A STUDY OF THE LIPOLYTIC ENZYMES OF COTTON SEEDS

III. ISOLATION AND PROPERTIES OF THE TRIBUTYRINASES

M. M. Rakhimov, V. M. Akhunov,
N. R. Dzhanbaeva, and P. Kh. Yuldashev

UDC 577.153+577.15.04+582.796

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

$$E + S \rightleftarrows ES_a \rightleftarrows ES_M \rightleftarrows E + P_1 + P_2,$$

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

Institute of the Chemistry of Plant Substances, Academy of Sciences of the Uzbek SSR. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 228-234, March-April, 1972. Original article submitted October 19, 1971.

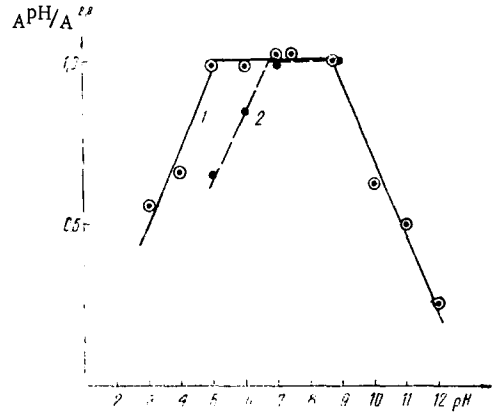

Fig. 1

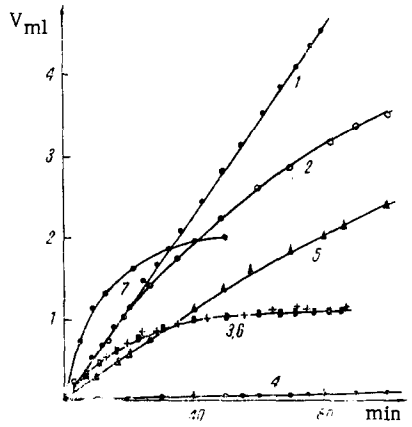

Fig. 2

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) $5 \cdot 10^{-3}$ 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).

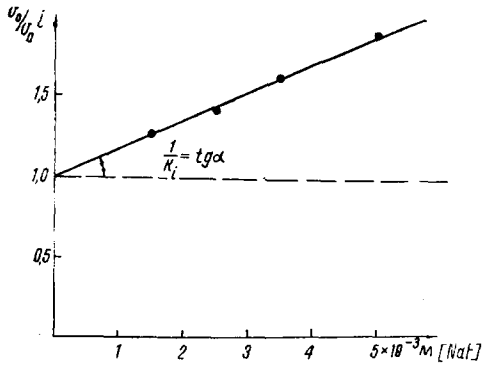

Fig. 3. Dependence of the rate of inhibition on the concentration of sodium fluoride (inhibition was performed at pH 5.0, and the activity was measured at pH 8.8).

with NaF in an acid medium, it may be considered that at pH values below 7.2 the reaction $E+I \rightleftharpoons EI$ 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

$$v_0 = \frac{k_2[E_0][S]}{K_M + [S]}.$$

Here, K_M is the Michaelis constant.

TABLE 1. Results of the Separation of Tributyrinase by Gel Chromatography on Sephadex G-100

Stage of purification	Amt. of protein, mg	Activity		Yield (of activity), %	Deg. of purification
		specific	total lipase		
Water-soluble fraction	6250	18	113 000	100	1
0-55% $(NH_4)_2SO_4$ fraction	2100	35	73 500	65	2,0
Gel chromatography on a column of Sephadex G-100					
Fractions					
111–113	32	540	17 000	15	30
119–122	38	330	13 500	12	18,0
129–133	36	340	12 000	10,7	19,0
135–137	20	390	8 000	7,2	22,0
162–164	21	185	4 000	3,6	10
179–181	12	385	4 500	4,0	19
Chromatography of fractions 119-122 on a column of Sephadex G-200*	3,7	1500	5 550	9,8	84

* On the column was deposited 19 mg of protein with a total activity of 6250 units.

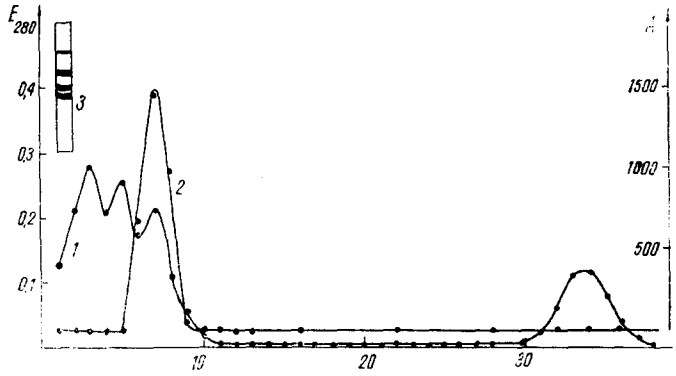

Fig. 4. Results of the purification of tributyrinase on a column of Sephadex G-200: 1) concentration of protein in the eluate; 2) lipase activity; 3) electrophoregram of the protein deposited on the column.

