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Purification and Kinetic Properties of Serine Acetyltransferase Free of *O*-Acetylserine(thiol)lyase from Spinach Chloroplasts[1]

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Serine acetyltransferase, a key enzyme in the L-cysteine biosynthetic pathway, was purified over 300,000-fold from the stroma of spinach (*Spinacia oleracea*) leaf chloroplasts. The purification procedure consisted of ammonium sulfate precipitation, anion-exchange chromatography (Trisacryl M DEAE and Mono Q HR10/10), hydroxylapatite chromatography, and gel filtration (Superdex 200). The purified enzyme exhibited a specific activity higher than 200 units mg^{-1} and a subunit molecular mass of about 33 kD upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Moreover, the purified serine acetyltransferase appeared to be essentially free of *O*-acetylserine(thiol)lyase, another enzyme component in the L-cysteine biosynthetic pathway. A steady-state kinetic analysis indicated that the mechanism of the enzyme-catalyzed reaction involves a double displacement. The apparent K_m for the two substrates, L-serine and acetyl-coenzyme A, were 2.29 ± 0.43 and 0.35 ± 0.02 mM, respectively. The rate of L-cysteine synthesis in vitro was measured in a coupled enzyme assay using extensively purified *O*-acetylserine(thiol)lyase and serine acetyltransferase. This rate was maximum when the assay contained approximately a 400-fold excess of *O*-acetylserine(thiol)lyase over serine acetyltransferase. Measurements of the relative level of *O*-acetylserine(thiol)lyase and serine acetyltransferase activities in the stroma indicated that the former enzyme was present in much larger quantities than the latter. Thus, the activity ratio for these two enzymes [*O*-acetylserine(thiol)lyase activity/serine acetyltransferase activity] measured in the stromal protein extract was 345. This strongly suggested that all the *O*-acetylserine(thiol)lyase and serine acetyltransferase activities in the stroma are involved in bringing a full synthesis of L-cysteine in the chloroplast.

It is known that in microorganisms the synthesis of L-Cys from L-Ser proceeds via a two-step pathway involving two enzymes (Kredich and Tomkins, 1966; Kredich et al., 1969; Cook and Wedding, 1978). Serine acetyltransferase (EC 2.3.1.30) catalyzes the acetylation of L-Ser (Eq. 1). Sulfhydrylation of *O*-acetylserine in the presence of sulfide (Eq. 2) is catalyzed by *O*-acetylserine(thiol)lyase (EC 4.2.99.8).

$$\text{L-Ser} + \text{acetyl-CoA} \rightarrow \textit{O}\text{-acetylserine} + \text{CoA} \quad (1)$$

$$\textit{O}\text{-acetylserine} + S^{2-} \rightarrow \text{L-Cys} + \text{acetate} \quad (2)$$

Both enzymes have been extensively purified and characterized in *Salmonella typhimurium* and *Escherichia coli* (Kredich and Tomkins, 1966; Kredich et al., 1969; Cook and Wedding, 1977, 1978; Baecker and Wedding, 1980). In these cells all the serine acetyltransferase activity is associated with a small portion (5%) of the total *O*-acetylserine(thiol)lyase in a complex called cysteine synthetase (Kredich and Tomkins, 1966). Resolution of this complex into its components is specifically accomplished in vitro by the addition of small amounts (0.1 mM) of the intermediate product, *O*-acetylserine. The reaction yields one molecule of serine acetyltransferase (molecular mass 160 kD) and two molecules of *O*-acetylserine(thiol)lyase (molecular mass 68 kD) (Kredich et al., 1969). This resolution is prevented by sulfide (Kredich et al., 1969). Reconstitution of the complex occurs spontaneously upon mixing solutions of serine acetyltransferase and *O*-acetylserine(thiol)lyase. The kinetic properties of resolved serine acetyltransferase are identical with those of the bound enzyme (Kredich et al., 1969). In contrast, these researchers showed that the turnover number for *O*-acetylserine(thiol)lyase and the apparent K_m for *O*-acetylserine were increased 2- and 4-fold, respectively, when this enzyme was resolved from serine acetyltransferase. It is also known that a single specific point mutation in the structural gene for serine acetyltransferase results in diminished catalytic activity of both components of the complex (Becker and Tomkins, 1969), which points out the potential importance of specific protein-protein interactions within the bifunctional complex for the regulation of the cysteine synthetase activity.

It is established that plant cells from either photosynthetic or nonphotosynthetic tissues contain all the enzymic equipment of the sulfate assimilation pathway leading to the synthesis of sulfur-containing amino acids (Anderson, 1980; Giovanelli et al., 1980; Brunold and Suter, 1989). Compared with the considerable information accumulated for more than 25 years with prokaryotes, the mechanism of L-Cys biosynthesis is not defined as well in plant systems. Another complexity with the plant systems arises from the compartmen-

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tation of enzyme activities within cellular organelles and cytosol. Such compartmentation plays an important role in many plant metabolic pathways (ap Rees, 1987).

In previous work we analyzed the intracellular compartmentation of some enzymes of the sulfate assimilation pathway in spinach (*Spinacia oleracea*) leaves (Lunn et al., 1990; Droux et al., 1992). These studies disclosed the existence of several isoforms of *O*-acetylserine(thiol)lyase, each of which is specific for each cell compartment involved in protein biosynthesis, namely, plastids, mitochondria, and cytosol. Several attempts have been made to purify serine acetyltransferase from plants, including *Phaseolus vulgaris* leaves and roots (Smith and Thompson, 1971), *Brassica chinensis* leaves (Nakamura et al., 1988), and *Allium tuberosum* leaves (Nakamura and Tamura, 1990). The enzyme was reported to be associated with plastids (Brunold and Suter, 1982; Droux et al., 1992), mitochondria (Smith and Thompson, 1969; Smith, 1972), and the cytosol (Brunold and Suter, 1982). However, all these previous attempts have been conducted with crude extracts, and, therefore, it is difficult to assign the isolated form of the enzyme to a given intracellular compartment. Furthermore, all of the serine acetyltransferases purified so far from plants contain variable amounts of tightly bound *O*-acetylserine(thiol)lyase.

