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Two-enzyme system for the synthesis of 1-lauroyl-*rac*-glycerophosphate (lysophosphatidic acid) and 1-lauroyl-dihydroxyacetonephosphate

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Abstract

A combination of two enzymes, phospholipase D (PL D) and C (PL C), was investigated for the production of two lysophospholipids, 1-lauroyl-*rac*-glycerophosphate (1-LGP) and 1-lauroyl-dihydroxyacetonephosphate (1-LDHAP). The high transphosphatidylation ability of phospholipase D from *Streptomyces* sp. allowed the formation of 1-lauroyl-phosphatidylglycerol (1-LPG) and 1-lauroyl-phosphatidyldihydroxyacetone (1-LPDHA) from phosphatidylcholine (PC) and 1-monolauroyl-*rac*-glycerol (1-MLG) and 1-lauroyl-dihydroxyacetone (1-MDHA), respectively. A two-phase system, diethyl ether/water, was chosen for the convenience of the recovery of the water insoluble products. A similar two-phase system was used for hydrolysis of the complex phospholipids by phospholipase C form *Bacillus cereus*, which released both lysophospholipids. Only trace amounts of phosphatidic acid (PA) were detected showing that the enzyme is highly selective for the release of the diacylglycerol and 1-lauroyl-*rac*-glycerophosphate and 1-lauroyl-dihydroxyacetonephosphate.

Keywords: Phospholipase D; Phospholipase C; Acyl-phosphatidylglycerol; Acyl-phosphatidyldihydroxyacetone; Acyl-*rac*-glycerophosphate; Acyl-dihydroxyacetonephosphate

1. Introduction

The combination of phospholipases D and C for the formation of phosphorylated organic water-soluble molecules has been reported (Shinitzky et al., 1993; Takami and Suzuki, 1994; D'Arrigo et al., 1995). The authors used phosphatidylcholine and glycerol or dihydroxyacetone for the synthesis of phosphatidylglycerol and phosphatidyldihydroxyacetone with PL D. A solvent/aqueous two-phase system was found to be convenient for the easy separation and recovery of the water insoluble products. After purification, the obtained phospholipids were treated with a PL C from *Bacillus cereus* in a similar two-phase system which rendered glycerophosphate and dihydroxyacetophosphate, this time as water-soluble products.

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The unique transphosphatidylation activity of the phospholipase D has been extensively studied, and their applications are multiple (for a review see Servi, 1999). Plant phospholipases D, from cabbage, castor bean or peanut seed, p.g., have proven to be effective catalysts in transphosphatidylation reactions (Heller, 1978). However, the simultaneous formation of the phosphatidic acid by-product has focused the investigators on the search for more selective enzymes on microorganisms. The results have been various, showing that there are enzymes possessing high transphosphatidylation activities and others with a solely hydrolitic ability (Nakajima et al., 1994; Juneja et al., 1988, 1989).

Phospholipase C is a very well studied enzyme that catalyses the hydrolysis of the phosphoester bond on phospholipids, such as phosphatidylcholine, into the phosphorylated polar head group and the diglyceride. The applications of this enzyme are somewhat limited, since it does not have a very broad selectivity and synthetic phospholipids are poor substrates for the enzyme (Ries et al., 1991; Servi, 1999). Ries et al. (1991) showed that for optimal activity, the phospholipids must contain an ester or ether bond in the sn 1 and an ester bond in the sn 2 position of the glycerol backbone and with a phosphocholine esterified in the sn 3 position. Anything out of this pattern resulted in a drop in the hydrolytic activity.

Enzymatic synthesis of lysophosphatidic acid has been achieved using different aproaches, such as the esterification of glycerophosphate and fatty acid or fatty acid vinyl ester with lipases (Han and Rhee, 1995; Virto et al., 1999). However, some phosphatidic acid was also formed in most reaction conditions. Lysophosphatidic acid can be also synthesised with a combination of two enzymes. Thus, phospholipase A_2 (PL A_2) can be used for the hydrolysis of phosphatidylcholine to form lysophosphatidylcholine, which can be further hydrolysed by PL D to give lysophosphatidic acid (Long et al., 1967). If natural PC is used, LPA with different fatty acid composition is formed. Van der Bend et al. (1992) reported the synthesis of 1-oleoyl-LPA via phosphorylation of dioleoylglycerol with diacylglycerol kinase forming phosphatidic acid, which was then hydrolysed with PL A_2.

