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ISOLATION OF MYO-INOSITOL FROM A MIXTURE OF ISOMERS

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The transfer reaction of phosphatidylcholine and aqueous solutions of inositols that is catalyzed by phospholipase D is studied. Optimal conditions for synthesis of posphatidylinositol in a water—organic-solvent heterogeneous system are found. Addition of the alcoholic substrate methanol to the reaction medium is demonstrated to liberate myo-inositol from the phosphatidylinositol in hexane—water. Myo-inositol is isolated from a mixture of isomers by carrying out the double transfer reaction using phospholipase D.

Key words: myo-inositol, isolation.

Inositol is a cyclic alcohol (cyclohexahexanol) that is one of the most important biologically active compounds, especially due to its involvement in Ca^{2+}, phospholipid, and phosphatidylinositide metabolism [1].

Of the nine isomers of this compound, only myo-inositol is considered to be biologically active. Therefore, the preparation of the biologically active isomer from a mixture of isomers is problematical. A method is known for isolating myo-inositol from a mixture of isomers on chiral columns [2]. This approach is labor-intensive and requires large expenditures. A microbiological method of analysis and preparation of myo-inositol in microquantities using yeast is also known [3]. This method is useful mainly on an analytical and micropreparative scale.

The isolation of myo-inositol from a mixture of isomers was accomplished by us using phospholipase D in a biotechnological approach.

Analogous problems in several instances were successfully solved using immobilized enzymes, e.g., isolation of L-aminoacids from a mixture of the D,L-isomers using acylase [4]. Lipolytic enzymes demonstrated the effectiveness of such an approach for the preparation of the active L-form of menthol from a mixture of the D,L-isomers [5].

Figure 1 presents results from the reaction of egg lecithin with aqueous solutions of inositols in diethylether—water (2:1). As the inositol concentration increases, the amount of phosphatidylinositol formed increases (curve 1) and the lecithin content decreases (curve 2). The decrease of lecithin content does not correspond with the amount of synthesized phosphatidylinositol. Apparently some of the lecithin is converted to phosphatidic acid through the hydrolase reaction that occurs in parallel (curve 3). Total phospholipids from egg yolk can be used instead of lecithin as the starting phospholipid substrate but the degree of conversion is lower (curve 4). Apparently this is due to other phospholipid components besides lecithin that are not highly reactive. This was found previously during a study of phospholipid hydrolysis by phospholipase D [6].

The transfer reactions for formation of phosphatidylinositol from lecithin or total phospholipids that were studied also occur if a mixture of inositol isomers is used as the alcohol substrate. However, the extent of conversion is much less (curves 5 and 6).

Figure 2 shows the rate of the transfer reaction as a function of alcohol substrate concentration in Lineweaver—Burke coordinates. The transfer reaction between phospholipids and inositol occurs at the diethylether—water phase boundary and is catalyzed by phospholipase D in various substrate systems. The experimental data obtained for phosphatidylcholine (PC) and myo-inositol give a linear plot (Fig. 2a, curve 1) from which the maximal reaction rate and Michaelis—Menten constant can easily be calculated. It is equal to 0.25 mM in alcohol substrate. This value is almost 5-8 times less than the analogous value

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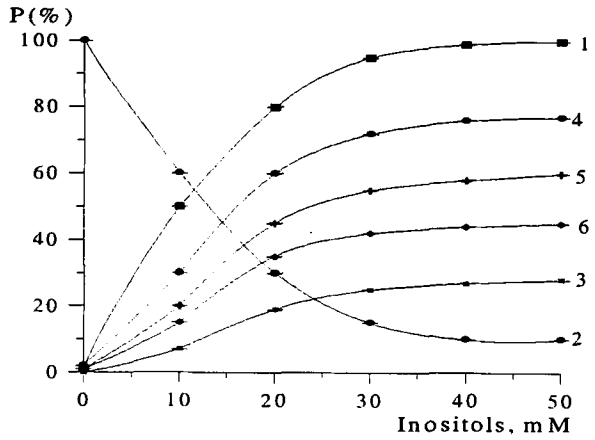

Fig. 1. Formation of PI (phosphatidylinositol) in transfer reaction of PL and inositols (diethylether—water, 2:1; Ca^{2+}, 50 mM; acetate buffer, 0.1 M, pH 5.8): PI formation (1), decrease of PC content in reaction with myo-inositol (2), PA content (3), formation of PI on reaction of total PL of egg yolk with myo-inositol (4), formation of PI on reaction of PC with a mixture of inositols (5), the same as in 5 but with total PL (6).

