STUDY OF THE LIPOLYTIC ENZYMES OF COTTON SEEDS

IV. TRIACETINASE

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The present paper gives the results on the isolation of the enzyme triacetinase with mol. wt. 36,000 – one of the representatives of the alkaline lipases [1, 2]. The protein from an acetone powder of cotton seeds was extracted with 0.1 M phosphate buffer, pH 7.4. With such a procedure, the specific activity of the enzyme was 40 units/mg (μmoles/min), and the degree of extraction was 64-66% (pH optimum 9.5).

In order to concentrate the enzyme and free it from the bulk of the ballast proteins we used ammonium sulfate as precipitant (55% saturation).

When the fraction precipitated by ammonium sulfate was chromatographed on a column of Sephadex G-100, six peaks possessing enzymatic activity in the hydrolysis of triacetin were observed on the chromatogram [1]. The first two of them had a low specific activity and were associated with the appearance of the nonspecific activity of tributyrinases. The enzymatic activities of the other four peaks corresponded to triacetinases with molecular weights of 74,000, 38,000, 18,000, and 12,000 (determined by thin-layer gel filtration on Sephadex G-200). The fractions corresponding to the enzyme with mol. wt. 38,000 were combined and dialyzed against 0.01 M phosphate buffer with one change of dialysis liquid (pH 7.5) for 16 h, and then the solution was deposited on a column of DEAE-cellulose and was eluted with the same buffer. The small amounts of protein impurities possessing no lipase activity were separated off and, with the application of an NaCl gradient, the active fractions were eluted, and then they were then combined and dialyzed. The dialyzate was freeze-dried. The final purification of the enzyme was achieved by chromatography on an analytical column of Sephadex G-100 (Fig. 1). The peak of the activity of the enzyme corresponded to the peak of the protein (mol. wt. 36,000) and its specific activity had not decreased in comparison with the preceding stage (Table 1).

The degree of purification of the enzyme amounted to a factor of 1050, the yield with respect to the activity of the initial material was 14%, and yield with respect to protein was 0.012%. The specific activity was 42,000 units at 25°C. This activity is the limiting value. With further purification, the specific activity of the enzyme did not increase.

TABLE 1

Stage of purification	Protein, mg	Activity: specific, units/mg	Activity: total units	Yield, %	Deg. of purification
Extraction	11 200	40	440 000	100	1
Fractionation with ammonium sulfate	1 000	370	390 000	84	9
Gel filtration on Sephadex G-100					
Fraction with mol. wt. 74,000	11,1	6700	76 000	17	170
Fraction with mol. wt. 38,000	8,2	19 500	164 000	37	490
Fraction with mol. wt. 18,000	2,4	26 000	60 000	14	650
Ion-exchange chromatography on DEAE-cellulose	4,2	38 000	160 000	35	950
Gel filtration on Sephadex G-100	1,4	42 000	59 000	14	1050

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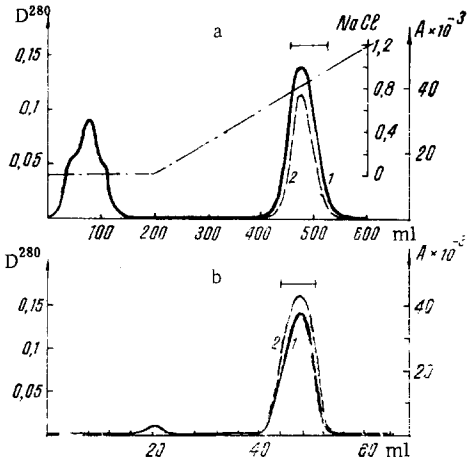

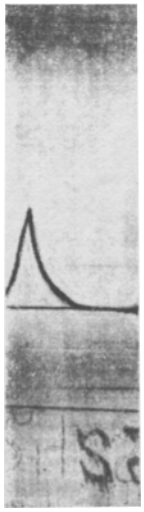

Fig. 1 Fig. 2

Fig. 1. Elution profiles of the chromatographic stages of the purification of the triacetinase: a) ion-exchange chromatography of the triacetinase on a column of DEAE-cellulose; b) gel filtration of the triacetinase on a column of Sephadex G-100; 1) optical density at 280 nm; 2) specific activity (units/mg of protein).

