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Control of Lipid Synthesis during Soybean Seed Development: Enzymic and Immunochemical Assay of Acyl Carrier Protein

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ABSTRACT

During soybean seed (*Glycine max*, var Am Soy 71) development, the rate of lipid biosynthesis per seed increases greatly. As the seed reaches maturity, lipid synthesis declines. To study the controls over the oil synthesis and storage process, we have chosen acyl carrier protein (ACP) as a representative marker for the fatty acid synthetase pathway. We have quantitated soybean ACP levels by both enzymic and immunochemical methods. *Escherichia coli* acyl-ACP synthetase was used as an assay for enzymically active ACP. Total ACP protein was determined by immunoassay using antibodies prepared in rabbits against spinach ACP. These antibody preparations also bind ACP isolated from soybeans, allowing development of a radioimmunoassay based on competition with [^{3}H]palmitoyl-ACP. The enzymic and immunochemical measurement of ACP at various stages of seed development have indicated that ACP activity and ACP antigen increase markedly in correlation with the *in vivo* increase in lipid synthesis. These results indicate that a major control over the increase in lipid synthesis arises through regulation of the levels of the fatty acid biosynthetic proteins. However, as the seed reaches maturity and lipid biosynthesis declines, ACP per seed remains relatively high. In the mature seed, we found that more than 95% of the ACP is localized in the cotyledons, less than 5% is in the axis, and less than 1% is in the seed coat.

During soybean seed development the rate of lipid biosynthesis per seed increases markedly, resulting in a mature seed containing 20 to 25% lipid by weight (12). Between 15 and 45 DAF,[1] the rate of fatty acid accumulation per seed increases 10 to 20 fold (9). Most of the lipid in soybean seeds is stored in the cotyledons in the form of triglycerides packaged in specialized structures, often referred to as oil bodies or spherosomes (3). Cytological studies of soybean embryogenesis have shown that cell division in the seed is completed at an early stage of development (by 20–25 DAF) while the embryo is still quite small (2). The major increase in seed size which occurs between 25 to 60 DAF is brought about through enlargement of preexisting cells. During this period of cell enlargement, the majority of the oil, protein, and carbohydrate synthesis and storage in the seed occurs. Thus the enlarging seed must simultaneously partition its photosynthate among three major reserves. Although the biochemistry and molecular biology of protein synthesis in seeds has been extensively studied, the regulation of lipid biosynthesis in seeds is largely unexplored. Acetyl-CoA carboxylase is the only individual enzyme in the fatty acid biosynthesis pathway whose activity has been examined throughout oilseed development. In castor bean (13) and rape-seed (16), large increases in the activity of acetyl-CoA carboxylase coincide with the period of increasing lipid synthesis. However, these studies did not examine whether control over changes in acetyl-CoA carboxylase activity resulted from new enzyme synthesis or from posttranslational modifications of the enzyme.

We are interested in the developmental control of seed lipid biosynthesis. ACP plays a central role in fatty acid biosynthesis as a cofactor to which acyl chains are attached via thioester linkage. ACP participates in at least eight reactions of fatty acid biosynthesis (15) and may also serve as an acyl donor for glycerolipid biosynthesis (1). We have chosen to study this protein as a representative marker for the oil storage process. In this paper we have asked whether the activation of seed fatty acid biosynthesis is brought about by changes in levels of the fatty acid biosynthetic proteins or by posttranslation mechanisms. As a preliminary approach to this question, we have determined ACP levels during soybean seed development by both enzymic and immunochemical methods and have compared these data to *in vivo* rates of fatty acid biosynthesis.

