Purification and Characterization of a Palmityl-CoA Thioesterase from *Rhodopseudomonas sphaeroides*

by

Stephen Glenn Boyce
Department of Biology

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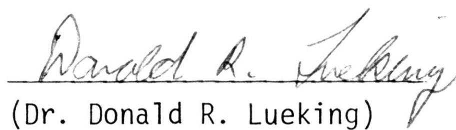
Approved by:



(Dr. Donald R. Lueking)

April 1982

Abstract

A palmityl-CoA thioesterase from Rhodopseudomonas sphaeroides has been purified 625-fold. The enzyme was homogenous as judged by both a constant ratio of thioesterase activity to absorbing material at 280 nm in fractions collected during gel filtration, and by the presence of a single major band on SDS polyacrylamide gels. The enzyme molecular weight is estimated at 21,000 D from its electrophoretic mobility on SDS polyacrylamide gels and its relative elution volume on a standardized gel filtration column of G-100. The thioesterase apparently is a single polypeptide and catalyzes the hydrolysis of long-chain fatty acyl thioesters of coenzyme-A whose acyl moiety is 12 to 18 carbons in length. With palmityl-CoA as a substrate, the enzyme exhibited a Km of 4.2 μM, and a Vmax (calculated) of 13.9 μ moles of palmityl-CoA hydrolyzed per min per mg protein. The enzyme was one of at least three thioesterases separable by gel filtration and ion exchange chromatography from the soluble protein fraction of R. sphaeroides. Its characteristics with respect to molecular weight, activity on palmityl-CoA, and substrate specificity indicate that it is very similar to the palmityl thioesterase II from Escherichia coli.

Acknowledgments

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Introduction[1]

In prokaryotic organisms, the intermediates of fatty acid metabolism exist as acyl thioester derivatives of both coenzyme A (CoA) and acyl carrier protein (ACP). ACP is a low molecular weight protein commonly associated with the fatty acid synthetase (FAS) complex in prokaryotic organisms possessing type II synthetases (1). Both ACP and CoA contain 4'-phosphopantetheine as a prosthetic group, and serve as acyl carriers during fatty acid synthesis and catabolism, respectively (2).

The primary products of *de novo* bacterial fatty acid synthesis are the vaccenyl ($C^{\Delta 11}_{18:1}$) and palmityl ($C_{16:1}$) thioester derivatives of ACP. These derivatives are freely dissociable from the bacterial FAS and are capable of serving as immediate substrates for phospholipid biosynthesis. Additionally, in *Escherichia coli*, acyl-CoA's have also been shown to be capable as serving as an acyl donor for phospholipid biosynthesis. Furthermore, investigations in *E. coli* have revealed that the acyl group may be removed from CoA and to a much lesser extent from ACP by enzymes termed thiosterases (3-6). Two distinct acyl thioesterases capable of catalyzing the hydrolysis of long chain fatty

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[1]The format followed in this text is that of the Journal of Bacteriology, a publication of the American Society for Microbiology.

acyl thioesters of CoA and ACP are present in the soluble protein fractions of *E. coli*, and have been purified to homogeneity (4,5). These thioesterases are designated as thioesterases I and II in the present paper.

In previous studies the nomenclature of the thioesterases in *E. coli* has been confusing since the 22,000 molecular weight enzyme has been referred to as both thioesterase I (3,4,6) and thioesterase II (5). Bonner and Bloch's nomenclature (5) is adopted here for they designated the thioesterases in accordance with the order in which they eluted during gel filtration from a column of G-100. Thus, the 122,000 molecular weight enzyme is thioesterase I, and the 22,000 molecular weight enzyme is thioesterase II.

E. coli thioesterase I exhibits a preference for long chain saturated fatty acyl thioesters as substrates with palmityl-CoA supporting optimal activity (Km=6.2 μM, and Vmax = 277 n moles of palmityl-CoA hydrolyzed per min per mg protein); however, it is capable of hydrolyzing acyl thioesters of CoA with acyl groups ranging from C_6 to C_{18} (5). When *E. coli* thioesterase I was examined utilizing palmitoeyl ($C^{\Delta 9}_{16:1}$), oleyl ($C^{\Delta 9}_{18:1}$), and cis-vaccenyl ($C^{\Delta 11}_{18:1}$) acyl-CoA's, the enzyme displayed similar kinetic constants and activities with all three substrates (Km=4.0 μM, and Vmax=140 n moles acyl-CoA hydrolyzed per min per mg protein) (5). This thioesterase was also found to catalyze the hydrolysis of β-hydroxyacyl-CoA derivatives with D(-) β-hydroxymyristyl-CoA being the preferred substrate (Km= 0.85μM, and Vmax= 110 n moles of β-hydroxymyristyl-CoA hydrolyzed per min per mg

protein) (5).

Acyl-ACP derivatives were also shown to be hydrolyzed by thioesterase I, but at a rate much reduced (3 to 7%) relative to the coresponding acyl-CoA derivatives (3,5). This reduction of enzyme activity was presumed to be due to the acylation of amino groups on ACP which occurred during the chemical synthesis of the acyl-ACP substrates (3,5); however, thioesterase hydrolysis of native acyl-ACP substrates prepared enzymatically using acyl-ACP synthetase proved to be even more resistant to hydrolysis by both thioesterase I and II (6). Thioesterase I hydrolysis of enzymatically synthesized palmityl-ACP was shown to be only 0.01% of that observed with palmityl-CoA (6). The enzyme was insensitive to diisopropyl fluorophosphate (DFP); was optimally active at a pH of 8.4; and preferred substrates whose acyl moiety was C_{10} or longer (3,4,5). After an 8,400 fold purification, thioesterase I was shown to be pure on polyacrylamide gels. It's molecular weight, determined by gel filtration, was 122,000 D (assuming a globular protein). The enzyme is thought to exist as a tetramer composed of four identical subunits each having a molecular weight of 30,000 D (5). The purified thioesterase I exhibited a specific activity of 2.3×10^5 n moles palmityl-CoA hydrolyzed per min per mg protein (5).

One early proposed role for thioesterase I was that it interrupted fatty acid synthesis to provide β-hydroxymyristic acid for the production of the lipopolysaccharide component of the outer membrane of Gram-negative organisms (3).

