SPECIFICITY OF THE LIPASE FROM COTTONSEED

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The seeds of various plants contain one or more enzymes possessing lipase activity [1-5]. Cottonseed has been found to contain lipases which have optimum activity at pH 4.75 and 8.75 [6]. This paper gives the results of a study of the substrate specificity of the action of the soluble lipase which has optimum activity pH 8.75.

At pH 8.75, the lipase catalyzes the hydrolysis not only of triglycerides but also of linear esters and Tweens, and also of emulsified oils (Table 1). Such broad specificity is somewhat unusual [5]. The rate of hydrolysis of triglycerides depends on the length of the carbon chain of the fatty acid. On passing from tributyrin to triolein, the activity rises by a factor of 1.8, which shows the importance of the size of the hydrophobic moiety for the occurrence of lipolysis. The activity of the enzyme with respect to linear esters, on the other hand, decreases on passing from acetoacetic ester to isoamyl acetate. A similar relationship is found for the Tweens.

For a more detailed interpretation of the results it was necessary to determine which bond in the molecule of the triglyceride is hydrolyzed first. An analysis of the hydrolysis products during the hydrolysis of triolein using thin-layer chromatography [7] has shown that the first products of lipolysis are oleic acid and 2,3-diolein. Monoolein is formed only on prolonged incubation (Fig. 1). 1,3-Diolein is not found during the reaction. Thus, the bond in position 1 is the most reactive with respect to lipase. Since positions 1 and 3 in triolein are equivalent, this conclusion means that the bond in position 3 in the 2,3-diolein hydrolyzes more slowly. On comparing the rates of hydrolysis of a monoglyceride and a triglyceride, it can be seen that under the same conditions the rate of cleavage of the triglycerides is higher. This means that, although the bonds in positions 2 and 3 are not hydrolyzed directly in the first stage, they are bound to the active center of the enzyme. Since, with the appearance of two bonds in the enzyme–substrate complex, the structure becomes more rigid, the activation energy of the reaction decreases in comparison with the reaction with the monoglyceride. In its turn, this leads, according to the Arrhenius equation $v = Ae^{-E/kT}$ (where E is the activation energy) to an increase in the reaction rate.

TABLE 1. Specific Activities of the Hydrolysis of Various Substances by Cottonseed Lipase

Substrate	Spec. activity, m μmole per min per mg	% with respect to tributyrin
Tributyrin	12,0	100
Tripalmitin	16,8	140
Tristearin	19,6	164
Triolein	21,6	180
Acetoacetic ester	40,0	332
Methyl butyrate	12,0	100
Isoamyl acetate	5,5	46
Tween 21 (monolaurate)	21,0	175
Tween 80 (monooleate)	12,0	100
Tween 85 (trioleate)	21,0	175
Oils		
cottonseed	8,1	67
groundnut	8,1	67
olive	11,1	89
castor	11,5	95
sunflower seed	13,8	115

Consequently, the hydrolysis of a triglyceride takes place with the participation of at least three groups of the enzyme. Let us denote these arbitrarily by –XH, –YH, and –ZH. The structure of such an enzyme–substrate complex is illustrated in Fig. 2. Since the hydrolysis of triglycerides by cottonseed lipase takes place as a zero-order reaction (the amount of acid liberated is proportional to the time, at all concentrations of enzyme and substrate, Fig. 3), the limiting stage is the monomolecular decomposition of the enzyme–substrate complex (see Fig. 2). Such a model satisfactorily explains the specificity of cottonseed lipase with respect to triglycerides. As experimental material is accumulated, the mechanism of the reaction can be

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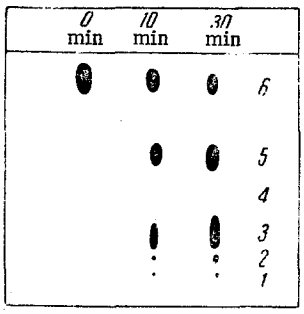

Fig. 1. Thin-layer chromatography of the products of the hydrolysis of triolein by lipase: 1) start; 2) monoolein; 3) oleic acid; 4) 1,3-diolein; 5) 2,3-diolein; 6) triolein.

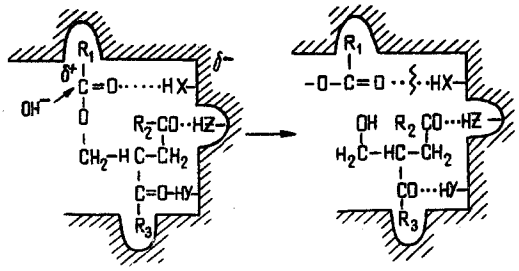

Fig. 2. Scheme of the decomposition of the triglyceride–lipase complex.

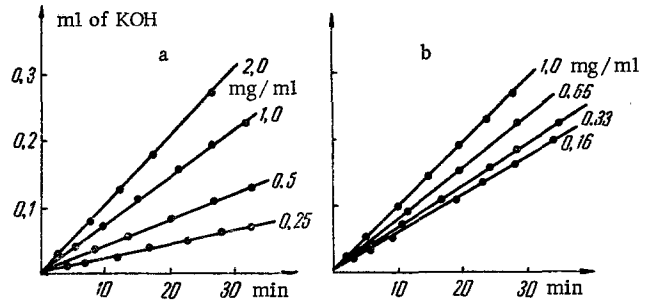

Fig. 3. Kinetic curves of the hydrolysis of tributyrin at pH 8.75 in the presence of cottonseed lipase: a) at various concentrations of substrate; b) at various concentrations of lipase.

refined. In this connection it is most important to study the nature of the –XH, –YH, and –ZH groups. Since lipase is inhibited by sulfhydryl reagents [8], it may be assumed that at least one of them is an –SH group.

