SEPARATION AND IDENTIFICATION OF MOLECULAR SPECIES OF PHOSPHOLIPIDS FROM A GRAM-NEGATIVE MODERATELY HALOPHILIC BACTERIUM

Yoshimi OHNO, Ikuya YANO and Masamiki MASUI
Department of Bacteriology, Osaka City University, Medical School, Abeno, Osaka, Japan

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1. Introduction

It is well known the hydrophobic structures of the phospholipid molecules are closely related not only to the fluidity but also to the physiological functions of the biological membrane systems. Recently, the separation and analysis by gas chromatography-mass spectrometry [1–5] of the highly unsaturated molecular species of the mammalian phospholipid classes have been reported. That work has been mainly concerned with the fatty acid structures and their positional distribution on the glycerol moiety.

In bacteria, the detailed structural analysis of phospholipid molecular species by using lipases and phospholipases has been described [6]. However, the convenient gas chromatographic and mass spectrometric analysis of the intact molecular species has not been reported yet.

The present paper first reports that the monoacetyldiglycerides obtained from phospholipids of a Gram-negative moderately halophilic bacterium (no. 101-W3) can be separated into at least 7 species according to the carbon numbers and the component fatty acids can be identified by the combined gas chromatography-mass spectrometry. The major species of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), cardiolipin (CL), and unknown phosphoglycolipid (PGL) were similar in patterns of diglyceride species, and they were 16:0-16:0*, 16:0-17cpa*, 16:0-18:1*, 16:0-19cpa*, 18:0-18:1*, 18:0-19cpa*, and 19cpa-19cpa*. It was noted that the 4 species containing C_{17} and C_{19} cyclopropanoic (cpa) fatty acids predominated and the amounts varied dramatically with the growth stages and concentrations of NaCl in the culture medium.

* Expressed as total carbon numbers of long-chain fatty acids.

2. Materials and methods

Cultures of a Gram-negative moderately halophilic bacterium (No. 101-W3) were incubated at 30°C for 48 hr in a medium containing 2 M NaCl [7]. After the cells were harvested by centrifugation, lipids were extracted with chloroform–methanol (2:1, v/v) and washed repeatedly by the method of Folch et al. [8]. The bacterial phospholipids were separated and purified by thin-layer chromatography on a plate of Silica gel G (Merck) with a solvent of chloroform–methanol–water (65:25:4, v/v/v) or chloroform–methanol–acetic acid–water (100:20:12:5, v/v/v/v). The acetolysis of the isolated phospholipids was performed essentially by the method of Renkonen [9].

The reaction mixture contained a few milligrams of phospholipids in 2 ml of a mixture of acetic anhydride and acetic acid (2:3, v/v) in a sealed tube. After reaction for 5 to 14 hr at 150°C, the resultant monoacetyldiglycerides were extracted with hexane–ether (1:1, v/v) and developed on a TLC plate with a solvent of hexane–ether (80:20, v/v).

After the completion of the reaction was confirmed, the monoacetyldiglyceride mixture was analyzed by gas chromatography or combined gas chromatography–mass spectrometry.

The gas chromatographic and mass spectrometric analysis was carried out with a Shimazu-LKB, model 9000 apparatus, equipped with a 2 m × 3 mm (i.d.) glass coiled column packed with 1% OV-1 on silanized Chromosorb W (60–80 mesh). The column tempera-

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ture was maintained at 295°C, the molecular separator 310°C, and the ion source at 330°C, respectively. The ionizing voltage was 22.5 eV, the accelerating voltage 3.5 kV, and the trap current 60 μA.

3. Results and discussion

From the chemical analysis of isolated lipids, the major phospholipids of a Gram-negative moderately halophilic bacterium (No. 101-W3) were revealed to be phosphatidylethanolamine, phosphatidylglycerol, cardiolipin, and unknown phosphoglycolipid [10], this being similar to the results reported earlier by Tietz et al. [11–13].

The fatty acid analysis of individual phospholipids after phospholipase A_2 digestion followed by methylation showed about 60% or more of saturated fatty acids attached to the 1-position while 70% or more of C_{17} and C_{19} cyclopropanoic (or C_{16} and C_{18} monoenoic) acids attached to the 2-position.

