Simultaneous Preparation of Purified Plasmalogens and Sphingomyelin
in Human Erythrocytes with Phospholipase A₁ from Aspergillus orizae

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A method for the simultaneous purification of plasmalogens and sphingomyelin (SM) in human erythrocytes is described. Treatment of total lipids with n-hexane/acetone (1:1 v/v) resulted in selective precipitation of SM. Both the supernatant and the precipitate fractions were incubated with a phospholipase A₁ (PLA₁) from Aspergillus orizae for 3.5 h. The PLA₁-treated lipids were extracted with n-hexane/isopropanol, the hexane layer was obtained using a Na₂SO₄ solution, and the hexane layer was further washed with water. At this step, the relative concentration of the plasmalogens was 92% of the total phospholipids in the supernatant fraction, and that of SM was 97.7% in the precipitate fraction. Each fraction was applied to high performance liquid chromatography (HPLC) for further purification. The plasmalogens and SM obtained were almost free of the other lipids. The purity of the plasmalogens and SM was monitored by HPLC, which can separate intact plasmalogens from their diacyl analogs.

Key words: human erythrocytes; plasmalogens; sphingomyelin; phospholipase A₁

Plasmalogens are glycerophospholipids containing a vinyl ether bond at the sn-1 position of the glycerol backbone. They are found in all animal tissues.1) They are not only structural membrane components and a reservoir for second messengers, but also can be involved in membrane fusion, ion transport, and cholesterol efflux.1,2) The vinyl ether bond at the sn-1 position makes plasmalogens more susceptible to oxidative stress than the corresponding ester glycerophospholipids,3) and hence plasmalogens can also act as antioxidants, protecting cells from oxidative stress.4) A causal relation of ethanolamine plasmalogen (pl-PE) to Alzheimer type dementia is also possible.11–13)

Sphingomyelin (SM) is another structural cell membrane component and a reservoir of second messengers. It is known to form a “lipid raft” together with cholesterol in the membrane. Lipid rafts are thought to be “hubs of signal transduction” in membranes.14–16) Highly purified plasmalogens are still difficult to obtain commercially. The structures of plasmalogens and SM can differ among the animal species, and may not be same even in different tissues of the same animal. Therefore, in some studies of phospholipids, there is a need to prepare purified plasmalogens or SM from each tissue as a standard or a substrate for some enzymes. The ether bond at the sn-1 position of plasmalogens is resistant to phospholipase A₁ for diacylphospholipids. Many attempts have been made to purify plasmalogens using various phospholipases: snake venom phospholipase A₁;17) purified phospholipase from porcine pancreas;18) purified phospholipase A₁ from guinea pig pancreas;19) Rhizopus delemar lipase;20) and a combination of Rhizopus delemar lipase and N. naja naja phospholipase A₂.21) To our knowledge, the most recent study on the purification of plasmalogens using lipases was done with commercially available phosphatidylcholine and phosphatidylethanolamine from porcine heart.21) Hydrolysis of diacylphospholipids with R. delemar lipase took 18 h,21) and thin-layer chromatography (TLC) was used to monitor purity and in the quantification of plasmalogens.21)

The present method includes n-hexane/acetone treatment of total lipids for selective precipitation of SM and hydrolysis of diacylphospholipids with phospholipase A₁ from Aspergillus orizae (PLA₁). PLA₁ is commercially available, and the hydrolysis of diacylphospholipids with PLA₁ took less than 4 h. Plasmalogen was collected from an HPLC with ultraviolet (UV) detection. The purity of the plasmalogens and SM was monitored by HPLC with evaporative light scattering detection (ELSD), which can separate intact plasmalogens from their diacyl analogs.22) Most of the procedures presented here have never been used in the purification of plasmalogens from biological tissues. The procedure is much simpler and requires less time than the reported procedures.

Materials and Methods

Chemicals. Phospholipase A₁ (EC. 3.1.1.32), from Aspergillus orizae, was purchased from Mitsui Chemicals and Foods (Tokyo). The

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Abbreviations: ELSD, evaporative light scattering detector; HPLC, high performance liquid chromatography; pl-PE, ethanolamine plasmalogen; pl-PC, choline plasmalogen; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; PE, diacylphosphatidylethanolamine; PC, diacylphosphatidylcholine; PS, diacylphosphatidylserine; PLA₁, phospholipase A₁; SM, sphingomyelin; UV, ultraviolet light; Hct, hematocrit
brochure of the manufacturer stated that the specific activity of PLA1 was 10,000 to 13,000 units/g. Authentic free fatty acids and phospholipids were purchased from Sigma (Tokyo), 9-Anthrildiazomethane was from Funakoshi (Tokyo). Reagent grade chemicals and HPLC grade solvents were from Wako Pure Chemical Industries (Osaka).