In this paper, we report the utilisation of a phospholipase D from *Streptomyces* sp. for the formation of a synthetic phospholipid (1-lauroyl-phosphatidylglycerol, acyl-phosphatidylglycerol) and a novel phospholipid (1-lauroyl-phosphatidyldihydroxyacetone, acyl-phosphatidyldihydroxyacetone). The enzyme has proven to be very selective for the transphosphatidylation reaction. The nuclophile 1-monolauroyl-*rac*-glycerol appeared to be preferred to the ketone counterpart. Acyl-phosphaditylglycerol has been shown to exist as a minor component of the phospholipids in microorganism membranes (Makula et al., 1978; De Siervo and Homola, 1980; Beach et al., 1991). Furthermore, in lysosomes together with bis (monoacylglycerol) phosphate it participates in the turnover of fatty acids in the metabolism of complex phospholipids (Waite et al., 1990; Huterer and Wherret, 1990). The physical and chemical properties of these phospholipids should be of fundamental, and may be of practical, interest.

The phospholipids were readily hydrolysed by a phospholipase C form *B. cereus* giving 1-lauroyl-*rac*-glycerophosphate (lysophosphatidic acid) or 1-lauroyl-dihydroxyacetonephosphate and diacylglycerol. The enzymatic rates were similar suggesting that the hydroxyl or ketone group in the sn 2 position is of minor relevance. However, the enzyme appeared to be somewhat more selective for the hydrolysis of the 1-lauroyl-phosphatidylglycerol, since the formation of phosphatidic acid from the hydrolysis of the terminal phosphoester bond on the molecule was lower than with 1-lauroyl-phosphatidyldihydroxyacetone. Increasing amounts of phosphatidic acid in the media did not inhibit the hydrolytic reaction.

2. Materials and methods

2.1. Materials

Phospholipase D from *Streptomyces* sp. and phospholipase C from *B. cereus*, phosphatidylcholine ($>99\%$) and N-(2-Hydroxyethylpiperazine-N′-(2-ethanesulfonic acid) (HEPES), were products from Sigma Chemical Co. (St. Louis,

MO). Monolaurin was purchased from Larodan AB (Malmö, Sweden), phosphatidic acid and lysophosphatidic acid were obtained by the action of phospholipase D from cabbage towards phosphatidylcholine and lysophosphatidylcholine, respectively. Diacylglycerol was obtained by the action of PL C on phosphatidylcholine. Sodium acetate trihydrate, copper (II) sulphate pentahydrate, calcium chloride dihydrate, silica gel 60 and all the solvents were purchased from Merck (Darmstadt, Germany) and were of analytical grade. 1-lauroyl-dihydroxyacetone was produced in our laboratory (Virto et al., 1999).

2.2. Methods

2.2.1. Phospholipase D catalysed reactions

Typically, phosphatidylcholine to a concentration of 10 mM and different concentrations of 1-monolauroyl-*rac*-glycerol or 1-monolauroyl-dihydroxyacetone (from 5 to 200 mM) were dissolved in 0.4 ml diethyl ether. Added to the solution were 0.4 ml Na-acetate buffer (0.2 M), pH 5.5 containing 40 mM $CaCl_2$, with the subsequent formation of a two-phase system. The reaction media were incubated in an orbital shaker (100 r.p.m.) at 25°C. The reactions were started by the addition of 0.1 units PL D. Samples of 10 μl were withdrawn from the ether phase at intervals and the solvent was allowed to evaporate before the addition of 25 μl tetrahydrofuran. For the final quantification of the products, the remaining reaction mixture was mixed with 50 μl of 1 N HCL and 500 μl of chlorofrom:methanol (2:1). The mixtures were thoroughly vortexed and centrifuged at 6000 r.p.m for 10 min. The lower chloroform rich part was collected for analysis. No phospholipids were detected in the upper methanolic phase which was discharged. Obtained samples were then analysed by HPTLC.

2.2.2. Preparation of 1-LPG and 1-LPDHA

50 mg of phosphatidylcholine were dissolved on 2 ml of diethyl ether containing 300 mM 1-monolauroyl-*rac*-glycerol or 1-monolauroyl-dihydroxyacetone. Two ml of Na-acetate buffer (0.2M), pH 5.5 containing 40 mM $CaCl_2$, were added and the mixtures were incubated at 25°C in an orbital shaker. The reactions were started by the addition of 4 units of phospholipase D. After the completion of the reaction the mixtures were centrifuged at 6000 r.p.m. The upper ether phase, containing products, was collected and the solvent evaporated in a rotavapor. If no phosphatidic acid was present, the products were separated from the 1-monolauroyl-*rac*-glycerol or 1-monolauroyl-dihydroxyacetone by simple precipitation with acetone. For the purification of reactions containing phosphatidic acid the mixtures were dissolved on chloroform and separated by chromatography on silica gel. The solution was applied to the column and eluted with chloroform until no more 1-MLG or 1-MLDHA was detected in the eluate. 1-LPG and 1-LPDHA were then eluted with a mixture of chloroform:methanol:NH_3 (70:21:2). Under these conditions phosphatidic acid is retained on the column.

