LIPOLYTIC ENZYMES OF COTTON SEEDS

V. TRIACETINASE

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

UDC 577.153+582.796

Among the alkaline lipases of cotton seeds a group of enzymes with a pH optimum of 9.5 stands out by its high activity [1, 2]. The present paper describes the results of the isolation of triacetinase with mol. wt. 18,000.

The extraction of the enzyme from an acetone powder of cotton seeds was performed with 0.1 M phosphate buffer, pH 7.4, as in the case of other alkaline lipases [1-4]. The main amount of ballast proteins was separated by acidifying the solution to pH 3.8. At this pH about 90% of the protein precipitates, while practically all the triacetinase remains in solution. Prolonged incubation at pH 3.8 is undesirable, since it leads to the inactivation of the enzyme. Consequently, the solution after the separation of the ballast proteins was centrifuged at 6000 rpm, neutralized, and dialyzed against 0.01 M phosphate buffer, pH 7.4. The precipitate that deposited on dialysis was separated off, and the solution was passed through a column of DEAE-cellulose for concentration. A column with a volume of 1.5 liter and height of 40 cm is capable of concentrating about 55 g of protein in a volume of 4-5 liters. After the adsorption of the protein, the triacetinase was eluted with a molarity gradient of sodium chloride up to 1.0 M. The solution was desalted by dialysis, and ion-exchange chromatography was performed on a column of DEAE-cellulose equilibrated with 0.01 M phosphate buffer, pH 7.4 (Fig. 1a).

The active fraction was eluted at a concentration of sodium chloride of 0.8 M. The fractions posessing triacetinase activity were collected and desalted by dialysis and were then freeze-dried.

The following stage of purification was gel filtration in a column of Sephadex G-50. Here the bulk of the protein having no activity was separated, but the triacetinase fraction still contained small amounts of protein impurities (Fig. 1b). Final purification was achieved by rechromatography on a column of Sephadex G-50 (Fig. 1c). The enzyme obtained in this stage possessed a high activity (70,000 units) and showed constancy of the D^{280}/D^{260} ratio on a chromatogram. The yield of protein by the method described was 30 mg from 330 g of acetone powder, or 0.025% calculated on the total protein. The yield with respect to activity was 35%. A 1400-fold purification was achieved.

The homogeneity of the enzyme obtained was checked by rechromatography on DEAE-cellulose and Sephadex G-50 (three times). The protein was eluted in all cases as a single fraction, and the activity peak on the elution diagram coincided with the protein peak. No increase in specific activity on rechromatography was found. The results of sedimentation analysis also showed the homogeneity of the protein. The molecular weight calculated from the sedimentogram was 19,200. The molecular weight determined by gel filtration on Sephadex G-100 was 18,000. An electrophoretic map obtained on disk electrophoresis is shown in Fig. 1c. An analysis of the amino-acid composition of the enzyme gave the following results (%): lysine 1.54, histidine 6.15, arginine 4.48, aspartic acid 1.78, threonine 3.26, serine 6.5, glutamic acid 9.35, proline 1.7, glycine 2.4, alanine 8.45, semicysteine 2.23, valine 5.4, methionine 4.8, isoleucine 1.87, leucine 6.54, tyrosine 9.5, phenylalanine 5.1, and tryptophan 2.6. The molecular weight calculated from the amount of each amino acid was 18,500 ± 1200.

The homogeneity of the protein was confirmed satisfactorily by the results of a determination of the N-terminal group of the enzyme. The dansylation method showed a high degree of purity of the triacetinase, and methionine was found as the only N-terminal group.

Institute of the Chemistry of Plant Substances, Academy of Sciences of the Uzbek SSR. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 767-770, November-December, 1974. Original article submitted June 29, 1973.