MATERIALS AND METHODS

Purification of Spinach ACP. The purification of spinach ACP has been modified from the method of Simoni *et al.* (14) to accommodate the processing of large amounts of spinach tissues. In a typical preparation, 50 kg of frozen fresh spinach with stems partially removed were homogenized in a Cowles Dissolver[2] (type 7VT, The Cowles Co., Cayuga, NY) with 25 liters of cold 0.1 M K-phosphate, pH 7.5, containing 10 mM $Na_2S_2O_5$, 10 mM β-mercaptoethanol, 0.1% Triton X 100, and 1 mM EDTA. After debris was removed with a Sharples centrifuge, the homogenate was heated to 85°C for 10 min in a 30-gallon heating tank and then cooled. The clear supernatant obtained after centrifugation was adjusted to 70% $(NH_4)_2SO_4$ saturation, centrifuged, and then acid-precipitated at pH 4.0. The acid precipitates, recovered from a small Sharples centrifuge, were suspended in 50 mM K-phosphate, pH 7.5. A second acid precipitation was carried out in the presence of 80% $(NH_4)_2SO_4$ mainly to reduce volume of the solution. The pellets were resuspended in 10 mM MeS, pH 6.1 (pH adjusted with KOH), with the conductivity of the solution below that of 0.1 M NaCl. Ion exchange chromatography was carried out using DE52 (Whatman) equilibrated with 10 mM MeS, pH 6.1, containing 1 mM β-mercaptoethanol and a linear gradient of 0.1 to 0.5 M NaCl in the same buffer. Major ACP-containing fractions were identified by an enzymic assay method as described below, and ACP was acid-precipitated from the

[1] Abbreviations: DAF, days after flowering; ACP, acyl carrier protein.

[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the United States Department of Agriculture over other firms or similar products not mentioned.

pooled solution containing 1 M NaCl (as a general practice, we add additional salt to facilitate precipitation of ACP from the acid solution). The ACP yield was similar to that reported in previous work (14) but the preparation was only 50% homogeneous, as judged by thin-layer isoelectric focusing on pH 3 to 6 Servalyt Precotes. To achieve homogeneity, ACP samples were reduced with 5 mM DTT and subjected to two cycles of HPLC with a Synchropak AX-300 column (25 cm × 10 mm i.d., SynChrom, Inc., Linden, IN). A linear gradient of 0.2 to 1.0 M NaCl in 50 mM K-phosphate, pH 6.8, was used for the first cycle and 0.2 to 0.6 M NaCl in the same buffer for the second (flow rate 3 ml/min).

Production of Specific Spinach ACP Antibodies. Because spinach ACP is a weak antigen (6), the purified ACP was polymerized to egg albumin (OA) using glutaraldehyde as a coupling agent as described by Reichlin (10). In this study, the polymerization was carried out in 0.1 M sodium phosphate, pH 7.5, containing spinach ACP and OA (0.15 mM each) in the presence of glutaraldehyde (25 mM). Two young New Zealand white male rabbits (8.8–11.0 kg) were injected subcutaneously at multiple sites with a sonicated emulsion containing approximately 0.4 mg of ACP equivalent of ACP-OA polymer in 1.0 ml of Freund's complete adjuvant. Two weekly booster injections were carried out after 4 weeks in the same manner, but using incomplete adjuvant. Serum collected after 3 weeks contained only weak titer, as monitored by its ability to inhibit ACP acylation in the ACP enzymic assay. Two additional booster injections were then given at 2-week intervals with incomplete adjuvant, using an emulsion containing an equal molar ratio of ACP (0.4 mg) and BSA or Cyt *c* (horse heart). Serum was collected 2 weeks after the final injection and stored at −15°C.