Fig. 2. Sedimentogram of the triacetinase in 0.1 M phosphate buffer, pH 7.4.

The homogeneity of the protein obtained was analyzed by rechromatography on a column of Sephadex G-100 (three times), rechromatography on a column of DEAE-cellulose, and ultracentrifugation. Rechromatography on a column with Sephadex G-100 gave a symmetrical profile of the curve of the elution of the protein corresponding to the activity peak. The ratio of the optical densities at 280 and 260 nm was constant at 1.52. On rechromatography on a column of DEAE-cellulose equilibrated with 0.01 M phosphate buffer, pH 7.4, the enzyme was eluted by the application of an NaCl gradient up to 1 M, and the maximum on the elution curve corresponded to 0.8 M NaCl.

The results of an analysis of purified enzyme by the ultracentrifugation method are shown in Fig. 2. The sedimentation coefficient was 2.18 S and the diffusion coefficient was $5.97 \cdot 10^{-7}$ cm^2/sec^{-1}.

The molecular weight of the protein determined from the results of ultracentrifugation was 35,600. This figure agrees well with those obtained by other methods. Gel filtration on a column of Sephadex G-100 calibrated with standard proteins gave a value of the molecular weight of 36,000, and thin-layer chromatography on plates with Sephadex G-200 gave a figure of 38,000. The pH optimum of the purified enzyme was 9.5 and the temperature optimum 40°C. Calcium ions do not show an activating effect, but activation by ions of divalent iron is possible.

EXPERIMENTAL

Isolation of the Enzyme. The acetone powder obtained by the method described previously [3] (18-20 g) was stirred with a tenfold amount of 0.1 M phosphate buffer, pH 7.4, at 0-6°C for 1 h. The suspension was centrifuged at 18,000 rpm on a TsLR-1 centrifuge for 30 min. The supernatant was treated with ammonium sulfate to 55% saturation and the precipitate was separated off by centrifuging. Then it was dissolved in 20 ml of 0.1 M phosphate buffer (pH 7.4), using a Potter homogenizer, and the solution was clarified by centrifugation. The supernatant, with a protein concentration of 50-56 mg/ml was deposited on a column of Sephadex G-100 (3.4 × 70 cm) equilibrated with the same buffer. The rate of elution was 24 ml/h. Fractions were collected every 15 min. The optical densities at 280 nm and the lipase activities with triacetin at pH 9.5 were measured. The active fractions corresponding to the enzyme with mol. wt. 36,000 (the column was previously calibrated with standard proteins) were combined and freeze-dried. Yield 7-8 mg.

The freeze-dried protein was dissolved in 3-5 ml of double-distilled water, and the solution was dialyzed against 0.01 M phosphate buffer, pH 7.5. The dialyzate was deposited on a column of DEAE-cellulose (1.0 × 20 cm) equilibrated with 0.01 M phosphate buffer, pH 7.5, and was eluted with the same buffer (200 ml). The rate of elution was 12 ml/h and the volume of the samples 3 ml. Then the material was chromatographed with a linear gradient of NaCl from 0 to 1.2 M (total volume 400 ml). The active fractions were eluted at 0.8 M NaCl. They were combined and dialyzed against 0.1 M phosphate buffer, pH 7.4. The dialyzate was freeze-dried. Yield 3.5-4 mg. Then the enzyme was dissolved in 2 ml of H_2O and the solution was deposited on a column of Sephadex G-100 (1.0 × 22 cm) equilibrated with 0.1 M phosphate buffer, pH 7.4, and the column was washed with the same buffer. The rate of elution was 6 ml/h and the volume of the samples 1.5 ml. The solution of the enzyme was freeze-dried, giving a yield of 1.2-1.5 mg.

