Glycerol-3-phosphate acyltransferase has been purified from the post-microsomal supernatant of cocoa seeds using differential ammonium sulfate solubility and gel filtration chromatography. Chromatofocusing and isoelectric focusing revealed a series of proteins with acyltransferase activity having isoelectric points close to 5.2.

Gel filtration on Sephacryl S-300 in 500 mM NaCl, along with polyacrylamide gel electrophoresis (denaturing and non-denaturing) and immunological analysis, gave evidence that the native enzyme has a molecular weight of \(2 \times 10^6\) and consists of an aggregate of \(10^5\), 20,000 subunits.

The highly purified enzyme carries an acyl donor, probably acyl-CoA, although this is not firmly established. The hydrophobic nature of the purified enzyme was demonstrated by its firm binding to octyl-Sepharose.

Mass spectrometric analysis of reaction products revealed the presence of both palmitic and stearic acids. Considering that 1) the fatty acids were derived from the purified enzyme; 2) they were found exclusively in the 1-position of glycerol 3-phosphate; 3) the fatty acid positioning and composition is consistent with that found in cocoa butter, the major storage product of cocoa seeds; and 4) the enzyme is found in the post-microsomal supernatant, it seems reasonable to conclude that the first step in cocoa butter biosynthesis is catalyzed by glycerol-3-phosphate acyltransferase in the cytoplasm of cocoa cotyledon cells.
Cocoa Soluble Glycerol-3-phosphate Acyltransferase

Table I
Purification of glycerol-P acyltransferase from 350 g of cocoa beans

| Step                              | Total activity (nmol/min/ml × total volume) | Total protein (g) | Yield (%) | Specific activity (nmol/min/mg protein) | Enrichment |
|-----------------------------------|--------------------------------------------|------------------|-----------|----------------------------------------|------------|
| Post-microsomal supernatant       | 19,200                                     | 16.6             |           | 1.2                                    |            |
| Ammonium sulfate 25-50% saturated | 12,000                                     | 3.7              | 63        | 3.3                                    | 2.8        |
| DEAE step gradient                | 7,700                                      | 315 mg           | 40        | 24.5                                   | 20         |
| DEAE linear gradient              | 1,708                                      | 14.4 mg          | 8.9       | 119                                    | 99         |
| Sephacryl S-300 1                 | 1,450                                      | 3.2 mg           |           | 455                                    | 378        |
| Sephacryl S-300 2                 | 1,245                                      | 1.8 mg           | 6.5       | 892                                    | 577        |

Fig. 1. Chromatofocusing chromatography of cocoa seed glycerophosphate acyltransferase. A 1 × 45-cm column was packed with Pharmacia polybuffer exchanger (PBE 94) pH range 6-4, and equilibrated with 0.025 M histidine-HCl, pH 6.2. Post-S-300 glycerophosphate acyltransferase was equilibrated with histidine buffer by passage over a Sephadex G-50 column then applied to the column. The column was eluted with Pharmacia Polybuffer 74, pH 4.0. 1-ml fractions were collected.

Elutes in two peaks at isoelectric points 5.2 and 5.3. Two smaller peaks are seen at isoelectric points 4.9 and 4.8.

Isoelectric focusing using vertical tube polyacrylamide gels confirmed the results of the chromatofocusing experiment (Fig. 2). Thus, we conclude that cocoa seed glycerophosphate acyltransferase exists in several molecular forms distinguishable by small charge differences.

Isoelectric focusing of purified pea chloroplast acyltransferase activity demonstrated the existence of two forms of the enzyme with apparent isoelectric points of 6.3 and 6.6 (8). The two forms co-purified through all other purification steps. In contrast, spinach chloroplast acyltransferase was found to exist in a single molecular form with isoelectric point 5.2 (8).

Hydrophobic Chromatography

Detection of enzyme activity in all preliminary purification steps, as well as in the chromatofocusing and isoelectric focusing experiments, was achieved without addition of a fatty acid donor to reaction mixtures. In addition, it was determined that, even with highly purified glycerophosphate acyltransferase preparations, addition of either acyl-CoA or acyl-ACP had no effect on enzyme activity. We reasoned that if the enzyme was the source of the fatty acid donor, it should be possible to demonstrate this by hydrophobic chromatography. Post-S-300 glycerophosphate acyltransferase was subjected to hydrophobic chromatography on an octyl-Sepharose column as described by Rock and Garwin (11). The procedure resulted in considerable loss of enzyme activity but the major recoverable activity was found in the fraction (peak C) eluted from the column with 25% isopropanol (Fig. 3). Peak C was 10 times more active than peak B and, in common with all prior samples in the purification, did not require addition of an exogenous fatty acid donor to the reaction mixture. That is to say, the enzyme preparation itself was the source of the fatty acid transferred to glycerol 3-phosphate. Protein present in peak A, on the other hand, had essentially no enzyme activity when palmitoyl-CoA was not added to the reaction mixture. However, a small amount of activity was seen when palmitoyl-CoA was added.