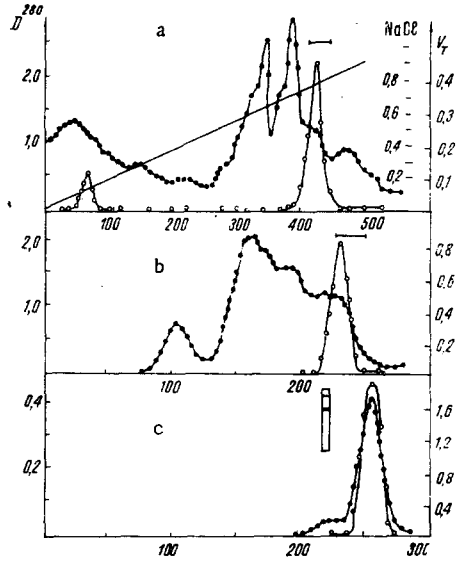

Fig. 1. Results of the purification of triacetinase: a) ion-exchange chromatography on DEAE-cellulose; b) gel filtration on Sephadex G-50; c) rechromatography on Sephadex G-50.

EXPERIMENTAL

The methods of measuring the triacetinase activity and the preparation of the solutions of the substrates and buffers have been described previously [1, 3, 4].

Isolation of the Enzyme. An acetone powder [4] from bare cotton seeds of the variety 108-F (330 g) was extracted with 0.1 M phosphate buffer, pH 7.4 (6 : 1) with stirring in the cold for 16 h. The suspension was centrifuged at 2000 rpm for 30 min in a TsLR-1 centrifuge, and the supernatant liquid was separated off and acidified with concentrated hydrochloric acid to pH 3.8 with continuous stirring. The precipitate that had deposited was separated off by centrifuging at 6000 rpm for 30 min, and the supernatant was neutralized with 6 M KOH and dialyzed against 0.01 M phosphate buffer, pH 7.4, with two changes of the dialysis liquid for a day. For the preliminary purification and concentration of the enzyme from the dilute solution we used ion-exchange chromatography. Two liters of solution was passed through a column of DEAE-cellulose (4.5 × 100 cm) at the rate of 100 ml/h. Then the column was washed with another 400 ml of buffer and a sodium chloride gradient up to 1 M in a total volume of four liters was applied to the column. The active fractions, with the maximum activity at 0.8 M NaCl, were combined and were desalted by dialysis against 0.01 M phosphate buffer, pH 7.4. After dialysis for 16 h, the protein fraction was deposited on a column of DEAE-cellulose (3.0 × 55 cm) at the rate of 45 ml/h, and elution was performed with 0.01 M phosphate buffer having a molarity gradient of sodium chloride up to 1 M. The rate of elution was 60 ml/h and the fraction volume 10 ml. The fractions containing the triacetinase were combined (250 ml), dialyzed against two liters of buffer, and freeze-dried. The yield of dry powder was 200 mg. The freeze-dried powder was dissolved in 2 ml of double-distilled water and deposited on a column of Sephadex G-50 (3.0 × 65 cm) equilibrated with 0.1 M phosphate buffer, pH 7.4, and elution was performed with the same buffer. A fraction with an elution volume of 275 ml possessed triacetinase activity. The active fractions were collected and freeze-dried. Yield 50 mg. Gel filtration was repeated on a column with a size of 2.0 × 120 cm filled with Sephadex G-50. The rate of elution was 12 ml/h and the volume of the samples taken was 3 ml. The protein fraction containing triacetinase was freeze-dried. Yield 30 mg.

Molecular Weight Determination. Column gel filtration was performed under the conditions described by Andrews [5]. A column of Sephadex G-100 (3.0 × 65 cm) was calibrated with proteins having known molecular weights: catalase – 300,000; lactate dehydrogenase – 140,000; malate dehydrogenase – 124,000 (dimer) and 62,000 (monomer); bovine serum albumin – 60,000; hemoglobin – 68,000; trypsin – 24,000; and cytochrome – 13,000. The molecular weight of the enzyme under investigation was found to be 18,000.