E. coli thioesterase II also catalyzes the hydrolysis of long chain fatty acyl thioesters of CoA and ACP to form free fatty acids and the respective thiols. Acyl-CoA thioester substrates with acyl moieties of C_{10} or less were not hydrolyzed, and the enzyme was inactive on all β-hydroxyacyl-CoA compounds tested (3). Palmityl-CoA (Km=3.9μM, and Vmax=4.8μM palmityl-CoA hydrolyzed per min per mg protein) was preferred over lauryl ($C_{12:0}$), myristyl ($C_{14:0}$), and stearyl-CoA's ($C_{18:0}$). With unsaturated fatty acyl CoA's as substrate, thioesterase II displayed slightly higher rates of hydrolysis than with the saturated fatty acyl CoA's as substrates. Cis-vaccenyl and palmitoleyl-CoA's were preferred to oleyl-CoA (4). Chemically synthesized palmityl-ACP was hydrolyzed at 7% (4), while enzymatically synthesized palmityl-ACP was hydrolyzed at only 0.6% (6) of the rate that palmityl-CoA was hydrolyzed by thioesterase II. The enzyme is thought to exist as a single ploypeptide with a molecular weight of 22,000 D as determined by SDS polyacrylamide gel electrophoresis and gel filtration on standardized columns of Sephadex G-100. Sensitivity to DFP was also reported (3-6). After a 260-fold purificaiton the enzyme was pure and displayed a specific activity of 3700 n moles of palmityl-CoA hydrolyzed per min per mg protein (4). This enzyme has been shown to be capable of mediating the release of free fatty acids form the *E. coli* FAS by terminating chain elongation (4). Other proposed roles for thioesterase II include the conservation of ACP *in situ* and controlling the levels of palmityl thioesters of CoA and ACP which may inhibit some enzymes due to their detergent properties (4).

The functions of the thioesterases in *E. coli* are simply not known. A more recent proposal suggested that thioesterases are required for fatty acid oxidation (William Nunn, unpublished). Nunn's studies show an increase (4 to 5 fold) of thioesterase activity in cultures of *E. coli* grown on medium containing free fatty acids as the sole carbon source. This role, however, seems unlikely because the substrate required by both the β-oxidation and thioesterase enzymes is the acyl-CoA form of the fatty acid; therefore, it is paradoxical that thioesterase activity would increase under these conditions.

The *E. coli* system, from which the thioesterase have been purified and characterized, is not the most desirable model system for determining whether or not the thioesterases play a role in prokaryotic phospholipid metabolism. The true substrate for phospholipid biosynthesis in *E. coli* has not been determined. Both the acyl-ACP and acyl-CoA thioester derivatives are capable of serving as the acyl donor for phospholipid biosynthesis. Also, *E. coli* cannot be manipulated to control the amount and time phospholipid biosynthesis occurrs under normal physiological conditions. Thus, it is impossible to monitor thioesterase activity under conditions where differing ammounts of phospholipids are required. In addition, no information is available regarding the genetics of the thioesterases for, in *E. coli*, the phenotype of a thioesterase negative mutant is unknown.

For these reasons, the Gram-negative, facultatively photohetero-trophic, non-sulfur-purple bacterium *Rhodopseudomonas sphaeroides* has been employed for the purification and characterization of a palmityl-CoA thioesterase. The utility of *R. sphaeroides* is seen in that one

can easily control both the time and the amount of phospholipids produced by this organism (8). It is also known that only the acyl-ACP thioester derivative serves as the acyl donor in phospholipid biosynthesis (7). Finally, the selection of a putative thioesterase negative mutant is made possible by the discovery that the normal prototroph *R. sphaeroides* can resume growth in the presence of exogenously supplied fatty acids when *de novo* fatty acid synthesis is completely inhibited with the antibiotic cerulenin.

Materials and Methods

Chemicals: All materials utilized were analytical grade. These compounds were purchased form the following sources: bisacrylamide, acrylamide, and tetramethylethylenediamine (TEMED) from Bio Rad; hydroxylapatite from LKB; Sephadex G-100, and G-200 from Pharmacia Fine Chemicals; nicotinic acid, thiamin-HCl, and biotin from Eastman Kodak; 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), lauryl-CoA, myristyl-CoA, palmityl-CoA, oleyl-CoA, stearyl-CoA, vaccenic acid, linoleic acid, linolenic acid, coenzyme-A, bovine serum albumin, ovalbumin, pepsin, soybean trypsin inhibitor, lysozyme, and 5'-adenosine tri-phosphate from Sigma.

Organism, Medium, and Growth Conditions: *Rhodopseudomonas sphaeroides* strain M29-5 (leu^-,met^-), derived from strain 2.4.7, was obtained from Samuel Kaplan, University of Illinois. The organism was routinely grown in a minimal medium (initial pH 6.8) containing per liter: 3.0g succinic acid, 0.1g glutamic acid, 40 mg aspartic acid, 0.8g ammonium sulfate, and additional inorganic salts as well as trace elements and vitamins as specified by Sistrom (9). Sistroms medium was typically prepared in a 10-X concentrated form and stored frozen in 100ml aliquots. Medium employed for the growth of strain M29-5 was supplemented with 0.2% (w/v) Casamino acids. Stock cultures were maintained at -20° C in the above medium adjusted to 10% (w/v) glycerol.

Incubations were conducted photoheterotrophically (anaerobic in the light) in completely filled screw cap vessels (16 to 160 mls),

in flat walled vessels (2 to 5 liters), or in 20 liter carboys completely filled or under an atmosphere of 95% N_2 and 5% CO_2. Either procedure created an environment with less than 0.1 part per million O_2 as determined with a YSI model 54S oxygen meter.

Cells previously adapted to logarithmic phase growth were used as the inoculum source for all studies. Incubations were performed at 30 to 32° C with continious saturating illumination (500 ft-c) provided by a bank of 60-Watt Lumiline (Sylvania) lamps. Culture growth was followed turbidimetrically using a Klett-Summerson colorimiter equipped with a number 66 filter. Cells were harvested at 450 Klett units with a typical cell yield from one liter of 5.0 grams (wet weight).

