Isolation and purification of cardiolipin from beef heart

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Summary A simple and efficient procedure is described for the preparation of cardiolipin sodium salt from beef heart. A crude phospholipid fraction is isolated by chloroform–methanol extraction of the homogenized tissue, followed by acetone precipitation and reprecipitation in 4% aqueous $CaCl_2$–methanol. Cardiolipin is separated from the calcium salts of the acidic phospholipids by partition column chromatography on silica gel (Polygosil 60-63100) using 2-propanol–cyclohexane–water 50:43:7 (v/v/v) as eluent. Further purification of the cardiolipin is achieved by high performance liquid chromatography of the calcium salt on silica gel (Lichrosorb Si 60-5) with a neutral eluent (2-propanol–cyclohexane–water 45:50:5 (v/v/v)), followed by quantitative conversion to the sodium salt. The yield of this procedure is 1.5–2.1 g of pure 99% sodium salt of cardiolipin per kg of moist ventricular tissue. —**Smaal, E. B., D. Romijn, W. S. M. Geurts van Kessel, B. de Kruijff, and J. de Gier.** Isolation and purification of cardiolipin from beef heart. *J. Lipid Res.* 1985. **26:** 634–637.

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In the past, several isolation procedures of beefheart cardiolipin have been reported (1). For the use of cardiolipin as a serological reagent (Wasserman test) Pangborn (2) developed a method that involved repeated precipitation of barium and cadmium salts from methanol. Further purification of this crude cardiolipin was described by several investigators, using adsorption, gel filtration or anion exchange column-chromatography (1), or thin-layer chromatography (1, 3). Most of these procedures yield products that are contaminated with traces of other (acidic) phospholipids, lysocardiolipins, free fatty acids, or oxidized and polymerized cardiolipin. In addition, the product is often of an undefined salt form. This is particularly complicating in studies of cardiolipin-containing model membranes, as the macroscopic organization of cardiolipin-containing dispersions is dependent on the salt form (4). Up until the present no satisfactory large scale isolation procedure has been presented. Moreover, commercially available cardiolipins often contain contaminants (3).

There are several reasons for difficulties in the purification of beef heart cardiolipin. Beef heart cardiolipin is very susceptible to peroxidation and polymerization (1), because all four acyl chains of the natural occurring lipid are highly unsaturated (5). In addition, cardiolipin is readily deacylated under acidic or basic conditions (1). Furthermore, separation of the different beef heart phospholipids by (column) chromatography is complicated by the presence of divalent cations in the chromatographic supports (1). The affinity of divalent cations (particularly Ca^{2+}) for cardiolipin is very high and the chromatographic behaviors of the various salt forms of acidic phospholipids are quite different (6, 7).

In the present method decomposition of the cardiolipin during the isolation procedure is prevented by carrying out all steps in a nitrogen atmosphere at 0–4°C, by keeping the intermediate products in benzene under argon at −80°C, and by doing partition column chromatography with a neutral eluent in a relatively short time. In the last purification steps the cardiolipin is chromatographed as the calcium salt to yield the pure lipid. Finally the calcium salt of cardiolipin is quantitatively converted to its sodium salt form by organic solvent–water partitioning against the sodium salt of EDTA.

MATERIALS AND METHODS

Materials

The cardiolipin that was used as referent was obtained from Avanti Polar Lipids (Birmingham, AL) (lot #C8-79); it was stored for about 5 months in benzene under argon at −80°C in well-sealed glass bottles. Benzene (Uvasol) and EDTA (analytical grade) were from Merck. Cyclohexane (99.5%) and 2-propanol (99.8%) were from Janssen Chimica (Beerse, Belgium). All other chemicals were of analytical grade.

Analytical methods

The sodium and calcium content of the phospholipids was measured by atomic emission and atomic absorbance spectrophotometry (Varian Techtron 1200), respectively, after destruction of about 3 μmol of material in 0.5 ml of 70% perchloric acid at 180°C and subsequent dilution with distilled water to 20 ml. Lipid phosphorus was determined in the same sample according to Böttcher, Van Gent, and Priest (8).

The fatty acid composition and content were determined by gas–liquid chromatography (Packard Becker 419, 1.8 m × 2 mm column packed with 12% polyethyleneglycol-adipate) of the fatty acid methyl esters, prepared by incubation of the lipid in H_2SO_4–methanol 5:95 (v/v) (2 hr, 70°C). For the fatty acid-phosphorus ratio deter-

Abbreviations: Tris, tris(hydroxymethyl)aminomethane; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid.

mination, margaric acid (17:0) was added to the sample as internal standard.

