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CHARACTERIZATION OF ACETYL-CoA CARBOXYLASE IN THE SEED OF TWO SOYBEAN GENOTYPES*

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Key Word Index—*Glycine max*; Leguminosae; seed development; acetyl-CoA carboxylase.

Abstract—Changes in the activity of acetyl-CoA carboxylase was followed during the formation of two lines of soybean (*Glycine max.*) seeds. The acetyl-CoA carboxylase activity expressed as units/seed was found to be higher in the experimental variety 9686 than in the cultivar, Wayne. The maximum activity in 9686 was around 40 days after flowering (DAF), while in Wayne it was around 30 DAF. The enzyme was active over a wide pH range, with an optimum at 8.2 for 9686 and 7.5 for Wayne. Citrate below 3 mM had no effect on 9686 enzyme, but higher concentrations were inhibitory. In Wayne, an increase in citrate up to 5 mM slightly stimulated the enzyme but higher concentrations were strongly inhibitory. Mn^{2+} could not replace Mg^{2+} as an essential activator of the carboxylase in both Wayne and 9686. A differential regulation of acetyl-CoA carboxylase in the two lines of soybean is suggested.

INTRODUCTION

Acetyl-coenzyme A carboxylase (acetyl-coenzyme A: bicarbonate ligase [ATP], EC 6.4.1.2) catalyzes the first committed step in the synthesis of fatty acids, the carboxylation of acetyl-CoA to form malonyl-CoA. The two-step reaction proceeds through a carboxylated enzyme intermediate, the carboxybiotinyl prosthetic group. Covalently bound biotin is carboxylated during the first half-reaction which utilizes bicarbonate and ATP. This one-carbon carrier then serves as the carboxy donor to acetyl-CoA in the second half-reaction to produce malonyl-CoA [1]. Acetyl-CoA carboxylase is required to supply malonyl-CoA for both the *de novo* synthesis and the elongation reactions. Studies of acetyl-CoA carboxylase from plant sources were initially hampered by low activities of this enzyme in extraction preparations. Recently, researchers have achieved stabilization and purification of this enzyme *in vitro* from a number of tissues [2–4].

Acetyl-CoA carboxylase has been shown to catalyze a regulatory step in the synthesis of fatty acids in plants [5–7] and animals [8–11]. In castor bean [5] and rape seed [6] large increases in the activity of acetyl-CoA carboxylase is correlated with lipid deposition.

During soybean seed development the rate of lipid biosynthesis per seed increases markedly, resulting in a mature seed containing 20–25% lipid by dry weight [12]. Between 15 and 45 days after flowering (DAF), the rate of fatty acid accumulation per seed increases 10–20 fold [13]. Recently, Cherry *et al.* [14], studied the pattern of fatty acid deposition in seven lines of soybean during seed maturation. Since experimental genotype, 9686, produces significantly less linoleate, linolenate and total lipid (approximately 30% less in all cases) than commercial genotype, Wayne, the regulation of total lipid synthesis could be due to changes in acetyl-CoA carboxylase. In the present study we have shown the changes in the activity of the enzyme acetyl-CoA carboxylase during seed maturation in two different genotypes of soybean (*Glycine max.* L. Merr.); the breeding line, 9686 and commercial genotype, Wayne.

RESULTS AND DISCUSSION

Significant differences exist in fatty acid composition of storage lipids between 9686 and Wayne [14]. As such we have studied the acetyl-CoA carboxylase activity during seed formation in these two lines of soybean. The pattern of acetyl-CoA carboxylase activity in the two lines is shown in Figs 1 and 2. The acetyl-CoA carboxylase activity (units/seed) in the experimental line was found to be higher than in the commercial line. The activity of acetyl-CoA carboxylase in 9686 increased until the seeds were around 400 mg fresh weight approximately 40 DAF and then decreased. In Wayne the activity increased until the seeds were around 225 mg fresh weight approximately 30 DAF and then decreased. The decrease in activity in 9686 appeared more abrupt while in Wayne it was gradual. We thus wanted to characterize the enzyme from the two lines of soybean. In rape seed [16], castor seed [5] and cultures of oil palm [6], the accumulation of lipid coincided with an increase in acetyl-CoA carboxylase activity.