Growth of Plants. *Rhizobium japonicum*-inoculated soybeans (*Glycine max*, var AmSoy 71) were grown in Redi-earth (W. R. Grace & Co., Cambridge, MA) in growth chambers under the following conditions: Daylength 14 h, 26°C, 5 to 10,000 foot candles, 80% RH, and night temperature of 20°C. Plants were fertilized once each week with Hoagland solution (4). Flowers were tagged 2 or 3 times each week to allow the age of developing seeds to be estimated. Total fatty acid per seed was determined by homogenization of 2 to 3 seeds in 10 ml of hexane:isopropanol 3:2 (v/v). The homogenate (1 ml) was mixed with heptadecanoic acid as an internal standard and evaporated to dryness under N_2; methyl esters were prepared by heating at 65°C for 2 h in 4 ml of 5% HCl in methanol:benzene 1:1 (v/v). After adding 2 ml H_2O, methyl esters were extracted with petroleum ether and analyzed by GC on a 305 cm × 2 mm OV-275 column at 190°C. For ACP assay, seeds were extracted by homogenization with a Brinkman Polytron homogenizer in 10 ml per g wet tissue of 0.1 M Tris, pH 8.0, 1% Triton X-100, 15 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulphonyl fluoride, 10 μM leupeptin, and 1 mM sodium metabisulfite. The homogenate was centrifuged at 10,000*g* for 15 min. This supernatant was assayed directly, using the radioimmunoassay. For enzymic assay of ACP levels, the supernatant was mixed with an equal volume of saturated $(NH_4)_2SO_4$ (pH 8), and after 5 to 10 min at 4°C was centrifuged at 10,000*g* for 10 min. The supernatant was heated at 60°C for 5 min and then mixed with 0.05 volume of 50% TCA. After 5 to 10 min at 4°C, the acid precipitate was centrifuged at 10,000*g* for 10 min and redissolved in 0.1 M Tris-HCl, pH 8.0, 1% Triton X-100. These additional treatments required for the enzymic assay caused negligible loss of ACP as evidenced by radioimmunoassay before and after the treatments.

Enzymic Assay of ACP. ACP levels were determined enzymically by modification of the method of Rock and Cronan (11). *E. coli* acyl-ACP synthetase (EC 6.2.1.3) was purified as described except that the hydroxyapatite step was replaced by Sephadex G-25 gel filtration to remove KSCN. The reaction mixture contained Tris-HCl (0.1 M, pH 8.0), ATP (5 mM), $MgCl_2$ (10 mM), LiCl (0.4 M), Triton X-100 (2%), DTT (2 mM), [^{3}H]palmitic acid (3 μM, 15 Ci/mmol) and acyl-ACP synthetase (2 milliunits) in a final volume of 50 μl. After 1 h at 37°C, 40 μl of the reaction mixture was transferred to a 1 × 3 cm piece of Whatman 3 mm filter paper and allowed to air dry. The filter papers were then washed with four changes of chloroform:methanol:acetic acid (3:6:1, v/v/v) to remove unreacted palmitic acid. The papers were placed in scintillation vials containing 0.8 ml of 1 M hyamine hydroxide and heated at 65°C for 15 min to hydrolyze the [^{3}H]palmitoyl ACP formed during the reaction. This hydrolysis step was necessary to obtain good scintillation counting efficiency of the ^{3}H.

Immunochemical Assay of ACP. Soybean [^{3}H]palmitoyl-ACP was prepared by enzymic acylation of partially purified soybean ACP. The enzymic assay reaction mixture described above was scaled up to 0.5 ml, and the [^{3}H]palmitate concentration was increased to 7.5 μM (57 μCi/0.5 ml). Soybean ACP (12 μg) was added, and the reaction was allowed to proceed for 16 h at 37°C. The reaction mixture was then diluted to 5.0 ml with 20 mM MeS, pH 6.3, and applied to a 0.8-ml column of DE52 (Whatman). After washing with 5 ml 20 mM MeS, pH 6.3, and 3 ml 80% isopropanol in 20 mM MeS, pH 6.3, 23 μCi of [^{3}H]palmitoyl ACP was eluted with 0.6 M NaCl in 10 mM MeS, pH 6.1.

For radioimmunoassay, 0 to 10 μl of soybean seed extract was mixed with 1 μl of anti-ACP serum in a final volume of 80 μl (adjusted with 0.01 M K-phosphate pH 6.8/0.15 M NaCl) and allowed to react for 2 h at 4°C. Then [^{3}H]palmitoyl-ACP (8-10 × 10^3 dpm) was added and allowed to react for an additional hour before addition of 4 μl preimmune rabbit serum and 0.2 ml of 20% polyethylene glycol 6000. After 15 min at 4°C, the immunoglobin-ACP complex was centrifuged and the radioactivity in 0.2 ml aliquots of the supernatant was determined. In preliminary experiments, we confirmed that less than 5% hydrolysis of the [^{3}H]palmitoyl-ACP by acyl-ACP hydrolase (7) occurred under these assay conditions. Unless otherwise indicated, the source of biochemicals was Sigma; [^{3}H]palmitic acid (15 Ci/mmol) was obtained from New England Nuclear.

