CHARACTERISTICS OF THE LIPOLYTIC ENZYMES OF COTTON SEEDS

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Lipases and phospholipases are enzymes playing an important role in the metabolism of lipids and also in the formation of some enzyme systems [1-3]. The study of the properties and behavior of these enzymes in plant seeds, in which lipids form the bulk of the reserve substances, is of particular interest, since the development of the seeds in the early stage is accompanied by the intensive hydrolysis of the plant oils [4, 5]. In our work we have characterized the properties of the lipases and phospholipases of dormant cotton seeds.

A suspension of a defatted powder of cotton seeds in water is capable of hydrolyzing cottonseed oil, tributryin, and egg lecithin. The hydrolysis of tributyrin and cottonseed oil takes place at the maximum rate in the pH ranges from 4 to 6 and 7.5 to 9.5, and that of lecithin at pH 4.5-6.5. The enzymatic activities mentioned are absent from the mitochondrial and microsomal fractions and are concentrated entirely in the soluble fraction (Table 1). When the conditions for the extraction of the lipase active in the acid region are varied, it is extracted best in a buffer containing sucrose and in 0.025 M ammonia; the lipase active at alkaline pH values is best extracted in 0.1 M phosphate buffer, pH 7.4, and the enzyme producing choline from lecithin [6] is best extracted in 0.1 M acetate buffer, pH 5.6 (Table 2). In the buffers mentioned, the optimum pH values of the enzymes differ: for the acid lipase 4.75, for the alkaline lipase 8.75, and for phospholipase D 5.6. The results obtained agree with those given in the literature. In the seeds of wheat [7], soya [8], and the fir [9], two pH optima in the acid and alkaline regions are found. The optimum pH for phospholipase D isolated from cabbage [6], groundnuts [10], and other plants [11] is in the range from 5.6 to 6.0. The acid lipase of cotton seeds resembles the acid lipase found in the seeds of the castor plant [12, 13].

A study of the heat stability of the enzymes has shown that the phospholipase D retains its activity even after being heated to 50°C for 30 min. At 0°C, the activity does not change for a week for incubation in 0.1 M acetate buffer, pH 5.6. The heat stability of the lipase depends on the medium in which the enzyme is dissolved. In water, the lipase rapidly loses its activity: the activity has fallen to one half after 12 h at 0°C and after 4 h at 20°C. The stability of the enzyme is substantially higher in phosphate buffer (0.1 M, pH 7.4) and in 0.1 M tris buffer containing sucrose (0.5 M), pH 7.4. In these buffers the lipase is stable even at 50°C, and at 0°C the activity does not change in two weeks. The fall in activity is connected with the aggregation of the lipase in an aqueous medium (see below). Aggregation is prevented in phosphate and tris buffers.

Interesting results were obtained in a study of the action of calcium ions on the lipolytic enzymes of cotton seeds. Calcium ions are specific activators for the majority of lipases [14, 15] and phospholipases [10, 16]. In many cases it may be considered as demonstrated that the activation by calcium ions is connected with the preliminary interaction of Ca^{2+} with the lipid micelles [14, 17, 18].

The phospholipase D and the lipase from cotton seeds react with calcium ions in different ways. The hydrolysis of lecithin by the phospholipase D is impossible in the absence of calcium ions. The optimum concentration depends on the purity and concentration of the lecithin. Apparently, the reaction is preceded by the interaction of the calcium ions with the emulsified substrate [10, 17, 18]. Conversely, the activity

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TABLE 1. Subcellular Distribution of the Lipolytic Enzymes in Dormant Cotton Seeds

Fraction	Acid lipase, tributyrin, pH 4.75	Alkaline lipase		Phospholipase D (lecithin*), pH 5.6
		tributyrin, pH 8.75	cottonseed oil, pH 8.75	
Homogenate	1,0	2,4	1,9	3,5
Aqueous-fatty layer	0,8	0	0	0
Mitochondrial fraction	0	0	0	0
Microsomal fraction	0	0	0	0
Soluble fraction	1,8	8,0	5,2	10,8

*The results are given with the free choline taken into account.