The optimum conditions for *in vitro* assay of acetyl-CoA carboxylase activity were determined to ensure that the enzyme activity was measured under non-limiting conditions for the amount of substrate. For each optimization the other variables were maintained at their optimal values. The results are shown in Figs 3 and 4. The assay system for 9686 saturated at approximately 5 mM HCO_3^-, 0.1 mM acetyl-CoA, 5 mM Mg^{2+}, 2.0 mM ATP. The

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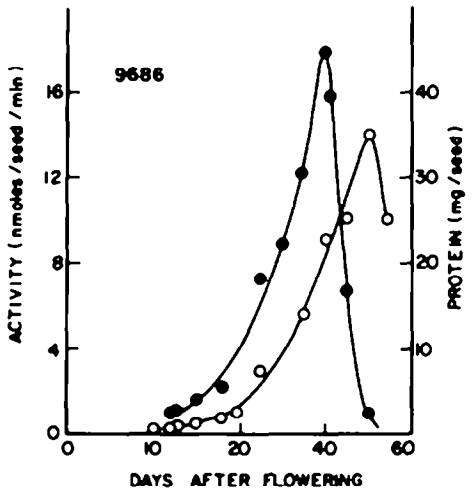

Fig. 1. Changes in the activity of acetyl-CoA carboxylase (●) and protein content (○) during seed formation in 9686.

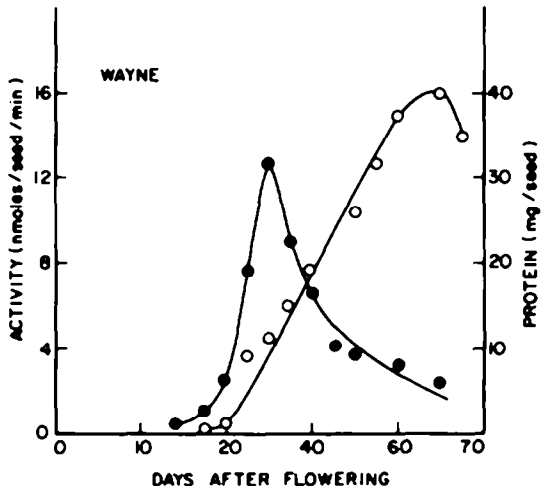

Fig. 2. Changes in the activity of acetyl-CoA carboxylase (●) and protein content (○) during seed formation in Wayne.

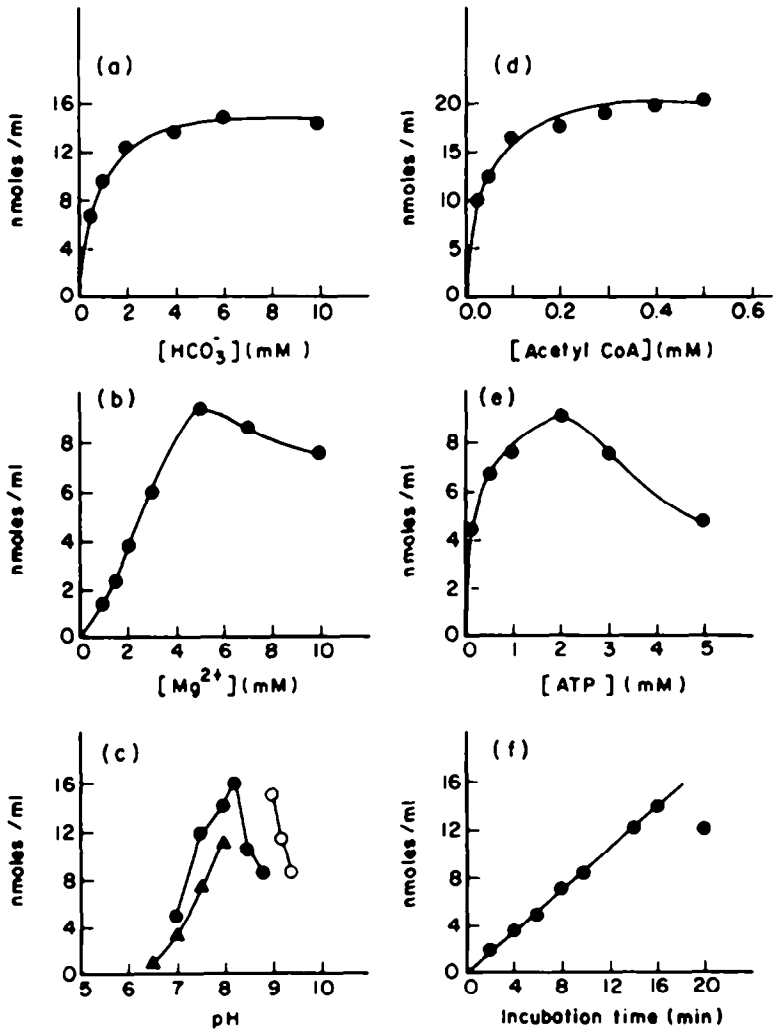

Fig. 3. The optimum conditions of the acetyl-CoA carboxylase assay in 9686. (a) HCO_3^- concentration; (b) Mg^{2+} concentration; (c) pH (▲, phosphate buffer; ●, Tris–HCl buffer; ○, glycine buffer); (d) acetyl-CoA concentration; (e) ATP concentration; (f) time.