Thin-layer chromatography was carried out on 8 × 8 cm HPTLC plates (Merck, Kieselgel 60) with chloroform–methanol–acetic acid–water 90:40:12:2 (v/v) as developing solvent. The chromatograms were rendered visible by spraying them with 70% H_2SO_4 and charring for 3 hr at 180°C.

Isolation procedure

To prevent degradation of the phospholipids, the isolation procedure was carried out as much as possible in a nitrogen atmosphere at 0–4°C.

Extraction of the lipids from beef heart

One heart (~2.5 kg) of freshly slaughtered beef was trimmed of fat and connective tissue. The ventricular tissue (~1000 g) was washed thoroughly with ice-cold demineralized water, minced and suspended in 1000 ml of buffer (0.5 M NaCl, 50 mM Na_2 EDTA, and 50 mM Tris-HCl; pH 8.4). The suspension (2 l) was homogenized with an Ystral Ultraturrax homogenizer (type 40/43, Dottingen, FRG), while the pH was kept constant (8.4) by adding small amounts of 1 M NaOH. Then, successively, 4 l of methanol and 2 l of chloroform were added to the stirred suspension and the mixture was homogenized. This resulted in a one-phase extraction system according to Bligh and Dyer (9). The non-extractable tissue residues were pelleted by centrifugation (15 min, 1000 *g*; 4°C) and re-extracted with 1 l of buffer, 2 l of chloroform, and 4 l of methanol. The clear extracts were pooled and 2 l of buffer and 2 l of chloroform were added to induce phase separation. The lipid fraction was collected after rotary vacuum evaporation of the chloroform phase.

Precipitations

The dry lipid from the Bligh and Dyer extract (~20 g) was dissolved in a minimal volume of chloroform (~40 ml). This solution was added dropwise to 1.5 l of stirred ice-cold acetone. After 30 min the white precipitate was collected by centrifugation (15 min, 1000 *g*; 4°C) and washed with 1 l of cold acetone.

The acetone precipitate (~16 g) was dissolved in 1.6 l of methanol. Then, 66 ml of 1 M $CaCl_2$ was added dropwise to the stirred phospholipid solution (0°C). After 30 min of stirring, the precipitate (calcium salts of the acidic phospholipids) was pelleted by centrifugation (20 min, 4000 *g*; 4°C) and redissolved in 300 ml of chloroform. The acidic phospholipids were converted to the sodium salt forms by adding 600 ml of methanol and 300 ml of 0.1 M EDTA, 0.1 M NaCl, 0.05 M Tris-HCl (pH 8.2) buffer. The mixture was shaken for 5 min and phase separation was induced by adding 200 ml of chloroform and 200 ml of buffer. Then the chloroform phase was separated and evaporated to dryness. A second precipitation with $CaCl_2$ in methanol was carried out in the same way.

Column chromatography

The precipitated calcium salts (~6 g) of the acidic phospholipids were dissolved in 30 ml of eluent (2-propanol–cyclohexane–water 50:43:7 (v/v/v)) and isocratically eluted over a 135 × 3 cm column, packed with Polygosil 60-63100 (Marchery-Nagel and Co.), with a flow rate of 10 ml/min. The column was packed and equilibrated in the same eluent system. The eluate was collected in fractions of about 200 ml; the content of lipid was determined by HPTLC. Cardiolipin was the first component in the eluate. Fractions 5–10, containing cardiolipin and some contaminants with a high R_f value, were collected. Following fractions, containing other acidic phospholipids, were discarded. The yield was about 2.5 g.

HPLC

About 300 mg of the chromatographed cardiolipin was dissolved in 3 ml of eluent (2-propanol–cyclohexane–water 45:50:5 (v/v/v)) and eluted over a 250 × 22 mm HPLC column, packed with Lichrosorb Si 60-5 (Merck), with a flow rate of about 17 ml/min. The eluate was detected with a Melz differential refractometer (LCD 201). The fractions that contain pure cardiolipin (elution time: 10–20 min) were pooled and evaporated to dryness. After each elution the column material was rinsed with 500 ml of methanol and re-equilibrated with the eluent until the refractive index of the eluate was constant. The total yield was 1.5–2.1 g.