TLC of acetolysis products from each phospholipids gave almost a single spot corresponding to authentic 1,2-dipalmitoyl-3-acetylglycerol (acetyl-1,2-dipal-

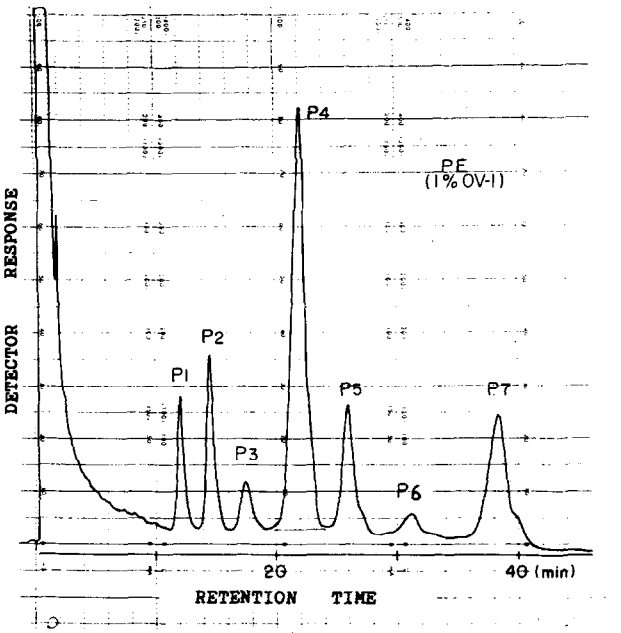

Fig.1. Gas-liquid chromatogram of the monoacetyldiglycerides from phosphatidylethanolamine of a moderately halophilic bacterium (No. 101-W3). Conditions for gas–liquid chromatography are described in the text.

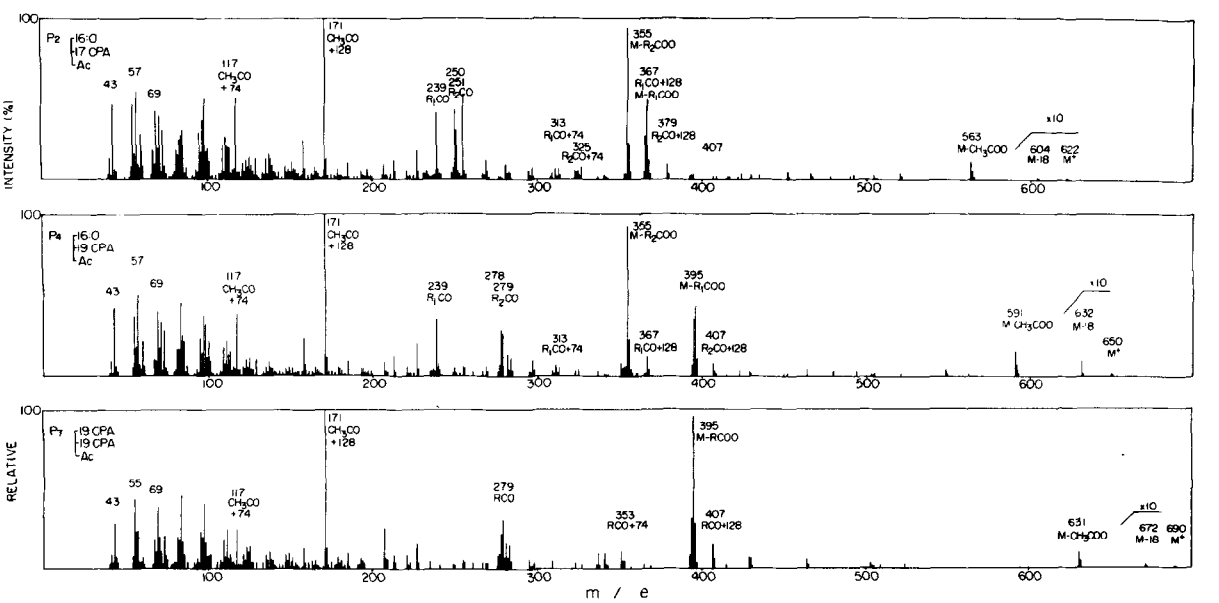

Fig.2. Mass spectra of peaks P_2, P_4 and P_7 of fig.1. Conditions for mass spectrometry are described in the text.

mitin). The monoacetyldiglycerides thus obtained were submitted to gas chromatography.