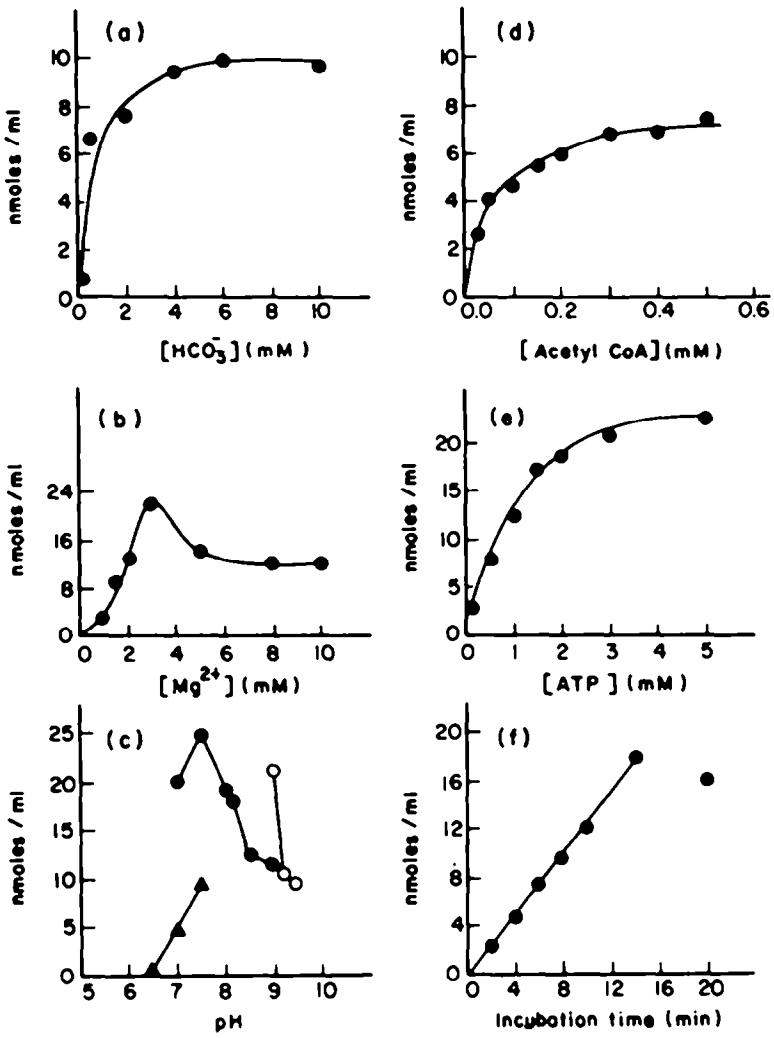

Fig. 4. The optimum conditions of the acetyl-CoA carboxylase assay in Wayne. (a) HCO_3^- concentration; (b) Mg^{2+} concentration; (c) pH (▲, phosphate buffer; ●, Tris–HCl buffer; ○, glycine buffer); (d) acetyl-CoA concentration; (e) ATP concentration; (f) time.

assay system for Wayne saturated at approximately 5 mM HCO_3^-, 0.3 mM acetyl-CoA, 3 mM Mg^{2+}, 3 mM ATP. The optimum pH for the assay was pH 8.2 in Tris buffer for 9686 and pH 7.5 in Tris buffer for Wayne. Under optimum conditions the assay was linear up to 50 μg of protein in both Wayne and 9686, and for at least 15 min.

Affinity chromatography purified acetyl-CoA carboxylase from developing seeds of Wayne and 9686 was found to be active over a wide pH range, with an optimum at 8.2 for 9686 and 7.5 for Wayne. The wheat germ enzyme had an optimum of 8.8 [17], that from avocado plastids an optimum of 7.5 [18] and that from castor oil endosperm an optimum of 8.0 [19]. The activity in glycine–NaOH at pH 9.6 and 9.2 was higher than the activities in Tris-HCl buffer at pH 8.5 and 8.8 in 9686. In Wayne, activity in glycine–NaOH buffer at pH 9.0 was higher than at pH 8.0 to 8.8 in Tris-HCl buffr. Glycine (5 mM) stimulated the activity of affinity-purified acetyl-CoA carboxylase from both Wayne and 9686. Also, when potassium phosphate buffer was used in place of sodium phosphate buffer, there was a stimulation of activity and this was due to potassium (Table 2).