A consideration of the possible roles attributable to protein-protein interactions in the regulation of L-Cys biosynthesis in plants requires a detailed characterization of the purified components involved in the pathway. To this aim we previously purified to apparent homogeneity *O*-acetylserine(thiol)lyase from the stroma of spinach chloroplast (Droux et al., 1992). We also cloned a cDNA coding for this enzyme and overexpressed the plant enzyme in *Escherichia coli* (Rolland et al., 1993). In this study we have purified for the first time the serine acetyltransferase from chloroplasts of spinach leaves, free of *O*-acetylserine(thiol)lyase. We have also characterized some catalytic properties of this purified enzyme.

MATERIALS AND METHODS

Plant Material

Spinach (*Spinacia oleracea*) leaves were purchased from a local market. The amount of serine acetyltransferase that could be extracted from spinach chloroplasts varied considerably depending upon the season at which plants were cultivated. Spinach plants cultivated from April to June yielded the highest enzyme amounts.

Enzyme Assay

O-Acetylserine(thiol)lyase Assay

Activity was measured in a volume of 0.1 mL containing 50 mM Tris-HCl buffer (pH 7.5), 5 mM DTT, 5 mM *O*-acetylserine, 1 mM sodium sulfide, and known amounts of enzyme. Reaction was initiated by adding sodium sulfide. After incubation for 10 min at 25°C, the reaction was stopped by adding 50 μL of 20% (w/v) TCA, and the precipitated protein was removed by centrifugation at 15,000*g* for 2 min. The supernatant was transferred to a glass tube containing 100 μL of concentrated acetic acid and 200 μL of ninhydrin reagent (250 mg of ninhydrin dissolved in 10 mL of concentrated acetic acid:concentrated HCl [60:40, v/v]). The mixture was boiled for 10 min, then cooled rapidly before addition of 600 μL of ethanol (Droux et al., 1992). L-Cys was determined by measuring the A_{560} (Gaitonde, 1967). One unit of enzyme activity is equivalent to the formation of 1 μmol of L-Cys per min. Product formation was linear for at least 30 min, and velocities were proportional to enzyme concentration in the assay, which indicates adherence to steady-state conditions.

Serine Acetyltransferase Assay

Serine acetyltransferase was assayed by measuring its capacity to promote L-Cys synthesis in the presence of L-Ser, acetyl-CoA, sulfide, and *O*-acetylserine(thiol)lyase, as described by Nakamura et al. (1987) (cysteine synthetase assay). Therefore, in this assay *O*-acetylserine(thiol)lyase was used as a coupling enzyme. In previous work a cDNA coding for the entire mature *O*-acetylserine(thiol)lyase from spinach chloroplasts had been cloned and overexpressed in *E. coli* (Rolland et al., 1993). The recombinant protein was purified to homogeneity (Rolland, 1992; a full description of the purification procedure will be published elsewhere) and used as a source of coupling enzyme for the serine acetyltransferase assay. The specific activity of this enzyme preparation was 500 units/mg. Activity was measured in a volume of 100 μL, containing 50 mM Tris-HCl buffer (pH 7.5), 5 mM L-Ser, 1 mM acetyl-CoA, 1 mM sodium sulfide, 5 mM DTT, and a saturating amount (5 μg) of *O*-acetylserine(thiol)lyase. Following a 2-min incubation at 25°C, reactions were initiated by adding either sodium sulfide or L-Ser. They were first incubated for 30 min at 25°C, and then processed as described above for the *O*-acetylserine(thiol)lyase assays. L-Cys was determined as described above. There was no significant variation in serine acetyltransferase activity when the first incubation was conducted for longer periods of time. One unit of enzyme activity is equivalent to the formation of 1 μmol of L-Cys per min. Product formation was linear for at least 30 min, and velocities were proportional to the concentration of serine acetyltransferase in the assay.

Isolation of Intact and Purified Chloroplasts

Chloroplasts were isolated from 10 kg of spinach leaves and purified as described by Douce and Joyard (1982), using discontinuous Percoll gradients formed by two layers of 50 and 20 mL of 40 and 80% (v/v) Percoll solutions, respectively. After centrifugation at 6000*g* for 20 min (JS-7.5 rotor, Beckman), the intact chloroplasts were recovered in a dark-green band near the bottom of the tube. They were suspended in a solution containing 10 mM phosphate buffer (pH 7.8) and 0.3 M mannitol (final volume 1 L), and then centrifuged at 2500*g* for 5 min.

Preparation of Soluble (Stroma) Proteins from Spinach Leaf Chloroplasts

All steps were performed at 4°C unless otherwise indicated. The intact chloroplasts (about 10 g of protein) were

lysed in 250 mL of lysis buffer containing 10 mM Tricine-NaOH buffer (pH 7.8) and 4 mM $MgCl_2$. The lysate was loaded on a 0.6 M Suc cushion and then centrifuged at 72,000*g* for 60 min (Beckman SW-27 rotor) to remove all chloroplast membranes (envelope membranes and thylakoids). The amber-colored supernatant (approximately 5 g of protein in 250 mL of lysis buffer) contained both serine acetyltransferase and O-acetylserine(thiol)lyase activities. Enzyme purification was performed after pooling two preparations of stromal proteins, thus corresponding to a total amount of about 10 g of soluble (stroma) proteins from 20 kg of spinach leaves.