The molecular weight was also determined by thin-layer chromatography on plates of Sephadex G-200 (14 × 18 cm^2) with an angle of slope of 15° [6]. The same proteins were used for calibration as for column chromatography. The molecular weight of the triacetinase was found to be 18,200 with an error of the determination of ±8%.

Sedimentation analysis was performed on a MOM 120 ultracentrifuge at 50,000 rpm in 0.1% NH_4HCO_3, pH 8.0 in the absence of NaCl. The molecular weight calculated from the sedimentogram was 19,200.

Disk Electrophoresis. The concentration of polyacrylamide in the concentrating gel was 2.5% and in the separating gel 7.5%. The medium was tris-glycine buffer, pH 8.0. The size of the tubes was 0.5 × 6 cm, the current 2-3 mA per tube, and the voltage 400 V. To stain the proteins, the gels were treated with a solution of Coomassie Blue.

Amino-Acid Analysis. The enzyme was hydrolyzed in 6 N HCl at 110°C for 24 and 72 h and at 145°C for 4 h [7]. The hydrolyzates were investigated on a AAA-881 amino-acid analyzer. The amount of protein necessary for one analysis was 3 mg. Tryptophan was determined by the spectrophotometric method [8].

Rechromatography. In an investigation of the homogeneity of the triacetinase, the enzyme was passed three times through a column of Sephadex G-50, with the collection each time of only those samples the op-

tical density of which exceeded 0.3 D^{280}_{max} (the maximum value of the optical density was at 280 nm). The "tails" of the protein peaks were combined and were chromatographed on a column of Sephadex G-50 (2.0 × 120 cm) and on a column of DEAE-cellulose (1.0 × 22 cm). With this procedure for rechromatography, no separation of the protein peak on the elution diagram or change in its symmetry was observed.

Determination of the N-Terminal Amino-Acid Residue. In order to determine the purity of the enzyme reliably, the amount of N-terminal amino acid was determined by the sensitive dansyl method [9, 10]. To 5-10 mmole of the enzyme was added 0.5 ml of 8 M urea, 0.15 ml of 0.4 M phosphate buffer, pH 8.9, and 0.25 ml of dimethylformamide. Dansylation was performed with a 1% solution of dansyl chloride in acetonitrile (0.2 ml) at room temperature for 24 h [11]. The reaction was stopped by the addition of 5 ml of a 10% solution of trichloroacetic acid, and the protein precipitate was separated by centrifuging at 5000 rpm for 15 min. The precipitate was washed twice with 4 ml of cold acetone, and the protein was hydrolyzed in 0.3 ml of 6 N HCl at 110°C for 4 h. The hydrolyzate was dried in vacuum and the dansylamino acid was dissolved in 0.1 ml of a mixture of acetone and 1 M HCl (19 : 1).

The terminal amino acid in the form of the dansyl derivative was identified by two-dimensional thin-layer chromatography on plates (6 × 6 cm^2) of silica gel (1.2 ml of a 40% suspension of silica gel in water per plate). Chromatography was performed in the first direction in the chloroform–tert-butanol–acetic acid (6 : 3 : 1) system and in the second direction in the chloroform–benzyl alcohol–acetic acid (70 : 30 : 3) system twice, R_f 0.20 and 0.59 for the N-terminal amino acid and 0.18 and 0.6 for dansylmethionine.

SUMMARY

A triacetinase has been isolated from cotton seeds by the methods of gel filtration and ion-exchange chromatography. The homogeneity of the enzyme was shown by rechromatography, electrophoresis, ultracentrifugation, and a determination of the terminal amino acid. The molecular weight of the triacetinase obtained by four different methods (column gel filtration, thin-layer chromatography, ultracentrifugation, and amino-acid composition) is 18,000 ± 1200. The amino-acid composition and the N-terminal amino-acid residue – methionine – have been determined.

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