THE EFFECT OF LIPOSOMIZATION ON REACTION CHARACTERISTICS OF PROSTAGLANDIN SYNTHETASE

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Introduction

Prostaglandins, C_{20} carboxylic acids containing a cyclopentane ring, have been recognized to have significant pharmaceutical potential, such as in the treatment of high blood pressure, asthma, ulcers and the induction of labor and abortion. These arachidonic acid derivatives are synthesized and released in low concentration by many tissues and organs—unlike hormones, which are produced by specific glands.[1,2] Their low concentrations in tissues and their multiple asymmetric carbon centers make the cost of their extraction or chemical synthesis prohibitively expensive. So, it is important to clarify the feasibility of the commercial production of these compounds by means of fermentation and enzyme engineering processes. Since PG-synthetase is one of the membrane-bound enzymes, liposomization—a procedure by which liposomes can be prepared from microsomes by the use of phospholipids—is expected to improve its reaction characteristics. In this work the effect of liposomization of PG-synthetase derived from sheep seminal vesicular glands on the rate of prostaglandin E_2(PGE_2) biosynthesis was studied.

1. Materials and Methods

1.1 Enzyme preparations

Acetone-pentane powder of microsomal precipitate containing PG-synthetase was prepared according to the procedure described by Wallach and Daniels.[5] Frozen glands were blended in a Waring Blender with 200 cm^3 of 0.154 mol/dm^3 KCl per 100 g of glands, and the resulting homogenate was then centrifuged at $4000 \times g$ for 5 min. The supernatant solution was brought to pH 4.9 by the addition of crystalline citric acid after filtration through cheesecloth. The resulting beige-colored precipitate was recovered by centrifugation at $4000 \times g$ for 5 min. All further operations were conducted at $-25°$ to $-30°C$. The precipitate was blended in a Waring Blender in approx. 15 vol. of acetone (v/w). After filtration, the cake was resuspended in half the volume of acetone previously used. The mixture was again filtered, and this time the cake was resuspended in 5 vol. of pentane (v/w). Pentane was removed by drying in a vacuum evaporator, and acetone-pentane powders in yield of 2 g/100 g of frozen glands were obtained.

1.2 Enzymatic assay

A buffer solution containing 0.0312 mol/dm^3 of sodium EDTA, 0.0005 mol/dm^3 of hydroquinone and 0.002 mol/dm^3 of glutathione was adjusted to pH 8.0 by the addition of sodium hydroxide. In most of the experiments, acetone-pentane powder (50 mg) was added to 10 cm^3 of a reaction mixture prepared by adding sodium arachidonate to the buffer solution at a concentration of 1 mg/cm^3 buffer. The reaction mixture in a test tube was then incubated in a water bath at 30°C with agitation by a magnetic stirring bar. At the end of the incubation period, the mixture was acidified by 2 mol/dm^3 citric acid (about 1 cm^3) to pH 2–3. PGE_2 produced in the reaction mixture was extracted directly with 25 cm^3 of methylene chloride in a separatory funnel. The solvent was then removed with a rotary evaporator, and the residue was dissolved in 20 cm^3 of methanol. PGE_2 was converted to prostaglandin B_2 (PGB_2) by the addition of 0.08 cm^3 of 3 mol/dm^3 KOH per 2 cm^3 of methanolic solution and further incubation at 50°C for 30 min. The concentration of PGB_2 in 2 cm^3 of methanol was measured by a HPLC system (HITACHI 655) with a Zorbax ODS column (15 cm, Du Pont Instr.) at a wave length of 278 nm. The solvent system used was 1% aqueous acetic acid–methanol (35 : 65) with a flow rate of 1 cm^3/min.

1.3 Liposomization of PG synthetase

Twenty mg of DMPC (D,L-α-dimyristoylphosphatidylcholine) was dissolved in 2 cm^3 of chloroform. Chloroform was then removed by an aspirator using a water bath at 40°–45°C above the phase transition temperature (T_c=23°C). To the residue

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(DMPC), a mixture of acetone-pentane powder (50 mg) and buffer solution (2 cm^3) was added. After being well shaken in a lukewarm water bath, the resulting milky solution was stored in a cool place for 2–3 h. Further operations were carried out in a similar manner to free enzyme assays, only instead of acetone-pentane powder, the milky solution was added to the reaction mixture.