Triton X-100, at a concentration of 0.2% in the homogenizing media, was essential to obtain maximal activity (Table 1). This could be due to the increased solubilization of the enzyme by the detergent.

The effect of the various assay constituents on the enzyme activity showed an absolute requirement for ATP, $MgCl_2$, acetyl-CoA and this is in agreement with the known cofactor requirements for acetyl-CoA carboxylase activity [1, 20]. Mild stimulation by DTT was also observed.

The inhibition by citrate (Table 3), an allosteric activator of the animal carboxylase [1], can be attributed to Mg^{2+} chelation since the inhibition can be relieved by the addition of equimolar concentrations of Mg^{2+}. The same effect was observed for the wheat germ enzyme [19, 21]; however, the activity of the carboxylase isolated from either spinach or avocado is stimulated by 3 mM citrate [18]. In parsley, citrate stimulated enzyme activity only

Table 1. Effect of triton on the extraction of acetyl-CoA carboxylase activity in Wayne and 9686

	Activity (nmoles/min/g fr. weight)	
	Wayne	9686
+ Triton X-100	15.3	12.0
− Triton X-100	5.4	4.0

Table 2. Effect of KCl on acetyl-CoA carboxylase activity

	Activity (nmoles/ml/5 min)			
	− KCl	20 mM KCl	40 mM KCl	80 mM KCl
Wayne	10.0	11.5	14.5	15.0
9686	13.1	26.5	28.5	30.0

Affinity chromatography-purified enzyme was used.

Table 3. Effect of varying concentrations of citrate on acetyl-CoA carboxylase activity in Wayne and 9686

Citrate (mM)	Relative rate	
	Wayne	9686
0	100	100
0.5	110	100
1.0	115	100
3.0	115	53
4.0	125	51
5.0	128	50

Affinity chromatography purified enzyme was used.

slightly at about 2 mM but strongly inhibited the activity at higher concentrations with either Na^+ or K^+ as the counter ion [22]. Citrate at 0.5 mM and 1.0 mM had no effect on the 9686 enzyme, but concentrations above 3.0 mM strongly inhibited the enzyme (Table 3). However, acetyl-CoA carboxylase from Wayne was stimulated slightly up to 5.0 mM citrate, but strongly inhibited above 5.0 mM citrate (Table 3).

Sodium pyrophosphate had no effect on the enzyme activity in both Wayne and 9686. Acetyl-CoA carboxylase from developing castor oil seeds was inhibited by pyrophosphate [19] and this was attributed to the formation of a dead-end complex. In addition, it was shown that ADP and phosphate act as product and dead end inhibitors [19].

Acetyl-CoA carboxylase activity both from Wayne and 9686 was stimulated by the presence of KCl in the assay system (Table 2). Acetyl-CoA carboxylase activity from Wayne was stimulated approximately 1.2 fold while the activity from 9686 was stimulated almost 2 fold. Nielsen and Stumpf [23] showed the stimulation of wheat germ acetyl-CoA carboxylase by potassium. In wheat germ, maximal stimulation took place between 30 and 60 mM KCl. In both Wayne and 9686, maximal stimulation took place between 20 and 40 mM KCl. The degree of stimulation by 20 mM KCl was similar both in the presence and absence of dithiothreitol, suggesting that K^+ stimulation was not related to a sulphydryl site activation. This was true in the case of wheat germ enzyme too. The maximum K^+-induced activation of both the castor oil seed enzyme (1.5-fold), the wheat germ enzyme (2.9-fold) and the soybean seed enzyme (1.2-fold for Wayne and 2-fold for 9686) is substantially less than that observed for the enzyme isolated from *T. aceti* (32-fold).

There was a marked inhibition of 9686 acetyl-CoA carboxylase activity at higher ATP concentrations. However, the Wayne acetyl-CoA carboxylase activity was not inhibited at higher ATP concentrations. The acetyl-CoA carboxylase activity from both Wayne and 9686 was inhibited at higher Mg^{2+} concentrations. It has been shown earlier [18] that $MgATP^{2-}$ is the reactive species in the carboxylation reaction, while both free ATP^{4-} and Mg^{2+} are inhibitory. Magnesium was essential for activity as shown in Fig. 2. Manganese could not replace Mg^{2+} in both Wayne and 9686 as an essential activator of the carboxylase (Table 4). For the 9686 enzyme, 15–20% of the activity could be obtained by 2.0 mM Mn^{2+}; concentrations above were inhibitory (Table 4). The enzyme from developing castor oil seeds [19] showed maximum velocity at a Mn^{2+} concentration of 1.5 mM, whereas with Mg^{2+}, the same velocity is only achieved at a concentration of 5 mM. For the wheat germ enzyme, the maximal activity obtained with Mn^{2+} was only 40–50% that obtained with Mg^{2+} [24].