**HPLC system.** The HPLC system used was an Agilent 1100 system (Agilent Technologies, Tokyo) equipped with a four-solvent delivery system, a degasser, and a UV detector. The system was connected to an evaporative light scattering detector (ELSMD) (Sedere-55, Sedere, Vitry sur Seine, France). The system was also connected to ChemiStation (Agilent Technologies) for the control and analysis of chromatograms.

**Monitoring of the purity of the plasmalogens.** Monitoring of the relative concentrations of the phospholipid classes, including plasmalogens, was done by the HPLC method, which was developed recently by us, with minor modifications. The HPLC column was a Lichrosphere Diol, 250 x 4 mm, 5 μm (Agilent). Mobile phase A was n-hexane/2-propanol/acetic acid (82:17:1 v/v) with 0.08% triethylamine (TEA), and mobile phase B was 2-propanol/water/acetic acid (85:14:1 v/v) with 0.08% TEA. Mobile phase A was 95% at 0 min, and decreased linearly to 63% in 21 min. The gradient continued from 63% to 15% A in 4 min, and 15% A was maintained for 1 min. Mobile phase A was increased to 95% in 3 min, then 95% A was maintained for 5 min for pre-conditioning to the next injection. The column temperature was 50°C and the flow rate was 1 ml/min. The phospholipid class was detected by ELSD, which was set at 50°C. The detection and was dried under N2 gas.

**Acid hydrolysis of plasmalogens.** Acid hydrolysis of plasmalogens in the total lipids was done by a method reported previously. The hydrolyzed lipids were re-suspended in 0.5 ml of n-hexane/2-propanol (3:2 v/v). After filtration through a 0.45-μm filter, an aliquot of the suspension (usually 10 μl) was injected into the HPLC system. Each phospholipid class was collected from the HPLC with 205 nm detection and was dried under N2 gas.

**Fatty acid analysis of the phospholipid class.** The fatty acid compositions of the phospholipids were analyzed by an HPLC method described previously. Briefly, each phospholipid class was dried under N2 gas and hydrolyzed with 1 ml of 0.5 M KOH in 70% methanol at 75°C for 60 min. After the addition of 0.3 ml of 2 M HCl, the fatty acids were extracted with n-hexane. The fatty acids in n-hexane were dried under N2 gas and labeled with 9-anthrildiazomethane. After filtration through a 0.45-μm filter, an aliquot of the labeled fatty acid was injected into the HPLC system.

**Results and Discussion**

A diagram of the procedure for the preparation of purified plasmalogens and purified SM of human erythrocytes is shown in Fig. 1. The steps of the procedure were named tentatively A, B-1, C-1, B-2, C-2, B-3, and C-3. These names correspond to those in figures and tables.
It is known that the addition of acetone to total lipid extract precipitates all kinds of phospholipids, but to our knowledge, it has not been reported that treatment of total lipids with n-hexane/acetone (4:6 to 6:4 v/v) causes selective precipitation of SM. The relative concentration of SM after treatment with n-hexane/acetone (1:1 v/v) was about 93% of the total phospholipids in the precipitate (C-1 in Fig. 2 and Table 1). The precipitate of SM by hexane/acetone (1:1) is accompanied by 5 to 11% of other phospholipids (mainly PC) in our experience. The amount of SM in the human erythrocyte membranes was about one-third of the phospholipids on the chromatogram as detected by ELSD (A in Table 1 and Fig. 2). SM is not prone to decomposition by phospholipase A\textsubscript{1}, and hence the precipitate fraction containing SM was also applied to the PLA\textsubscript{1} treatment for further purification of SM. On the other hand, other phospholipids, including plasmalogens, were found in the supernatant of n-hexane/acetone treatment (B-1 in Fig. 2 and Table 1). Hence, purification of plasmalogens with PLA\textsubscript{1} was started from this fraction.