2. Results and Discussion

The rates of PGE_2 synthesis showed no appreciable change in a pH range from 7.5 to 8.5, and proceeded to a plateau above a sodium arachidonate concentration of 0.5 mg/cm^3 for the cases of both free and liposomized enzyme. Therefore, in the present experiments, the rates were measured at pH 8.0 and 1 mg/cm^3 of sodium arachidonate as the optimal conditions.

Figure 1 shows the effect of increasing amount of DMPC for liposome formation on the rate of PGE_2 biosynthesis. Prostaglandin yields increased with the amount of DMPC and reached a plateau at 20 mg of DMPC per 50 mg of the enzyme.

Figure 2 shows time courses of PGE_2 formation with free and liposomized PG-synthetase at 30°C. The asymptote was reached slowly in the case of liposome, and it is of importance that liposomization has improved the prostaglandin yields. The effects of enzyme concentration on synthesis of PGE_2 for the cases of both liposome and free enzyme are shown in **Fig. 3**. Stability assays were run at 4°C within two days. The liposome sample retained its initial activity for two days while the free enzyme sample lost 25% of its initial activity. These results show that liposomization increased prostaglandin yields to more than double those obtainable by the free enzyme method with good stability during storage. For reactions involving non-polar substrates, a phospholipid requirement for membrane-bound enzymes have been observed.[3] When phospholipids associate with enzymes, these amphipathic compounds may prevent them from aggregation and denaturation. Phospholipid phase changes from crystalline state to liquid-crystalline state increase the mobility of the lipid-crystalline state increase the mobility of the lipid fatty acid chains, bilayer permeability, and the capacity of foreign compounds.[4]

As seen in **Fig. 4**, further addition of either enzyme or substrate increased the amount of PGE_2 formed, though it was much smaller than what was obtained in the first incubation. These results show the inhibition of the synthetic reaction by some by-products, and probably some denaturation of the enzyme.

Although DPPC (D,L-α-dipalmitoylphosphatidyl choline) also showed a PG-yield increasing effect, it

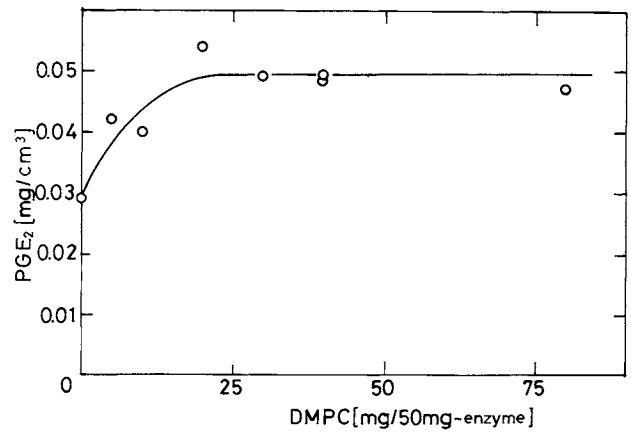

Fig. 1. Effect of DMPC concentration. Incubation time: 20 min.

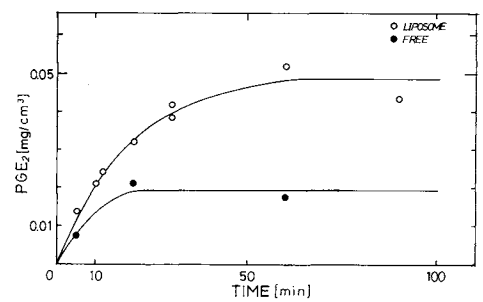

Fig. 2. Time course of PGE_2 synthetase reaction at 30°C. Incubation conditions were the same as outlined in methods.