Purification of Serine Acetyltransferase

Step I: $(NH_4)_2SO_4$ Precipitation

Crystalline $(NH_4)_2SO_4$ was added slowly to the above supernatant with stirring until 40% (w/v) saturation was achieved. The mixture was stirred for 30 min at 4°C and then centrifuged at 40,000*g* for 20 min. The resulting precipitate was resuspended in 20 mL of buffer A containing 20 mM phosphate buffer (pH 8.4), 1 mM EDTA, 10 μM pyridoxal phosphate, 1 mM DTT, and a mixture of protease inhibitors (5 mM ε-aminocaproic acid, 1 mM benzamidine). All subsequent chromatographic steps were performed at 25°C.

Step 2: Trisacryl M DEAE Chromatography

The above protein extract (20 mL) was desalted by passage through a G25 column (2.6 × 25 cm) equilibrated in buffer A and then clarified by centrifugation (40,000*g*, 30 min, 20°C). The clear eluate (30 mL, 1 g of protein) was applied to a Trisacryl M DEAE column (2.6 × 25 cm; Pharmacia) that had been previously equilibrated in buffer A. After the column had been washed with 1 L of the equilibrating buffer, elution was carried out with 50 mL of buffer A containing 0.2 M NaCl (flow rate 2 mL/min, fraction size 5 mL). Both serine acetyltransferase and O-acetylserine(thiol)lyase activities emerged from the column at the same elution volume. Fractions containing the highest serine acetyltransferase specific activity were pooled (20 mL, 150 mg of protein) and desalted by passage through a GF05 column (2.6 × 25 cm) equilibrated in buffer B containing 20 mM Tris-HCl buffer (pH 7.8), 1 mM DTT, and 10 μM pyridoxal phosphate.

Step 3: Mono-Q HR10/10 Chromatography

This solution (150 mg of protein, 25 mL) was applied to a Mono-Q HR10/10 column (1 × 10 cm; Pharmacia) that had been previously equilibrated in buffer B. After the column had been washed with 100 mL of buffer B, elution was carried out with a first 50-mL linear gradient of NaCl (0–0.3 M) in the same buffer, a 30-mL wash with buffer B containing 0.3 M NaCl, and a second 70-mL linear gradient of NaCl (0.3–1 M) in buffer B (flow rate 1.5 mL/min; fraction size 3 mL). O-Acetylserine(thiol)lyase activity was recovered in the fractions eluting with the first NaCl gradient (0.18 M NaCl), whereas the fractions eluting with the second NaCl gradient (0.45 M NaCl) contained the serine acetyltransferase activity free from O-acetylserine(thiol)lyase.

Step 4: Hydroxylapatite Chromatography

The fractions containing the highest serine acetyltransferase specific activity were pooled (approximately 8 mL, 600 μg of protein). This solution was applied to a hydroxylapatite column (0.6 × 3 cm; Pharmacia) that had been previously equilibrated in buffer B containing 0.4 M NaCl. After the column had been washed with 50 mL of the equilibrating buffer, serine acetyltransferase was eluted as a single sharp peak with buffer B containing 0.4 M NaCl and 20 mM phosphate buffer (pH 7.8) (flow rate 0.5 mL/min; fraction size 1 mL).

Step 5: Superdex 200 Chromatography

The fractions containing the highest serine acetyltransferase specific activity were pooled (1.5 mL, approximately 50 μg of protein). This solution was applied to a Superdex 200 column (2 × 61.5 cm; Pharmacia) that had been previously equilibrated in buffer B containing 0.4 M NaCl. The enzyme was eluted with the equilibrating buffer (flow rate 1 mL/min; fraction size 1.5 mL).

Electrophoresis

SDS-PAGE was carried out at room temperature in SDS-polyacrylamide slab gels containing a 10 to 20% (w/v) linear acrylamide gradient. The experimental conditions for gel preparation, sample solubilization, electrophoresis, and gel staining were as described by Chua (1980).

Protein Determination

Protein was measured either by the method of Bradford (1976) using the Bio-Rad protein reagent with γ-globulin as the standard or by measuring A_{205} (Scopes, 1974). The latter method was used to calculate the final specific activities of O-acetylserine(thiol)lyase and serine acetyltransferase.

Curve-Fitting Analyses

The kinetic data were analyzed with a program providing an iterative fit to the appropriate equation by using a nonlinear curve-fitting method (KaleidaGraph, Abelbeck software) and a Macintosh IIsi computer. The saturation curve for serine acetyltransferase activity was fitted to the following equation:

$$v = \frac{V_m^{app} \cdot [\text{acetyl-CoA}]}{K_m^{app} + [\text{acetyl-CoA}]} \quad (3)$$

where V_m^{app} and K_m^{app} are the apparent V_m and K_m values for acetyl-CoA at different fixed values of [L-Ser], respectively. The V_m^{app} and K_m^{app} data were fitted to the equations

$$V_m^{app} = \frac{V_m \cdot [\text{L-Ser}]}{K_m^{LS} + [\text{L-Ser}]} \quad (4)$$

and

$$K_m^{app} = \frac{K_m^{CoA} \cdot [\text{L-Ser}]}{K_m^{LS} + [\text{L-Ser}]} \quad (5)$$

where V_m, K_m^{LS}, and K_m^{CoA} are the maximum rate of L-Cys

synthesis at saturating [L-Ser] and [acetyl-CoA], the K_m for L-Ser, and the K_m for acetyl-CoA, respectively.