The hydrophobic chromatography experiment clearly demonstrates that long chain fatty acids are associated with the
Cocoa Soluble Glycerol-3-phosphate Acyltransferase

Analysis of Reaction Products

We conducted experiments to determine the chemical nature of the products formed when glycerol 3-phosphate was incubated with cocoa seed extracts. Peak C from the octyl-Sepharose column (Fig. 3) was the enzyme source used for these experiments. Detailed protocols for the analyses are given under "Experimental Procedures."

First, it was determined by thin layer chromatography that a single fatty acid was transferred to glycerol 3-phosphate forming monoacylglycerol 3-phosphate. An important property of acyltransferase is the positional specificity. To determine whether the fatty acid transferred was in position 1 or 2 of glycerol 3-phosphate, the reaction product, monoacylglycerol 3-phosphate, was isolated and dephosphorylated by alkaline phosphatase, and the resulting monoacylglycerol analyzed for isomeric composition by thin layer chromatography. The results clearly established that acylation occurred exclusively in the 1-position.

The pea and spinach chloroplast glycerophosphate acyltransferase also possess high positional specificity, both directing acyl groups into the C-1 position of glycerol with negligible acylation of C-2 (8).

Finally, the fatty acids transferred were shown by mass spectrometric analysis to be palmitic and stearic. All these observations are consistent with the conclusion that the enzyme purified from the soluble fraction of cocoa seed extracts is the first enzyme in the biosynthetic pathway leading from glycerol 3-phosphate to cocoa butter.

Antibodies to Cocoa Seed Glycerophosphate Acyltransferase

Radioimmunoassay to Demonstrate Presence of Antibodies—Antibodies to cocoa seed glycerophosphate acyltransferase were induced in a New Zealand White rabbit by injecting isoelectric focusing purified enzyme homogenized with polyacrylamide gel slices (see Fig. 2). Detailed protocols describing the immunological procedures are given under "Experimental Procedures." Ordinate represents amount of [125I]-labeled protein A bound to antigen-antibody complex in microtiter wells (II and III) compared to amount bound when preimmune serum was added (I), B, specificity. Using 500-μl Eppendorf tubes, 0.05 μl of post-S-300 2 (Table I) glycerophosphate acyltransferase was mixed with 12.2 μl of antisem or preimmune serum dilutions containing 11.4, 114, and 1140 μg of protein. The mixtures were incubated at room temperature for 30 min, then overnight at 4 °C. The samples were centrifuged for 15 min in a microfuge and the supernatants were assayed for glycerophosphate acyltransferase activity.

Kinetic Data

Typical Michaelis-Menten kinetics were obtained when a glycerol 3-phosphate concentration curve was run in the absence of added fatty acid donors. A $K_m$ of 4.2 mM was found, a value similar to that reported for the spinach chloroplast enzyme (8). Reaction velocity was linear for 10 min, then slowed, and finally stopped after 2-3 h.

Purified soluble cocoa seed glycerophosphate acyltransferase could be made responsive to added acyl-CoA by first incubating the enzyme with glycerol 3-phosphate, then passing the incubation mixture over a Sephadex G-50 column. The enzyme recovered after this treatment gives a typical Michaelis-Menten substrate concentration curve with a $K_m$ for stearoyl-CoA of about 4 μM, again similar to values for the spinach and pea chloroplast enzymes.