Acetyl-CoA carboxylase from plant tissues has been reported to be a very labile enzyme [18, 23, 25, 26]. The activity of acetyl-CoA carboxylase in extracts of developing soybean seeds declined by 80% in 10 hr at 4° in the presence of 1 mM phenylmethanesulphonyl fluoride/5 mM DTT/0.2% Triton-X-100. However, the ammonium sulphate purified enzyme in the presence of 1 mM phenylmethanesulphonyl fluoride/5 mM DTT/0.2% Triton X-100 retained 80% of the activity after 24 hr at 4°.

Our research on the comparison of the experimental genotype (9686) with Wayne has provided a unique foundation to explore the biochemical pathways in lipid synthesis. The present data suggest that it is reasonable to assume that acetyl-CoA carboxylase may play a key regulatory role in fatty acid biosynthesis. Further studies

Table 4. Effect of replacing Mg^{2+} with Mn^{2+} on the activity of acetyl-CoA carboxylase in Wayne and 9686

Mn^{2+} (mM)	Relative rate	
	Wayne	9686
0 + Mg^{2+} (5 mM)	100	100
1.0	5	29
2.0	0	25
4.0	0	13
5.0	0	8
10.0	0	8

being carried out on the kinetic properties of the affinity purified enzyme could possibly shed some light on the differential regulation of the enzyme in the two lines of soybean. Antisera to acetyl-CoA carboxylase is also being used to study the regulation of acetyl-CoA carboxylase in developing soybean seeds.

EXPERIMENTAL

Two lines of soybean plants varying in linolenic acid content from 4 to 10‰ were grown in the greenhouse, and seeds selected at different stages of development from one low linolenic acid experimental line (9686) and one high linolenic acid commercial variety (Wayne) were used in this study. Seed pods were harvested from between 12 and 70 days after flowering.

$NaH^{14}CO_3$ (2.07 GBq/mmol) was purchased from Amersham International, Amersham. ATP and Acetyl-CoA were from Sigma Chemical Co.

Enzyme preparation. Seeds were removed from the pods and enzyme extracts prepared by grinding in a chilled mortar with pestle at 4°. Seeds were homogenized in 100 mM Tris–HCl, pH 8.2, containing 1 mM phenylmethanesulphonyl fluoride, 2 mM EDTA, 5 mM DTT and 0.2% Triton X-100. The homogenate was filtered through two layers of muslin cloth and centrifuged at 5000 *g* for 20 min. Proteins were precipitated from the supernatant by using $(NH_4)_2SO_4$ 90% saturation and pelleted at 15 000 *g*. The pellet was redissolved in a small vol of homogenization medium. For other experiments, the 5000 *g* supernatant was brought to 10% saturation with $(NH_4)_2SO_4$ and centrifuged at 15 000 *g* for 20 min. The supernatant was brought to 40% saturation with $(NH_4)_2SO_4$ and pelleted at 15 000 *g*. The pellet was redissolved in homogenization medium. This gave about 5-fold purification. For some of the experiments, the enzyme purified by affinity chromatography (not shown) was also used. The affinity column was prepared and the enzyme purified according to [27] with some modifications.

Acetyl-CoA carboxylase assay. Acetyl-CoA carboxylase activity was determined by measuring the incorporation of $NaH^{14}CO_3$ *into* malonyl-CoA. The incubation mixture contained, in a total volume of 150 μl, 44 mM Tris–HCl, pH 8.2 or 7.5, 2 mM Na_2 ATP, 5 mM $MgCl_2$, 1 mM DTT (dithiothreitol), 10 mM $NaH^{14}CO_3$ (1 μCi/μmol), 0.5 mM acetyl-CoA and enzyme protein (about 30–40 μg). The mixture was preincubated for 4 min at 37°. The reaction was started by the addition of AcCoA preincubated for 1 min at 37°. After 5 min at 37°, the reaction was terminated by adding 60 μl of 6 M HCl. A portion of the mixture (100 μl) was dried on glass fibre disks for 1 hr and the acid stable radioactivity was counted using a scintillator (4 g and 50 mg POPOP in 1L Toluene) in a Beckman liquid scintillation counter. Blanks without acetyl-CoA were included in every assay.

Protein determination. Protein was determined by the method of Bradford [15] after precipitation with trichloroacetic acid using bovine serum albumin as a standard.

All the experiments were repeated five times and the values reported are of a typical experiment.

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