Decreased Growth Temperature Increases Soybean Stearoyl-Acyl Carrier Protein Desaturase Activity

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ABSTRACT

Developing soybean (Glycine max) seeds respond to a change in growth temperature by changing the level of stearoyl acyl carrier protein desaturase activity in the tissue. After 20 hours in liquid culture, seeds grown at 20°C show an increase in activity while seeds grown at 35°C show a decrease in activity, relative to their preculture levels. Analysis of the enzyme from both growth conditions shows the change not to be due to induction of kinetically distinct isoenzymes: desaturase activities from both 20°C and 35°C have identical behavior with regard to pH, temperature optimum, substrate concentration and cofactor requirements. Experiments with boiled extracts indicate that the modulation is not caused by induction of metabolic effectors. From these data, it appears that stearoyl-acyl carrier protein desaturase responds to changes in growth temperature by altering the level of active enzyme present in the tissue. The magnitude of this response is a function of the developmental stage of the seed and not a function of the growth conditions of the parent plant. Changing the age of the seeds from early late RS changed the ratio of 20:35°C activity from 3.8:1 to 1.2:1. Changing the temperature at which the parent plants were grown over a range from 20/12°C to 34/28°C (day/night) produced only minor, and inconsistent, changes in the ratio of 20:35°C activities.

Plants respond to changes in environmental temperature by altering their fatty acid composition (7-9). In mature soybeans the fatty acid composition of both phospholipids (4) and triglycerides (18) is altered by changes in growth temperature. When soybeans are grown under cool conditions the level of polyunsaturated fatty acids increase relative to seeds from warm-grown plants (4, 18). When the enzymes of fatty acid synthesis and desaturation were examined, several of the enzymes showed increased activity in developing seeds cultured at 20°C compared with seeds cultured at 35°C (2). Among the altered enzymes was stearoyl-ACP desaturase. Desaturase activity was five-fold higher in 20°C-grown seeds than in 35°C-grown seeds.

Stearoyl-ACP desaturase is a plastidial enzyme that catalyzes the formation of oleoyl-ACP from stearoyl-ACP. The enzyme is present in all plants; however, its potential role differs depending on whether the plant is a 16:3 or 18:3 type plant (16, 17). Higher plants can be divided into two types; those that only desaturate stearic acid and its derivatives, the 18:3 type, and those that possess an additional plastidial pathway for desaturation of palmitic acid and its derivatives, the 16:3 type (16, 17). Thus, all unsaturated fatty acids in 18:3 type plants, like soybean, are synthesized by the pathway that begins with stearoyl-ACP desaturase. Therefore, any changes in this enzyme could lead to a change in the ratio of saturated to unsaturated fatty acids in the tissue (6). In developing soybean seeds, stearoyl-ACP desaturase activity is low compared to the in vitro activity of the other enzymes of fatty acid synthesis and desaturation (2).

The absence of other Δ-9 desaturases in soybean (17), the low activity in vitro, and the modulation of activity by growth temperature (2) all suggest that this enzyme may play an important role in the regulation of fatty acid desaturation in developing soybean seeds. More information is needed on how this enzyme is regulated and what role it may play in controlling fatty acid desaturation. This report concerns the mechanism by which stearoyl-ACP desaturase responds to changes in growth temperature and some possible factors that may affect the magnitude of this response.

MATERIALS AND METHODS

Materials

The [9,10-3H]stearic acid was purchased from Amersham. All other reagents and solvents were from Sigma Chemical Co. or EM Science. ACP was purified from log phase Escherichia coli (Grain Processors Inc.) by the method of Rock and Cronan (14). [9,10-3H]Stearoyl-ACP was synthesized by the procedure of Rock and Garwin (15). Ferredoxin was purified by the method of Yocum (19).

Plant Material

Soybean plants (Glycine max L. var Williams 82) were grown either in a greenhouse with 32°/26°C day/night, or in a growth chamber at 34°/28°C day/night with a 12 h photo-period. Developing soybean pods of stage R5 (3, 5) were harvested from the plants, surface sterilized and transferred to liquid culture media (13). The resulting pod cultures were incubated for 20 h at either 20°C or 35°C. Seeds were harvested from the cultures and crude enzyme extracts were prepared as previously described (2). Extracts were either used directly or stored at -80°C. Frozen extracts were stable for more than 1 month at -80°C. For induction experiments seeds were harvested after 0, 1, 5, 10, 20, or 40 h in culture. In developmental studies R5 pods were cultured for 20 h, and

1 Abbreviation: ACP, acyl carrier protein.
the resulting seeds were grouped by size (5, 6, and 7 mm) before homogenation.

