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ENZYMATIC SYNTHESIS OF TRIGLYCERIDES CATALYZED BY LIPASES FROM *Penicillium sp.*

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Two lipases have been isolated from Penicillium sp. *and purified. A synthetase activity of the total lipase preparation has been shown in an aqueous medium and in a system of reversed micelles (RMs) of a surface-active agent (SAA) in benzene, and advantages of the reversed-micelle system over an aqueous medium have been demonstrated.*

The lipases form a unique group of enzymes that are capable of performing catalytic reactions at a lipid—water phase-separation boundary. The processes catalyzed by lipolytic enzymes include the hydrolysis of esters and triglycerides [1], and acidolysis, alcoholysis, and transesterification reactions [2]. It is in fact the synthetase capacity of these biocatalysts that is currently attracting the firm attention of scientists throughout the world [3]. This is due to the fact that in synthesis and transesterification processes it is possible to obtain lipids with required, predetermined, properties. In view of this, the development of the optimum condition for the performance of such processes is an extremely urgent task. One of the new directions is the performance of the reaction in hydrated reversed micelles of SAAs in an organic solvent [4].

As we have shown previously, a complex preparation of lipases from *Penicillium sp.* partially purified on Sephadex G-75 (Superfine) possesses synthetase activity [5]. This total preparation consists of two lipases and some ballast proteins. The synthesis of glycerides from oleic acid and glycerol catalyzed by these lipolytic enzymes takes place both in an aqueous medium and in a system of reversed micelles (RMs) [5]. The aim of the present work was a more detailed study of the synthetase properties of the lipases from *Penicillium sp.* and the selection of the optimum conditions for the performance of the reaction.

The chromatogram in Fig. 1 demonstrates the products of a synthetic reaction in an aqueous medium (Fig. 1, *c*) and in an RM system (Fig. 1, *d-h*) [5], the reaction in the RM system taking place even in the absence of exogenously added water. The main reaction products proved to be triglycerides, which shows the nonspecificity of the enzyme preparation. Even though, in hydrolysis, the lipase from *Penicillium sp.* behaves as a 1,3-specific enzyme [6], the same lipase in the immobilized state forms monoglycerides during synthesis [7]. The manifestation of different position specificities is apparently connected with a substantial influence of the physicochemical state of the substrate, which leads to different conformational changes in the enzyme molecule.

We have studied the kinetics of the formation of the products of the synthetase reaction in an aqueous medium and in an RM system (Fig. 2). In an aqueous medium the formation of triglycerides took place very slowly; thus, for example, after incubation for 4, 6, and 8 h, no glycerides were detected among the reaction products. After 16 h the degree of conversion did not exceed 20%, and after 24 h triglycerides had disappeared again, which showed not only the complete cessation of the synthetase reaction but also, possibly, its reversal in the direction of hydrolysis.

The kinetics of the formation of the products of the synthetase reaction in the RM system were studied during the first 4 h of incubation, when the maximum accumulation of products took place at a constant rate. The degree of conversion reached 48-50%. After this, the amount of glycerides scarcely changed. After 24 h, the synthetase activity had fallen sharply and no triglycerides were detected in the system.

It must be mentioned that, on the performance of the process in an aqueous medium, when an acetate buffer was replaced by distilled water practically no reaction took place, which confirmed results obtained previously on the inhibiting

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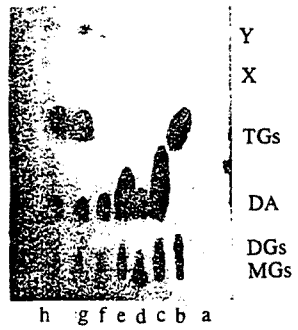

Fig. 1. Chromatogram of the products of synthesis from glycerol and oleic acid by a lipase from *Penicillium sp.*: MGs) monoglycerides; DGs) diglycerides; TGs) triglycerides; OA) oleic acid; *a*) OA; *b*) olive oil; *c*) syn-thesis in an aqueous medium; *d-h*) synthesis in a system of reversed micelles; *X* and *Y*) unidentified components.

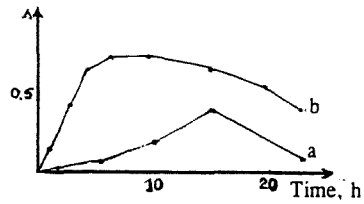

Fig. 2. Kinetics of the formation of products: *a*) in an aqueous medium; *b*) in an RM system.

role of oleic acid in relation to some fungal lipases and the protective action of an acetate buffer [8] (Fig. 3). Performing the reaction in a system of reversed micelles did not require the presence of a buffer or water at all.

We studied some properties of the lipases in a system of reversed SAA micelles in benzene.

Kinetic investigations with an excess of one of the substrates (glycerol) enabled us to calculate for oleic acid the values of K_m — 5 mM — and V_{max} — 0.62 mg per 1 mg of enzyme —, since under the given conditions the reaction can be regarded as a one with a single substrate.