2.2.3. Fatty acid analysis of 1-LPG and 1-LPDHA

For the verification of the structure the products were subjected to fatty acid analysis (Svensson et al., 1992). Bands on TLC plates containing 1-LPG and 1-LPDHA, and their products after hydrolysis with phospholipase C, were scraped and mixed with 2 ml of 0.5 M sodium methoxide in methanol. After incubation for 10 min at 50°C, 200 μl of hexane and 4 ml of saturated NaCl solution were added. The mixture was vortexed and centrifuged at 6000 r.p.m. for 10 min. The fatty acid methyl esters formed were recovered in the hexane phase and analysed by gas chromatography (GC) on a Varian 3400 gas chromatograph equipped with a septum-equipped programmable injector and a flame-ionisation detector. The column was a SP-2380 (30 m, 0.32 mm i.d., 0.20 μm film thickness, Supelco, Bellafonte, PA). The injector was programmed from 50 to 200°C at 100°C/min and held at 100°C for 3 min. The column temperature was programmed between 75 and 190°C at 10°C/min and held at 190°C for 8 min. The chromatograms obtained were compared with the fatty acid methyl esters from phosphatidylcholine produced in the same way and with lauric acid methyl ester.

2.2.4. ^{1}H-NMR analysis

Structural verification of 1-LPG and 1-LPDHA was also done by ^{1}H-NMR analysis on a DRX 500c spectrometer.

2.2.5. Phospholipase C catalysed reactions

Typically, 1-lauroyl-phosphatidylglycerol or 1-lauroyl-phosphatidiyldihydroxyacetone in concentrations between 1 and 10 mM, were dissolved in 0.2 ml diethyl ether. To the solution, 0.2 ml HEPES buffer, pH 7.5 containing 50 mM $CaCl_2$, were added, with the subsequent formation of a two-phase system. The reaction media were incubated in an orbital shaker (100 r.p.m.) at 25°C. The reactions were started by the addition of 1 unit PL C. Samples of 5 μl were withdrawn from the ether phase at intervals and the solvent was let to evaporate before the addition of 25 μl tetrahydrofuran. For the final relative quantification of the products the remaining media was mixed with 50 μl of 1 N HCl and 500 μl of chlorofrom:methanol (2:1). The mixtures were thoroughly vortexed and centrifuged at 6000 r.p.m. for 10 min. The lower chloroform rich part was collected and evaporated to a final volume of around 30 μl. No phospholipids were detected in the upper methanolic phase which was discharged. Obtained samples were then analysed by HPTLC.

2.2.6. HPTLC analysis

Thin-layer chromatography was performed on 20×10 cm silica gel 60 HPTLC plates (Merk, Darmstadt, Germany). Then 2–15 μl samples were applied as 6 mm bands using an autosampler (Camag ATS3, Muttenz, Switzerland). The plates were eluted in an unsaturated automatic development chamber (Camag ADC, Muttenz, Switzerland) with the upper organic phase of a two-phase mixture of ethyl acetate:isooctane:acetic acid:water (13:2:2:10) (Van Blitterswijk and Hilkmann, 1993) for the PL D reaction samples, and ethyl acetate:isooctane:acetic acid:water (13:13:4:10) for the PL C reaction samples. Developed and dried plates were immersed for 5 s in a dipping tank (Camag chromatogram-immersion device III, Muttenz, Switzerland) containing a 0.1% aqueous solution of 8-anilino-1-naphthalenesulfonate (ANS) and densitometrically evaluated with a Camag TLC scanner 3 (Muttenz, Switzerland) in fluorescence/reflection mode. The slit dimensions were set at 5×0.3 mm, the excitation wavelength was 376 nm and the emission cut-off filter was 420 nm. The relative amounts of the substrates and the products formed were calculated in each chromatographic plate from their peak areas. Substrates and products detected from PL D reaction samples were phosphatidylcholine, phosphatidic acid, 1-lauroyl-phosphatidylglycerol and 1-lauroyl-phosphatidyldihydroxyacetone with a relative Rf of 0.10, 0.25, 0.45 and 0.65, respectively. Only the ether soluble substrates and products were analysed for the PL C reactions and these were 1-lauroyl-phosphatidylglycerol, 1-lauroyl-phosphatidyldihydroxyacetone, 1-monolauroyl-*rac*-glycerol, 1-monolauroyl-dihydroxyacetone and diacylglycerol with a relative Rf of 0.10, 0.15, 0.45, 0.60 and 0.70, respectively. Calibration curves for all purified substrates and products showed the detector response was linear in the concentration range used. Relative response factors were calculated for the quantification of the lipids.