Spectrophotometric Assay for Thioesterase Activity: Acyl-CoA's are hydrolyzed in the presence of thioesterase to form the free fatty acid and CoA-SH. The assay system is based upon the quantitation of the free sulfhydryl moiety of CoA-SH employing Ellman's (10) thiol reagent DTNB. The release of CoA-SH is measured by monitoring the increase in optical density at 412 nm resulting from the production of the colored 2-nitro-5-thiobenzoate anion (10). A recording Beckman split-beam spectrophotometer was used for these measurements (ε_{412}=13,600), while each sample was blanked with a reference solution prepared exactly as the sample (minus substrate). A typical assay contained: 0.5ml of 0.1M Tris-HCl buffer (pH 8.0 at room temperature); 12µl of 2.5mM palmityl-CoA; 10µl of 10mM DTNB; with protein and distilled H_2O being added to a final volume of 1.0 ml. The initial

velocity of the reaction was taken as a measure of thioesterase activity with an enzyme unit being defined as that amount of enzyme necessary to catalyze the hydrolysis of 1.0 pmole of palmityl-CoA per minute. Specific activity was defined as nanomoles of palmityl-CoA hydrolyzed per minute per milligram protein. All assays were performed at room temperature.

Preparation of Acyl-CoA Thioesters: The CoA thioesters of vaccenic, linoleic, and linolenic acids were prepared as described by Merrill et al. Six adult female rats (Charles River strain or equivalent) were killed by decapitation and livers removed (0°C). The livers were washed, minced, and homognized in buffer "A" containing; Tris-HCl (pH 7.4), sucrose, EDTA, and DTT. The homogenate was centrifuged at 24,000 x g for 15 min., then for 1 hr. at 105,000 x g. The high speed pellet produced was resuspended in 100 mls of the above buffer (minus sucrose) and again centrifuged at 105,000 x g for 1 hr. producing a washed pellet. This pellet was resuspended in buffer "A" (minus sucrose), and 4.0 mls of 20% (w/v) Triton x-100 were added at 4°C. After mixing for one hour the insoluble material was removed by centrifugation at 105,000 x g for 1 hr. and the resulting supernatant (containing the detergent solublized acyl-CoA synthetase was stored frozen in 25ml aliquots at -70°C.

The Acyl-CoA synthetase was immobilized on a column of matrix red (Amicon) prepared according to manufacturers recomendations and equilibrated in buffer "B" containing; Tris-HCl (pH 7.4), Triton X-100, and DTT. The synthetase was further pruified by washing the column in succession with buffer "C" (containing Tris-HCl

pH 8.0, Triton x-100, and DTT), buffer "C" plus 0.25 MNaCl, buffer "C" plus 10mM ATP, and again with buffer "C".

A reaction mixture containing the free fatty acid, Triton x-100, DTT, Tris-HCl, ATP, $MgCl_2$, and CoA was then cycled over the column for 12 hrs. at room temperature, after which time, the eluent was collected. The eluent was then adjusted to 1.0 M LiCl and applied to a column of octyl-sepharose. Unbound material was removed, and a decreasing linear gradient (1.0 to 0.0 M LiCl) was applied to the column and 5.0 ml fractions collected. Fractions displaying an increased absorbance at 232nm, 259nm, and 280nm were pooled. Soluble material in these pooled fractions was precipitated with 1.0% (v/v) $HClO_4$ and trace impurities were removed by washing the precipitable material in acetone, then in diethyl ether. The ether pellet was then resuspended in H_2O.

All substrate concentrations were determined by monitoring total adenine (for CoA) at 259nm employing a molar extinction coeficient of 15,900 (11). The substrates were also monitored for absorbance of the thioester bond at 232nm (ε_{232} = 9,400) (12). CoA free sulfhydyl was determined using the normal assay conditions (minus enzyme), and any excess CoA present as determined by abosrbance at 259nm, and not accounted for as thioester bond or free sulfhydryl, was presumed to be in the form of a CoA disulfide.

<u>Protein Determination</u>: Protein was determined by the method of Lowry et. al. (13), employing bovine serum albumin as standard.

SDS Ployacrylamide Disc Gel Electrophoresis: The method of Laemmli (14) was used for sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoresis. A stacking gel of 3% acrylamide (160mm x 10mm) and a resolving gel of 7.5% to 15% acrylamide (160mm x 150mm) were run at a constant current of 25 mA at 10°C with a Tris-glycine electrode buffer (pH 8.3 at 10°C). All wells contianed 30μg total protein. Lysozyme (14,314D), Soybean Trypsin Inhibitor (20,095D), Pepsin (34,700D), Ovalbumin (45,000D), and Bovine Serum Albumin (66,296D) were used as standards for molecular weight determinations.

Purity and Molecular Weitht Determination of Native Protein: The molecular weight of the native protein was determined by its behavior on a column of Sephadex G-100 standardized with the above molecular weight standards. Two criterion for judging pruity was the presence of a single band on SDS gels (IIB was a single polypeptide), and a constant ratio of units of activity/absorbance at 280nm in fractions collected from a column of G-100.

Preparation of Cell Free Extract: Soluble protein fractions possessing the enzyme thioesterase were prepared from cells in the stationary phase of growth (450 Klett units) which were harvested either at 10,000 x g for 10 minutes in 250 ml bottles, or at 15,000 rpm's using a SS 34 rotor equiped with the Szent-Gyorgyi and Blum continous flow aparatus for the Sorval RC2-B centrifuge at a flow rate of 150 mls per min. The cells were washed once in 20 mMKH_2PO_4 pH 7.0 at 4°C and stored frozen at -20°C. Average cell yields, depending upon method used in harvesting, were approximately 5.0 grams cells wet weight per liter. 160 grams of R. sphaeroides

M29-5 were thawed in 20mM KH_2PO_4 at 4°C, and disrupted by passage (2 times) through a French Pressure Cell at 16,000 psi. The resulting suspension was freed of whole cells and cellular debris by centrifugation at 10,000 x g for 15 minutes. The resulting crude supernatant was freed of particulate matter by centrifugation at 150,000 x g for 1 hr. (w^2t = 6084) resulting in the high speed supernatant. The pellets obtained at this step were resuspended in a minimal volume of 20 mM KH_2PO_4 pH 7.0 and centrifuged for 1 hr. at 150,000 x g. The supernatant was decanted and pooled with the high speed supernatant. This high speed supernatant was utilized as the immediate source of thioesterase activity.

Preparation Of Palmityl-CoA Thioesterase IIB:

Streptomycin Sulfate: The high speed supernatant was treated with streptomycin sulfate as described by Majerus et. al. (15). Ten percent streptomycin sulfate (pH 7.0), ten mililiter per gram of protein, was added dropwise with constant stirring to the high speed supernatant at 4°C. This solution was mixed for 30 min, and the insoluble mater was precipitated by centrifugation at 10,000 x g for 15 min. This resulted in the streptomycin sulfate supernatant containing the thioesterase activity, and the pellet was discarded.