![Graph showing kinetic data](image-url)
Cocoa Soluble Glycerol-3-phosphate Acyltransferase

The molecular weight of non-denatured glycerophosphate acyltransferase as determined by gel-filtration chromatography on Sephacryl S-300 columns in 500 mM NaCl is estimated to be about 200,000. Enzyme assay of gel slices obtained after polyaclamydyl gel electrophoresis under non-denaturing conditions confirmed this estimate. However, when gel electrophoresis was performed in the presence of sodium dodecyl sulfate, the vast majority of protein, estimated by Coomassie Blue staining, representing all proteins present except for minor contaminants, was concentrated in a single area of the gel with electrophoretic mobility corresponding to a molecular weight of about 20,000. Antibodies to cocoa seed glycerophosphate acyltransferase formed immune complexes only with this polypeptide (Fig. 5), confirming that the M, 20,000 protein was an enzyme subunit. Hence, we conclude that the native molecular weight of cocoa seed glycerophosphate acyltransferase is $2 \times 10^6$ and that the enzyme is composed of 10 M, 20,000 peptides. Both molecular forms of pea chloroplast glycerophosphate acyltransferase had molecular weights of 42,000, whereas the spinach enzyme was slightly larger (8). No subunit determinations were reported for these enzymes.

**Stoichiometry**

Lamb and Fallon (12) reported that 1 mg of rat microsomal protein could bind about 68 nmol of palmitoyl-CoA, all of which could be removed by incubation with high concentrations of albumin. If we assume an average molecular weight of 100,000 for the rat microsomal protein, it can be calculated that each nanomole of protein binds 6.8 nmol of palmitoyl-CoA. Incubation of highly purified cocoa seed glycerophosphate acyltransferase under assay conditions for up to 3 h, long enough to allow transfer of all bound fatty acid, resulted in formation of 100 nmol of product for each nanomole of enzyme, or 10 nmol of bound fatty acid donor/subunit. The amount of product formed was a linear function of protein concentration.

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Cocoa Soluble Glycerol-3-phosphate Aciytransferase

EXPERIMENTAL PROCEDURES

Materials

L-([1-14C]glycerol) 3-phosphate was obtained from Amersham. Apo-Oleos were from ProBiochimie. AcpApolys and A-re-sulphated-lys-acyltransferases were from Bio-Rad. Ampholytes were from LKB. Oval-Deoxyrase was from Pharmacia.

Cocoa (Theobroma cacao L) seeds were obtained from a grower generously provided to us by R.M. Melick, Dr. R.A. Severson, or Gordon Pattersones of the Nestle Foods Corporation. The pods had been picked at 6 days previously at the Nestle Foods Corporation experimental research facility in Belo Horizonte, Brazil.

Buffer Mixtures

Buffer mixtures containing (in a final volume of 25 ml): 100 mM, Tris [pH 8.0], and sodium succinate-3-phosphate disodium salt-HCl buffer, pH 7.4, 7 mM [32P] glycerol-3-phosphate (10 000 Ci/mmol) and various amounts of enzyme. Incubations were carried out at 30°C for variable periods of time to determine initial velocity for activity determinations during purification. The reaction was stopped by addition of 75 µl glycerol-3-phosphate (100 g/l) followed by vortexing and 2 minute centrifugation at 15 000 rpm in a Sorvall Microfuge. Twenty µl of the (glycerol) phase were counted in 1 ml of either Aquasol (New England Nuclear) or ECF-25 (Amersham) in a Beckman LS-500 liquid scintillation counter with about 85% efficiency for [32P]. Note that the enzyme preparation was the source of fatty acids.

Protein was determined using the Pierce Chemical Company protein assay reagent with bovine serum albumin as the standard.

Purification of glycerol-3-acyltransferase (G3AT)

1. Initial extraction and preparation of post micellar supernatant.

Unless otherwise indicated, all steps were carried out at 4°C. Seeds from 6 mature pods were stripped of their tests and placed in liquid nitrogen. The seeds (150 g) were powdered in liquid nitrogen then mixed with 1 liter of extraction buffer and dispersed in a Waring blendor (1 x 30 second bursts). Extraction buffer, pH 6.0, contained: sodium, 200 mM Tris-Cl buffer, 40 mM EDTA, 5 mM, mercaptoethanol, 15 mM, polyvinylpyrrolidone, 0.001% SDS, 0.01M dithiothreitol, 100 µM. The dispersion was filtered through layers of cheesecloth and the filtrate centrifuged for 30 minutes at 100,000 x g to produce a micellar pellet and a post micellar supernatant. Small aliquots (10 µl) were saved at each step for enzyme and protein analyses.