For the final relative quantification of the products after extraction of the reactions with PL C, 1-lauroyl-*rac*-glycerophosphate, 1-lauroyl-dihydroxyacetonephosphate and phosphatidic acid, the plates were eluted with the organic phase of a two-phase mixture of ethyl acetate:isooctane:acetic acid:water (13:2:5:10). The calculated Rf were 0.15, 0.20 and 0.40, respectively. The Rf for 1-lauroyl-*rac*-glycerophosphate and 1-lauroyl-dihydroxyacetonephosphate were considered to be the same.

3. Results and Discussion

3.1. Preparation of 1-LPG and 1-LPDHA and structural characterisation

The transphosphatidylation of phosphatidylcholine with 300 mM of 1-monolauroyl-*rac*-glycerol or 300 mM of 1-monolauroyl-dihydroxyacetone proved to be very selective and no phosphatidic acid was de-

tected. The simple purification method, in which 1-LPG and 1-LPDHA precipitated in acetone, enabled the preparation of highly pure products and the reuse of the unreacted 1-MLG and 1-MLDHA.

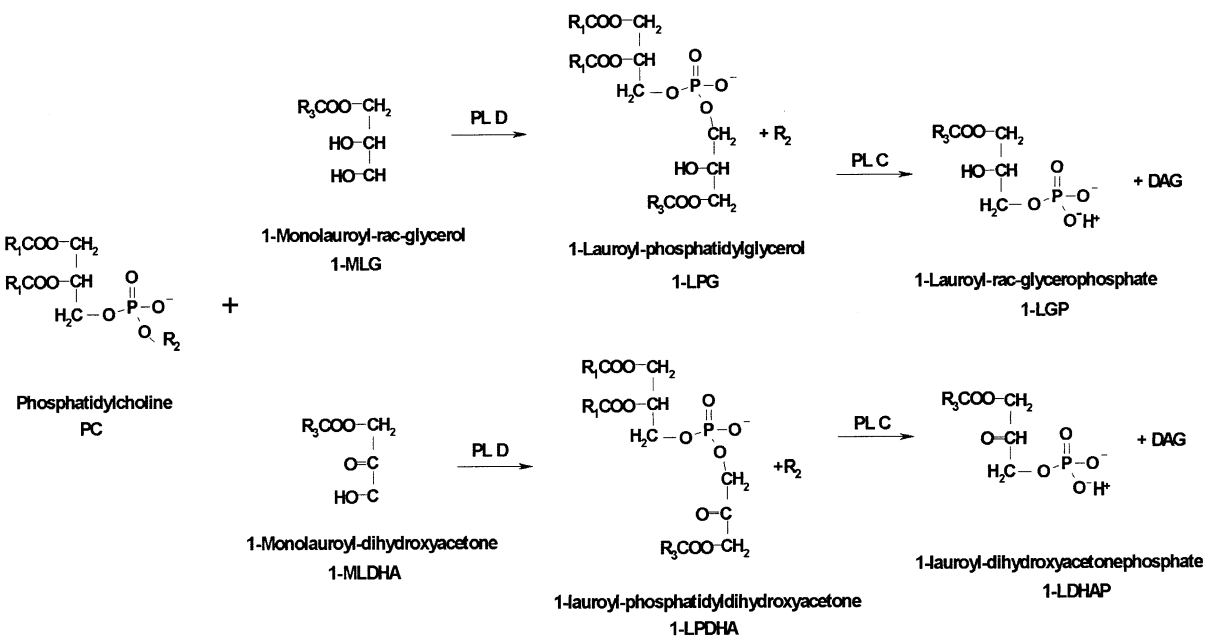
Structural verification was done by 1H-NMR and fatty acid analysis. 1H-NMR of 1-LPG and 1-LPDHA in CDCl3 gave the following structural information:

- 1-Lauroyl-phosphatidylglycerol,0.88 (9H, m, 3 CH_3 of fatty acids); 1.28 (60H, m, CH_2 of fatty acids); 1.6 (6H, m, 3 CH_2 of fatty acids); 2.3 (6H, m; 3 $COCH_2$ of fatty acids); 3.9–4.2 (6H, 2H, m, 4 CH_2 of both glycerol molecules); 4.4 (1H, m, CH glycerol of 1-LG); 5.22 (1H, m, CH glycerol).
- 1-Lauroyl-phosphatidyldihydroxyacetone, 0.88 (9H, m, 3 CH_3 of fatty acids); 1.28 (60H, m, CH_2 of fatty acids); 1.6 (6H, m, 3 CH_2 of fatty acids); 2.25–2.5 (4H, 2H, m; $COCH_2$ of fatty acids); 3.9–5.0 (2H, 1H, 1H, 1H, 2H, m, 4 CH_2 and 1 CH of glycerol and 1-LDHA); 5.22 (1H, m, CH glycerol).

Chromatograms of the fatty acid methyl ester analysis of the products showed the presence of the characteristic fatty acids in phosphatidylcholine and lauric acid, which accounted for one third of the sum of the peak areas indicative for one lauric acid in each phospholipid molecule. After hydrolysis with PL C, the bands with an Rf corresponding to diglyceride and lysophosphatidic acid showed the presence of the characteristic fatty acids from PC and methyl laurate, respectively.

3.2. Reaction progress

Scheme 1 shows the reaction pathways for the formation of the lysophospholipids by the combination of the enzymes phospholipase D and C. The conditions in which the reactions are carried out are very similar, a diethyl ether-aqueous two-phase system for both enzymes. Phospholipase D from *Streptomyces* sp. is very selective for the formation of this transphosphatidylation product in the presence of an alcohol. It should be mentioned that neither PL D from savoy cabbage nor



Scheme 1. Reaction pathways for the enzymatic two-step preparation of 1-lauroyl-*rac*-glycerophosphate (lysophosphatidic acid) and 1-lauroyl-dihydroxyacetonephosphate. R_1: fatty acids, R_2: choline, R_3: lauric acid.

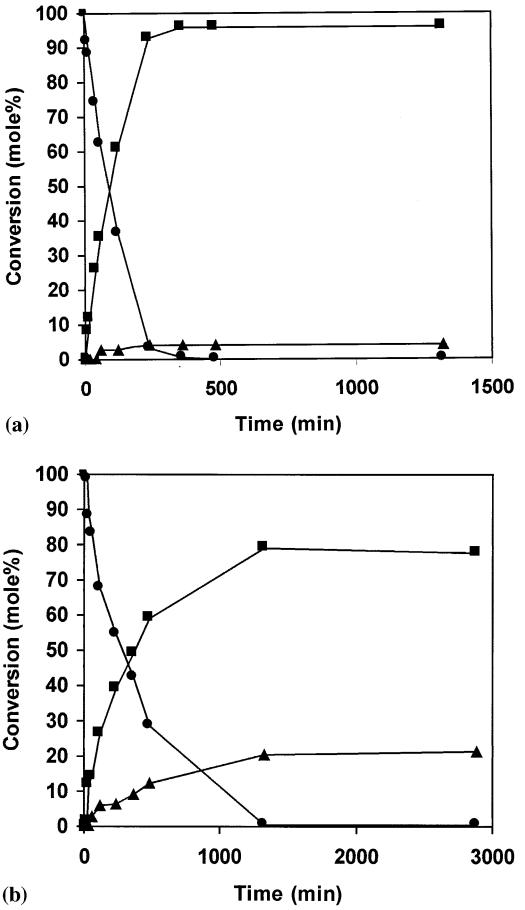


Fig. 1. (a) PL D catalysed transphosphatidylation of PC with 1-monolauroyl-*rac*-glycerol in a two-phase system. Reaction media containing 10 mM PC and 75 mM 1-MLG in 0.4 ml diethyl ether, 0.4 ml Na-acetate buffer (0.2 M), pH 5.5, 40 mM $CaCl_2$ and 0.1 units PL D. Reaction temperature 25°C (●) PC, (■) 1-LPG and (▲) PA; (b) PL D catalysed transphosphatidylation of PC with 1-monolauroyl-dihydroxyacetone in a two-phase system. Reaction media containing 10 mM PC and 75 mM 1-MLDHA in 0.4 ml diethyl ether, 0.4 ml Na-acetate buffer (0.2 M), pH 5.5, 40 mM $CaCl_2$ and 0.1 units PL D. Reaction temperature 25°C. (●) PC, (■) 1-LPDHA and (▲) PA.