RESULTS AND DISCUSSION

Assay Systems for ACP. We have measured acyl carrier protein levels during soybean seed development by both enzymic and immunochemical techniques. Enzymic measurement of ACP was performed using the acyl-ACP synthetase isolated from *E. coli*. This enzyme will acylate ACP of both bacterial and plant origin (Kuo and Ohlrogge, unpublished). In Figure 1, a standard curve for acylation of soybean ACP with [^{3}H]palmitic acid is shown. We found it was necessary to heat-treat and fractionate soybean extracts to obtain a low background and a linear response to added extract in this assay. Under the conditions shown in Figure 1, this assay is able to detect ACP levels of 1 ng/10 μl extract (10^{-8} M).

Immunochemical measurement of ACP levels was performed using a radioimmunoassay. Antibodies were prepared against acyl carrier protein isolated from spinach rather than from soybean, because this protein is more easily purified from spinach. Antibodies raised against spinach ACP will cross-react with soybean ACP as shown in Figure 2. In this experiment, increasing levels of antiserum were incubated for 2 h with 2.7 ng of either spinach or soybean [^{3}H]palmitoyl-ACP. The free and bound ACP were then separated by polyethyleneglycol precipitation of the immunoglobulins. As shown in Figure 2 this anti-spinach ACP serum effectively binds soybean ACP although an approximately 4-fold higher level of antiserum is required to bind equivalent levels of soybean *versus* spinach ACP.

The results in Figure 2 indicated that a radioimmunoassay for soybean ACP could be developed based on competition for the

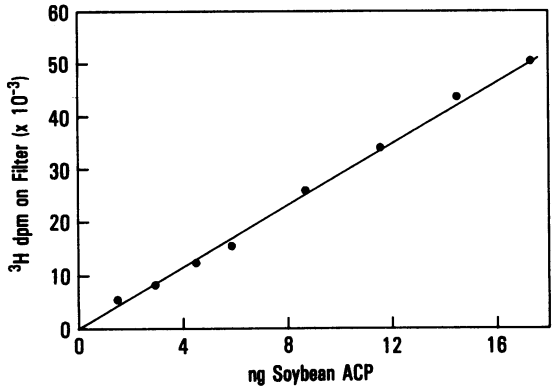

FIG. 1. Enzymic assay of soybean acyl carrier protein using *E. coli* acyl-ACP synthetase (11). Crude soybean ACP was incubated 1 h with 2.5 μM [^{3}H]palmitic acid and 0.5 milliunits of acyl-ACP synthetase. The level of ACP in extracts is estimated from the total radioactivity recovered on filter paper after extractions of unreacted fatty acid, as described in "Materials and Methods."

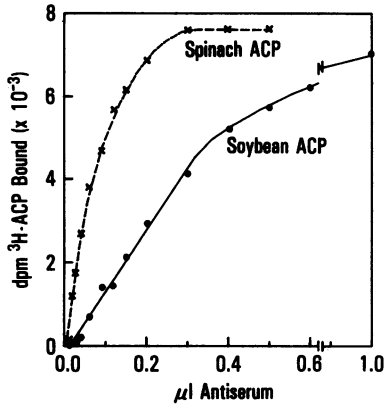

FIG. 2. Binding of spinach and soybean [^{3}H]palmitoyl ACP by antibodies raised in rabbits against spinach ACP. 2.7 ng of spinach (X——X) or soybean (●——●). [^{3}H]Palmitoyl ACP (3700 dpm/ng) was incubated for 2 h at 4°C with antiserum, and then the immunoglobin fraction was precipitated with polyethylene glycol. [^{3}H]ACP bound was calculated from assay of radioactivity in the supernatant (unbound) fraction.

antibody-combining sites between soybean [^{3}H]palmitoyl-ACP and ACP in the seed extracts. In Figure 3 a standard curve for this assay is plotted, indicating that this assay is also able to detect soybean ACP levels in the nanogram range.