RESULTS

Isolation of Serine Acetyltransferase Free from *O*-Acetylserine(thiol)lyase from Spinach Leaf Chloroplasts

Spinach chloroplasts were purified on Percoll gradients. Control experiments were carried out by measuring the activity of several marker enzymes including pyrophosphate-fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90) for the cytosol, fumarase (EC 4.2.1.2) for the mitochondria, and catalase (EC 1.11.1.6) for the peroxisomes. The results disclosed that extrachloroplastic contaminations were negligible (not shown). The isolated chloroplasts were lysed and membranes were separated from soluble proteins (stroma) by ultracentrifugation. Each fraction was assayed for serine acetyltransferase and *O*-acetylserine(thiol)lyase activities. Over 98% of both activities were found in the soluble fraction (Table I), confirming previous findings (Droux et al., 1992). Table I shows that considerable purification was needed to obtain a homogeneous preparation of serine acetyltransferase. Previous work suggested the existence in spinach chloroplasts of a bifunctional protein complex composed of serine acetyltransferase and *O*-acetylserine(thiol)lyase (Droux et al., 1992). A tight interaction between the two enzymes was confirmed in the present study when we observed that, upon gel filtration of the soluble protein extract from spinach chloroplasts on Ultrogel ACA 34 (IBF), *O*-acetylserine(thiol)lyase activity eluted in two distinct peaks. A minor peak of molecular mass approximately 300 kD contained both *O*-acetylserine(thiol)lyase and serine acetyltransferase activities and a major peak (molecular mass approximately 68 kD) was essentially free of serine acetyltransferase activity (not shown).

Details on the purification of serine acetyltransferase from the chloroplast protein extract are given in Table I. The key steps were: $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography on Trisacryl M DEAE and Mono-Q HR10/10, hydroxylapatite chromatography, and gel filtration on Superdex 200. $(NH_4)_2SO_4$ precipitation allowed the elimination of the major part (90%) of the free *O*-acetylserine(thiol)lyase activity contained in the stromal extract, with a minimal loss of the total serine acetyltransferase activity. Following this step the activity ratio [*O*-acetylserine(thiol)lyase activity/serine acetyltransferase activity, *R*] decreased from a value of 345 in the chloroplast extract to a value on the order of 25 in the precipitated fraction. During Trisacryl M DEAE chromatography the two enzyme activities still co-eluted, and the value of the activity ratio, *R*, was on the order of 25 in the eluted fractions. Complete separation of serine acetyltransferase and *O*-acetylserine(thiol)lyase activities was achieved by anion-exchange chromatography on Mono-Q HR10/10 (Fig. 1). Under these conditions the activity ratio, *R*, decreased to a negligible value smaller than 10^{-3} in the eluted fractions. SDS-PAGE analysis disclosed, however, that this enzyme preparation still contained many polypeptides (Fig. 2A, lane 4). Further purification was achieved by hydroxylapatite chromatography and gel filtration on Superdex 200 (Table I). The procedure resulted in an approximately 300,000-fold purification. Protein was not detectable by Bradford's method following the Superdex 200 gel-filtration step.

The yield obtained from 11 g of chloroplast stromal protein was less than 2 μg and the overall yield of activity was about 7% (Table I), due in large part to losses during desalting steps and hydroxylapatite chromatography. We estimate that the specific activity of purified serine acetyltransferase is greater than 200 units/mg.

Properties of the Purified Serine Acetyltransferase

Purity of the Enzyme

Using SDS-PAGE, the purified chloroplast serine acetyltransferase showed a predominant band corresponding to a molecular mass of 33 kD (Fig. 2A, lane 6), substantially smaller than that of *O*-acetylserine(thiol)lyase purified from spinach chloroplast (Droux et al., 1992; Rolland et al., 1993). This latter enzyme exhibits a molecular mass of 35 kD on

Table I. *Purification procedure of serine acetyltransferase from soluble proteins (stroma) of spinach leaf chloroplasts*

Protein was determined with the Bio-Rad protein assay up to the Mono-Q HR10/10 column and thereafter according to Scopes (1974). O-Acetylserine(thiol)lyase (O-ASTL) and serine acetyltransferase (SAT) activities were determined as described in "Materials and Methods." *R* stands for the ratio [*O*-acetylserine(thiol)lyase activity/serine acetyltransferase activity]. The table refers to a starting quantity of spinach leaves of 20 kg. Assuming that 1 g of spinach leaves contains 20 mg of chloroplastic proteins (1 mg Chl) and that the stromal proteins represent half of the total amount of chloroplastic proteins, the yield of intact chloroplasts was 5%.

Step	Total Protein	Total Activity		Specific Activity SAT	Recovery SAT	*R*
		SAT	O-ASTL			
	mg	*units*	*units*	*units/mg protein*	%	
Stroma	10,200	5.4	1,865	0.53 10^{-3}	100	345
Ammonium sulfate 0–40%	970	4.35	112	4.5 10^{-3}	80.6	25
Trisacryl M DEAE pool	180	3.5	86	19 10^{-3}	64.8	25.5
Mono-Q HR10/10 pool	0.62	1.05	0.094	1.69	19.4	$<10^{-3}$
Hydroxylapatite pool	0.02	0.530	0	26.5	9.8	0
Superdex 200 pool	0.002	0.41	0	205	7.6	0

SDS-PAGE (Fig. 2B, lane 1) [note the different migration positions on SDS-PAGE of the polypeptides assigned to serine acetyltransferase in lane 6 (Fig. 2A) or lane 2 (Fig. 2B), and to O-acetylserine(thiol)lyase (Fig. 2B, lane 1) relative to that of the marker proteins]. Control experiments showed that when the two purified proteins were mixed prior to electrophoresis they remained clearly separable by SDS-PAGE, using a gel containing a 10 to 20% polyacrylamide gradient.

Titration of Serine Acetyltransferase with O-Acetylserine(thiol)lyase

Due to the very low abundance of serine acetyltransferase in the stromal chloroplast extract (Table I) and owing to the high sensitivity of the spectrophotometric assay developed by Nakamura et al. (1987) (cysteine synthetase assay), during this study spinach chloroplast O-acetylserine(thiol)lyase was used as a coupling enzyme to detect the serine acetyltransferase activity. Therefore, it was important to avoid complication in the interpretation of the results to rule out the possibility that the coupling enzyme was contaminated by serine acetyltransferase. This condition was fulfilled by using as a coupling enzyme the spinach chloroplast O-acetylserine(thiol)lyase that had been cloned and overexpressed in *E. coli* (Rolland et al., 1993). The recombinant enzyme exhibits an identical amino acid sequence as well as identical physicochemical and kinetic properties compared with the enzyme purified from spinach leaf chloroplasts (Droux et al., 1992; Rolland, 1992; Rolland et al., 1993).