PL D from *Streptomyces chromofoscus* were effective catalysts for this transphosphatidylation reaction.

As seen in Fig. 1a and b, the formation of 1-LPG and 1-LPDHA was predominant over the hydrolysis of the phosphatidylcholine when 75 mM 1-MLG or 1-MLDHA was used as nucleophile. Nevertheless, the selectivity seems to be lower for the ketone substrate and under the same conditions more hydrolytic product is formed.

The products of the transphosphatidylation were purified and then treated with phospholipase C from *B. cereus*. Fig. 2 illustrates the consumption of 1-LPG and 1-LPDHA and the formation of diacylglycerol. Both substrates are readily hydrolysed by the enzyme at similar rates. It should be mentioned that mainly diglyceride was detected indicating a high selectivity of the enzyme towards the phosphoester bond between the diglyceride and the phosphate moieties.

3.3. Kinetics of the transphosphatidylation reaction with PL D

The transphosphatidylation of phosphatidylcholine with 1-monolauroyl-*rac*-glycerol or 1-monolauroyl-dihydroxyacetone followed the Michaelis–Menten equation profile as seen in Fig. 3. The activity of the enzyme decreased at concentrations higher than 75 mM 1-monolauroyl-*rac*-glycerol, probably a result of substrate inhibition. With 1-monolauroyl-dihydroxyacetone the reaction rates increased in the range of concentrations tested (5–200 mM).

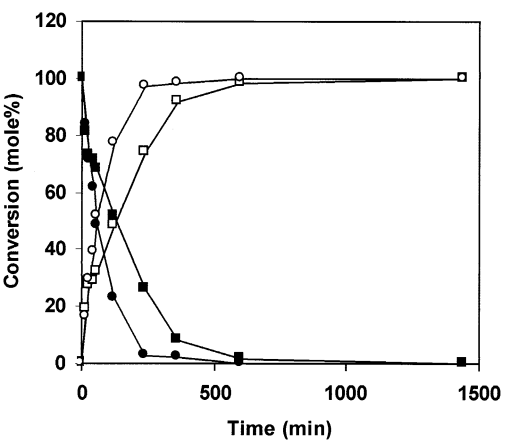


Fig. 2. Diacylglycerol formation (open symbols) in the hydrolysis of 4 mM 1-lauroyl-phosphatidylglycerol and 4 mM 1-lauroyl-phosphatidyldihydroxyacetone (closed symbols) in diethylether-0.1 M HEPES buffer, pH 7.5 and 50 mM $CaCl_2$ two-phase system catalysed by phospholipase C (1 U) from *Bacillus cereus* (□, ■) 1-LPG and (○, ●) 1-LPDHA.

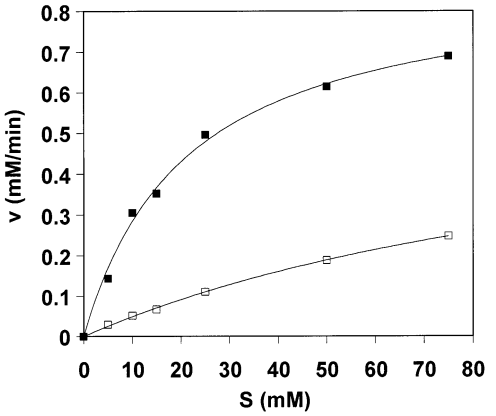


Fig. 3. Substrate effect on the initial rates for the PL D catalysed transphosphatidylation of PC (10 mM) with different concentrations of 1-monolauroyl-*rac*-glycerol (■) and 1-monolauroyl-dihydroxyacetone (□) in a diethyl ether-Na-acetate buffer (0.2M), pH 5.5, 40 mM $CaCl_2$ and 0.1 units PL D. Reaction temperature 25°C.

