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QUATERNARY STRUCTURE OF COTTONSEED TRIACETINASE

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A method of isolating cottonseed triacetinase, an enzyme catalyzing the hydrolysis of short-chain triglycerides that are substrates of esterases [2] has been described previously [1]. We give the results of an investigation of the subunit structure of this enzyme.

Esterases are oligomeric proteins [2]. By the titration of mammalian esterases with organophosphorus compounds [3-8] and carbamates [5, 6, 9] it has been established that there are two active centers in the esterase molecule. Under various conditions the dissociation of each of the esterases into two active half-molecules has been observed: a) on far-reaching dilution [7-9]; b) in an acid medium at pH 4.5 [7]; and c) at high concentration of salts (0.5 M NaCl; 0.5 M LiCl) [7]. When an esterase was incubated in a 0.2% solution of sodium dodecyl sulfate (SDS) [3], however, or in solutions of dissociating agents, it split into four polypeptide chains. The presence of two active centers in the molecule of the enzyme presupposes that the subunits are heterogeneous, but up to the present time there has been no proof of this statement.

The triacetinase was isolated by a modification of the previous method [1] in the following way: an acetone powder of cotton seeds was extracted with 0.1 M phosphate buffer, pH 7.4, and the extract was fractionated with ammonium sulfate and, after dialysis, it was chromatographed twice on DEAE-cellulose. The homogeneity of the preparations obtained was checked by disc electrophoresis and with respect to the N-terminal amino acid.

The active fraction (A) (Fig. 1) eluted from the DEAE-cellulose at a concentration of NaCl of 0.8 M [1] was dialyzed against 0.01 M phosphate buffer, pH 7.4 and was rechromatographed on DEAE-cellulose. Thereupon, in addition to fraction A, another active fraction, B, eluted at an NaCl concentration of 0.2 M was found (see Fig. 1), and in a series of experiments it was observed that the amount of protein in fraction A decreased with the simultaneous increase in the amount of protein in fraction B, and that the specific activity of A and B was approximately constant, amounting to 4500 activity units with respect to tributyrin. Similar results were obtained in an investigation of the esterase from bovine liver [8].

It is natural to assume that fraction B is formed from fraction A. The molecular weight of A determined by ultracentrifugation was 37,000 and by gel filtration 38,500, which agrees satisfactorily with literature information [1]. It was shown by ultracentrifugation and by gel filtration on Sephadex G-100 that the molecular weight of B is 20,000, which is approximately half the molecular weight of fraction A. The markers in gel filtration were

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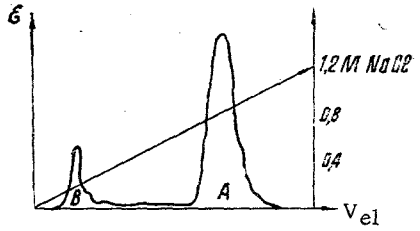


Fig. 1. Elution profile of triacetinase from DEAE-cellulose.

bovine serum albumin (mol. wt. 67,000), pepsin (mol. wt. 35,000), trypsin (mol. wt. 23,000), and cytochrome C (mol. wt. 12,000).

In an acid medium at pH 4.2, fraction A dissociated completely into two active subunits with mol. wt. 20,000, and at pH 7.4 the subunits slowly associated to form the native molecule. These results were obtained by gel filtration and on the ultracentrifuge. The same dissociation into subunits is observed in the presence of high concentrations of salts — in 1 M NaCl. This explains the appearance of fraction B with mol. wt. 20,000 on the rechromatography of fraction A. On further acidification, at pH below 3.8, the enzyme denatures irreversibly. When fraction A was subjected to gel filtration G-100, part of the molecule (∿20%) dissociated into active half-molecules.

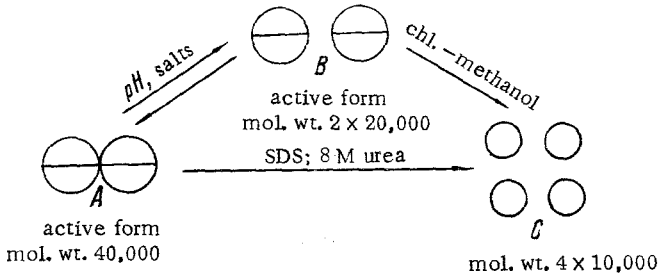
On the basis of the facts presented it may be concluded that A consists of two B subunits.

When fraction A was incubated in a 2% solution of SDS in 8 M urea, it decomposed into four polypeptide chains (we shall denote them by C) with mol. wt. 11,000 according to the results of ultracentrifuging and 10,000 according to gel filtration through a column of Sephadex G-75 equilibrated with 0.1% SDS solution using as markers adrenocorticotropic hormone (mol. wt. 4000), insulin (mol. wt. 6000), and cytochrome C (mol. wt. 12,000).

Various esterases are isolated from tissue homogenates in the form of lipid-containing aggregates [2, 10]. In view of this, it was of interest to determine whether lipids were present in our enzyme preparations and if so, what their role is.

