, the chemical structure of this compound could not be determined because the molecular ion peak was not detectable. The long-chain bases of OEBP-derived CAEP were mostly composed of d16:1 and d18:2, which are commonly found in bivalve-derived CAEP. However, the long-chain bases of OEBP-derived CAEP were also composed of d20:1 and d20:2. Most of the long-chain bases of marine-derived CAEP found to date are composed of up to 19-carbon chain, and few long-chain bases with 20-carbon chains have been discovered. The results of this study indicated that OEBP-derived CAEP characteristically contains long-chain bases with 20-carbon chains. Further investigation is required to determine the factors responsible for these differences in the carbon chain length.

Moreover, it has been suggested that the chemical structures of ceramides are similar in the same taxonomic order[7]. Although further information is not available on CAEP in the order Oysteri, to which the Pacific oyster belongs, it is possible that CAEP in other bivalves belonging to the order Oysteri also have a chemical structure similar to that mentioned in the present results.

MALDI-TOF MS analysis

There are two measurement modes for MALDI-TOF MS: positive-ion mode and negative-ion mode, depending on the functional group of the analyzed sample. For measurement of lipids with acidic functional groups in the molecule of the sample to be measured, the ionization efficiency is extremely poor in the positive-ion mode[7]. In addition, it is effective for analyzing phospholipids with free amino groups such as *N*-acetyl derivatives[12]. Therefore, for OEBP-derived CAEP, MALDI-TOF MS analysis was performed in negative-ion mode after *N*-acetylation of free amino groups in the molecule. The MS spectra are shown in Fig. 6. Forty-two masses (CH_3COO = 42) of more spectra based on each molecular species [M-H] were observed after N-acetylation, corresponding to the major components of the ceramides shown in Table 1. The main spectra were as follows: a, m/z 656.71 (16:0 fatty acid-d16:1 long chain base, calculated value 656.49); b, m/z 670.59

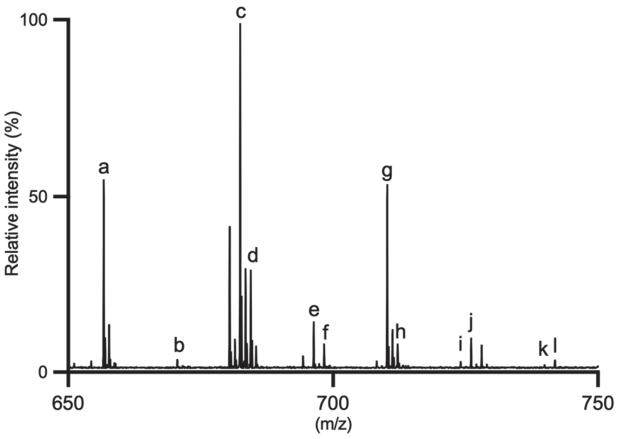

Fig. 6. Negative-ion mode of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry spectra of *N*-acetylated OEBP-derived CAEP
a, m/z 656.71 (16:0 fatty acid-d16:1 long chain base, calculated value 656.49); b, m/z 670.59 (17:0-d16:1, 670.50); c, m/z 682.49 (16:0-d18:2, 682.52); d, m/z 684.46 (16:0-d18:1 and 18:0-d16:1, 684.52); e, m/z 696.34 (17:0-d18:2, 696.53); f, m/z 698.33 (17:0-d18:1, 698.53); g, m/z 710.21 (16:0-d20:2 and 18:0-d18:2, 710.55); h, m/z 712.22 (16:0-d20:1 and 18:0-d18:1, 712.55); i, m/z 724.09 (17:0-d20:2, 724.57); j, m/z 726.12 (17:0-d20:1, 726.57); k, m/z 739.94 (18:0-d20:2, 738.58); l, m/z 741.90 (18:0-d20:1, 740.58).
CAEP, ceramide aminoethyl phosphonate; OEBP, oyster extract by-product.

(17:0-d16:1, 670.50); c, m/z 682.49 (16:0-d18:2, 682.52); d, m/z 684.46 (16:0-d18:1 and 18:0-d16:1, 684.52); e, m/z 696.34 (17:0-d18:2, 696.53); f, m/z 698.33 (17:0-d18:1, 698.53); g, m/z 710.21 (16:0-d20:2 and 18:0-d18:2, 710.55); h, m/z 712.22 (16:0-d20:1 and 18:0-d18:1, 712.55); i, m/z 724.09 (17:0-d20:2, 724.57); j, m/z 726.12 (17:0-d20:1, 726.57); k, m/z 739.94 (18:0-d20:2, 738.58); l, m/z 741.90 (18:0-d20:1, 740.58), confirming the presence of the spectra attributed to the product. These results indicated the presence of CAEP molecular species with all fatty acids and long-chain bases listed in Table 1.

In this study, we found that OEBP contained CAEP (26 wt% phospholipids). In addition, the chemical structure of this CAEP was revealed, particularly, it characteristically contained a long-chain base with a carbon number of 20. These results provide information necessary for the effective utilization of OEBP in the future.

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