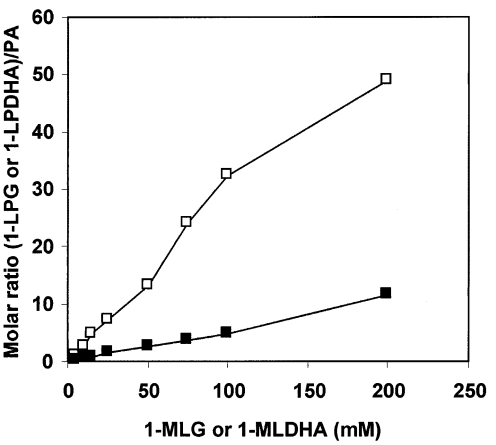


Fig. 4. Nucleophile concentration effect on the molar ratio of the transphosphatidylation and hydrolysis products. Reaction conditions containing 10 mM PC and different concentrations of 1-MLG (□) or 1-MLDHA (■) on 0.4 ml diethylether, 0.4 ml Na-acetate buffer (0.2M), pH 5.5, 40 mM $CaCl_2$ and 0.1 units PL D. Reaction temperature 25°C.

The kinetic parameters were determined by a non-linear regression using Kaliedagraph™, Abelbeck Software. Km (app) and V_{max} (app) of 1-MLG were 23 ± 7 and 0.90 ± 0.01 mM/min, respectively and Km (app) and V_{max} (app) for 1-MLDHA were 104 ± 5 and 0.55 ± 0.04 mM/min, respectively. From this data it is clear that 1-MLG is better substrate than 1-MLDHA. The low Km of specially 1-MLG, but also of 1-MLDHA is remarkable. Km of nucleophiles which are not amphiphilic, but present almost exclusively in the aqueous phase, are usually much higher. A value of 1.3 M has been reported for 3-dimethylamino-1-propanol (Carrea et al., 1997). The low Km values of the nucleophiles used in this study may indicate that the PL D is present either in the mixed micelles formed by the substrate or closely associated with those micelles. The local concentration of the nucleophile in the vicinity of the enzyme would thus be high so that it can react effectively. The high reactivity of the 1-MLG with respect to 1-MLDHA may be explained considering their ability of solvation and association with the phosphatidylcholine substrate. While 1-MLG is more soluble than 1-MLDHA in the water saturated diethyl ether and will probably easily form micelles with the phospholipid, 1-MLDHA is a somewhat more rigid molecule and with less possibilities of hydrogen bonding, and thus less association ability.

Fig. 4 shows the final composition of the products at different 1-MLG and 1-MLDHA concentrations. The enzyme is very selective for 1-MLG, so that at almost all substrate concentrations tested the formation of the transphosphatidylation product was favoured. It is also noted that PL D is not steroespecific in the synthesis of 1-LPG with respect to 1-MLG, since the nucleophile, which is a racemic mixture, is almost totally consumed at equimolar substrate concentrations.

The selectivity of the enzyme towards the other substrate is lower but still high considering that at 200 mM 1-MLDHA, only 8% of the product was phosphatidic acid. Higher concentrations of water-soluble alcohols such as glycerol or dihydroxyacetone are reported to be necessary for the minimisation of phosphatidic acid formation (Takami and Suzuki, 1994; D'Arrigo et al., 1995).

3.4. Hydrolysis of 1-LPG and 1-LPDHA by PL C

Fig. 5a and b illustrate the time course for the hydrolysis of both substrates in the presence of

PL C. The calculation of the activities from the formation of diglyceride was not accurate, since the curves deviated greatly from linearity and thus the determination of the kinetic parameters was not possible. Nevertheless, the substrates were 100% hydrolysed at all concentrations tested, although a sign of possible substrate inhibition was detected with the highest concentrations. 1-LPDHA seems to be hydrolysed more effectively