The concentration of the protein in the first stages of purification was determined by the biuret method and, subsequently, after the separation of the pigment components, spectrophotometrically by measuring the optical density at 280 and 260 nm on an SF-4a spectrophotometer.

Substrates. Tributyrin and triacetin were purified by redistillation. Cottonseed oil was extracted from the seeds with petroleum ether, and the solvent was distilled off under vacuum. The other substrates were not purified additionally. The insoluble substrates were suspended in double-distilled water in a ratio of 15:85, and the suspension was stabilized with the nonionic detergent Triton X-100 (from 0.1 to 1%).

In the experiments to detect lipolytic enzymes we used tributyrin, which can easily give stable emulsions with aggregated molecules and at the same time possesses a high solubility [4]. The activities were measured at pH 8.8. At this pH it is possible to detect both tributyrinases [5] and triacetinases with respect to their nonspecific activity [1].

In the experiments on the purification of the triacetinase we used triacetin (kh. ch. ["chemically pure"]) and measured reactivity at pH 9.5 [1, 2]. The capacity of the enzymes for hydrolyzing lipids was monitored from the rate of cleavage of cottonseed oil at pH 9.5.

Measurement of Enzymatic Activities. To determine the lipase activity we used the titrometric method with the aid of a TTT1 pH-stat or a BAT-12 LMZ + pH 340 automatic titration unit. The amount of enzyme was chosen in such a way that the titration of the reaction product formed consumed 0.05 ml of the titrant (0.1 M KOH) in 1 min. The concentration of substrate of 0.1-6 mg/ml was chosen in accordance with the saturation curve for the substrate [3]. In the activity measurements, buffer solutions were not used, the pH being checked by LPM-60 M and pH-340 pH-meters. The total volume of the cell was 20 ml, and the temperature 25°C.

Determination of Molecular Weights by Column Gel Chromatography. The molecular weights of the lipases were determined by Andrews' method [6]. A column of Sephadex-100 (3.0 × 60 cm) was calibrated with standard proteins: lactate dehydrogenase from porcine liver (140,000), malate dehydrogenase (124,000, dimer), hemoglobin (68,000), bovine serum albumin (60,000), trypsin (23,800), and cytochrome c from horse heart (12,500). The free volume was found by the use of dextran blue (116 ml).

Determination of Molecular Weights by Thin-Layer Chromatography. Sephadex G-200 was left to swell in 0.05 M phosphate buffer containing 0.15 M of potassium chloride (pH 7.4) for 2 days and was then centrifuged at 3000 rpm on a T-30 centrifuge for 10 min. The precipitate was spread in a uniform layer (0.8-0.9 mm) on a 14 × 18 cm plate. The gel was equilibrated in a closed chamber with the buffer for 24 h. The samples of protein were deposited on the plate by means of a calibrated capillary. The angle of slope of the plate was 20° [7]. Under these conditions the rate of migration of the hemoglobin band was 12 mm/h. As standard proteins we used the same proteins as in the calibration of the Sephadex G-100 column. For all the proteins a linear dependence of R_{Hb} (ratio of the distances migrated by the protein under investigation and by hemoglobin) on the logarithm of the molecular weight was observed.

Sedimentation Analysis. The analysis of homogeneity and the determination of molecular weights by the ultracentrifugation method was performed on an MOM-120 instrument. The speed of rotation of the centrifuge was 50,000 rpm, 20°C. The concentration of protein was 40 mg/ml in 0.1 ml of phosphate buffer, pH 7.4.

SUMMARY

A triacetinase with mol. wt. 36,000 has been isolated from cotton seeds by ion-exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-100.

The homogeneity of the protein has been confirmed by the results of rechromatography and ultracentrifugation.

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