Figure 3 depicts the dependence of the rate of L-Cys synthesis from L-Ser, acetyl-CoA, and sodium sulfide with a constant amount of purified serine acetyltransferase and increasing amounts of pure O-acetylserine(thiol)lyase. In these experiments all reaction components, except L-Ser, were incubated in a total volume of 100 μL for 10 min at 25°C. Then, reactions were initiated by adding L-Ser. When 2.1×10^{-4} units of serine acetyltransferase was used, the rate of L-Cys synthesis first increased linearly with O-acetylserine(thiol)lyase concentration and then reached a plateau value. Full functional saturation of this amount of serine acetyltransferase was achieved at about 0.08 unit of O-acetylserine(thiol)lyase. Also, these data proved that the preparation of serine acetyltransferase was essentially free from O-acetylserine(thiol)lyase activity. Results identical to those presented in Figure 3 were obtained when the first 10-min incubation was conducted in the absence of sodium sulfide, but in the presence of L-Ser, and then the reaction was started by adding sodium sulfide (not shown).

Stability of the Purified Serine Acetyltransferase

When the final enzyme preparation was stored for 2 weeks at 20°C in buffer B containing 0.4 M NaCl, there was no loss of activity. There was no significant effect of BSA (up to 5 mg/mL) on the stability of the enzyme. The thermal stability of serine acetyltransferase was investigated by incubating purified enzyme for 5 min at various temperatures in the buffer used for the activity measurements prior to assaying at 25°C in the presence of L-Ser, acetyl-CoA, sodium sulfide, and O-acetylserine(thiol)lyase. There was no change in the enzyme activity when the first incubation was conducted over the temperature range 15 to 70°C. However, when the preincubation was carried out at 80 and 90°C, 50 and 100%, respectively, of the enzyme activity was lost. Neither O-acetylserine(thiol)lyase nor BSA introduced into the preincubation mixture at a concentration of 0.2 mg/mL prior to assaying the enzyme protected serine acetyltransferase against heat inactivation above 70°C.

Catalytic Properties of the Purified Serine Acetyltransferase

The purified serine acetyltransferase was used for kinetic experiments using the coupled assay system containing an excess of homogeneous spinach chloroplast O-acetylserine(thiol)lyase (i.e. the cloned enzyme that had been overexpressed in *E. coli* [Rolland et al., 1993]). The optimum pH range measured at 25°C was quite broad, from 8.0 to 8.5.

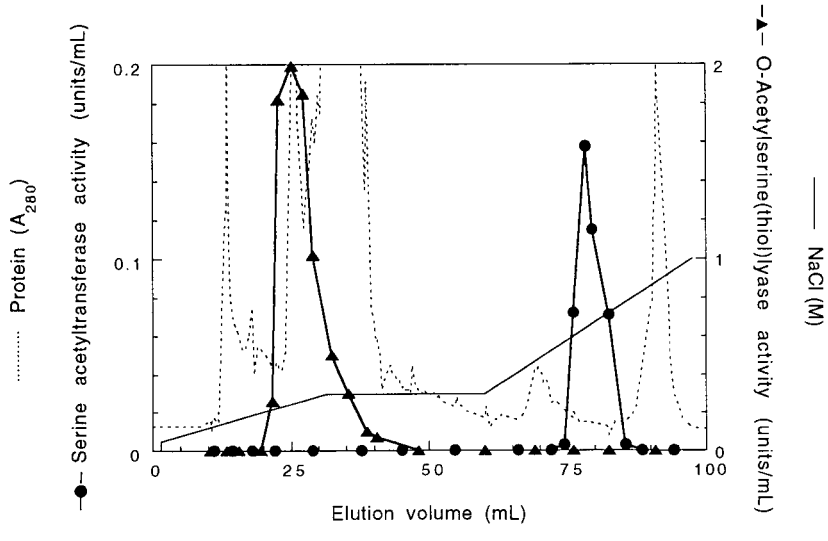

Figure 1. Purification of serine acetyltransferase by Mono-Q HR10/10 chromatography. Approximately 180 mg of the desalted DEAE pool (see Table I) was applied to the Mono-Q HR10/10 column equilibrated in buffer B (see text). A first 50-mL linear gradient of NaCl (0–0.3 M) in the same buffer, followed by a 50-mL wash with buffer B containing 0.3 M NaCl, and a second 70-mL linear gradient of NaCl (0.3–1 M) in buffer B caused the elution of O-acetylserine(thiol)lyase activity in the first NaCl gradient and elution of serine acetyltransferase activity in the second NaCl gradient. The broken line represents protein concentration measured by A_{280}, the continuous line shows the salt concentration, the black circles indicate serine acetyltransferase activity, and the black triangles represent O-acetylserine(thiol)lyase activity.

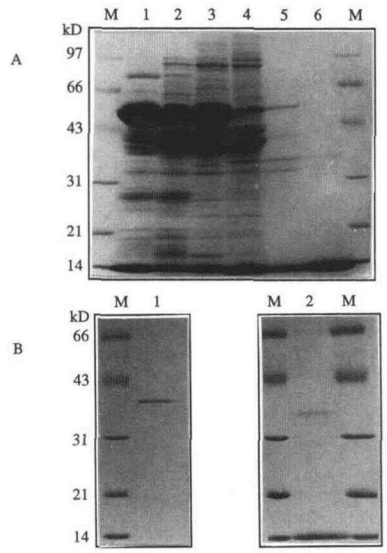

Figure 2. Documentation of purification procedure for serine acetyltransferase by SDS-PAGE. Proteins were separated on an SDS/10 to 20% (w/v) gradient polyacrylamide gel stained with Coomassie blue R-250. A, Electrophoretic migration of protein fractions. Lane 1, Stromal extract (see Table I), 150 μg; lane 2, $(NH_4)_2SO_4$ precipitate (see Table I), 150 μg; lane 3, Trisacryl M DEAE pool (see Table I), 150 μg; lane 4, Mono-Q HR10/10 pool (see Table I and Fig. 1), 50 μg; lane 5, hydroxylapatite pool, 2 μg; lane 6, Superdex 200 pool (see Table I), approximately 500 ng (one-fourth of the total preparation). B, Comparison of electrophoretic mobilities of spinach chloroplast *O*-acetylserine(thiol)lyase overexpressed in *E. coli* (lane 1) and purified serine acetyltransferase (lane 2, as in lane 6 of A). The fast-migrating heavy band in A (lanes 4–6) and B (lane 2) corresponds to purified Cyt *c* added as carrier protein (10 μg). Lanes M, Molecular mass standards as indicated.