Fig.1 shows that the monoacetyldiglycerides from phosphatidylethanolamine consist of 7 major peaks designated P_1 to P_7.

Mass spectra of each component were recorded and 3 of them (P_2, P_4, and P_7) are reproduced in fig.2.

Mass spectra of P_2 showed the presence of a very intense peak at m/e 171 due to $[CH_3CO + 128]^+$, m/e 117 $[CH_3CO + 74]^+$, and m/e 43 $[CH_3CO]^+$, these being characteristic and common for monoacetyldiglycerides [2]. On the other hand, a very weak molecular ion peak at m/e 622 $[M]^+$ and more intense peaks at m/e 604 $[M-18]^+$ due to loss of water and m/e 563 $[M-59]^+$ due to loss of acetoxy group $[M-CH_3COO]^+$ were detected.

Furthermore, the mass ion peaks due to acyl moiety $[RCO]^+$ were observed at m/e 239 ($C_{16:0}$) and m/e 251 ($C_{17:1}$), this indicating that peak 2 is palmitoyl-methylene hexadecanoyl-acetyl-glycerol (16:0–17cpa), exclusively. The mass ion peaks at m/e 355 and m/e 367 due to $[M-RCOO]^+$ also supported this structure. Similarly, peaks 4 and 7 were identified as palmitoyl-methylene octadecanoyl-acetylglycerol (16:0–19cpa) and di-methylene octadecanoyl-acetylglycerol (19cpa–19cpa), respectively. From the mass ion peaks due to $[M-R_1COOCH_2]^+$, saturated fatty acids appeared to be linked mainly to the 1-position while monoenoic and cyclopropanoic fatty acids to the 2-position of the glycerol moiety [2]. All of the other components were identified similarly and the results are summarized in table 1.

It was noted that the molecular species containing cyclopropanoic fatty acids (C_{17} or C_{19}) were present abundantly and in higher concentrations (up to 90%) particularly in the organism at the late stages of growth

Table 1
Mass fragment ions and relative amounts of monoacetyldiglycerides

peak No / fragment ions	P_1	P_2	P_3	P_4	P_5	P_6	P_7
$[M]^+$	610	622	636	650	664	678	690
$[M-18]^+$	592	604	618	632	646	660	672
$[M-CH_3COO]^+$	551	563	577	591	605	619	631
$[M-R_1COO]^+$	355	367	381	395	381	395	395
$[M-R_1COOCH_2]^+$	341	353	367	381	367	381	381
$[M-R_2COO]^+$	355	355	355	355	383	383	395
$[R_1CO]^+$	239	239	239	239	267	267	279
$[R_1CO + 74]^+$	313	313	313	313	341	341	353
$[R_1CO + 128]^+$	367	367	367	367	395	395	407
$[R_2CO]^+$	239	251	265	279	265	279	279
$[R_2CO + 74]^+$	313	325	339	353	339	353	353
$[R_2CO + 128]^+$	367	379	393	407	393	407	407
molecular species C–1	16:0	16:0	16:0	16:0	18:0	18:0	19cpa
C–2	16:0	17cpa	18:1	19cpa	18:1	19cpa	19cpa
phospholipid classes (%)							
cardiolipin	2.2	11.5	2.8	55.7	6.5	3.4	17.9
phosphatidyl-ethanolamine	6.4	10.2	4.5	44.4	11.8	3.0	19.5
phosphatidyl-glycerol	3.7	12.7	1.8	57.8	5.9	3.0	15.1
unknown phosphoglyco-lipid	6.2	9.1	4.1	54.2	5.8	3.1	18.1

(40–90 hr) or grown at high NaCl concentration (from 0.5 to 3.0 M).

These findings, obtained by the direct gas chromatography–mass spectrometry, agree well with those from gas chromatographic analysis of fatty acid methyl esters obtained after phospholipase A_2 treatment of the original phospholipids, thus suggesting that the analytical method used above is widely applicable to the analysis of the other membrane phospholipid molecular species of biological sources.

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