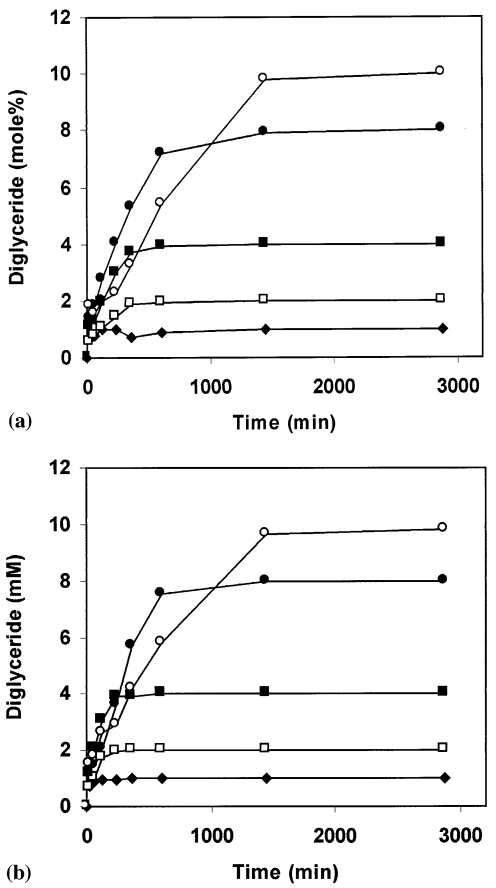


Fig. 5. (a) Diacylglycerol formation in the hydrolysis of 1-lauroyl-phosphatidylglycerol by PL C form *Bacillus cereus*. Reaction media containing 1-LPG (◆)1 mM, (□) 2 mM, (■) 4mM, (●) 8 mM and (○) 10 mM, in 0.2 ml diethylether, 0.2 ml HEPES buffer (0.1 M), pH 7.5, 50 mM $CaCl_2$ and 1 unit PL C. Reaction temperature 25°C; (b) Diacylglycerol formation in the hydrolysis of 1-lauroyl-phosphatidyldihydroxyacetone by PL C form *Bacillus cereus*. Reaction media containing 1-LPDHA (◆) 1 mM, (□) 2 mM, (■) 4mM, (●) 8 mM and (○) 10 mM, in 0.2 ml diethylether, 0.2 ml HEPES buffer (0.1 M), pH 7.5, 50 mM $CaCl_2$ and 1 unit PL C. Reaction temperature 25°C.

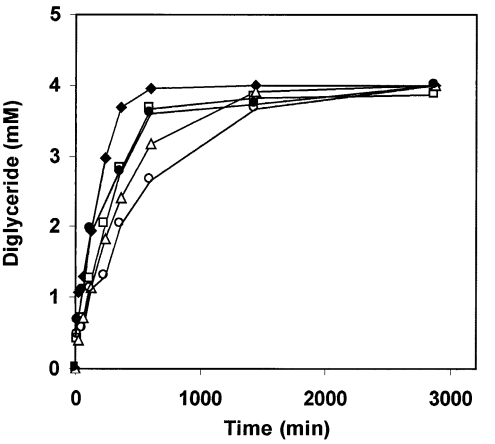


Fig. 6. Effect of phosphatidic acid on the hydrolysis of 1-lauroyl-phophatidylglycerol by PL C form *Bacillus cereus*. Reaction media containing 1-LPG (4 mM) and PA (◆) 0 mM, (□) 1mM, (▲) 2 mM, (○) 3 mM and (●) 4 mM, in 0.2 ml diethylether, 0.2 ml HEPES buffer (0.1 M), pH 7.5, 50 mM $CaCl_2$ and 1 unit PL C. Reaction temperature 25°C.

than 1-LPG at low substrate concentrations. The final relative product quantification showed clearly that 1-lauroyl-*rac*-glycerophosphate and 1-lauroyl-dihydroxyacetonephosphate were formed as main products and that the amount of phosphatidic acid detected was somewhat higher (4–5%) with 1-LPDHA than with 1-LPG (1–2%), suggesting that the hydrolysis of the former substrate is less selective.

3.5. Effect of phosphatidic acid

Phosphatidic acid was reported to completely inhibit the hydrolysis of phosphatidylcholine and phosphatidyldihydroxyacetone with PL C (D'Arrigo et al., 1995). Since the phosphatidic acid is a possible product in the transphosphatidylation of PC with 1-MLG and 1-MLDHA, it was considered of interest to investigate its effect on the hydrolysis of 1-LPG and 1-LPDHA. Fig. 6 shows the time course for the hydrolysis of these substrates in the presence of increasing amounts of PA. As above, the determination of activities was not possible, but clearly the lipids are hydrolysed by the enzyme at all PA concentrations tested. However, there is a retardation of the reaction which could be a result of an interfacial dilution

effect (Dennis, 1983) as both substrates and PA are surface-active substances.

4. Conclusions

The combination of phospholipase D and C for the preparation of lysophosphatidic acid and 1-lauroyl-dihydroxyacetonephosphate is simple and effective. PL D has proven to be very selective for the organic amphiphilic nuclophiles and conversions of 100% can be achieved at low substrate alcohol concentrations. The easy purification of the transphosphatidylation product and the possibility of reutilization of the unreacted substrates make the system very attractive. The enzymatic synthesis of 1-LPG and 1-LPDHA has not been reported before and it could be of interest to investigate their properties and possible applications. Both substances are hydrolysed by PL C in a very selective way. The enzymatic reaction was not affected by the presence of phosphatidic acid, rendering unnecessary the previous purification of the substrates. It should be the case that it is possible to simplify the two-step reaction by the exchange only of the aqueous phase and the enzyme catalyst after the completion of the first reaction.

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