**Enzyme Assay**

Stearoyl desaturase activity was determined by a modification of the procedure of McKeon and Stumpf (11). The assay measures the amount of $^3$H release during the formation of the double bond at position 9,10 of stearic acid. The $^3$H removed from carbons 9 and 10 of stearoyl-ACP by the desaturase is exchanged into water, which can then be measured by liquid scintillation counting. Comparison of the activity measured by $^3$H release with oleic acid formed (using [1-14C]stearoyl-ACP) showed that the two methods gave equivalent results (data not shown). The reaction contained: 0.6 $\mu$M [9,10-3H]stearoyl-ACP (320 $\mu$Ci/μmol), 50 mM Mes (pH 6.5), ferredoxin, ferredoxin-NADPH oxidoreductase, catalase, 300 $\mu$M NADPH, 0.2 mg/mL BSA, 1 mM DTT, and enzyme in a final volume of 125 μL. Ferredoxin, ferredoxin-NADPH oxidoreductase, catalase, NADPH, BSA, and DTT were first mixed and incubated for 10 min at 15°C; the reaction was then started by addition of stearoyl-ACP, Mes buffer, and enzyme. After incubating 10 min at 15°C, the reaction was stopped by addition of 140 μL of 1.2 M HCl and 560 μL of chloroform:methanol (2:1 v/v). The samples were mixed, centrifuged 20 s in a microfuge, and 300 μL of the aqueous phase were removed for quantitation of the product by liquid scintillation counting.

**RESULTS**

**Kinetic Analysis**

Extracts from beans cultured at 20°C or 35°C were analyzed to determine if the previously observed (2) differences in stearoyl desaturase activity were due to changes in the level of the activity present, or to changes in the characteristics of the enzyme(s) present.

Preparations from both culture conditions showed the same response to pH. Enzyme prepared from seeds cultured at either 20°C or 35°C had a distinct pH optimum of 6.5 with activity decreasing three to fivefold at pH values of 7.5 and 5.5 (Fig. 1A). In contrast to developing soybeans, this same enzyme from safflower seeds still retained half maximal activity at pH values of 5.5 and 8.5 (11). Further experiments with purified soybean stearoyl-ACP desaturase should indicate whether these results reflect true differences in the two enzymes or are caused by interaction with other components present in the soybean extract. Assays of the soybean extracts using PIPES buffer showed a lower activity and a broader pH optimum; this dependence of activity on buffer composition was also found by McKeon and Stumpf (11) with the enzyme from safflower seeds.

Extracts from seeds cultured at 20°C and 35°C showed similar responses to assay temperature. The optimum temperature for stearoyl-ACP desaturase activity was 15°C for enzyme from both 20°C- and 35°C-grown seeds (Fig. 1B). Activity remained high at the lowest temperature tested. In both preparations activity rapidly decreased with increasing assay temperatures; no activity was detected in assays at 40°C or 50°C.

Stearoyl-ACP desaturase from both 20°C and 35°C cultures showed maximum activity with 1.2 $\mu$M stearoyl-ACP (Fig. 1C). Higher concentrations of substrate caused a marked inhibition of activity; this inhibition was not seen with the purified enzyme from safflower (12). Lacking purified enzyme from soybeans, it can not be determined if the inhibition is a property of the desaturase or caused by interaction of the enzyme with other components, such as acyl-ACPs and polar lipids, in this plastidial fraction. Enzyme prepared from both 20°C and 35°C showed an apparent $K_m$ of 0.3 $\mu$M for stearoyl-ACP. Though slightly lower in activity, assays with 0.6 $\mu$M substrate were linear for at least 20 min (data not shown). Because of the inhibition at high substrate concentrations 0.6 $\mu$M stearoyl-ACP was routinely used for enzyme assays.

Enzyme preparations from both 20°C- and 35°C-grown seeds required ferredoxin, ferredoxin-NADPH oxidoreductase, catalase and NADPH for activity. Removal of DTT or BSA from the assay resulted in a 25 to 40% reduction in the activity of the enzyme.

Based on the above data, the changes induced by altered growth temperature do not involve induction of kinetically distinct isoenzymes during growth at 20°C and 35°C. If iso-enzymes were involved in the response, differences in kinetic characteristics should have been detected.