Ammonium Sulfate Fractionation: The streptomycin sulfate supernatant was adjusted to 30% $(NH_4)_2SO_4$ with saturated $(NH_4)_2SO_4$ pH 7.0 at 4.0°C. After mixing for one hour at 4.0°C this suspension was centrifuged at 10,000 x g for 15 min. and the pellet discarded. This 30% supernatant was adjusted to a final $(NH_4)_2SO_4$ concentration of 80% with either saturated ammonium sulfate pH 7.0 or solid ammonium sulfate

(after adjusting the buffer concentration to 0.2 M KH_2PO_4 pH 7.0 with 1.0 M KH_2PO_4 pH 7.0), mixed for 1 hr. at 4°C, and centrifuged at 10,000 x *g* for 1 hr. The supernatant was discarded, and the pellet was re-suspended in a minimal volume of 20 mM KH_2PO_4 buffer.

Gel Filtration: Two Pharmacia Fine Chemical Co. K50/60 and one K50/100 columns were packed with pre-swollen (as recommended by the manufacturer) Sephadex G-200 in 20mM KH_2PO_4 buffer. A constant pressure and flow rate (33 mls/hr) were maintained during packing and use with a P-3 peristaltic pump. The columns were hooked in tandem to give a column 5.0 cm in diameter with an effective bed heighth of 187 cm. The void volume (1,600 mls) and column packing were checked with blue dextran. No more than 200 mls of the ammonium sulfate resuspended 80% pellet (5% of the bed bolume) was loaded on the column at one time. 15 ml. fractions were collected.

Ion Exchange: Diethyaminoethyl (DEAE) Sephadex A-25 preswollen in 20mM KH_2PO_4 buffer (conductivity of 1.2 mmho's at 5°C) was packed under a flow of 33.0 mls/hr. in a 2.5 cm diameter column to give a final bed heighth of 40 cm. The column was washed with buffer until the conductivity of the eluent equaled that of the buffer. The second peak of thioesterase activity (G-200) was then diluted with distille d H_2O to a final conductivity of 1.2 mmho's or less, then applied to the column at the same flow rate. A linear gradient of increasing NaCl concentration 0.0 to 0.5M NaCl) was applied to the column, 2.0 ml fractions were collected and fractions exhibiting activity were pooled.

Hydroxylapatite: The second peak of thioesterase activity eluted from

the DEAE column was then applied to a column of hydroxylapatite (equilibrated in 20mM KH_2PO_4 buffer 4°C) without destalting, and the material which did not bind was collected.

Octyl-Sepharose: All previous steps were performed at 4°C; however, this step was performed at room temperature. All solutions used were extensively degassed. A small column of octyl-sepharose was equilibrated in 20mM KH_2PO_4 buffer and packed under a flow of 33.0 mls/hr. The hydroxylapatite fraction was loaded onto the column and material not binding was removed. The column was then washed with 20mM KH_2PO_4 buffer until the absorbance at 280 mm reached baseline. The thioesterase activity was then eluted with 3 bed volumes of the same buffer adjusted to 35% (w/v) 2-propanol onto a small column (in tandem) of DEAE-A-25 which was equilibrated at room temperature in the 20mM KH_2PO_4 buffer. After washing octyl-sepharose with the buffer-35% 2 propanol solution (onto DEAE), the columns were separated and the DEAE was washed with 25 bed volumns of 20mM KH_2PO_4 buffer to remove any remaining 2-propanol. The DEAE column with bound thioesterase was then equilibrated in the cold (4°C) and eluted with buffer adjusted to 25 mmho's conductivity with NaCl at 4°C (o.53 M NaCl).

G-100: The octyl-sepharose material removeable with 2-propanol was applied to a column of G-100. Fractions were monitored for absorbance at 280nm and those exhibiting a constant ratio of activity/absorbance at 280nm were pooled and utilized for further studies.

RESULTS

Purification of palmityl-CoA thioesterase II B from R. sphaeroides— After disrupting *R. sphaeroides* as described previously, the suspension was centrifuged at 10,000 x g for 10 min. No thioesterase activity was localized in the pellet and the supernatant typically exhibited a specific activity of 5 to 6 n moles of palmityl-CoA hydrolyzed per min per mg protein. The activity localized in this crude soluble fraction was then calculated to be 100%.

The crude soluble fraction was freed of particulate material by centrifugation at 150,000 x g for 1 hr. yielding a clarified soluble protein fraction. In this high speed supernatnat, greater than 95% of the total thioesterase activity was localized. The high speed supernatant possessed a thioesterase specific activity of 7 to 8 n moles of palmityl-CoA hydrolyzed per min per mg protein.

At this point the nucleic acids were precipitated with streptomycin sulfate. This step did not give a significant increase in specific activity, however, it did decrease the viscosity of the high speed supernatant and eliminated the possibility of the interference due to nucleic acids later in the purification scheme. In order to eliminate the heavily pigmented material, which possessed no thioesterase activity, the streptomycin sulfate supernatant was then adjusted to 30% ammonium sulfate and the insoluble material was discarded. Greater than 80% of the remaining units of activity could be precipitated by adjusting the 30% $(NH_4)_2 SO_4$ supernatant to 80% $(NH_4)_2 SO_4$ and harvesting the precipitated material.

After resuspending the 80% ammonium sulfate pellet into a minimal volume of buffer the material was then applied to a 5 x 187 cm column of G-200, eluted and 15 ml fractions collected. We repeatedly observed a symetrical distribution of protein in the fractions collected. (Fig. 1). The thioesterase activity, however, was shown to elute as two distinct peaks totally separable by gel filtration. Each peak of thioesterase activity contained approximately 50% of the total activity recovered. Fractions containing the separate activities were pooled for the quantitation of enzyme yields. Up to 90% of the total units applied to the column were localized in these two pooled fractions. These peaks were designated thioesterase I and thioesterase II based the order in which they were eluted during gel filtration. Thioesterase I possessed a specific activity of 5 to 6 n moles of palmityl-CoA hydrolyzed per min per mg protein and thioesterase II possessed a specific activity of 17 to 20 n moles palmityl CoA hydrolyzed per min per mg protein.