TABLE 2. Yield of Lipolytic Activities under Various Conditions

Conditions of extraction	1	2	3	4	5	6	7
Amount of protein extracted	312	390	420	320	540	280	460
Specific activity							
pH 4.75 lipase	—	0,16	1,0	0,9	0,21	0,22	0,41
pH 8.75 lipase	1,9	2,0	1,2	1,8	1,0	0,58	0,61
Phospholipase D	3,0	4,2	—	4,0	—	4,8	—

Note. 1) H_2O; 2) 0.1 M phosphate buffer, pH 7.4; 3) 0.025 M ammonia; 4) 0.1 M tris hydrochloride containing 0.5 M sucrose, pH 7.2; 5) 10% sodium chloride; 6) 0.1 M acetate buffer, pH 5.6; 7) 50% glycerol.

of the lipase is inhibited by the presence of calcium chloride. However, the inhibiting action of calcium ions is not shown in all cases. Thus, $CaCl_2$ completely suppresses the lipase activity in water and 0.1 M tris buffer but at the same concentration of calcium ions lipase is active in 0.1 M phosphate buffer. This is the result of a specific interaction of phosphate ions with the lipase. In the phosphate buffer, the lipase possesses no hydrolytic activity in the absence of Ca^{2+}. An increase in the concentration of phosphate ions in the aqueous solution of lipase completely inhibits lipolysis. The addition of a solution of calcium chloride to a medium containing the substrate, the enzyme, and phosphate in an amount sufficient for complete inhibition restores the lipase activity (Fig. 1). Activation is not connected with the interaction of calcium and phosphate ions since 33.3 μmoles of $CaCl_2$ is necessary completely to nullify the inhibiting action of 2.3 μmoles of phosphate. The addition of an excessive amount of $CaCl_2$ acts in the same way as in the absence of phosphate (Fig. 1, curve 2). This specific action of calcium and phosphate ions is apparently explained by conformational states of the lipase and requires an accumulation of experimental facts for its explanation.

The lipase has an enhanced heat stability in 0.1 M phosphate buffer, which we used to develop a method of purifying the lipase. The lipase was precipitated quantitatively from an 0.1 M phosphate buffer, pH 7.4, in the range of concentrations of ammonium sulfate of from 10 to 55% saturation. The precipitate was dissolved in the same buffer and fractionated on a column of Sephadex G-100. Analysis of the fractions issuing from the column for tributyrinase activity (the activity was measured with the addition of the calculated amount of $CaCl_2$ solution) showed the presence of six zones containing lipases differing from one another in molecular weight (Fig. 2). The number of microequivalents of butyric acid formed by the action of 1 ml of the fraction issuing from the column on emulsified tributyrin (2 mg/ml, pH 8.75) has been plotted along the axis of ordinates and the volume of eluate issuing from the column along the axis of abscissas. Figure 2 gives a zymogram of the fraction deposited on the column (the gel was colored with the dye Amido Black).

The relationships that were found in the distribution of the tributyrinase activities in these zones permit the assumption that two enzymes are present which exist in the monomeric, dimeric, and tetrameric forms (see Fig. 2).

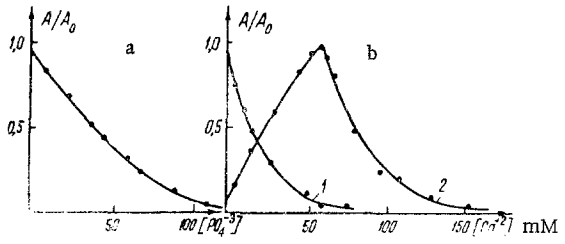

Fig. 1

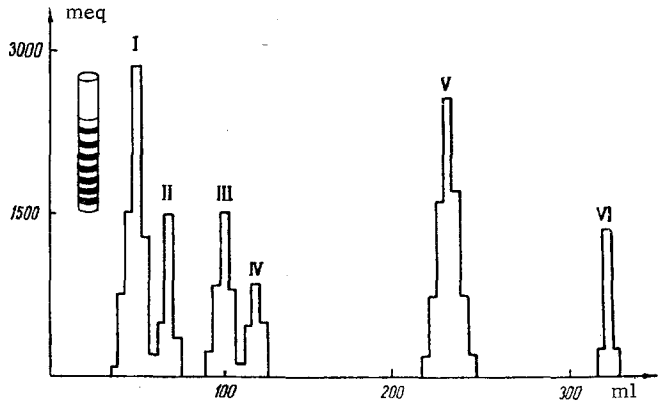

Fig. 2

Fig. 1. Influence of phosphate ions (a) and calcium ions (b) on the activity of the lipase at pH 8.75 and 25°C with concentrations of protein and tributyrin of 2 mg/ml. 1) Inhibition of the lipase activity by Ca^{2+} ions; 2) influence of calcium ions on the lipase activity in the presence of phosphate buffer (100 mmoles). A = specific activity, $A_0 = 8.2$.

