A STUDY OF THE LIPOLYTIC ENZYMES OF COTTON SEEDS

III. ISOLATION AND PROPERTIES OF THE TRIBUTYRINASES

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One of the distinguishing features of the hydrolysis of lipids by the enzyme lipase (hydrolysis of glycerol esters, EC 3.1.1.3) is the occurrence of the reaction at the phase-separation boundary. The substrate forms an insoluble phase emulsified or micellarized in water, and the enzyme is water-soluble. A number of natural or synthetic emulsifying agents are used for emulsification [1, 2]. Since, because of their hydrophobic nature, proteins represent a microheterogeneous system in solution, it is natural that the first stage of the enzymatic hydrolysis of lipids is an interaction between the protein and the lipid micelles [3, 4]. In a previous paper [5] it was reported that the following scheme applies to hydrolysis by lipases

\[ E + S \rightleftharpoons ES_a \rightleftharpoons ES_M \rightleftharpoons E + P_1 + P_2, \]

where \( E \) represents the enzyme; \( S \) the substrate; \( ES_a \) the adsorption complex; \( ES_M \) the Michaelis complex; and \( P_1 \) and \( P_2 \) the hydrolysis products.

Starting from this situation, it may be assumed that an active configuration of the enzyme is created only when the enzyme is adsorbed on the micellarized or emulsified substrate. We have obtained a proof of this hypothesis by studying the inhibition of the alkaline lipase of cotton seeds with sodium fluoride. As the substrate for the lipase we selected tributyrin, which is convenient because it possesses a fairly low solubility, ensures a high activity of the lipase, and at the same time, forms stable emulsions [6]. The optimum pH of the lipase in the reaction with tributyrin is 8.8.

The inhibition process was studied at pH 5.0-9.0, i.e., in the range where the lipase is stable to the action of the medium (Fig. 1, curve 1). At pH values below 5.0 above 9.0 the lipase is inactivated, undergoing denaturation. In this pH range the inhibiting action of sodium fluoride is shown only at the acid pH values. In the pH range from 7.2 to 9.0, including the optimum pH, sodium fluoride has no inhibiting action (see Fig. 1, curve 2).

A completely different pattern is observed in the reaction of the inhibitor with the enzyme in the presence of the substrate (Fig. 2, curve 2). At pH 8.8, the rate of hydrolysis with sodium fluoride decreases with time. In this case, the nature of the inhibition differs sharply from the inhibition at pH 5.0 (see curves 3 and 5, Fig. 2). The shape of the curves resembles the case of inhibition by the reaction product. If a new portion of enzyme is added to the reaction mixture (after inhibition has taken place completely), the remainder of the substrate begins to be hydrolyzed again at the same initial velocity. As hydrolysis continues, the inhibiting action of the sodium fluoride reappears. This fact unambiguously permits the conclusion that inhibition at an alkaline pH value takes place only in the presence of the substrate and is irreversible.

The enzyme was kept with the inhibitor in the absence of the substrate at acid pH values - under these conditions the activity of the enzyme does not appear. Then the pH was raised to 8.8, the substrate was added, and the rate of lipolysis brought about by that fraction of the enzyme molecule which was not bound to the inhibitor was measured. Since the enzymatic activity falls after the lipase has been incubated


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Fig. 1. Inhibition of the activity of the alkaline lipase of cotton seeds by sodium fluoride as a function of the pH of the medium: 1) incubation of the enzyme at various pH values in the absence of inhibitor; 2) incubation in the presence of $5 \cdot 10^{-3}$ M NaF (25°C, 80 min.).

Fig. 2. Influence of sodium fluoride on the rate of formation of butyric acid in the hydrolysis of tributyrin by alkaline lipase at pH 8.8: 1) hydrolysis of tributyrin in the absence of additives; 2) hydrolysis in the presence of $5 \cdot 10^{-4}$ M NaF; 3) $5 \cdot 10^{-5}$ M; 4) activity measured after incubation of the enzyme, substrate, and inhibitor for 80 minutes at pH 8.8; 5) activity measured after the enzyme and inhibitor had been kept for 80 min at pH 5.0; 6) the enzyme was kept at pH 5.0 in the absence of the inhibitor; 7) inhibition by sodium fluoride of purified tributyrinase ($1 \cdot 10^{-3}$ M).

with NaF in an acid medium, it may be considered that at pH values below 7.2 the reaction $E + I \rightarrow EI$ takes place, where $I$ represents the inhibitor and $EI$ the enzyme-inhibitor complex, which possesses no lipase activity.

The constant of the binding of the enzyme and the inhibitor is

$$K_i = \frac{[E][I]}{[EI]}.$$

whence

$$[EI] = \frac{[E][I]}{K_i}.$$

Since $[E] = [E_0] - [EI]$,

$$[EI] = \frac{[E_0][I]}{K_i + [I]}.$$

The proportion of free enzyme reacting at pH 8.8 is

$$[E_0] = \frac{K_i[E_0]}{K_i + [I]}.$$

According to the Michaelis equation, the velocity of the reaction in the absence of the inhibitor has the form

$$v_0 = \frac{k_0 [E_0][S]}{K_M + [S]}.$$

Here, $K_M$ is the Michaelis constant.