The material comprising peak II from G-200, because it was more pure and corresponded roughly to the second thioesterase from E. coli, was then applied to a column of the ion-exchange resin, DEAE-A25. An increasing linear salt (NaCl) gradient was applied to the column and analysis of the fractions collected revealed that both the protein and thioesterase activities eluted as two distinct peaks (Fig. 2). These peaks of palmityl thioesterase activities were designated thioesterase II A and thioesterase IIB. The fractions of these activity peaks were pooled separately. Thioesterase II A eluted

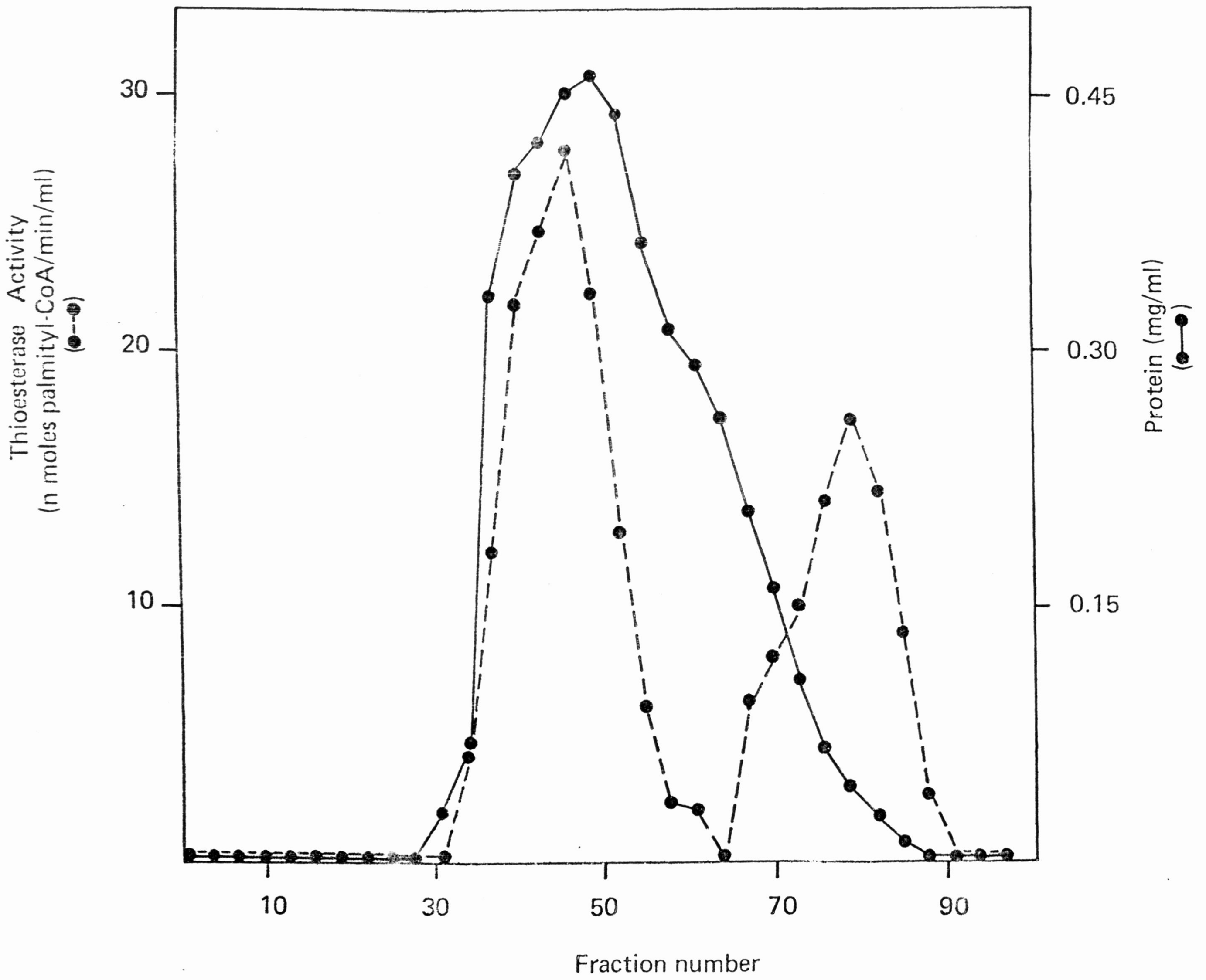
30
20
10
0.45
0.30
0.15
Thioesterase Activity
(n moles palmityl-CoA/min/ml)
Protein (mg/ml)
10
30
50
70
90
Fraction number

Figure 1 -- Soluble protein and thioesterase activity elution profile obtained from G-200. In the 15.0 ml fractions collected, the symmetrical protein distribution and resolution of two thioesterase activities (designated thioesterase I and II) were consistently obtained.