The active fraction B with mol. wt. 20,000 was repeatedly treated with chloroform—methanol (2:1). The protein was separated by centrifuging and the extract was investigated for the presence of lipids and phospholipids. The protein after this treatment was investigated on the ultracentrifuge. It was found that its molecular weight (10,000) was equal to that of fraction C formed after the incubation of fraction A in SDS, although the protein obtained was inactive. It is obvious that subunit B, having mol. wt. 20,000, consists of two polypeptide chains C with mol. wt. 10,000 connected to one another by means of lipids. The dry weight of the lipids amounted to ∿10% of the weight of the subunits. After the separation of the lipid fraction by TLC in the petroleum ether—diethyl ether (4:1) system it was found that the phospholipids made up 80% and the neutral lipids 20% of the lipid fraction. The phospholipid fraction was investigated by TLC in the chloroform—methanol—water (63:25:4) system and it was found that in R_f value it coincided with polyglycerophosphatides.

Thus, the cottonseed triacetinase is an oligomeric protein. At a neutral pH it exists in the form of a tetramer which is capable at low pH values and high concentrations of salts of decomposing into two active subunits. These subunits, in their turn, consist of two protomers connected with one another through lipids.



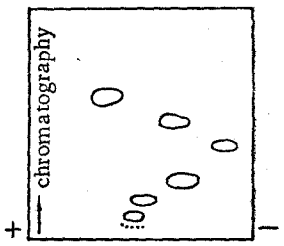


Fig. 2. Peptide maps of the BrCN-cleaved forms A, B, and C of the enzyme.

It follows from our results that in the process of binding the protomers into the half-molecule, a decisive role is played by hydrophobic interactions, and association of the active subunits into the native enzyme takes place mainly through hydrogen bonds. It has been established that the N-terminal amino-acid of fractions A, B, and C is methionine. All the fractions are very similar in amino-acid composition (mole/mole):

Amino acid	A	B	C
Aspartic acid	9.3	10.1	10.5
Threonine	7.2	7.8	7.0
Serine	10.1	10.6	9.8
Glutamic acid	13.4	13	15
Proline	3.5	4	3.3
Glycine	14.4	15	14
Alanine	10.2	11	11
Valine	3.1	3.6	4.0
Methionine	2.5	2.8	2.3
Isoleucine	3.9	4.1	3.6
Leucine	5.7	6.0	6.2
Phenylalanine	3.9	4.1	3.8
Histidine	2.0	1.8	2.0
Lysine	5.1	4.6	4.2
Arginine	7.2	7.7	7.8
Tyrosine	1.5	1.1	1.6
1/2 Cystine	2.0	1.7	1.8

A comparison of all the forms of the enzyme was made by the method of peptide maps. Reduced and carboxymethylated A, B, and C were cleaved with BrCN under similar conditions. All three maps proved to be completely identical, containing six ninhydrin-positive spots (Fig. 2).

EXPERIMENTAL

The triacetinase of the seeds of the cotton plant of variety 108-F was isolated from 30-150 g of an acetone powder of the seeds by the method described previously [1]. Nitrogen was passed through all the solutions to prevent the oxidation of the enzyme. The activity was determined as in [1] in a Radiometer (Denmark) TTT-1 automatic titrator at pH values of 7.4 and 8.8 using tributyrin as the substrate. Disc electrophoresis and the determination of the terminal amino acid were performed by standard methods.

The molecular weights were determined by gel filtration [12]. In each case, 10 mg of enzyme was deposited on a column (1.5 × 80 cm) containing Sephadex G-100 equilibrated with 0.01 M phosphate buffer. The rate of elution was 16 ml/h. Then a calibration graph of the dependence of V_e/V_o on log mol. wt. was plotted from which the molecular weights of A and B were calculated. The molecular weight of C was determined in the same way on a column (1.5 × 80 cm) of Sephadex G-75 equilibrated with a 0.1% solution of SDS.

Ultracentrifugation was performed by the method of unestablished equilibrium at 22,000 and 8000 rpm on a MOM 3170 ultracentrifuge.

The reduction and carboxylmethylation of A, B, and C were performed on 15 mg of the substance concerned by a known method [11]. After carboxymethylation, the solution was acidified to pH 4.0 and was desalted on a column of Sephadex G-25 fine equilibrated with 0.1 M acetic acid.

Cleavage with BrCN. The carboxymethylated protein was incubated in a 75% solution of formic acid with a 100-fold, calculated on the methionine, excess of BrCN at room temperature for 30 h. After this, the solution was diluted 10-fold with double-distilled water and was freeze-dried three times.

Peptide Maps. The BrCN hydrolyzate (100-200 γ) was deposited on plates 20 × 20 cm with a thin layer of cellulose of type FND (GDR) and chromatography was performed in the Bu—AcOH—Py—H_2O (15:3:10:12) system. Then electrophoresis was performed in pyridine acetate buffer, pH 6.5, and U = 940, J = 25 mA, for 35 min on a cooled plate. The spots were revealed with a 0.1% solution of ninhydrin.

SUMMARY

1. It has been established that the triacetinase of cotton seeds with mol. wt. 40,000 consists of two active subunits with mol. wt. 20,000, each of which in its turn consists of two protomers with mol. wt. ∿10,000 bound to one another by lipids.

2. The conditions for the dissociation and association of the subunits have been determined. It has been shown by the method of peptide maps that the subunits consists of identical polypeptide chains.

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