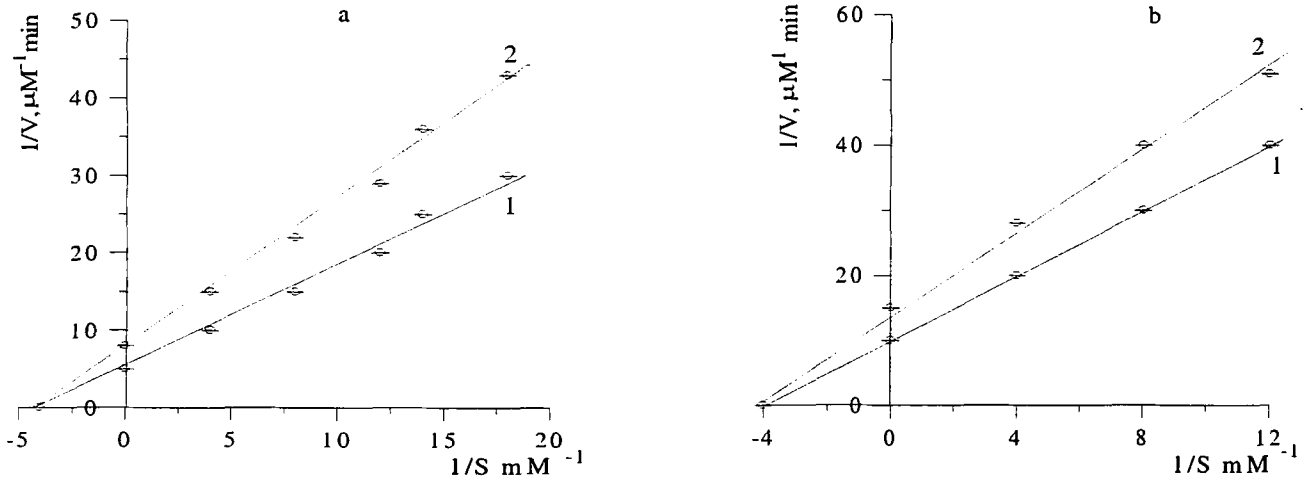

Fig. 2. Rate of PI formation as a function of alcohol-substrate concentration in Lineweaver—Burke coordinates: reaction with myo-inositol (a), reaction with a mixture of inositols (b); formation of PI from PC (1) and from total phopholipids (2).

K_M in hydrolase reactions catalyzed by phospholipase D [7]. Consequently the affinity of the enzyme for myo-inositol in the transfer reaction is much higher. The same value of K_M was obtained for total phospholipids (Fig. 2a, curve 2). The value of K_M does not change if a mixture of inositols is used as the alcohol substrate (Fig. 2b, curves 1 and 2). Regardless of the type of phospholipid substrate (lecithin or total phospholipids), the Michaelis—Menten constants determined from the intercept with the abscissa coincide although the maximal rates determined from the intercepts with the ordinate are different. They are higher for the reaction with PC. They are unchanged if myo-inositol is replaced by a mixture of inositols. These data indirectly indicate that only myo-inositol is involved in transfer reactions with phospholipids in the enzymatic reaction.

Thus, myo-inositol is confirmed in all experiments to be very reactive compared with the mixture of isomers. The greatest yield of phosphatidylinositol is observed for alcohol concentrations of 30-50 mM.

