Enzymatic synthesis of phosphatidylserine on small scale by use of a one-phase system

Paul Comfurius,[1] Edouard M. Bevers, and Robert F. A. Zwaal

Department of Biochemistry, University of Limburg, P. O. Box 616, 6200 MD Maastricht, The Netherlands

Summary A modification of the phospholipase D-catalyzed synthesis of phosphatidylserine is described, which allows the handling of small quantities of lipid without the need for an ether–water system. By using octylglucoside to disperse the lipid during the enzymatic conversion, it was possible to reduce the volume of the reaction mixture to 50–100 μl. The amount of lipid that can be handled in such small volumes is in the order of micrograms. This facilitates the synthesis of phosphatidylserine from rare or expensive phosphatidylcholine species. The yield of phosphatidylserine is increased by replacing phospholipase D from cabbage by the enzyme from Streptomyces *species*.—**Comfurius, P., E. M. Bevers, and R. F. A. Zwaal.** Enzymatic synthesis of phosphatidylserine on small scale by use of a one-phase system. *J. Lipid Res.* 1990. **31:** 1719–1721.

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In 1977 we described (1) the enzymatic conversion of phosphatidylcholine (PC) into phosphatidylserine (PS). The reaction, a base-exchange catalyzed by phospholipase D (phosphatidylcholine phosphatidohydrolase, E.C. 3.1.4.4), is carried out at 45°C in a two-phase system composed of a solution of PC in diethylether and a saturated solution of serine in acetate buffer. The purpose of developing this procedure was to prepare PS species with a defined fatty acid composition, starting from commercially available PC species and avoiding the laborious total chemical synthesis of PS. Since then, increasing interest has arisen in PS molecules containing groups whose fate can be easily assessed by various physical and chemical methods. For instance, in research aimed at elucidating mechanisms underlying transbilayer movement and the maintenance of phospholipid asymmetry in biological membranes, various labeled PS species are used. Some recent examples are the incorporation of radiolabeled PS in erythrocytes, followed by enzymatic degradation to determine the fraction that remains in the outer monolayer upon prolonged incubation (2). A similar technique uses a fluorescent analog of PS, where the fraction of label still present in the outer monolayer is assessed by exchange with phospholipid vesicles (3). Phosphatidylserine containing a spin-label has been used in several cells to evaluate their capacity to transport exogenously added aminophospholipids to the inner monolayer of the cells by an energy-dependent translocating system (4–6). Radioiodinated, photoactivatable lipid was used in an attempt to identify erythrocyte membrane proteins involved in such a phospholipid transport over the plasma membrane (7, 8). These labeled lipids are, in general, available only in small amounts and are rather expensive. To prepare PS from the respective phosphatidylcholines by the use of a two-phase system consisting of water and ether layers, incubated at a temperature well above the boiling point of ether, limits the minimal volume that can be handled conveniently. When small amounts of PC are used, the overall yield of the procedure will be lower, mainly because of adsorption problems. We found that ether could be replaced by detergent to disperse the lipids, which makes it possible to perform the synthesis in a one-phase system on a micro scale (volumes less than 1 ml). In such a system the lipids are presented to the phospholipase in the form of a mixed micelle of lipid and detergent. Using a suitable detergent, this micellar structure is such that the phospholipase D can modify the PC molecules at a rate comparable to that found in an ether–water system. Recently, it was reported by Juneja et al. (9) that phospholipases D from several Streptomyces strains are able to produce essentially complete conversion of PC to PS. Of two commercially available phospholipases D from Streptomyces strains, we found that one (from the strain *species*) is capable of producing higher amounts of PS compared to cabbage phospholipase D, while the other (from the strain *chromofuscus*) does not produce any PS, but only PA.

MATERIALS AND METHODS

Phosphatidylcholine (from egg yolk), dilauroyl phosphatidylcholine, 1-stearoyl-2-NBD-amino-hexanoyl-phosphatidylcholine, sodium dodecylsulfate (SDS), sodium deoxycholate (DOC), Triton X-100, and n-octyl-β-D-glucopyranoside were obtained from Sigma. Cabbage phospholipase D was partially purified from the inner leaves of Savoy cabbage as described (1). Phospholipase D from Streptomyces *species* and Streptomyces *chromofuscus* were obtained from Sigma. All other reagents were of the highest grade available. Incubations were carried out by first drying PC in a glass tube by a stream of nitrogen. The lipid was solubilized three times in pure chloroform and dried again, to remove traces of methanol or ethanol which could give rise to unwanted side-products such as methyl or ethyl phosphatidic acid (1). To the dried PC a solution is added, containing calcium, acetate, detergent, and serine. This solution is prepared at 45°C by mixing

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PA, phosphatidic acid; octylglucoside, n-octyl-β-D-glucopyranoside; NBD, 7-nitrobenz-2-oxa-1,3-diazole.

[1]To whom correspondence should be addressed.