Fig. 2. Chromatography of the alkaline lipase on Sephadex G-100.

The quantitative relationships in the maxima of the zones of tributyrinase activity obtained by chromatography on Sephadex G-100 are as follows:

Variety	Ratio of the maxima of lipase activity						
	I/III	I/V	II/IV	II/VI	I/II	III/IV	V/VI
2847	1,9	1,1	1,8	1,1	1,9	1,9	1,9
108-F	1,9	1,1	1,85	—	0,39	0,39	—
108-F	1,9	1,1	1,9	—	0,40	0,40	—

The lipases isolated by fractionation are stable in phosphate buffer. Prolonged dialysis against water leads to the formation of inactive aggregates constituting a white precipitate. The process is reversible: when the precipitate is dissolved in phosphate buffer, the activity is slowly restored. After 4, 12, 24, and 72 h in 0.1 M phosphate buffer, pH 7.4, 28, 42, 52, and 60% of the original activity, respectively, is restored.

The liberation of butyric acid in the hydrolysis of tributyrin by the lipase is proportional to the time at all concentrations of substrate. This relationship is found for other lipases, also [19, 20]. The activity of the lipase is proportional to the concentration of tributyrin at low concentrations of the latter and remains constant at high concentrations. This nature of the relationship can be explained on the basis of the reversible adsorption of the enzyme on the micelle of the substrate [21, 22]. In the hydrolysis of olive oil, the activity as a function of the concentration of the substrate is expressed in the form of a curve with a maximum, i.e., large concentrations of oil inhibit the reaction (Fig. 3). It is possible that the oils contain lipase inhibitors [23]. In all cases, the rate of hydrolysis rises with an improvement in the degree of dispersion of the substrate [22]. The rate of hydrolysis of lecithin by phospholipase D doubles if the emulsion is homogenized in a Potter homogenizer and quadruples if the detergent Triton X-100 (0.1 ml per 12.5 mg of lecithin) is added during homogenization.

In addition to tributyrin and oils, the lipase from cotton seeds is capable of cleaving other substrates. The specific activity obtained for various substrates using the combined lipases is as follows: tributyrin 18.0, triolein 28.1, olive oil 16.0, cotton seed oil 12.0, castor oil 17.1, apricot oil 12.0, Tween-21, 21.0, Tween-80, 12.0, Tween-85, 21.0, and acetoacetic ester 40.0 [22]. The specific activities of the enzyme with respect to various substrates depends on the concentration of the enzyme. At high concentrations of protein the activity is low, and it rises when the solution is diluted:

Concentration of protein, mg/ml	28,8	17,8	6,7	2,5	0,67	0,33	0,17	0,1	0,067
Specific activity	0,4	1,0	3,0	6,7	8,5	15,0	26,0	45	240

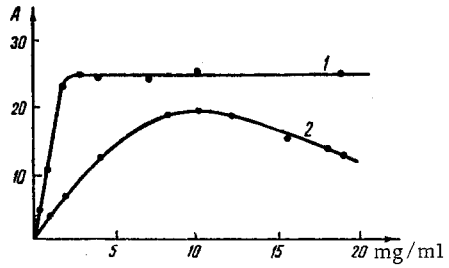

Fig. 3. Influence of the concentration of the substrate on lipase activity.

The results given show the presence in cotton seeds of three enzymes with lipolytic activity: an acid lipase with a pH optimum of 4.75, a lipase with a pH optimum of 8.75, and phospholipase D. The broad substrate specificity distinguishes the lipase from the corresponding enzymes of animal and microbial origin [20, 22]. The specific action of phosphate and calcium has been detected for the first time. How far this is shown in the case of other lipases is not yet known.

EXPERIMENTAL

Substrates. Lecithin was obtained from fresh egg yolks by the method of Singleton et al. [24]. Its purity was checked by thin-layer chromatography on silica gel G in the chloroform–methanol–water (65 : 24 : 4) system. Before use the tributyrin was distilled in vacuum at 10 mm Hg (bp 183-185°C). The triolein was chromatographically homogeneous in the hexane–diethyl ether (40 : 60) system. The oils were extracted with organic solvents from the appropriate sources. The substrates were emulsified in a Potter homogenizer with the addition of detergents where required.