Conversion of cardiolipin into the sodium salt form

During the chromatographic steps, a small part of the cardiolipin was converted from the calcium salt form into other undefined salt forms (see **Table 1**). That is why it was considered necessary that the HPLC-purified cardiolipin was first converted quantitatively into the calcium salt, in order to enable a more efficient and quantitative conversion into the sodium salt.

The HPLC-purified cardiolipin (2 g) was dissolved in 100 ml of chloroform, whereafter 200 ml of methanol and 100 ml of 0.1 M $CaCl_2$ were added. The mixture was shaken for 5 min and phase separation was induced by adding 50 ml of chloroform and 50 ml of 0.1 M $CaCl_2$. The chloroform phase (150 ml) was separated, 300 ml of methanol and 150 ml of a 0.1 M EDTA, 0.1 M NaCl, and 0.05 M Tris-HCl (pH 8.2) buffer solution were added, and the mixture was shaken for 5 min. Then 100 ml of chloroform and 100 ml of buffer solution were added and the chloroform phase was separated, washed twice with 10 mM NaCl, and subsequently evaporated to dryness. The

TABLE 1. Chemical analysis of beef heart cardiolipin

	½Ca:P	Na:P	Fatty Acid:P	Fatty Acid Residues (mol % of total fatty acids) 12:0[a]	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:3	20:4
Present cardiolipin preparation													
After HPLC	0.71	0.05											
Final product	0.00	0.99	2.03	trace	trace	1.0	0.5	0.9	5.5	90.7	1.0	trace	trace
Commercial cardiolipin (Avanti)	0.00	0.90	1.88		trace	0.5	1.0	0.6	8.2	84.6	4.6	trace	trace

[a]Number of carbon atoms:number of double bonds.

sodium cardiolipin was dissolved in benzene and kept under argon at −80°C in well-sealed glass bottles.

RESULTS AND DISCUSSION

In former isolation procedures (1), the total lipid fraction of beef heart was obtained by extraction with chloroform–methanol 2:1 in a two-phase system without addition of an aqueous buffer. An advantage of a simple one-phase Bligh and Dyer extraction, as we are using, is the optimal contact between the organic solute molecules and the amphipathic molecules in the tissue, because they are all in the same solvent phase (9). In addition, the pH of the system and the salt form of the acidic phospholipids (i.e., cardiolipin) can be more easily controlled by varying the composition of the buffer solution.

The yield of the extraction is about 20 g of dry lipid material per kg of moist tissue. The extract contained sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, cardiolipin, cholesterol, cholesteryl ester, free fatty acids, and pigments (**Fig. 1**). Most of the latter four components and some of the phosphatidylcholine were removed in the acetone precipitation (Fig. 1). Precipitation of the Ca^{2+} salts of the acidic phospholipids in 4% aqueous methanol resulted in the removal of some of the phosphatidylethanolamine and most of the phosphatidylcholine (Fig. 1). Lower or higher water content of the solvent in this step caused, respectively, incomplete precipitation of the cardiolipin or an inferior purification. The total yield of the phospholipid fraction after both precipitation steps was about 6 g per kg of moist tissue.

For the column chromatographic purification, we tested several solvent mixtures as eluting fluid. Elution of the calcium precipitate with solvents that contained acetic acid or ammonia resulted in a reasonable separation of cardiolipin from the other phospholipids, but most of the cardiolipin was converted from the calcium salt form into the free acid or ammonium salt form, respectively, resulting in broadening of the elution profile of cardiolipin. Furthermore, degradation of the cardiolipin on the column or during subsequent evaporation of the solvent was also possible.

Pilot experiments using HPTLC indicated that neutral chloroform–methanol–water mixtures, which are commonly used as eluting fluid in column chromatography of phospholipids, did not give a satisfactory separation of the different acidic phospholipids. In our hands 2-propanol–cyclohexane–water mixtures gave much better results. The most favorable separation of cardiolipin from the other phospholipids in the calcium precipitate was obtained with a 2-propanol–cyclohexane–water mixed in the ratio 50:43:7 (v/v/v).