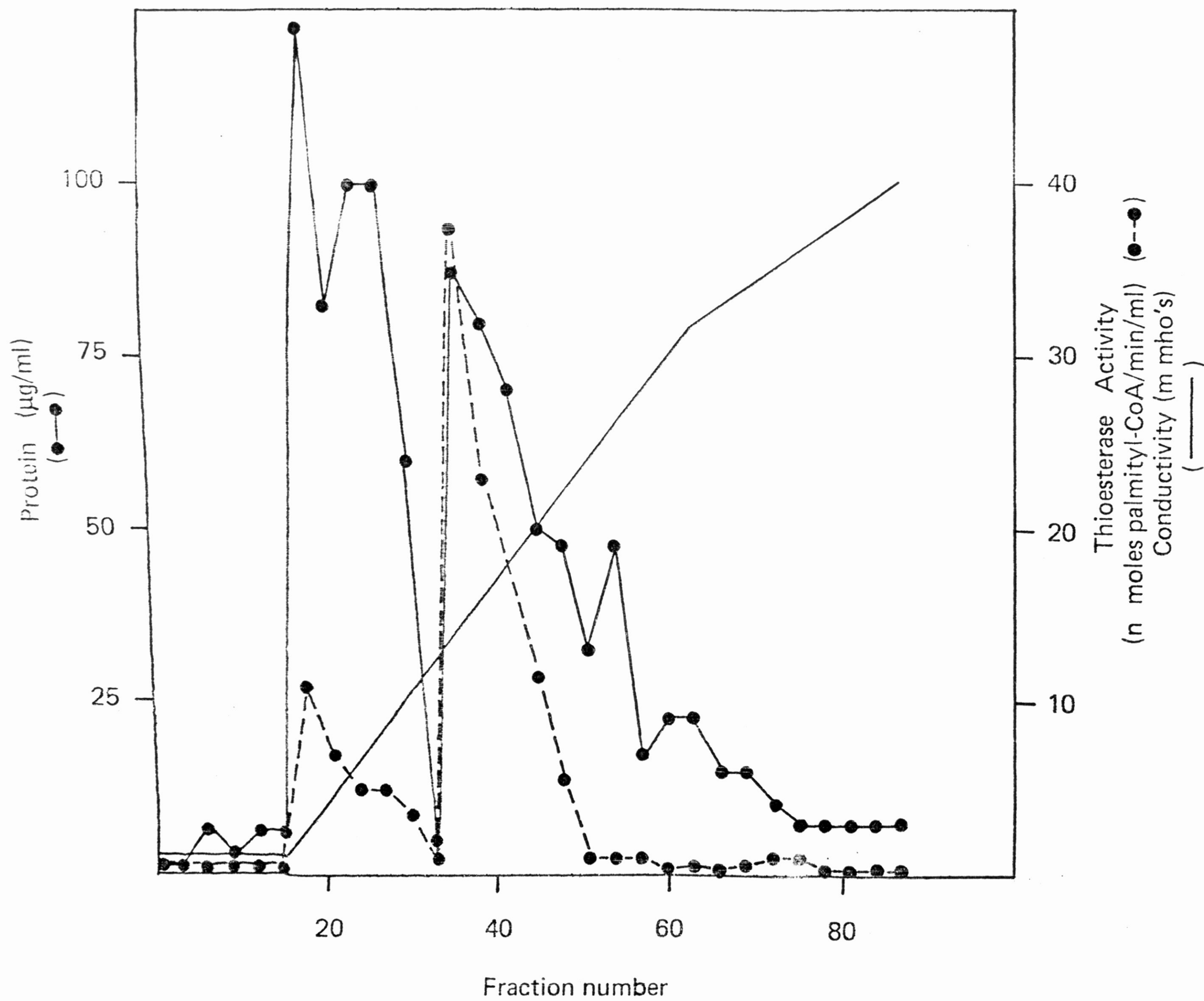
100
75
50
25
Protein (µg/ml)
(●—●)
40
30
20
10
Thioesterase Activity
(n moles palmityl-CoA/min/ml) (●--●)
Conductivity (m mho's)
(——)
20
40
60
80
Fraction number

Figure 2 -- Ion exchange (DEAE) chromatographic resolution of thioesterase activity peak II into thioesterases II A and II B.

from DEAE between 3 and 7 mmho's conductivity (.03 to 0.1 M NaCl) and possessed approximately 20% of the total units of activity recoverable. Thioesterase II A exhibited a specific activity of 18 n moles of palmityl-CoA hydrolyzed per min per mg protein. Thioesterase II B constituted the remaining 80% of the units obtained at this step and eluted between 12 to 20 mmho's conductivity (0.2 to 0.4 M NaCl) at 4°C. Thioesterase II B had a specific activity of 95 to 110 n moles of palmityl-CoA hydrolyzed per min per mg protein and represented an 18 fold purification over the crude supernatant. The activities in the pooled fractions of thioesterases II A and IIB accounted for greater than 90% of the total units of activity applied to the column.

The thioesterase II B pooled fraction containing a high NaCl concentration (0.3M NaCl) was then applied to a small column of hydroxylapatite. Under these conditions thioesterase IIB would not bind and 97% of the palmityl-CoA thioesterase activity was localized in the void fractions: Although thioesterase IIB did not absorb, a purification was obtained at this step since extraneous (non-thioesterase) proteins did absorb. The specific activity of the thioesterase in the pooled void fractions after hydroxylapatite was 172 n moles of palmityl-CoA hydrolyzed per min per mg protein and represented an overall purification of 31 fold over the crude supernatant activity.

The hydroxylapatite treated material, with high salt, was then applied to a small column of octyl-sepharose at room temperature. Proteins are generally bound to octyl-sepharose under high salt

concentrations in order to increase their hydrophobic interactions and are removed by decreasing the salt concentration of the elution buffer. Thioesterase II B however could only be eluted by decreasing the polarity of the elution buffer. The elution buffer adjusted to 35% 2-propanol could free the thioesterase II B from octyl-sepharose. After removing the 2-propanol, as described in Materials and Methods, up to 90% of the palmityl-CoA thioesterase II B activity could be recovered. The specific activity of this octyl-sepharose fraction was typically 1600 n moles of palmityl-CoA hydrolyzed per min per mg protein and represented a 290 fold purification over the crude supernatant.

A portion of the octyl-sepharose fraction was then applied to a previously standardized column of sephadex G-100. Most of the fractions containing thioesterase activity also displayed a constant ratio of thioesterase activity to absorbing material at 280 nm (Fig. 3). The relative elution volume of the thioesterase activity coresponds to that of a globular protein of 21,000 D (Fig. 4). Those fractions which displayed a constant ratio of thioesterase activity to absorbance at 280 nm were pooled and found to have a specific activity of 3441 n moles of palmityl-CoA hydrolyzed per min per mg protein. This pooled fraction was found to consist of one major band and three minor bands on SDS polyacrylamide gels. The primary band displayed a electrophoretic mobility corresponding to a molecular weight of 21,000 D (Fig. 5).