Enzymes. The enzymes were obtained from an acetone powder isolated from the kernels of the seeds which had been separated from the hulls by means of liquid nitrogen, by crushing in a mortar and grinding in a coffee mill without heating. The oil and the pigments were extracted successively with 85% aqueous acetone (twice), acetone, and a mixture of acetone and diethyl ether (1 : 1). The white powder obtained was stored in an evacuated vacuum desiccator at 0-4°C. The extracts for the production of the enzymes were prepared by triturating the acetone powder in a mortar with a tenfold amount of the required buffer solution together with quartz sand or ground glass. This operation was more effective than homogenization or prolonged extraction in the cold. Centrifuging was carried out at 18,000 rpm at 0°C for 30 min.

Measurement of Enzymatic Activities. The activity of the lipases was investigated titrimetrically. In standard experiments, the substrate was tributyrin emulsified in 0.1 M tris buffer. The nonionic detergent Triton X-100 (0.1% solution) was used to stabilize the emulsions. The medium for the activity measurements contained 60 mg of emulsified tributyrin, 10 mg of enzyme protein, 10 mM phosphate, and 90 mM $CaCl_2$ in a total volume of 30 ml. The amount of butyric acid liberated in the reaction was determined by titration with 0.01 M KOH at constant pH by means of a sensitive LPM-60MZ pH-meter with glass and silver-chloride electrodes. The other substrates were hydrolyzed under similar conditions. The optimum concentration of substrate was selected by studying the dependence of the lipase activity on the concentration of the substrate.

The activity of the phospholipase D was evaluated by measuring the amount of free choline formed in the hydrolysis of lecithin [25]. The medium for the activity measurements consisted of 0.1 M acetate buffer, 60 μmoles of $CaCl_2$, and 1.25 mg of lecithin suspended in 0.1 M acetate buffer containing 0.1% of Triton X-100. The total volume was 3 ml. The specific activities were expressed in μmoles of product formed in 1 min calculated to 1 mg of protein.

Chromatography on Sephadex G-100. A column with a porous base having dimensions of 25 × 1.8 cm was filled with Sephadex that had been swollen in 0.1 M phosphate buffer, pH 7.4, and was eluted overnight with the same buffer for compaction. The precipitate obtained after the fractionation of the extract of defatted powder of cotton seeds (10 g of powder suspended in 100 ml of 0.1 M phosphate buffer, pH 7.4) by ammonium sulfate in the range of concentrations from 10 to 55% saturation was dissolved in the buffer and centrifuged at 18,000 rpm at 0-2°C for 30 min. The concentration of protein in the supernatant was adjusted to 10 mg/ml by means of the phosphate buffer, and 10 ml of the solution was deposited on the column. Elution was carried out with 0.1 M phosphate buffer, pH 7.4. The rate of elution was 18 ml/h, 4.5-ml fractions being collected. The temperature was 0-5°C.

Disc Electrophoresis. Vertical electrophoresis was carried out on polyacrylamide gel. The concentration of the concentrating gel was 2.5% in 0.025 M tris-glycine buffer, pH 6.6, and the concentration of the separating gel was 7.5%, pH 8.6. The dimensions of the glass tubes for filling with the gel were 0.5 × 5.0 cm. Before deposition, the samples were saturated with sugar at the rate of 250 mg/ml. The current strength in electrophoresis was 3 mA for each tube.

SUMMARY

1. Three enzymes possessing lipolytic activity have been detected in dormant cotton seeds: a lipase active in acid media with a pH optimum of 4.75, a lipase active in alkaline media with a pH optimum of 8.75, and phospholipase D with a pH optimum of 5.6.

2. The localization of the enzymes in the seeds has been established and their properties (heat stability, stability in various media, nature of the hydrolysis of the lipids, necessity for phase separation for enzymatic action, etc.) have been described.

3. The alkaline lipase with a pH optimum of 8.75 is capable of catalyzing the hydrolysis of triglycerides, various oils, Tweens, and esters. It is specifically inhibited by calcium and phosphate ions. Chromatography on Sephadex G-100 has shown that the alkaline lipase represents a whole class of enzymes consisting of six proteins each of which is capable of hydrolyzing tributyrin. Definite quantitative relationships exist between the components.

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