Since the reaction proceeded in a system of reversed micelles, we examined the role and influence of micelle-filling agents on the synthetic reaction. Raising the amount of exogenously added water (we did not calculate the amount of endogenous water) to 2 μl led to an increase in the activity of the lipase, while a further increase in the concentration of water in the system led to a fall in the intensity of synthesis (Fig. 4, *a*).

A change in the amount of glycerol, which also affects the volume of the micelles, from 0.5 to 6 μl had practically no effect on the rate of synthesis (Fig. 4*b*).

Thus, water exerts an inducing effect more as an agent maintaining the active conformation of the enzyme than as a micelle-forming agent, while the lowering of the activity of the lipase when the concentration of exogenously added water was increased is possibly connected with a reversal of the reaction in the direction of hydrolysis, although it is possible that the interrelationship of the increase in the amount of water and the decrease in the intensity of synthesis has a different nature.

On the basis of the results obtained it is possible to draw the following conclusions:

1. The complex lipase preparation from *Penicillium sp.* is nonspecific in the synthesis of triglycerides;

2. An aqueous medium is unsuitable for performing synthetic reactions, although synthesis does take place in it; and

3. A system of reversed SAA micelles in an organic solvent is more suitable and convenient, since conversion in this system amounts to 40-50%, while in an aqueous sytem it is only 20%; in a system of reversed micelles constant stirring and the presence of a buffer are not required; oleic acid does not inhibit the lipase; and synthesis takes place far more rapidly than in an aqueous medium.

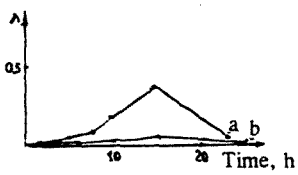

Fig. 3

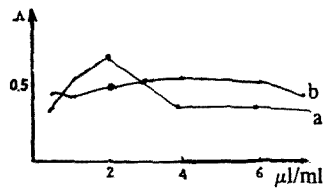

Fig. 4

Fig. 3. Influence of an acetate buffer on the synthesis of triglycerides in an aqueous medium: *a*) addition of 1 ml of 0.25 M acetate buffer; *b*) addition of 1 ml of water.

Fig. 4. Influence of micelle-forming agents on the synthesis of triglycerides in an RM system: *a*) influence of exogenous water; *b*) influence of glycerol.

EXPERIMENTAL

As the SAA we used AOT (sodium salt of dioctyl sulfosuccinate) (Serva); as the initial enzyme preparation, a total preparation of the lipases from *Penicillium sp.* obtained by a procedure described previously [9] and partially purified by gel filtration on Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) as also described previously [5]; and, as substrates, glycerol and oleic acid of chemically pure grade. Benzene of chemically pure grade was used without additional purification, the amount of water being 0.2%.

Synthesis of Glycerides in an Aqueous Medium. A mixture containing 5 mg of the enzyme preparation, 0.2 ml of glycerol, 1 ml of 0.25 M acetate buffer, pH 6.5, and 0.12 ml of oleic acid was incubated at 38°C with constant stirring for 16 h. The products were extracted with hexane and were analyzed by micro-TLC [10] in the petroleum ether—diethyl ether—acetic acid (80:20:1) system. The spots were revealed by spraying with 50% sulfuric acid and heating at 200°C for 20 min. Oleic acid and mono-, di- and triglycerides from olive oil were used as markers. The lipids on the microplates were determined quantitatively from the areas of the spots.

Preparation of Reversed Micelles of AOT in Benzene. To 2 mg of the enzyme preparation were added 2 μl of glycerol (water was added only when its influence was being studied, and buffer only in the determination of pH dependence), and then 2 ml of a 0.1 M solution of AOT in benzene, and the mixture was shaken vigorously until a clear system had been formed. The reaction was begun by the addition of 0.6 μl of oleic acid. The mixture was incubated at 38°C for 4 h. To eliminate the AOT, at the end of the process the reaction mixture was added to a 500-fold amount of distilled water and the resulting mixture was left for 24 h. Then the benzene layer was taken off and evaporated, and the residue was analyzed by micro-TLC, as described above. Quantitative determination was made densitometrically or from the areas of the spots.

The efficacy of the synthetase reaction was evaluated as percentage conversions of the oleic acid into triglycerides and in units of total and specific activity. Percentage conversion was calculated as the ratio of the triglyceride content to the total content of triglycerides and oleic acid on a chromatogram multiplied by 100% [5]. The total activity was calculated from the formula:

$$A = \frac{\text{Initial oleic acid content (mg)} \times \%\ \text{conversion}}{100\%}$$

and was expressed in milligrams of triglycerides synthesized on incubation for 4 h. The specific activity A_{sp} was determined as the ratio of the total activity to the amount of enzyme in the sample (mg).

The mean square error of the experiments did not exceed 7%.

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