To our knowledge, phospholipase A\textsubscript{1} from Aspergillus orizae has not been used for the purification of plasmalogens hitherto. The enzyme preparation consisted of 25% of PLA\textsubscript{1} and 75% of food materials, and is commercially available. In this study, 2 mg of material/1 mg of total lipids of erythrocytes was used, which was equivalent to 20 to 26 units/mg of the lipids. The diacylphospholipids in the supernatant fraction were almost completely destroyed within 4 h at 50 °C, and all of the plasmalogens appeared to be preserved. The shapes of pl-PE on the chromatogram did not change after treatment with PLA\textsubscript{1} (B-2 and C-2 in Fig. 2). Hydrolysis of bovine heart phosphatidylethanolamine and phosphatidylethanolamine by \textit{R. delemar} lipase took about 18 h.\textsuperscript{21} The hydrolysis time with lipase by the present method was only one-fourth of that by the reported method.\textsuperscript{21}

Alkylacyl analogs of phospholipids might not be destroyed by PLA\textsubscript{1} from \textit{Aspergillus orizae}, although this was not confirmed in the present study, but the content of alkylacyl analogs of ethanolamine phospholipids in the human erythrocytes has been found to be only 0.6% of total phospholipids.\textsuperscript{27}

The method for removing lysophospholipids after PLA\textsubscript{1} treatment is important, because large amounts of lysophospholipids from diacylphospholipids are generated by PLA\textsubscript{1}. We adapted the lipid extraction method reported by Hara and Radin,\textsuperscript{24} in which Na\textsubscript{2}SO\textsubscript{4} solution is used in partition of the n-hexane layer from n-hexane/2-propanol (3:2 v/v) solution. The method using Na\textsubscript{2}SO\textsubscript{4} solution effectively removed lysophospholipids as well as other substances, including non-lipid substances, but lysophospholipids remained. Hence, we further washed the hexane layer with water. The washing resulted in almost complete removal of lysophospholipids (B-2 in Fig. 2 and Fig. 3).

### Table 1. Changes in Relative Composition of Phospholipids of Human Erythrocyte Membrane Due to Treatment with Hexane/Acetone and Phospholipase A\textsubscript{1}

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B-1</th>
<th>B-2</th>
<th>B-3</th>
<th>C-1</th>
<th>C-2</th>
<th>C-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pl-PE</td>
<td>17.8</td>
<td>23.7</td>
<td>91.2</td>
<td>100</td>
<td>1.6</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>PE</td>
<td>17.9</td>
<td>24.3</td>
<td>0.5</td>
<td>0</td>
<td>1.4</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>pl-PC</td>
<td>31.2</td>
<td>39.2</td>
<td>0.4</td>
<td>0</td>
<td>3.8</td>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td>PC</td>
<td>31.2</td>
<td>39.2</td>
<td>0.4</td>
<td>0</td>
<td>3.8</td>
<td>1.4</td>
<td>0</td>
</tr>
<tr>
<td>SM</td>
<td>28.5</td>
<td>5.9</td>
<td>7.1</td>
<td>93.2</td>
<td>97.7</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>4.1</td>
<td>6.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are the means of two determinations and are expressed as % of the phospholipids detected by HPLC with ELSD detection. Fractions A to C-3 correspond to Fig. 1. A, total phospholipids; B-1 and C-1, after treatment with hexane/acetone (1:1, v/v); B-2 and C-2, after phospholipase A\textsubscript{1} treatment; B-3 and C-3, collected from HPLC with 205 nm UV detection. pl-PE, ethanolamine plasmalogen; PE, diacylphosphatidylethanolamine; pl-PC, choline plasmalogen; PC, diacylphosphatidylcholine; SM, sphingomyelin; PS, diacylphosphatidylserine

### Fig. 2. HPLC Chromatograms with ELSD Detection for the Phospholipids in the Human Erythrocyte Membranes.

The HPLC method is described in “Materials and Methods.” A, B-1, B-2, B-3, C-1 and C-2 correspond to Fig. 1. pl-PE, ethanolamine plasmalogen; PE, diacylphosphatidylethanolamine; pl-PC, choline plasmalogen; PC, diacylphosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine
The plasmalogen content (the sum of pl-PE and pl-PC) after treatment with PLA1 reached 92% of phospholipids, but the fraction still contained SM (7.1% of phospholipids), and other phospholipids. When this fraction (B-2) was further washed with n-hexane/acetone (1:1) to remove SM, the purity of pl-PE was increased to about 97% of phospholipids. Contamination by small amount of free fatty acids and cholesterol was reduced by further washing with acetone to precipitate the plasmalogens, but these washings with organic solvents caused large decreases in recovery of the plasmalogens. Therefore, instead of these washings with organic solvents, we used HPLC for further purification of pl-PE (Fig. 3). On the other hand, the SM fraction (C-2) contained little cholesterol or free fatty acids, and the purity of SM had already reached 97.7% at this step. If needed, 100% pure SM can be obtained by collection of the SM peak after applying the C-2 fraction (or total lipids) to HPLC with UV detection, as was done with pl-PE (Fig. 3).