2. Ammonium Sulfate Fractionation of post micellar supernatant.

The volume of the post micellar supernatant was measured and solid ammonium sulfate was added, while stirring, to make the solution 50% saturated (146 g/l). After the ammonium sulfate dissolved, the solution was allowed to stand in ice for 30 minutes, then centrifuged at 10,000 x g for 15 minutes. The pellet was discarded, the supernatant made 50% saturated with ammonium sulfate (158 g/l) and the solution processed as above. The pellet from this stage had a specific activity of 90 U/mg. This was dissolved in 0.02M Tris, pH 7.4, in order to be used for dialysis. This solution was dialyzed against 1 liter of buffer A x 2 x 1 hr. Then overnight.

3. Anion Exchange Chromatography

a. Step Gradient

The dialyzed supernatant fraction was layered on a 1 x 2 cm DEAE-cellulose column contained in a 1 x 2 cm diameter glass funnel. The DEAE-cellulose had been prepared by standard DEAE-cellulose washing procedures (16) and had been pre-equilibrated with buffer A. Three Fractions of 75 ml each were collected: (1) buffer A with no salt, (2) buffer A with 50 mM NaCl and (3) buffer A with 80 mM NaCl. Most of the enzyme activity was found in the 50 mM salt fraction. This fraction was concentrated by precipitation with 0.5% ammonium sulfate. The pellet after centrifugation was resuspended in 6 ml buffer A and dialyzed against buffer A as above. This step in the purification scheme removed a large amount of dark brown, blue and green pigmented material.

b. Linear Gradient (Fig. 1a)

The concentrated sample from the step gradient was pipetted onto a 1.2 x 15 cm Sephadex G-200 column previously equilibrated with buffer A. The ATRs were collected at a flow rate of 45 ml/hour. Enzyme activity was found in the early Fractions eluting from the column. The first indication that the enzyme might have a high molecular weight. Active Fractions were again pooled and concentrated with 0.5% ammonium sulfate. The pellet after centrifugation was dissolved in 200 µl buffer A and chromatographed a second time in the same Sephadex G-200 column.

4. Gel Filtration Chromatography (Figures 2a and 3a)

The dialyzed sample from the step gradient was placed on a 1 x 15 cm DEAE-cellulose column using a Harvard pump. The column was eluted by a linear gradient with buffer A until the 25 ml absorbance was zero (about 12 ml), then a linear NaCl gradient was begun. The gradient was 0-500 mM in buffer A and was carried out in a total volume of 250 ml. Two ml fractions were collected at a flow rate of 24 ml per hour. Salt concentrations were verified by conductivity measurements. Fractions with enzyme activity eluted early in the gradient, as expected, and were combined and concentrated with 0.5% ammonium sulfate as before. The pellet after centrifugation was dissolved in 1.5 ml of buffer A.

Figure 2a. Gel filtration chromatography of cocoa seed soluble glycerol-3-phosphate acyltransferase. First Sephadex G-200 column.

Figure 3a. Gel filtration chromatography of cocoa seed soluble glycerol-3-phosphate acyltransferase. Second Sephadex G-200 column.

The concentrated sample from the linear gradient was pipetted onto a 1.2 x 15 cm Sephadex G-200 column previously equilibrated with buffer A. The ATRs were collected at a flow rate of 45 ml/hour. Enzyme activity was found in the early Fractions eluting from the column. The first indication that the enzyme might have a high molecular weight. Active Fractions were again pooled and concentrated with 0.5% ammonium sulfate. The pellet after centrifugation was dissolved in 200 µl buffer A and chromatographed a second time in the same Sephadex G-200 column.
Analysis of Reaction Products

1. Identification of reaction product

Solvent phases from reaction mixtures were lyophilized, dissolved in 25 μl developing solvent (chloroform-methanol-acetic acid-water 80:20:1:1 v/v/v), spotted on 25x25 mm glass backed precast silica gel 60 plates (Merck) and developed by ascending chromatography. This system permitted a clear separation of glycerophosphate (RF 0.95) from lysophosphatidic acid (RF 0.81, Fig. 1b). Authentic standards (50–100 μg each) were developed in separate lanes (by addition of solvent) and developed with the reaction mixtures. The plate was dried at 80°C. The plate was exposed to autoradiography. After the plate was developed it was covered with x-ray film and placed in contact with the x-ray film for 16 hours. The developed film revealed an area of radioactivity in the sample lane corresponding to the expected area for the glycerophosphate and phosphatidic acid standards. Also shown in the figure are sketches of the phosphatidic and phosphatidic acid standards identified by exposure to an iodine vapor chamber.