In **Fig. 2**, a thin-layer chromatogram of the pooled

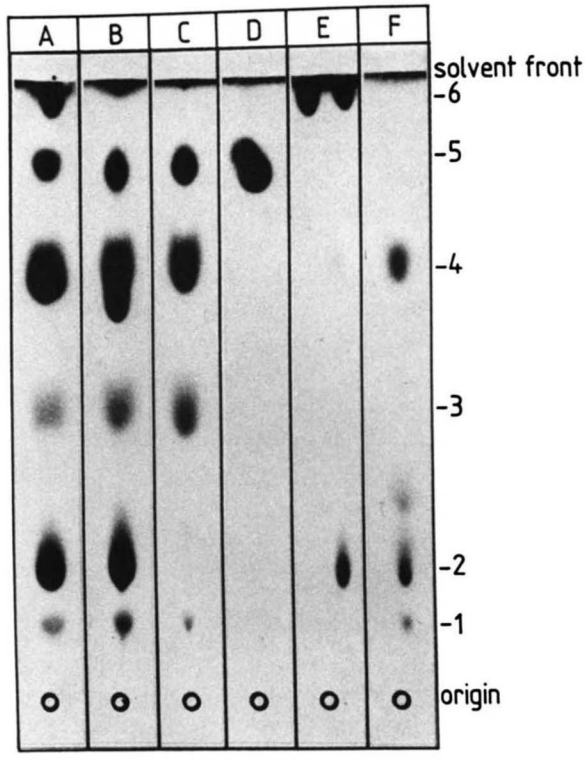

Fig. 1. HPTLC of total lipid extract (A), acetone precipitate (B), and supernatant (E), 4% aqueous $CaCl_2$–96% methanol precipitate (C), and supernatant (F), and reference cardiolipin (Avanti) (D). Each lane was loaded with 5–10 μg of material. Chloroform–methanol–acetic acid–water 90:40:12:2 (by vol) was used as developing solvent. The numbers correspond to the following compounds: 1, sphingomyelin; 2, phosphatidylcholine; 3, phosphatidylserine; 4, phosphatidylethanolamine; 5, cardiolipin; and 6, cholesterol (ester), fatty acids, neutral lipids, and pigments.

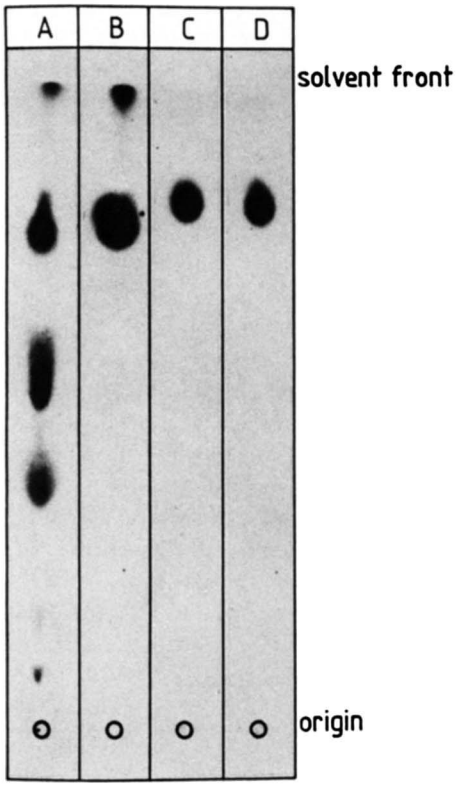

Fig. 2. HPTLC of 4% aqueous $CaCl_2$–96% methanol precipitate (A), pooled cardiolipin-containing fractions after Polygosil column chromatography (B), pooled cardiolipin-containing fractions after HPLC (C), and reference cardiolipin (Avanti) (D). Each lane was loaded with 5–10 μg of material. Chloroform–methanol–acetic acid–water 90:40:12:2 (by vol) was used as developing solvent.

cardiolipin fractions of the column chromatography with this solvent is shown. The cardiolipin was completely separated from the other phospholipids and was only contaminated with some material at the solvent front of the TLC. This material was easily removed by HPLC with 2-propanol–cyclohexane–water 45:50:5 (v/v/v) as eluting fluid (Fig. 2).

After conversion of the calcium cardiolipin to its sodium salt form, we obtained 1.5–2.1 g of pure cardiolipin. In Table 1 the chemical analysis of the product is shown. The cardiolipin was completely calcium-free and was 99% in the sodium salt form. The fatty acid ester content was 2.03 mole per atom phosphorus, which indicates that the cardiolipin contained no peroxidized fatty acids. The cardiolipin contained 90.7 mol % linoleic acid and totally 98 mol % unsaturated fatty acids. This is in agreement with earlier observations (1, 3).

The procedure described can easily be done within 3 days, mainly because of the short elution time for the column chromatography (1–4 hr) and HPLC (within 20 min).

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