The influence of acetyl-CoA and L-Ser substrate concentrations on enzyme activity was assessed by determination of apparent V_m and K_m values. When the concentration of acetyl-CoA was varied in the range 0 to 1.2 mM at fixed concentrations of L-Ser, the saturation curves shown in Figure 4A were obtained. Michaelis-Menten kinetic behavior was observed. This analysis allowed us to estimate the apparent V_m and K_m values, V_m^{app} and K_m^{app}, for acetyl-CoA (Eq. 3) at each fixed L-Ser concentration (10, 5, 1, 0.5, and 0.2 mM). A saturation behavior was obtained when the values of V_m^{app} and K_m^{app} were plotted as a function of [L-Ser] (Fig. 4B). Nonlinear regression analyses using Equations 4 and 5 yielded the values of the following kinetic constants: $V_m = 434 \pm 7$ units/mg; $K_m^{LS} = 2.80 \pm 0.15$ mM from Equation 4, and 2.29 ± 0.43 mM from Equation 5; $K_m^{CoA} = 0.35 \pm 0.02$ mM. These results indicate that the specific activity of purified serine acetyltransferase is greater than that inferred from the data in Table I. In the latter case enzyme activity was measured by using the standard conditions given in "Materials and Methods," i.e. the reaction assays contained unsaturating concentrations of L-Ser (5 mM) and acetyl-CoA (1 mM). Taking this into account, we calculate that the values given in Table I should be increased by a factor of about 2. This yields a maximum specific activity of the purified serine acetyltransferase on the order of 400 units/mg protein. This value compares well with that of 397 units/mg reported for highly purified serine acetyltransferase from *S. typhimurium* (Baecker and Wedding, 1980).

DISCUSSION

This report presents for the first time an extensive purification and characterization of serine acetyltransferase free of *O*-acetylserine(thiol)lyase from chloroplasts of plant cells. serine acetyltransferase was purified from spinach leaf chloroplasts by preparation of stromal proteins, ammonium sulfate precipitation, anion-exchange chromatography, hydroxylapatite chromatography, and gel filtration. The presence of serine acetyltransferase was previously noted in mitochondria (Smith and Thompson, 1969; Smith, 1972), plastids (Brunold and Suter, 1982; Droux et al., 1992), and in the cytosol (Brunold and Suter, 1982). It is not yet known, however, whether this enzyme exists in the form of several isoforms, each being specific for a cell compartment, as is the case for plant *O*-acetylserine(thiol)lyase (Lunn et al., 1990; Droux et al., 1992; Saito et al., 1992). All the previous attempts to purify serine acetyltransferase from plants were carried out from whole tissue extracts, which made it difficult to assign the isolated enzyme to a given intracellular compartment. Furthermore, these preparations were shown to contain significant amounts of contaminating *O*-acetylserine(thiol)lyase (Nakamura et al., 1990). The purification procedure we have developed provides chloroplastic serine acetyltransferase at a very high level of purity. The procedure resulted in an overall 300,000-fold purification. Based on SDS-PAGE, the major polypeptide in the highly purified serine acetyltransferase preparation has a molecular mass of 33 kD. A subunit value of 30.1 kD was reported by Baecker and Wedding (1980) for the *S. typhimurium* serine acetyltransferase, and a value of 34 kD was reported by Denk and Böck (1987) for the *E. coli* enzyme.

A remarkable feature of the present study is the very low amount of serine acetyltransferase contained in the chloroplasts. Based on the 7.6% yield of enzyme activity, the

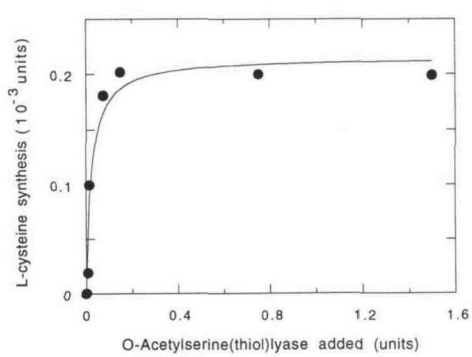

Figure 3. Effect of exogeneous *O*-acetylserine(thiol)lyase on serine acetyltransferase-dependent L-Cys synthesis. For each experiment standard conditions were used. Spinach chloroplast *O*-acetylserine(thiol)lyase overexpressed in *E. coli* (Rolland et al., 1993) was added as indicated and 2.1×10^{-4} units of purified serine acetyltransferase was used.