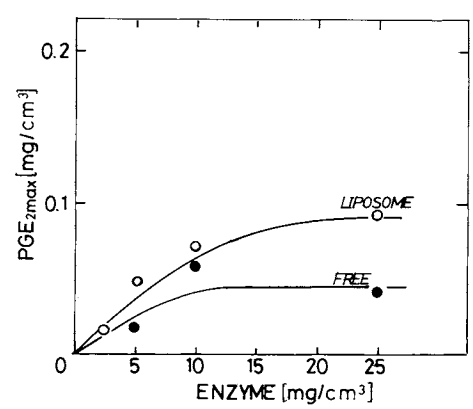

Fig. 3. Effect of enzyme concentration. Incubation time: 60 min.

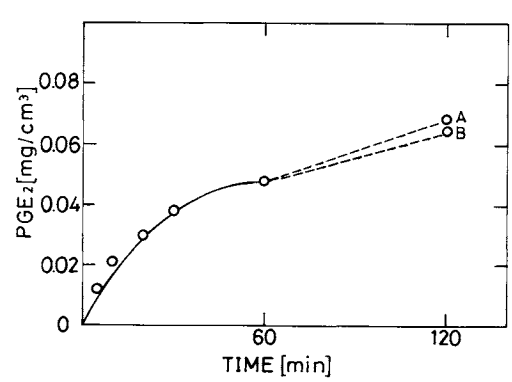

Fig. 4. Effect of further addition of either enzyme or substrate (pH 8). A, further addition of enzyme (liposome); B, further addition of substrate (AA).

was found that DMPC supports PG-synthetase better than DPPC. This difference might be caused by higher T_c of DPPC.

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CORRELATION OF DROP SIZES IN LIQUID–LIQUID AGITATION AT LOW DISPERSED PHASE VOLUME FRACTIONS

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Introduction

Under conditions where the volume fraction of dispersed drops in an agitated tank is sufficiently small, the sizes of the drops are controlled by drop breakup. Previously, the present authors carried out experiments at very low dispersed phase volume fractions and measured drop sizes to analyze the breakup mechanism.[1] In these experiments the viscosity of the dispersed phase was varied over a wide range. Recently, Calabrese *et al.*[3,4,8] conducted experiments in which the interfacial tension between continuous and dispersed phases and the viscosity of the dispersed phase were subject to extensive changes in geometrically similar mixing vessels. They have derived correlation equations of drop sizes.[3,4,8] However, their equations are not applicable to the whole region of the operational variables in either the experiments of Calabrese *et al.* or in those of our previous work. In the present study, drop size data of both groups of experiments are expressed by a single correlation equation.

1. Dimensionless Numbers for Correlation

The deformation and breakup of a dispersed drop are brought about by external forces acting on the surface of the drop. Although viscous and inertial forces due to shear flow of the continuous phase are both considered to be external forces, the viscous force may be ignored in the first approximation under ordinary conditions in liquid–liquid agitation.[6] On the other hand, during the process of drop deformation, the external force is counterbalanced by the sum of viscous force due to the internal flow of the drop and interfacial force due to interfacial tension between the continuous and dispersed phases.[1] Therefore, for the analysis of drop breakup three physical properties should be considered: the viscosity of the dispersed phase, μ_d, the interfacial tension, σ, and the density of the continuous phase, ρ_c. In geometrically similar agitated tanks, flow conditions are defined by agitation speed, N, and impeller diameter, L. Therefore we can assume that the five parameters μ_d, σ, ρ_c, N and L determine the maximum stable drop size for the breakup, d_{max}. Under this assumption, dimensional analysis allows the postulate that d_{max}/L should be expressed as a function of the following two dimensionless groups:

$$We = \rho_c N^2 L^3/\sigma \tag{1}$$

$$Re^* = \rho_c N L^2/\mu_d \tag{2}$$

The maximum stable drop size equals the largest size of drops which remain in steady dispersion after the processes of drop breakup under noncoalescence conditions.

The experiments of previous investigators[2,3] showed a linear relationship between the largest drop size and Sauter mean drop size, d_{32}. Since the measurements of the largest drop size tend to be accom-

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