The equation is valid if [E] ≪ [S].

For the fraction of the enzyme not bound to the inhibitor,

$$v_0^i = \frac{k_2 [E_0'] [S]}{K_M + [S]}.$$

or, substituting the expression for $[E_0']$, we obtain

$$v_0^i = \frac{k_2 [E_0] [S] K_i}{(K_i + [I])(K_M + [S])}.$$

Hence,

$$\frac{v_0}{v_0^i} = \frac{K_i + [I]}{K_i} = 1 + \frac{1}{K_i} \cdot [I],$$

i.e., the ratio of the initial velocities of the reactions in the absence of the inhibitor and in its presence must depend linearly on the concentration of inhibitor. A plot of v_0/v_0^i versus inhibitor concentration should

give a straight line with a slope $\tan \alpha = 1/K_i$, or $K_i = 1/\tan \alpha$. The experimental results confirm this conclusion (Fig. 3). The inhibition constant K_i determined graphically is $4.9 \cdot 10^{-3}$ M.

When the Michaelis equation is used, the expression for the velocity of the reaction contains the value of the concentration of the substrate, but not the value of the effective surface of the substrate as should be the case for an insoluble substrate [8]. This substitution is possible only when the dimensions of the substrate particles do not change during the experiment. We have observed the latter condition (the concentration of sodium fluoride did not exceed 10^{-2} M).

The inhibition of the reaction by sodium fluoride in the presence of the substrate takes place in a completely different manner. Since at pH 8.8 in the absence of substrate no inhibition is observed (see Fig. 1, curve 2), the inhibitor interacts with the enzyme only when the latter is present in a functioning state. This can be the case if, within the limits of the enzyme–substrate complex, a local acidified segment spatially separated from the medium is formed. The appearance of such a segment is completely probable, since the substrate consists of a micelle and the enzyme is located on the surface of the micelle so as to ensure the maximum hydrophobic bonding between the corresponding parts of the molecule of the enzyme and the substrate micelle. Under these conditions, a spatially limited cavity arises in which a molecule of substrate undergoing hydrolysis is located. Since the reaction product is butyric acid, this cavity is locally acidified. Then the reaction scheme can be written in the form

$$E + S \rightleftarrows ES_a \rightleftarrows ES_M \rightleftarrows EP_a \rightleftarrows E + P.$$
$$\begin{array}{c} +I \\ \downarrow \\ (EPI)_a \\ \downarrow \\ EI + P \end{array}$$

According to this scheme, the initial velocity of lipolysis should not change at any concentrations whatever of the inhibitor (see Fig. 2); butyric acid is formed in all cases. But as the reaction proceeds the rate must decrease, since some of the molecules of the enzyme are converted into the inactive complex EI and cannot take part in the repeat catalytic act. The number of enzyme–inhibitor complexes formed and, consequently, the degree of retardation must increase. The experimental results confirm these conclusions (see Fig. 2). Thus, our assumption of the formation of a lipid–protein complex with a locally limited reaction cavity is correct.

Summarizing what has been said, the following conclusion relating to the features of the enzyme reaction taking place at a surface of phase separation can be drawn: the surface of the substrate micelle is a component part of the active center of the enzyme, and the formation of an active cavity is due to the specific adsorption of the enzyme on the surface of the substrate.

In the experiments described, the tributyrinase used was a fraction obtained by precipitation with ammonium sulfate at concentrations of 10-55% of saturation. All the tributyrinase activity (see Fig. 3) present in this fraction is inhibited by sodium fluoride (100% inhibition at the 59th minute). This means that all the proteins possessing activity with respect to tributyrin behave similarly under the action of sodium fluoride. The difference can only be quantitative. We checked the inhibiting action of sodium fluoride for purified tributyrinase.

The tributyrinases were separated by the scheme described previously [9], but with a slight change: the fraction deposited on the column of Sephadex G-100 was precipitated by ammonium sulfate at a concentration of 55% of saturation (Table 1).

The greatest tributyrinase activity (15%) was concentrated in the highest-molecular-weight fractions (111-113). With a fall in the molecular weights of the tributyrinases, their concentration also decreased. The highest yield of protein was obtained for the second fraction (119-122). On electrophoresis on polyacrylamide gel, this fraction gave four bands (disc electrophoresis), one of which was active.

An attempt to effect the separation of the tributyrinase and the corresponding proteins on a column of DEAE-cellulose led to the adsorption of the enzyme on the support. Elution in a 0-3 M NaCl gradient gave no active fraction. A test of the protein adsorbed on the DEAE-cellulose for lipase activity after the removal of the packing from the column was positive. When a column of Sephadex G-200 (fine) was used for chromatography, the proteins of fraction 2 were separated (Fig. 4). Tributyrinase was detected in fractions 6-8 during elution. Its specific activity was 1500. The amount of protein was 3.7 mg.