The given scheme was drawn on the assumption that the rate of formation of the Michaelis complex is fairly high and that the process takes place in the stationary state. As expected, the reaction rate increases with a rise in the concentration of the substrate. However, the Michaelis curve (Fig. 4) has an unnatural form with a horizontal asymptote. Interpretation of such a curve can be based on the reversible adsorption of the enzyme on the micelle of the substrate. If all the external factors are kept constant, an increase in the volume of the emulsion arriving at the lipase in unit volume leads to the situation in which the rate of hydrolysis increases and tends to reach a limit. The adsorption of the enzyme and, consequently, the lipase activity, depend on the state of dispersion of the substrates, i.e., emulsions of tributyrin in water, in a 1% solution of dodecylsulfate, and in a 0.1% solution of Triton X-100. The specific activity of the lipase hydrolysis under these conditions is 7.4, 12.0, and 18.0, respectively. An increase in the total surface suitable for

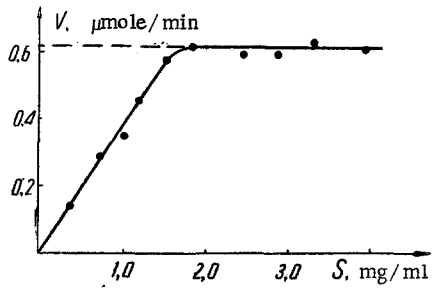

Fig. 4. Dependence of the rate of lipolysis of tributyrin on the substrate concentration.

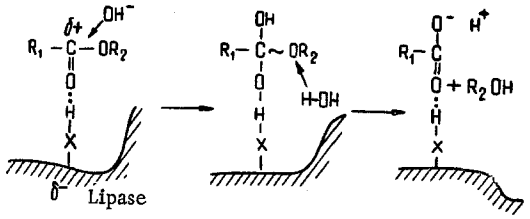

Fig. 5. Scheme of the hydrolysis of linear esters of cottonseed lipase.

adsorption by increasing the number of particles increases the rate of hydrolysis. The emulsification of cottonseed oil in a Potter homogenizer for 0-30 min increases the lipase activity.

Time of homogenization, min	1	2	5	10	15	30
Lipase activity	2.0	7.2	9.6	12.0	12.5	12.5

Thus, the overall scheme of the hydrolysis of lipids under the action of lipase can be written in the following manner:

$$E + S \rightleftarrows (ES)_a \rightleftarrows (ES)_M \rightleftarrows E + P_1 + P_2$$

where E represents the enzyme, S represents the substrate, $(ES)_a$ is the adsorption complex, $(ES)_M$ is the Michaelis complex, and P_1 and P_2 are the reaction products.

The hydrolysis rate is determined by the isothermal adsorption and the rate of migration of the enzyme to the surface of the micelle of the lipid. With an increase in the hydrophobic moiety of the lipid, its capacity for forming micelles and the proportion of active surface increases, and a rise in the rate of lipolysis must be found. In actual fact, the rate of hydrolysis increases by a factor of 1.6 in the sequence tributyrin–tripalmitin–tristearin (see Table 1). The limit of the increase will be determined by steric hindrance.

The esterolytic activity of cottonseed lipase with respect to soluble substrates depends on the reactivity of the –XH group alone (Fig. 5). The probability of the formation of enzyme–substrate complexes and their number and, consequently, also the rate of hydrolysis, will be determined by the mobility of the molecule undergoing hydrolysis. Ethyl acetate is hydrolyzed 7.2 times faster than isoamyl acetate (see Table 1). Within the limits of the experimental material presented it is impossible to establish quantitative relationships between the lipase and esterase activities of the cottonseed enzyme. They apparently represent different forms of tributyrinase with an optimum pH at 8.75 [6]. Kinetic experiments with highly purified preparations will probably enable us to obtain a knowledge of the detailed mechanisms of these reactions.

EXPERIMENTAL

Substrates. The tributyrin was purified by vacuum distillation; the triolein was chromatographically homogeneous [7]; the Tweens and plant oils were used without additional purification; the acetoacetic ester, methyl butyrate, and isoamyl acetate were purified by redistillation. Triton X-100 (0.1% solution in 0.1 M tris buffer, pH 7.4) was used for emulsification.

Enzyme. The lipase was obtained from an acetone powder of cotton seeds by the method described previously [6]. Medium: 0.1 M phosphate buffer, pH 7.4.

Measurement of Activity. The lipase and esterase activities were determined by the titrimetric method [6] at 25°C and at pH 8.75. The medium for the activity measurement contained 0.9 ml of 1 M $CaCl_2$ solution and 60 mg of the substrate, and the enzyme was added in a volume of 1 ml so that the final concentration was 0.33 mg/ml, the total volume being made up to 30 ml with double-distilled water. Titration was carried out with 0.01 M KOH solution (Fixanal).

Thin-layer Chromatography [7]. Silica gel (Kiselgel G) plates with dimensions of 9 × 9 cm were used for the analysis of triolein and the hydrolysis products. The solvent for chromatography was hexane–ether (60 : 40). The spots were revealed with iodine vapor. The layers were activated at 110°C for 30 min.

CONCLUSIONS

1. The lipase from cotton seeds possesses lipase, esterase, and tweenase activities.

2. The broad substrate specificity of the lipase is connected with its capacity for functioning both in homogeneous and in heterogeneous media. A mechanism of the action of the lipase explaining the specificity of its action is proposed.

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