The reaction occurs in the presence of Ca^{2+} (Fig. 3). Other ions are less activating. Several organic solvents that differ significantly in activating ability for lecithin hydrolysis can be used as the nonaqueous phase [8]. Significant differences in the three compounds, diethylether, hexane, and benzene, were not observed for the transfer reaction (Fig. 4). Therefore, hexane was used for convenience in further experiments. The aqueous and organic phase in the hexane—water system are practically completely immiscible. Phospholipid components transfer to the organic phase (the hexane solution was evaporated in a rotary

TABLE 1. Catalytic Activity of Phospholipase D in Transfer Reactions with Various Alcohols

Alcohol content in reaction medium, ml	Alcohols*					
	1	2	3	4	5	6
	catalytic activity, μmol/min mg protein					
0.025	0.17	0.12	0.17	0.1	0	0
0.05	0.27	0.25	0.31	0.05	0.023	0.022
0.1	0.52	0.41	0.38	0.025	0.020	0.016
0.2	0.75	0.70	0.21	0.018	0.018	0.014
0.3	0.84	0.62	0.11	0.012	0.013	0
0.4	0.76	0.44	0.08	0	0.011	0
0.5	0.48	0.19	0	0	0.06	0

*Methanol (1), ethanol (2), propanol (3), butanol (4), hexanol (5), octanol (6).

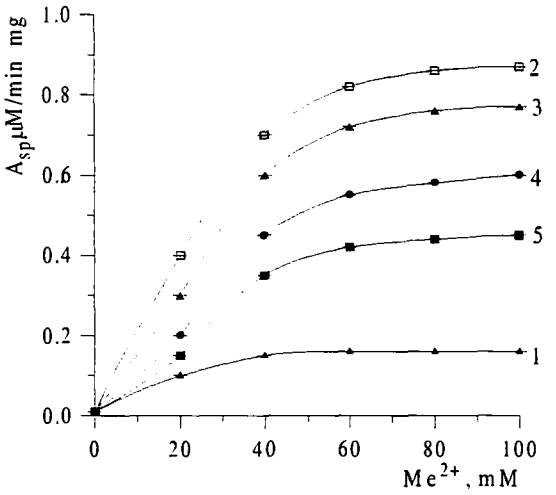

Fig. 3

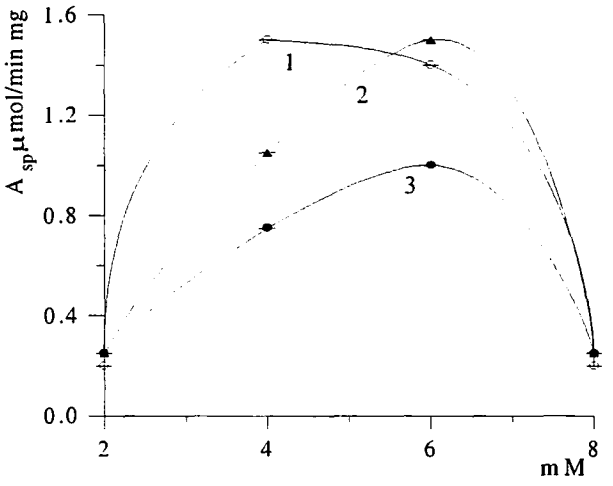

Fig. 4

Fig. 3. Effect of divalent metal (chloride) ions on rate of phosphatidylinositol formation from egg lecithin: without ions (1), Ca^{2+} (2), Sr^{2+} (3), Mg^{2+} (4), Ba^{2+} (5).

Fig. 4. Transfer reaction optimal pH values as functions of organic solvent: diethylether (1), hexane (2), benzene (3) (A is the specific activity in μmol per 1 min per 1 mg protein).

evaporator) and are dissolved in the minimal amount of $CHCl_3$—CH_3OH (1:1) for TLC analysis [9]. The analysis revealed phosphatidylinositol (80% by P), unreacted lecithin (4.5%), and phosphatidic acid (12.4%). Analysis of the reaction products showed choline and unreacted inositol in the aqueous phase. Thus, the principal component in the organic phase was phosphatidylinositol. Therefore, special experiments on the fractionation of phsopholipids were not performed further.