In the case of the purified tributyrinase, inhibition by sodium fluoride at pH 8.8 took place only in the presence of the substrate (see Fig. 2, curve 7). At an inhibitor concentration of $5 \cdot 10^{-3}$ M complete inhibition was observed after 25 min from the beginning of incubation. Consequently, the conclusions on the nature of the interaction of the proteins and the lipid micelles in the enzymatic hydrolysis of lipids are valid both for the individually purified enzyme and for the whole class of enzymes, and it has a general nature.

EXPERIMENTAL

The tributyrin and methyl butyrate were purified by redistillation. The sodium fluoride was twice recrystallized. The lipase was isolated from an acetone powder of cotton seeds. The enzymatic activities were measured by the titrimetric method using tributyrin as substrate [9, 10].

Inhibition in the absence of the substrate was performed in the following way: 10 mg of the enzyme was kept at various pH values in the absence and in the presence of the inhibitor in aqueous solution (total volume 25 ml). After 80 min, the pH was brought to 8.8 by the addition of 0.1 M KOH or HCl, depending on the initial pH. The reaction was begun by the addition of 5 ml of an emulsion of tributyrin in water (60 mg).

In the presence of the substrate, the sodium fluoride was added at pH 8.8. The nature and degree of inhibition were the same when the substrate was added to the mixture of enzyme and inhibitor and when the enzyme was added to the mixture of substrate and inhibitor. The concentration of sodium fluoride was varied from 10^{-5} M to $5 \cdot 10^{-3}$ M. In all the experiments, the concentrations of the enzyme and substrate were 0.33 and 1.78 mg/ml, respectively.

Gel chromatography on Sephadex G-100 was performed in a column (3.0 × 60 cm) equilibrated with 0.1 M phosphate buffer, pH 7.4. The rate of elution was 30 ml/h, the amount of protein deposited on the column being 2.0-2.1 g. Fractions were collected after each 15 min [11].

Ion-exchange chromatography in a column of DEAE-cellulose (1.0 × 15 cm) was performed in a concentration gradient of NaCl (0-3 M). The protein deposited on the column was previously dialyzed overnight against 0.01 M phosphate buffer and was concentrated by using Sephadex G-25. The rate of elution was 20.8 ml/h. The amount of protein was 15.5 mg. Fractions were collected after each 15 min. Gel chromatography with Sephadex G-200 to purify fraction 2 was performed in the same buffer. Size of the column 1.0 × 20 cm; rate of elution 6.6 ml/h; sample volume 3.3 ml; amount of protein deposited 19 mg.

In the measurement of the tributyrinase activity of the fractions eluted from the column, the concentration of protein was 0.003 mg/ml. Disc electrophoresis was performed as described by Fielding [12]. The time of electrophoresis was 2 h, the current 3 mA on each tube, and the voltage 400 V.

SUMMARY

1. The preparative separation of the tributyrinases of cotton seeds has been performed. Purified (electrophoretically homogeneous) fluorine-sensitive tributyrinase has been obtained.

2. The mechanism of the inhibition of tributyrinases by sodium fluoride has been studied. The hypothesis has been put forward that the surface of the micelle of the lipid is part of the structure of the active center of the enzyme.

LITERATURE CITED

1. P. Desnuelle and P. Savary, J. Lipid Res., 4, 369 (1963).
2. T. Ogiso and M. Sugiura, Chem. Pharm. Bull., 17, 1025 (1969).
3. R. L. Ory, A. J. Angelo, I. V. de Gruy, and A. M. Altschul, Can. J. Bioch., 45, 1445 (1967).
4. H. Brokerhoff, Arch. Biochem. Biophys., 134, 36 (1969).
5. M. M. Rakhimov, N. R. Dzhanbaeva, and P. Kh. Yuldashev, Khim. Prirodn. Soedin., 602 (1970).
6. B. Entressangles and P. Desnuelle, Biochim. Biophys. Acta, 159, 285 (1968).
7. L. Webb, Enzyme and Metabolism Inhibitors, Academic Press, New York, Vol. 1 (1963).
8. G. Benzonana and P. Desnuelle, Biochim. Biophys. Acta, 105, 121 (1965).
9. M. M. Rakhimov, N. R. Dzhanbaeva, and P. Kh. Yuldashev, Khim. Prirodn. Soedin., 642 (1970).
10. M. M. Rakhimov, Sh. R. Mad'yarov, N. R. Dzhanbaeva, and P. Kh. Yuldashev, Khim. Prirodn. Soedin., 738 (1970).
11. N. R. Dzhanbaeva, M. M. Rakhimov, and P. Kh. Yuldashev, Khim. Prirodn. Soedin., 226 (1972).
12. C. J. Fielding, Biochim. Biophys. Acta, 178, 499 (1969).