The HPLC method with ELSD detection can separate intact plasmalogens from its diacyl analogs. However, individual peaks could not be collected from the HPLC method, because all the substances were evaporated for detection with ELSD. On the other hand, the HPLC method with UV detection does not destroy phospholipids. Because the diacylphospholipids are completely removed after PLA1 treatment, the HPLC peaks of PE + pl-PE and PC + pl-PC of the fraction indicate pl-PE and pl-PC respectively, even if HPLC is not able to separate the plasmalogens from the diacylphospholipids. The HPLC method with UV detection clearly separated the phospholipid classes (Figs. 3 and 4). Thus plasmalogens can be collected by the HPLC with UV detection. The pl-pE collected from the HPLC was almost free of the other lipids. Because the size of the column used was for analytical use, only a small part of the pl-PE fraction was applied to the HPLC, and the recovery of pl-pE was not calculated. However, even a single chromatographic run provided enough pl-PE for further use for such fatty acid analysis.

Acid hydrolysis of total lipids selectively destroys the vinyl ether bond at the sn-1 position of plasmalogens.
and generates lysophospholipids of plasmalogens, which contain fatty acids at the sn-2 position.26) The treatment did not alter the fatty acid compositions of phospholipids.26) The HPLC method with UV detection showed baseline separation of the lysophospholipids of pl-PE and of other major phospholipids composed of the erythrocyte membrane (Fig. 4). Because acid hydrolysis of plasmalogens can be done with total lipid extract, the total lipid fraction was subjected to acid hydrolysis of pl-PE (LPE-1 and LPE-2 in Fig. 4). We cannot explain why lysophospholipids from pl-PE showed two peaks after acid hydrolysis. Detection of a phospholipid class with 205 nm UV depends primarily on the double bond of the lysophospholipids of pl-PE on the HPLC chromatogram was observed after acid hydrolysis of pl-PE (LPE-1 and LPE-2 in Fig. 4). We cannot explain why lysophospholipids from pl-PE showed two peaks after acid hydrolysis. Fatty acid analysis showed that both LPE-1 and LPE-2 are plasmalogens. PE + pl-PE indicate total ethanolamine phospholipids.

### Table 2. Fatty Acid Compositions of Glycerophospholipids in Human Erythrocyte Membrane

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>pl-PE (%)</th>
<th>LPE-1 (%)</th>
<th>LPE-2 (%)</th>
<th>PE (%)</th>
<th>PC (%)</th>
<th>PS (%)</th>
<th>PE + pl-PE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>3.5</td>
<td>0</td>
<td>26.0</td>
<td>37.4</td>
<td>0.6</td>
<td>17.3</td>
<td></td>
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<tr>
<td>18:0</td>
<td>2.1</td>
<td>0</td>
<td>9.9</td>
<td>12.0</td>
<td>44.8</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td>18:1</td>
<td>9.2</td>
<td>9.0</td>
<td>7.9</td>
<td>25.4</td>
<td>20.2</td>
<td>2.5</td>
<td>21.0</td>
</tr>
<tr>
<td>18:2</td>
<td>10.2</td>
<td>7.3</td>
<td>8.7</td>
<td>7.4</td>
<td>17.9</td>
<td>1.4</td>
<td>5.2</td>
</tr>
<tr>
<td>20:4</td>
<td>32.7</td>
<td>34.9</td>
<td>36.6</td>
<td>14.6</td>
<td>6.2</td>
<td>21.3</td>
<td>21.6</td>
</tr>
<tr>
<td>20:5</td>
<td>2.3</td>
<td>1.1</td>
<td>0.4</td>
<td>2.1</td>
<td>1.2</td>
<td>1.6</td>
<td>3.9</td>
</tr>
<tr>
<td>22:4</td>
<td>7.5</td>
<td>7.3</td>
<td>8.5</td>
<td>2.3</td>
<td>0.2</td>
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<td>10.2</td>
<td>10.5</td>
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<td>2.7</td>
<td>0.7</td>
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<tr>
<td>22:6</td>
<td>24.6</td>
<td>28.5</td>
<td>25.9</td>
<td>9.6</td>
<td>4.5</td>
<td>19.8</td>
<td>15.2</td>
</tr>
</tbody>
</table>

Each phospholipid class was collected by HPLC with 205 nm UV detection (Figs. 3 and 4). Values are the means of two determinations. Analysis of fatty acid composition was done by the HPLC method described in ‘‘Method and Materials.’’ LPE-1 and LPE-2 were lysophospholipids after acid hydrolysis of pl-PE and showed fatty acids in the n-2 position of pl-PE. PE, PC, and PS were diacylphospholipids after acid hydrolysis of plasmalogens. PE + pl-PE indicate total ethanolamine phospholipids.

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