The phospholipid composition in the organic phase was more varied if total phospholipids from egg yolk were used as the phospholipid substrate. The following phospholipids were observed by two-dimensional TLC after the reaction: PC (27.3%), cephalin (21.7%), phosphatidylinositol (38%), other components (~11.2%). Thus, the phosphatidylinositol that formed was separated from the other components by column chromatography on aluminum oxide before the next operations [10].

The enzymatic reaction was repeated in the next step. The phospholipids that were prepared in the first reaction were dissolved in hexane and treated with one of the alcohol substrates (methanol, ethanol, propanol, butanol, hexanol, octanol) (Table 1). The myo-inositol was liberated well from the phosphatidylinositol in the presence of the water-soluble alcohols and was practically not liberated if long-chain alcohols were used. The most effective was methanol although ethanol also gave a

high reaction rate and a rather good yield of target products.

The aqueous and organic phases were separated after the reaction and analyzed for reaction products. TLC of the organic phase revealed phosphatidylmethanol (for the reaction with methanol) (8.9%) and unreacted phosphatidylinositol (5.0%) in addition to a small amount of phosphatidic acid (2.4%). Some of the phospholipid components (~1.5%) did not migrate from the TLC origin. Their composition was not determined.

The aqueous phase contained myo-inositol and some unreacted methanol that were separated by TLC [11].

Thus, myo-inositol can be isolated from a mixture of isomers by carrying out a duplicate enzymatic transetherification using phospholipase D and phospholipid substrates in the first step and phospholipase D and an alcohol substrate in the second step.

EXPERIMENTAL

Phospholipase D that was isolated from roots of the middle Asian *Rhaphanus sativus* was used as the enzymatic preparation [7]. The protein content in the enzymatic preparation was determine by the Lowry method. The rate of the phospholipase-D-catalyzed reaction was followed using the amount of reaction product (phosphatidylinositol) produced (based on P [11]).

The phospholipid substrates in the transalkylation reactions were egg lecithin and total phospholipids from egg yolk that were isolated by the literature method [2].

Acid Hydrolysis of Inositols. The phytin extract that was obtained was subjected to acid hydrolysis by the Anderson method [12]. A sample of the extract was placed in a Pyrex glass tube (1.0-1.5). The end of the tube was drawn out beforehand. The hydrolysis was carried out in a sealed ampul heated with 2N HCl at 110°C for 4 h. The color of the hydrolysate was light yellow and transparent. The cooled hydrolysate was neutralized with base, adjusted to pH 5.6, and evaporated in a rotary evaporator at 40°C. The chemical destruction and phosphate migration produced two products, a complicated mixture of inositols and inorganic phosphates. The inorganic phosphate in the hydrolysate was determined using the Bartlett method [13].

Transalkylation Reactions were performed in the incubation medium (2.5 mM) containing lecithin (10 mM), Ca^{2+} (40 mM), acetate buffer (0.1 M, pH 5.8), and enzyme solution (0.1 ml, 5 mg/ml in protein). The reaction mixture was activated by adding organic solvent, diethylether (hexane or benzene). The reaction was carried out at 37°C for 60 min. The amount of myo-inositols and the mixture of isomers was varied (30-50 mM). The reaction conditions were the same when the transferase reaction was repeated but methanol (30 mM) was added to the reaction system.

Phospholipids from the extract were concentrated and dissolved in $CHCl_3$—CH_3OH (2:1). Aliquots were syringed onto a TLC plate as a band containing 2-3 μg of lipid phosphorus per 1 cm. The phospholipids migrated in the first direction using $CHCl_3$—CH_3OH—NH_4OH (65:25:5) and in the second using $CHCl_3$—$(CH_3)_2CO$—CH_3OH—CH_3CO_2H—H_2O (100:40:20:20:10) [4]. The amount of phosphatidylinositol was determined from the amount of P.

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