ACP Levels during Seed Development. Data in Figure 4 are plotted on a per seed basis rather than as specific activity (per mg protein). Because the seeds are accumulating large amounts of storage protein, the ACP specific activity does not change markedly during seed development.

In Figure 4A the accumulation of total fatty acids per seed is plotted *versus* seed age for plants grown in our growth chambers. In Figure 4B the rate of fatty acid synthesis per seed per day has been calculated from the slope of the curve drawn in Figure 4A. The *in vivo* rate of fatty acid synthesis increases sharply between 20 and 50 DAF and decreases to zero by about 70 d.

During soybean seed development, cell division stops at an early stage (20–25 DAF) of embryogenesis (2). Therefore, the major increase in seed size and the large increase in the rate of fatty acid synthesis shown in Figure 4B occurs without the production of new cells. In light of this, we have considered whether the increase in fatty acid synthesis activity might result from one or more of several posttranslational mechanisms. For example, perhaps the maximum level of the fatty acid synthetase

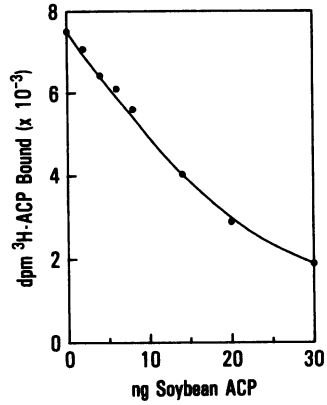

FIG. 3. Radioimmunoassay of soybean acyl carrier protein using antibodies raised in rabbits against spinach ACP. Antiserum (1.0 μl) was incubated first for 2 h with unlabeled soybean ACP and then for 1 h with 10,000 dpm of soybean [^{3}H]palmitoyl ACP, after which the immunoglobin fraction was precipitated with polyethyleneglycol. [^{3}H]ACP bound was calculated from assay of radioactivity in the supernatant (unbound) fraction.

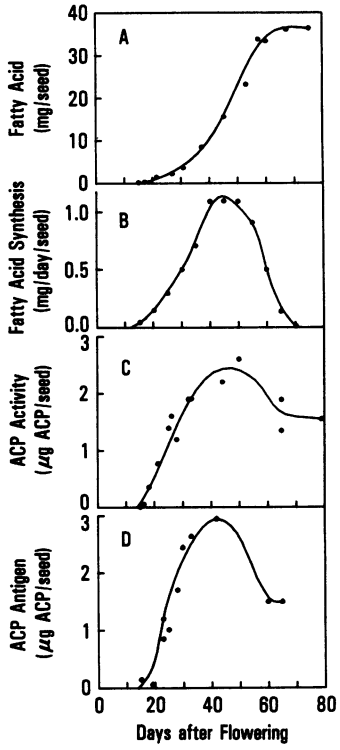

FIG. 4. Acyl carrier protein levels during soybean seed development. A, total fatty acid per seed; B, rate of fatty acid synthesis per day per seed; C, ACP levels per seed measured enzymically; D, ACP levels per seed measured immunochemically.

proteins is established during the cell division stage of seed development (before 20–25 DAF), but these proteins become maximally active only when optimal levels of photosynthate, cofactors (*e.g.* ATP, etc.) or hormones are available. In such cases the limiting process in oil storage would not be directly dependent on the level of the fatty acid synthetase proteins. As an alternative mechanism, the levels of the fatty acid synthetase proteins may continue to increase after cell division stops. In this case the quantity of the biosynthetic proteins could be considered as a limiting factor in fatty acid production and, therefore, their increase during seed development would be

needed for the increase in fatty acid synthesis. Similarly, at the later stages of seed development, we asked whether the decrease in fatty acid synthesis is related to decreases in the level of the biosynthetic proteins. Does lipid production decline as part of a developmentally programmed decrease in levels of the fatty acid synthetase proteins or is the decline in oil synthesis the result of other limitations, such as substrate supply or dehydration? The results in Figure 4 provide a preliminary resolution of these alternatives.