Chain length specificity - Thioesterase IIB was then assayed with the butyryl ($C_{4:0}$); hexanyl ($C_{6:0}$); decanyl ($C_{10:0}$); lauryl ($C_{12:0}$);

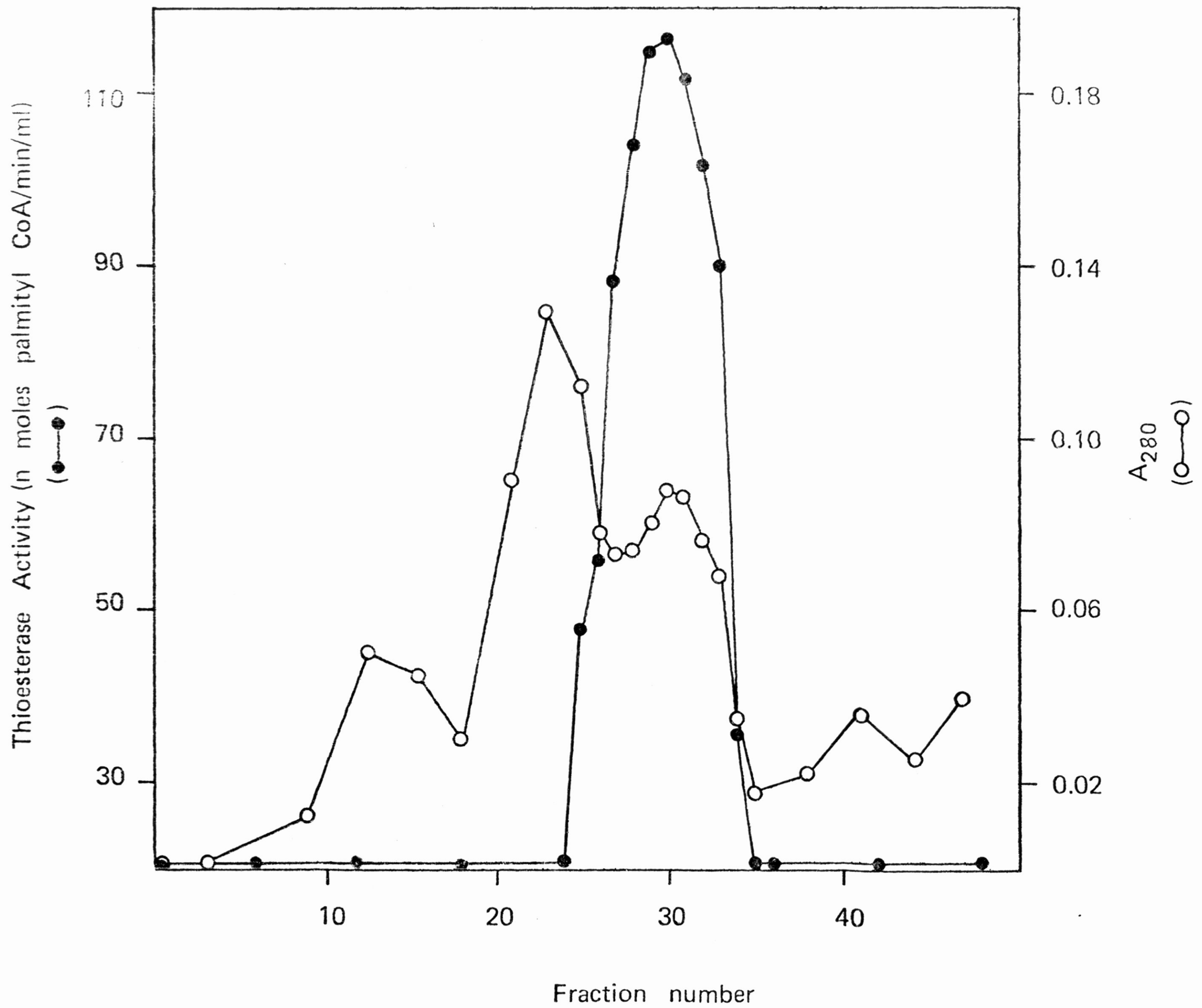
Thioesterase Activity (n moles palmityl CoA/min/ml)
(●—●)
110
90
70
50
30
0.18
0.14
0.10
0.06
0.02
A_{280}
(○—○)
10
20
30
40
Fraction number

Figure 3 -- Thioesterase II B activity peak and profile of 280 nm absorbing material elution profile from G-100. Fractions 27-32 displayed a constant ratio of thioesterase activity to absorbance at 280 nm

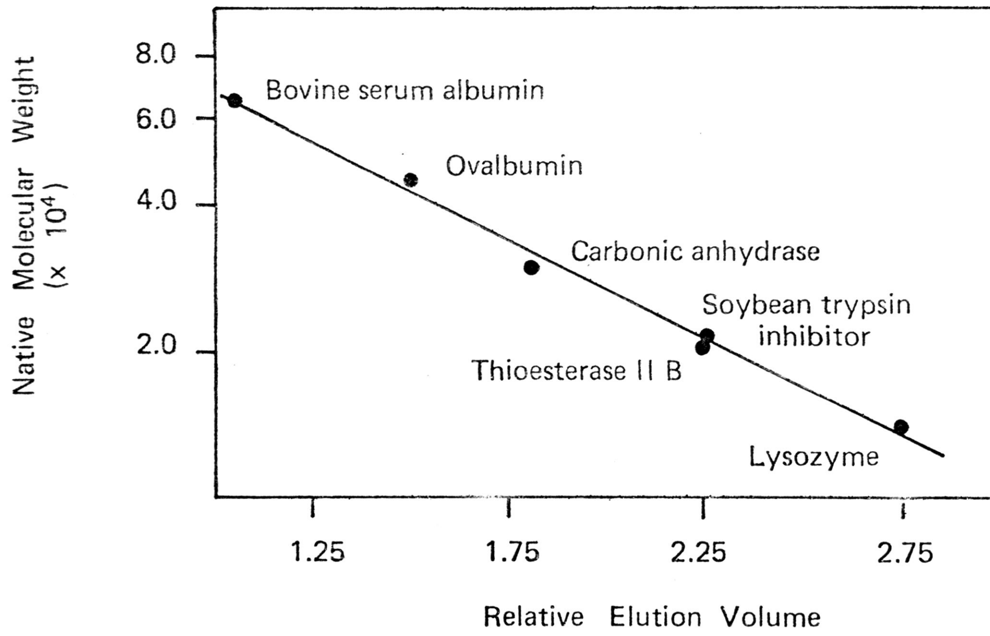
Native Molecular Weight
(x 10^4)
8.0
6.0
4.0
2.0
Bovine serum albumin
Ovalbumin
Carbonic anhydrase
Soybean trypsin
inhibitor
Thioesterase II B
Lysozyme
1.25
1.75
2.25
2.75
Relative Elution Volume

Figure 4 -- Molecular weight determination of native thioesterase II B. A column of G-100 was standardized with the designated standards. The thioesterase II B activity eluted at a relative elution volume of 2.26 corresponding to a molecular weight of 21,000 D. The column void was determined utilizing blue dextran.