**Metabolic Effectors**

One possible explanation for the difference in activity between desaturase preparations from 20°C and 35°C cultures is the presence of inhibitory or stimulatory factors in the extracts. This hypothesis was tested by addition of boiled extracts to assays containing enzyme from 20°C or 35°C cultures. Activities in 20°C enzyme preparations were not reduced when boiled extracts from 35°C seeds (both chloroplasts and post-chloroplastic supernatant (2) were added to the assay (data not shown). Thus, the change in activity cannot be due to production of heat-stable inhibitors at 35°C. Likewise, boiled extracts from 20°C did not stimulate 35°C enzyme preparations (data not shown); so the difference can not be due to production of heat-stable activators at 20°C. Therefore, the difference in activity between enzyme extracts from 20°C- and 35°C-grown seeds does not appear to be due to induction of metabolic effectors by altered growth temperature.

**Factors Affecting Response to Growth Temperature**

The ratio of stearoyl desaturase activity in extracts from seeds grown at 20°C and 35°C varied between preparations from as much as 7:1 to as little as 1.5:1. Experiments were conducted to determine what other factors might be influencing the response of this enzyme to changes in growth temperature.

Based on previous studies of soybean seed development (1, 5), the experiments reported here used seeds from early in growth stage R5 (5–7 mm). This growth stage represents ~2 days of a 70 day developmental period from flowering, R1,
changed during this stage of development, extracts were prepared from 5, 6, and 7 mm seeds cultured for 20 h at 20°C or 35°C. Assays of these extracts indicated that the response of developing seeds to growth temperature is strongly influenced by the age of the seed used (Fig. 2). The enzyme activity from seeds of stage R5-S, 5 mm, showed a ratio of 3.8:1 for 20°C versus 35°C preparations, while desaturase from the largest seeds (R5-L, 7 mm) had almost identical activity in 20°C and 35°C extracts (Fig. 2A). The same trend is found when stearoyl-ACP desaturase activities were compared on a total activity basis (Fig. 2B); the ratio of 20°C versus 35°C activities were 3.8:1, 2.0:1, and 1:1 for 5, 6 and 7 mm seeds, respectively. These data indicate the changes are not due to differential accumulation of proteins; this is further reported by the near equal amounts of protein per g fresh weight seen in these and all other experiments reported here. No changes were found in the rate of development under the two culture conditions (Table I). When 5, 6, and 7 mm seeds were weighed before and after 20 h in culture the seed weight increased by 27, 18, and 21%, respectively, in 20°C cultures and 27, 17, and 17%, respectively, in 35°C cultures. The seeds from both treatments had the same appearance and average size after culture. These data indicate that the developmental stage of the seed is a primary factor in determining the amount of modulation caused by altered growth temperature.

The degree to which a developing seed responds to changes in growth temperature could be a function of the temperature to mature seed, R8 (3). Because of the amount of seed needed for each experiment, most assays reported in this paper used seeds from the oldest segment of this growth stage (i.e. 6.5 mm). To determine if the response to growth temperature

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**Figure 1.** Comparison of stearoyl-ACP desaturase characteristics in extracts from 20°C and 35°C-grown seeds. (A) Effect of assay pH on stearoyl-ACP desaturase activity. The enzyme was assayed as described in the methods section with either 50 mM MES (-----) or 50 mM HEPES (- - - - -) buffer at the pH indicated. (B) Effect of assay temperature on stearoyl-ACP desaturase activity. Assays were performed at the indicated temperatures by using the standard reaction mixture. (C) Effect of stearoyl-ACP concentration on desaturase activity. Assays were run at 15°C using the indicated concentrations of stearoyl-ACP in the standard reaction mixture. Enzyme activity was measured in chloroplast fractions prepared from seeds cultured for 20 h at either 20°C (C) or 35°C (●).

**Figure 2.** Effect of developmental stage on the response of stearoyl-ACP desaturase to growth temperature. (A) Changes in specific activity. (B) Changes in total activity. The enzyme was assayed as described in the methods section. All seeds were from growth stage R5 (S): S, M, and L correspond to 5, 6 and 7 mm long seeds, respectively.
at which the plants were grown prior to culture. To test this hypothesis, plants were grown in a growth chamber or greenhouse at a series of different day/night temperatures. Seed pods were removed from these plants and cultured for 20 h at 20°C or 35°C. The resulting enzyme activities showed only minor changes between the different day/night regimes (Fig. 3). The ratio of stearoyl-ACP desaturase activities did increase slightly with increasing growth temperature of the parent plant (Fig. 3). However, the ratio of 20:35°C activity did not show a consistent pattern of increase with increasing plant growth temperature; the increased ratio was only seen in plants grown at the highest temperature. Whether the plants were grown in a growth chamber under artificial light or in the greenhouse with natural light made little difference in this ratio. Thus, prior growth conditions of the seeds do not appear to influence the response of the seed to altered growth temperature.