SOLVENT FRONT

PHOSPHATIDIC ACID

LYSOPHOSPHATIDIC ACID

ORIGIN

Figure 1a. Thin layer chromatography of cocoa seed glycerophosphate acyltransferase catalyzed reaction products.

2. Fatty Acid Positional Analysis

Phosphatidic acid esters, and thin layer chromatography were carried out on the presence of each ester and the migration of each FAME being eluted from the plate by radiocaesium (150 μl–200 μl). The mixture was allowed for 40 minutes at 37°C. The reaction mixture was extracted with 5 ml diethyl ether/acetone (1:1) by sonication in a glass-stopped vial. The ether was evaporated and the residue was dissolved in 50 μl methanol-water (9:1, v/v). Standards were loaded by spreading the plates with 256 nmol of each standard and allowing it to dry at 80°C for 2 minutes. Samples were divided into 5 ml-segments, scraped into scintillation vials and counted in 5 ml of scintillation fluid. The results are shown in Figure 1b.

In Figure 1a, lanes A, B, and C represent, respectively, 5 μg monoacylglycerol; 5 μg 2-monooacylglycerol; and 5 μg each 1- and 3-monooacylglycerols. The solvent system was used in each of the samples to achieve the proper solvent system for the separation of the reaction products.

3. Electron impact mass spectra of fatty acids transferred to glycerol-3-P by catalytic action of purified cocoa seed soluble glycerol-3-P phosphatase.

Fatty acid methyl esters were prepared by transesterification of sodium monoglycerides-3-phosphate into methanol. The reaction mixture was then dried by removal of the alcohol and the residue was dissolved in ethyl acetate and heated at 65°C in a glass-stopped tube. Methyl esters were extracted into 1 ml petroleum ether, dried in a nitrogen stream, dissolved in methylene chloride and analysed by gas chromatography. The retention times of 499 and 477 indicated the presence of methyl esters of stearic and palmitic acids, respectively.

Figure 1b. Fatty acid positional analysis of commercial glycerol-3-P product of cocoa seed soluble acyltransferase catalyzed reaction.

Experimental Procedures

1. Preparation of Antiserum

Antiserum to cocoa seed glycerophosphate acyltransferase were prepared in 5 male New Zealand White rabbits according to the following protocol: 0.5 ml aldehydes and hemoglobin (50 μM) in 1 M-β-glycerophosphate containing glycerophosphate acyltransferase (see Figure 2) was injected into a 1 ml syringe with an equal volume of complete Freund's adjuvant. The mixture was injected intravenously at three sites in the hind quarters. Two weeks later the procedure was repeated. Ten days after 50 ml of blood was collected from the ear artery and the animal was injected as before but using Freund's incomplete adjuvant. Blood was allowed to clot for several hours at 4°C and serum was collected by centrifugation for 15 minutes at 10,000 g. Propranolol tartrate was prepared prior to serum injection.

2. Solid Phase Fluorimunometric

Solid phase RIA was performed according to the following protocol:

1. Purified enzyme diluted to 30 μg/ml with buffer B (25 mM potassium phosphate, pH 7.5/150 mM NaCl/0.01% BSA).
2. 100 μl diluted enzyme diluted onto 400 μl capacity, flat-bottom plastic wells (Corning) strips (100 wells).
3. Incubate at 37°C for 3 hours in a humid chamber with gentle shaking.
4. Remove solutions from wells and wash with 4 x 100 μl.
5. Add 150 μl buffer A containing 14 μM/ml NaCl.
6. Incubate for 1 hr at room temp. x 1 washing plate for
7. Remove acid and add 100 μl NaCl diluted with buffer B. Buffer B (50 mM Tris-Cl, pH 7.4; 150 mM NaCl; 0.01% Tween 80; 0.5% bovine serum albumin; 0.1 μg/ml phenoxyethylthiourea; 0.1 μl/ml fluorochrome).
8. Incubate 1 hr at room temp.
9. Remove acid and add 4 with 200 μl buffer B.
10. Add 150 μl '3H' labeled protein A in buffer B (0.01 μg per well).
11. Incubate 1 hr at room temp.
12. Remove acid and add 4 with 100 μl buffer C (50 mM triethyl amine, 1 mM NaCl; 5 mM EDTA; 0.01% Tween 80; 0.1 μg/ml phenoxyethylthiourea; 0.1 μl/ml fluorochrome).
13. Wells counted in a gamma counter.