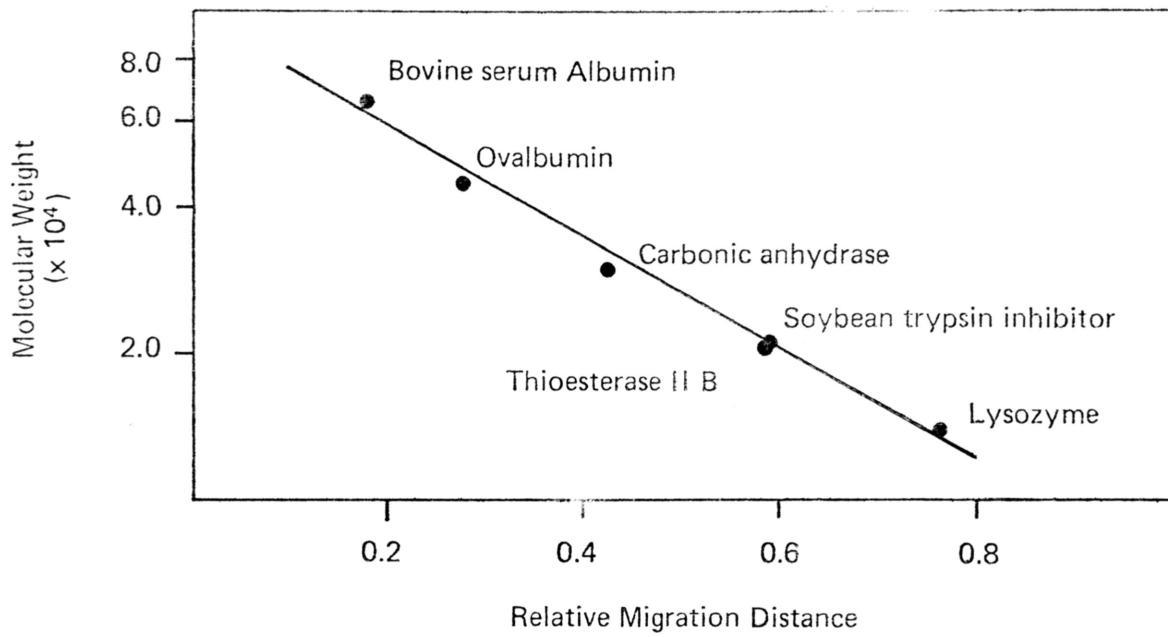
Molecular Weight
(x 10⁴)
8.0
6.0
4.0
2.0
Bovine serum Albumin
Ovalbumin
Carbonic anhydrase
Soybean trypsin inhibitor
Thioesterase II B
Lysozyme
0.2
0.4
0.6
0.8
Relative Migration Distance

Figure 5 -- SDS polyacrylamide gel electrophoresis of thioesterase II B. Relative to bromophenol blue, thioesterase II B exhibited a migration distance of 0.59. Thioesterase II B exists as a single polypeptide with a molecular weight of 21,000 D.

myrystyl ($C_{14:0}$); palmityl ($C_{16:0}$); stearyl ($C_{18:0}$); oleyl ($C^{\Delta 9}_{18:1}$); vaccenyl ($C^{\Delta 11}_{18:1}$); lenoleyl ($C^{\Delta 9,12}_{18:2}$); and lenolenyl ($C^{\Delta 9,12,15}_{18:3}$) thioester derivatives of CoA. Each assay contained saturating (25μM) substrate concentrations, and 0.3μg protein. The enzyme exhibited a strict requirement for the longer chain fatty acyl-CoA's. Thioesterase II B did not exhibit any activity on the acyl-CoA's whith acyl moieties C_{10} or less, and was capable of catalyzing the hydrolysis of substrates containing 12 to 18 carbons in their acyl chains. Thioesterase II B was optimally active on palmit-CoA and cis-vaccenyl-CoA. With palmityl-CoA as substrate, thioesterase II B displayed a Km of 4.2 μM and a V max of 13.9μmoles of palmityl-CoA hydrolyzed per min per mg protein. Under the conditions described, the above thioesterase II B was 40% more active on cis-vaccenyl-CoA than on palmityl-CoA.

DISCUSSION

The purification procedure described in the present paper (Table I) is reproducible with high yields at each step and allows for the purification of a long-chain fatty acyl thioesterase from *Rhodopseudomonas sphaeroides*. *R. sphaeroides* was found to possess at least three palmityl-CoA thioesterase activities localized in the cells soluble protein fraction. The enzyme designated palmityl thioesterase II B was purified 625-fold, and exhibited a specific activity of 3441 n moles of palmityl-CoA hydrolyzed per min per mg protein. The enzyme was pure as judged by a constant ratio of thioesterase activity to absorbance at 280 nm in fractions collected during gel filtration. On SDS polyacrylamide gels the protein ran as one major band with 3 minor contaminating bands.

R. sphaeroides thioesterase II B displayed a Km of 4.2 μM for palmityl-CoA, and Vmax (calculated) 13.9 μ Moles of palmityl-CoA hydrolyzed per min per mg proetin. It is believed to exist as a single polypeptide of 21,000 D as determined by gel filtration and SDS polyacrylamide gel electrophoresis.

The physiological role of these thioesterases is unknown. With the purification of thioesterase II B, however, a powerful tool is now available in terms of elucidating the role this thioesterase. Having antibody to the purified thioesterase II B will make possible the quantitation of thioesterase II B levels in cultures undergoing differing rates of phospholipid metabolism. It would also allow the quantitation of thioesterase levels with respect to

TABLE I

Procedure	Fraction	Specific Activity	Fold Purification	Yield	Protein
		n moles/min/mg		%	mg
Crude Supernatant		5.5	0.0	100.0	5187.0
High Speed Supernatant		7.7	1.4	95.6	3534.0
Streptomycin Sulfate		7.7	1.4	92.7	3428.0
Ammonium Sulfate	30-80%	9.0	1.5	76.0	2423.0
G-200	peak II	16.9	3.1	22.5	382.5
DEAE	peak B	102.3	18.5	17.0	31.1
Hydroxyl-apatite	void	172.8	31.2	16.5	27.4
Octyl-Sepharose	35% 2-propanol	1608.4	201.4	14.9	1.3
G-100		3441.6	625.7	10.9	0.4

the cell cycle in synchonously growing cultures of *R. sphaeroides*. These studies, and additional studies presently being initiated, should ultimately provide valuable information regarding the role(s) of long-chain acyl-CoA (ACP) thioesterases in cellular lipid metabolism.

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