Using in vivo labeling, MacCarthy and Stumpf (10) demonstrated that soybean cell suspension cultures could respond to altered growth temperature in 10 to 20 h. Therefore, 20 h was chosen as the incubation period for measuring changes in the enzymes of fatty acid synthesis and desaturation in pod cultures (2). The data reported here were also obtained from extracts of seeds cultured for 20 h at 20°C or 35°C. The time required to elicit a change in stearoyl-ACP desaturase activity was further analyzed to determine how duration of culture might effect the ratio of enzyme activity in 20°C versus 35°C extracts (Fig. 4). Enzyme was prepared from seeds cultured for 0, 1, 5, 10, 20, and 40 h; assay of these enzyme preparations indicated that the maximum response to altered growth temperature occurred 10 h after transfer to pod culture. This was true for both 20°C and 35°C growth temperatures. At all times after 0 h the activity in 20°C cultures was greater than in 35°C cultures. However, the difference in activities was greatest at 10 h and decreased slightly after 20 to 40 h. After 20 h the enzyme appears to reach new equilibrium levels. Adaptation to 20°C growth temperatures may occur through induction of increased desaturase activity, with levels in the 40 h cultures remaining higher than those in the starting material. Growth at 35°C shows the opposite response, i.e. after 10 h the enzyme activity declines to a new steady state with about half the activity found in the 0 h seeds.

All of the assays discussed above used a chloroplast fraction as the enzyme source. This leaves open the possibility that the changes found are a function of differences in chloroplast recovery between 20°C and 35°C grown seeds. Comparison of total homogenates and chloroplast fractions showed that 70% of the desaturase activity was in the chloroplasts from 20°C cultures, while 76% of the total activity was in the chloroplast fraction from 35°C cultures (data not shown). Thus, the differences found represent a real change in the amount of activity present after 20 h in culture at 20°C or 35°C. This conclusion is supported by previous work (2) which found that stearoyl-ACP desaturase activity changed with changes in growth temperature while the other enzymes in the chloroplast fraction (malonyl-CoA transacylase, fatty acid synthetase and palmitoyl-ACP elongase) did not change.

### CONCLUSIONS

The data presented here indicated that the activity of stearoyl-ACP desaturase is modulated by growth temperature. These results are consistent with a previous report from this laboratory (2) as well as with in vivo labeling studies on soybean suspension cultures (10) that showed the rate of fatty

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<th>Table 1. Changes in Seed Weight during Growth at 20°C or 35°C</th>
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<td>Developing seed pods were collected and sorted by seed size. Each size group was divided in thirds. One-third was harvested and weighed before culture (T₀). One-third was cultured for 20 h at 20°C and one-third was cultured for 20 h at 35°C then harvested and weighed.</td>
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<td>Seed Size (mm)</td>
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acid desaturation was changed with changes in growth temperature. The enzymatic data presented here indicate that the difference in stearoyl-ACP desaturase activity between seeds grown at 20°C and 35°C is not due to induction of kinetically distinct isoenzymes. Addition of boiled extracts to the assays failed to produce changes that would be expected if the modulation were due to induction of positive or negative effectors. These facts indicate that the change in stearoyl-ACP desaturase activity is due to a change in the level of active enzyme present in the tissue. Changes in the amount of active enzyme present in the tissue could be caused by any of three mechanisms: (a) increased synthesis of the enzyme, (b) changes in the rate of degradation of the enzyme, and (c) covalent modification of the enzyme leading to either activation or inactivation. Studies of soybean seed development have shown that desaturase levels peak at stage R5 (1); seeds either younger or older than stage R5 show little or no activity. The fact that the ratio of 20:35°C stearoyl-ACP desaturase activity was greatest in early R5 seeds, where enzyme synthesis should be highest, and lowest in mid R5 seeds, where enzyme synthesis should be decreasing, suggests that changes in synthesis may be involved. However, without data on the levels of desaturase protein present and the rate of enzyme and mRNA turnover no conclusions can be drawn about which of the three mechanisms discussed above is responsible for creating the observed change in activity.

**ACKNOWLEDGMENTS**

The author would like to thank Drs. H. Norman, T. Simpson, and C. G. Crawford for critical review of this manuscript.

**LITERATURE CITED**