In Figure 4C the level of the ACP per seed measured enzymically is shown. It can be seen from the comparison of Figure 4C with 4B that the increase in enzymically active ACP occurs in close coordination with increased lipid synthesis. This result indicates that the soybean seed continues to increase the level of active ACP even after cell division ceases. Thus, the large increase in lipid synthesis seen between 25 and 50 DAF is not solely the result of increased photosynthate supply to a preexisting fatty acid biosynthetic system. Furthermore, the close correlation between the level of active ACP and *in vivo* lipid biosynthesis indicates that the quantity of active fatty acid synthetase proteins present in the cell may be a rate-determining component of the cell's overall lipid biosynthetic capacity.

The increase in ACP activity as measured enzymically and shown in Figure 4C, could be the result of several processes, including *de novo* synthesis of ACP, activation of preexisting ACP precursors, or transfer of the 4′ pantothenate prosthetic group to apoACP. Antibodies often have the capability to recognize both active and inactive forms of protein. Therefore, to distinguish in part between active and inactive ACP, we measured ACP 'cross-reacting material' immunochemically. We use a radioimmunoassay because the levels of ACP in the seed are too low to detect by immunoprecipitation. In Figure 4D the level of ACP per seed measured by radioimmunoassay is shown. ACP 'antigen' also increases in parallel to lipid synthesis. We believe the most likely interpretation of these data is that the increase in enzymically active ACP seen in Figure 4C is produced through *de novo* synthesis of ACP rather than through posttranslational activation of this protein. This interpretation is preliminary because we are not yet able to completely rule out the existence of other possible precursor or inactive forms of soybean ACP which are not recognized by our immunochemical assay. We have tested the binding of spinach apoACP with our antibodies and find approximately 40% cross-reactivity compared to spinach holoACP (data not shown). Thus, if apoACP were a major component of the total ACP pool, we believe our methods could detect this difference. However, further studies of ACP expression at the mRNA level will be necessary to establish more completely the mechanism for the increase in ACP level.

In Figures 4, B to D, there is a close parallel between increases in lipid synthesis and ACP level. However, this parallel does not continue for the final stages of seed maturation. Between 50 and 70 DAF, *in vivo* lipid biosynthesis (Fig. 4A) and acetate incorporation into lipid (8) decrease to levels less than 10% of their maximum. In contrast, ACP levels decrease approximately one half. This result indicates that mechanisms such as desiccation or lack of substrates rather than decreased fatty acid synthetase proteins lead to the decrease in rate of lipid accumulation. Thus 'turning off' fatty acid biosynthesis may not be simply the result of a decrease in the levels of fatty acid synthetase proteins. However, it is not possible to rule out that the activity of lipid biosynthetic proteins other than ACP has become rate limiting at the final stages of seed maturation.

Because new membrane synthesis is one of the early events upon seed germination, it is logical that fatty acid biosynthetic proteins such as ACP are stored in the dry seed. Furthermore, it might be expected that the axis whose cells undergo rapid expansion and division during germination would contain a substantial proportion of the ACP in dry seeds. However, our measurements of the distribution of ACP between structures of the dry seed indicated that >95% of the ACP (1.2 μg per seed) was localized in the cotyledons and <5% was in the axis (0.03 μg per seed). Therefore, most of the ACP remaining in the soybean seed at dormancy is evidently part of the pool used in the biosynthesis of the storage lipids.

The approximately 50% decrease in ACP levels that occurs between 50 to 70 DAF implies that ACP undergoes turnover or degradation during seed development. This is in contrast to the stability of the 7S and 11S soybean storage proteins for which Madison *et al.* (5) were unable to detect any protein turnover. Evidently, the enzyme or biosynthetic proteins undergo different regimes of turnover than do the storage proteins.

In conclusion, our results indicate that a major control over the increase in lipid biosynthetic capacity in developing soybean seeds arises through regulation of the levels of at least one of the fatty acid biosynthetic proteins.

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