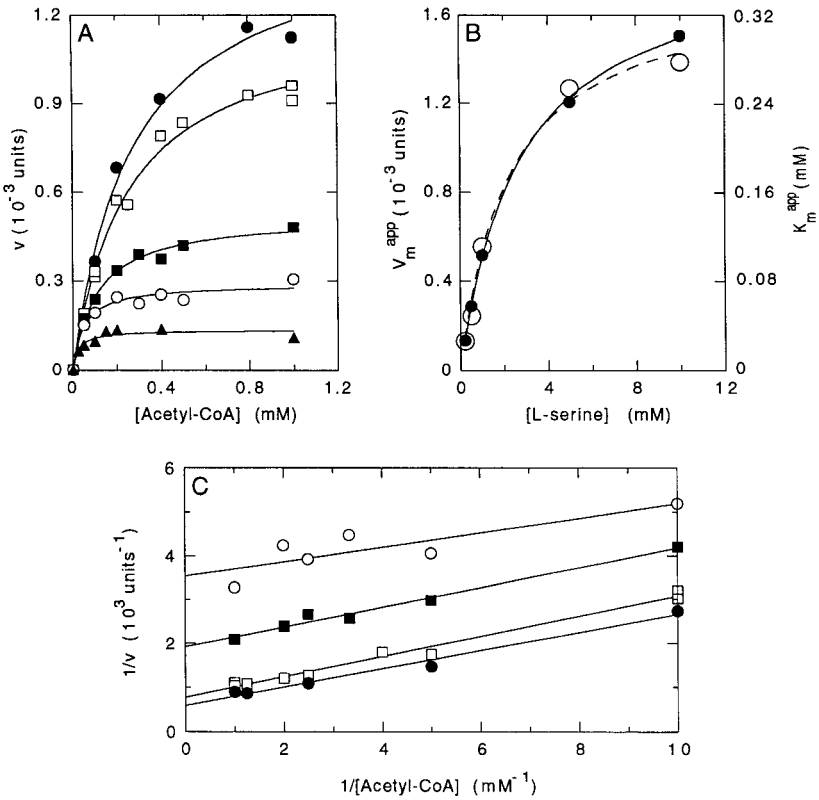

Figure 4. Dependence of serine acetyltransferase activity on the concentration of acetyl-CoA and L-Ser. Standard assay conditions were used. The assays contained 0.5 unit of *O*-acetylserine(thiol)lyase overexpressed in *E. coli* (Rolland et al., 1993) in a total volume of 100 μL. Reactions were initiated by addition of 4.4 ng of purified serine acetyltransferase. A, Velocity plots. The rate of Cys synthesis, v, was measured by varying acetyl-CoA concentrations, for the fixed L-Ser concentrations: ●, 10 mM; □, 5 mM; ■, 1 mM; ○, 0.5 mM; ▲, 0.2 mM. The points are experimental. The curves are the best fits obtained by nonlinear regression analysis of the experimental data to Equation 3. For each curve best-fit parameters V_m^{app} and K_m^{app} (Eq. 3) were calculated. B, Plot of V_m^{app} (●) and K_m^{app} (○) versus [L-Ser]. The curves are the best fits obtained by nonlinear regression analysis of the experimental data to Equation 4 (●) and Equation 5 (○). C, Double-reciprocal plots for the data shown in A. The lines were obtained by linear regression analysis. The symbols are the same as in A.

maximal expected amount of serine acetyltransferase in the stroma space of spinach leaf chloroplasts is approximately 2.5 ng/mg of protein. This value is much lower than the expected amount (700 ng/mg of protein) of *O*-acetylserine(thiol)lyase in the same compartment (Table I). Figure 3 shows that a large excess of *O*-acetylserine(thiol)lyase activity over serine acetyltransferase activity was required to obtain a maximum L-Cys synthesis in vitro. When 2.1×10^{-4} units of purified serine acetyltransferase was used, maximal L-Cys synthesis was achieved with about 0.08 unit of purified *O*-acetylserine(thiol)lyase, corresponding to an activity ratio *R* of 381. Remarkably, this value was not very different from the value of the activity ratio *R* found in the initial stromal protein extract ($R = 345$, Table I). This suggests that (a) the *O*-acetylserine(thiol)lyase and serine acetyltransferase contents of the chloroplasts are well adapted to bring a full synthesis of L-Cys from the two enzymes, and (b) even though plant serine acetyltransferases might be associated in vivo with a small portion of total *O*-acetylserine(thiol)lyase to form a bifunctional complex analogous to prokaryotic cysteine synthetase (Nakamura et al., 1988, 1990; Droux et al., 1992), the large excess of free *O*-acetylserine(thiol)lyase molecules could contribute significantly to the net rate of L-Cys synthesis. This suggestion is supported by kinetic evidence showing that for cysteine synthetase purified from *S. typhimurium* there is no channeling of the first product, *O*-acetylserine, from serine acetyltransferase to *O*-acetylserine(thiol)lyase, because the synthesized *O*-acetylserine is released in the bulk solution (Cook and Wedding, 1977). It is clear, therefore, that a characterization of the kinetic mechanism of L-Cys synthesis is required to determine whether L-Cys synthesis in higher plants proceeds via a tight complex between *O*-acetylserine(thiol)lyase and serine acetyltransferase.

The chloroplast serine acetyltransferase displayed normal Michaelis-Menten kinetic behavior. V_m^{app} and K_m^{app} parameters were calculated by varying the acetyl-CoA concentration and maintaining that of L-Ser. When these parameters were plotted versus [L-Ser] the experimental data could be fitted to the hyperbolic relationships represented by Equations 4 and 5. This pattern of behavior is typical for a Ping Pong Bi Bi mechanism (Segel, 1975), consistent with the finding that the double-reciprocal plots, 1/velocity versus 1/[acetyl-CoA], yielded a series of parallel lines when the concentration of L-Ser was varied (Fig. 4C). Since acetyl-CoA is the substrate with the group to be transferred and L-Ser is the acceptor, the results suggest that the enzyme first binds acetyl-CoA, forming an acetylated enzyme derivative with release of CoA, which then reacts with the acceptor substrate L-Ser. This analysis allowed us to calculate intrinsic K_m values for acetyl-CoA and L-Ser that are similar to those already reported for less-purified plant serine acetyltransferases. For example, for the enzyme purified from *A. tuberosum* leaves, K_m values for acetyl-CoA and L-Ser are 0.26 mM and 5.1 mM, respectively (Nakamura et al., 1990). These values, however, differ substantially from those determined for serine acetyltransferase from *S. typhimurium*. For this latter enzyme the K_m values for acetyl-CoA and L-Ser are 0.10 and 0.77 mM, respectively (Baecker and Wedding, 1980).