1 M $CaCl_2$, 1 M Na acetate, detergent, L-serine, and water. Final concentrations are 0.1 M Ca^{2+}, 0.1 M acetate, 50% (w/v) serine, and 2% (w/v) detergent. The resulting pH is between 5.4 and 5.8. The reaction is started by adding phospholipase D to the mixture. Of the cabbage phospholipase D, 1 I.U. is used per 10 mg lipid (1 I.U. converts 1 μmol of substrate per minute at 37°C). The bacterial phospholipases are added to a final concentration of 5 μg/ml. Extraction, purification, and analysis by two-dimensional thin-layer chromatography of the resulting lipid mixtures was performed as described before (1). When PS species are synthesized that are relatively water-soluble, it is preferable to use a one-phase extraction procedure as described by Reed et al. (10) to avoid loss of material by partitioning of lipid over two phases. Briefly, after complexing calcium by EDTA, five volumes of methanol are added to the resulting solution, followed by five volumes of chloroform. After stirring for 10 min the mixture is centrifuged to remove insoluble material. The supernatant is evaporated and dried by repeated evaporation from absolute ethanol. Finally, the lipid is extracted from the dried material with pure chloroform and purified by CM-cellulose chromatography or thin-layer chromatography.

RESULTS AND DISCUSSION

We compared the detergents sodium dodecylsulfate, sodium deoxycholate, Triton X-100, and octylglucoside in their capacity to replace diethyl ether as a solvent for PC in the enzymatic synthesis of PS. Of these detergents, octylglucoside proved to be the most effective, both with respect to the yield of PS and to the rate at which PS is formed (**Table 1**). We found that when 10 mg/ml of PC is used, at least 1% (w/v) of octylglucoside is needed to allow complete conversion of PC under the conditions used

TABLE 1. Conversion of PC to PS in the presence of detergents

Detergent	Incubation Time	PC Degradation	PS Formation
	h	%	%
TX-100	3	0	0
SDS	3	50	25
DOC	1	45	15
DOC	2	87	29
DOC	3	94	21
DOC	4	96	25
Octylglucoside	1	67	25
Octylglucoside	2	93	41
Octylglucoside	3	95	46
Octylglucoside	4	96	45

In all cases a detergent concentration of 2% (w/v) was used. Incubations were carried out at 45°C with stirring for the time indicated in the table. Degradation is expressed as the fraction of lecithin converted. The amount of PS formed is expressed as fraction of the amount of lecithin present at zero time.

TABLE 2. Effect of octylglucoside concentration on the efficiency of PC conversion

Octylglucoside	PC Degradation	PS Formation
% *w/v*	%	%
0.5	56	17
1.0	95	41
2.0	95	38
5.0	95	39

Incubations were carried out at 45°C with stirring for 3 h. Octylglucoside concentrations are expressed as weight per volume. Degradation and PS formation are expressed in the same way as stated in the legend to Table 1.

(**Table 2**). This is in line with the very high critical micellar concentration of octylglucoside, which implies a monomeric concentration of several mg/ml, which leaves very little detergent to allow formation of mixed micelles of detergent and lipid when less than 10 mg/ml octylglucoside is present. Based on the sudden increase in light scattering that is observed when increasing amounts of PC are dispersed in a solution of octylglucoside, up to 25 mg/ml of PC can be solubilized in the form of mixed micelles when 2% (w/v) octylglucoside is used (data not shown), before bilayer structures are formed in which PC cannot efficiently be converted by phospholipase D.

Using different lecithins (from egg yolk, diC 12:0, or NBD-labeled) a comparison was made between cabbage and bacterial phospholipase D. It is shown (**Table 3**) that, in agreement with the results of Juneja et al. (9), much higher amounts of PS can be obtained by the use of the bacterial phospholipase D from Streptomyces *species*, although we did not observe complete conversion of PC to PS which might be due to a difference in the Streptomyces strain. The enzyme from Streptomyces *chromofuscus* fails to produce any PS under our conditions. In contrast to phospholipase D from cabbage, the bacterial enzyme appears

TABLE 3. Conversion of some PC species to PS by phospholipase D from cabbage or bacterial origin

	Egg-PC	diC12:0 PC	NBD-PC
Ether-water system			
Cabbage PlD	35–40%	20–25%	5–10%
Octylglucoside system			
Cabbage PlD	35–40%	30–35%	25–30%
Streptomyces PlD	60–70%	65–75%	70–80%

Lecithin in a concentration of 10 mg/ml was incubated with 1 I.U. cabbage phospholipase D/ml or 5 μg/ml of enzyme from Streptomyces *species*. Incubations were carried out in an ether–water two-phase system or in a one-phase system containing 2% (w/v) octylglucoside for 2 h at 45°C with constant stirring. PS formation is expressed as stated in the legend to Table 1. Data represent the range of at least two different experiments.

to be able to hydrolyze PS to phosphatidic acid, making it essential to follow the reaction by thin-layer chromatography. Using acceptor concentrations as described before (1) we found no difference in yield of either phosphatidylethanolamine or phosphatidylglycerol, either in the traditional two-phase system or in the octylglucoside one-phase system.

In conclusion, a method is described for the enzymatic synthesis of PS in a one-phase system, using octylglucoside to disperse the lipids. One advantage of this system is that small quantities of expensive and rare species of PC can be handled in small volumes, thereby reducing losses from adsorption during the procedure. Also it appears (Table 3) that more polar PC species with a lower solubility in ether, like short chain lecithins or species containing bulky polar groups, are more efficiently converted to PS using this method compared to the two-phase system. Replacing cabbage phospholipase D by the commercially available enzyme from Streptomyces *species* increases the fraction of PC converted to PS, allowing overall recoveries of 60–65%, irrespective of the fatty acid composition of the lecithin used as starting material.

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