In conclusion, the present demonstration that chloroplasts

of plant cells contain a highly active serine acetyltransferase confirms our previous finding that these organelles are equipped with all the enzymes of the sulfate assimilatory pathway leading to L-Cys synthesis (Lunn et al., 1990; Droux et al., 1992). The availability of purified serine acetyltransferase, together with that of the previously described *O*-acetylserine(thiol)lyase (Droux et al., 1992; Rolland et al., 1993), will allow investigation of how these proteins interact to regulate L-Cys biosynthesis within chloroplasts.

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LITERATURE CITED

Anderson JW (1980) Assimilation of inorganic sulfate into cysteine. *In* BJ Miflin, ed, The Biochemistry of Plants, Vol 5. Academic Press, New York, pp 203–223

ap Rees T (1987) Compartmentation of plant metabolism. *In* DD Davies, ed, The Biochemistry of Plants, Vol 12. Academic Press, San Diego, CA, pp 87–115

Baecker PA, Wedding RT (1980) Purification of serine acetyltransferase, a component of a multienzyme complex, by immunoadsorption and selective dissociation of the complex. Anal Biochem **102:** 16–21

Becker MA, Tomkins GM (1969) Pleiotropy in a cysteine-requiring mutant of *Salmonella typhimurium* resulting from altered protein-protein interaction. J Biol Chem **244:** 6023–6030

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brunold C, Suter M (1982) Intracellular localization of serine acetyltransferase in spinach leaves. Planta **155:** 321–327

Brunold C, Suter M (1989) Localization of enzymes of assimilatory sulfate reduction in pea roots. Planta **179:** 228–234

Chua NH (1980) Electrophoresis analysis of chloroplast proteins. Methods Enzymol **69:** 434–436

Cook PF, Wedding RT (1977) Initial kinetic characterization of the multienzyme complex, cysteine synthetase. Arch Biochem Biophys **178:** 293–302

Cook PF, Wedding RT (1978) Cysteine synthetase from *Salmonella typhimurium* LT-2. J Biol Chem **253:** 7874–7879

Denk D, Böck A (1987) L-Cysteine biosynthesis in *Escherichia coli*: nucleotide sequence and expression of the serine acetyltransferase (*cysE*) gene from the wild-type and a cysteine-excreting mutant. J Gen Microbiol **133:** 515–523

Douce R, Joyard J (1982) Purification of the chloroplast envelope. *In* M Edelman, RB Hallick, NH Chua, eds, Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, Amsterdam, pp 239–256

Droux M, Martin J, Sajus P, Douce R (1992) Purification and characterization of *O*-acetylserine(thiol)lyase from spinach chloroplasts. Arch Biochem Biophys **295:** 379–390

Gaitonde MK (1967) A spectrophotometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. Biochem J **104:** 627–633

Giovanelli J, Mudd SH, Datko AH (1980) Sulfur amino acids in plants. *In* BJ Miflin, ed, The Biochemistry of Plants, Vol 5. Academic Press, New York, pp 453–505

Kredich NM, Becker MA, Tomkins GM (1969) Purification and characterization of cysteine synthetase, a bifunctional protein complex, from *Salmonella typhimurium*. J Biol Chem **244:** 2428–2439

Kredich NM, Tomkins GM (1966) The enzymatic synthesis of L-cysteine in *Escherichia coli* and *Salmonella typhimurium*. J Biol Chem **241:** 4955–4965

Lunn JE, Droux M, Martin J, Douce R (1990) Localization of ATP sulfurylase and *O*-acetylserine(thiol)lyase in spinach leaves. Plant Physiol **94:** 1345–1352

Nakamura K, Hayama A, Masada M, Fukushima K, Tamura G (1987) Measurement of serine acetyltransferase activity in crude plant extracts by a coupled assay system using cysteine synthase. Plant Cell Physiol **28:** 885–891

Nakamura K, Hayama A, Masada M, Fukushima K, Tamura G (1988) Purification and some properties of plant serine acetyltransferase. Plant Cell Physiol **29:** 689–693

Nakamura K, Tamura G (1990) Isolation of serine acetyltransferase complexed with cysteine synthase from *Allium tuberosum*. Agric Biol Chem **54:** 649–656

Rolland N (1992) Quelques observations sur l'*O*-acetylsérine(thiol)lyase des plantes supérieures: localisation, structure et caracteristiques biochimiques. Doctoral thesis. Université Joseph Fourier, Grenoble, France

Rolland N, Droux M, Lebrun M, Douce R (1993) *O*-Acetylserine(thiol)lyase from spinach (*Spinacia oleracea* L.) leaf: cDNA cloning, characterization, and overexpression in *Escherichia coli* of the chloroplast isoform. Arch Biochem Biophys **300:** 213–222

Saito K, Miura M, Hirano H, Murakohi I (1992) Molecular cloning and bacterial expression of cDNA encoding a plant cysteine synthase. Proc Natl Acad Sci USA **89:** 8078–8082

Scopes RK (1974) Measurement of protein by spectrophotometry at 205 nm. Anal Biochem **59:** 277–282

Segel IH (1975) Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. John Wiley & Sons, New York, pp 606–623

Smith IK (1972) Studies of L-cysteine biosynthetic enzymes in *Phaseolus vulgaris* L. Plant Physiol **50:** 477–479

Smith IK, Thompson JF (1969) The synthesis of *O*-acetylserine by extracts prepared from higher plants. Biochem Biophys Res Commun **35:** 939–945

Smith IK, Thompson JF (1971) Purification and characterization of L-serine transacetylase and *O*-acetylserine sulfhydrylase from kidney bean seedlings (*Phaseolus vulgaris*). Biochim Biophys Acta **227:** 288–295