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PURIFICATION AND CHARACTERIZATION OF L-SERINE TRANSACETYLASE AND *O*-ACETYL-L-SERINE SULFHYDRYLASE FROM KIDNEY BEAN SEEDLINGS *(PHASEOLUS VULGARIS)*

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SUMMARY

1. Serine transacetylase and *O*-acetylserine sulfhydrylase have been purified over 50-fold from kidney bean seedlings (*Phaseolus vulgaris*) by ammonium sulfate precipitation, and chromatography on DEAE-cellulose and Agarose gel filtration. Each enzyme was free of the other.

2. Serine transacetylase has a pH optimum of 8.25–8.5, is specific for serine and is inhibited by sulfhydryl reagents and L-cysteine. The calculated K_m's for serine and acetyl-CoA were $6 \cdot 10^{-4}$ M and $2 \cdot 10^{-4}$ M, respectively.

3. *O*-Acetylserine sulfhydrylase has a broad pH optimum (7.5–8.5). In addition to the synthesis of cysteine, it catalyzes the formation of *S*-methylcysteine from methylmercaptan and *O*-acetylserine. No requirement for pyridoxal phosphate could be demonstrated, though it would be expected to be a cofactor.

INTRODUCTION

The synthesis of cysteine from serine in diverse organisms involves two enzymes. One enzyme, serine transacetylase, is responsible for the acetylation of serine (Eqn. 1).

L-Serine + acetyl-CoA → *O*-acetyl-L-serine + CoA (1)

Serine transacetylase has been found in bacteria[1,2] and in higher plants[3]. This enzyme has been extensively purified from *Salmonella typhimurium*[2].

The enzymatic synthesis of cysteine from *O*-acetylserine and sulfide (Eqn. 2) has been reported in bacteria[2], yeast[4], Neurospora[4,5] and in higher plants[6,7].

O-Acetyl-L-serine + S^{2-} → cysteine + acetate (2)

The present communication reports the partial purification and characteri-

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethylsulfonate.

zation of serine transacetylase and *O*-acetylserine sulfhydrylase from kidney bean seedling extracts.

MATERIALS AND METHODS

Uniformly ^{14}C-labeled serine was obtained from International Chemicals and Nuclear Corp., Irvine, Calif. $Na_2{}^{35}S$ was purchased from New England Nuclear Corp.

Purification of serine transacetylase and O-acetylserine sulfhydrylase

Serine transacetylase and *O*-acetylserine sulfhydrylase have been isolated from kidney bean seedlings grown in the dark at 30° for six days. The cotyledons were removed prior to homogenization. Operations were carried out at 0–4°. All solutions contained 0.5 mM dithioerythritol, unless otherwise stated.

Homogenate. Seedlings (250 g), were homogenized in a Waring blender with 250 ml of 0.1 M potassium phosphate (pH 8.0) containing 1 mM dithioerythritol. The homogenate was squeezed through cheese cloth and the filtrate centrifuged at 100000 $\times$ *g* for 45 min. An aliquot of the supernatant was dialyzed against 0.05 M potassium phosphate buffer (pH 7.5) for 16–18 h prior to the determination of initial enzymatic activity.

$(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to the bulk of the supernatant to give 40% saturation, protein precipitation was allowed to proceed for 15 min, and the precipitated protein was collected by centrifugation at 10000 $\times$ *g* for 15 min. Additional $(NH_4)_2SO_4$ was added to the supernatant to give 40–50% and 50–80% saturation fractions. Precipitated protein was dissolved in 10 ml of 0.05 M potassium phosphate, (pH 7.5) and dialyzed overnight against the same buffer.

DEAE-cellulose fractionation. DEAE-cellulose (DE-52) was equilibrated with 0.5 M potassium phosphate (pH 7.5), packed into a column (20 cm $\times$ 1.5 cm) and washed overnight with 0.05 M phosphate buffer (150 ml). Dialyzed protein (20 ml) (0–40% $(NH_4)_2SO_4$ fraction for serine transacetylase or 50–80% $(NH_4)_2SO_4$ fraction for *O*-acetylserine sulfhydrylase) was applied to the column and washed with 50 ml of 0.05 M phosphate. The remaining protein was eluted using a linear gradient prepared from 100 ml of 0.05 M potassium phosphate (pH 7.5) and 100 ml of 0.5 M potassium phosphate (pH 7.5); 2.5 ml fractions were collected and assayed for enzymatic activities.

Agarose gel filtration. An agarose gel (Biogel A-0.5 m) column (49 cm $\times$ 2.9 cm) was prepared and equilibrated with 0.02 M *N*,*N*-bis(2-hydroxyethyl)glycine buffer (pH 8.0) containing 0.1 M NaCl. One or two fractions from the DEAE-cellulose column with peak activity were applied to the column and eluted with the same buffer at 9.4 ml/h; fractions were collected every 15 min.

Protein was assayed either by the method of LOWRY *et al.*[8] or by measurement of the absorbance at 260 nm and 280 nm, using bovine serum albumin as standard.

Assay procedures

Serine transacetylase. Reaction mixtures (1 ml) contained 60 μmoles *N*-2-hydroxyethylpiperazine-*N*-2-ethylsulfonate (HEPES) buffer (pH 8.5), 1 μmole uniformly ^{14}C-labeled-L-serine 1 μmole (0.5 μC/μmole), *S*-acetyl-CoA and protein. Reaction mixtures were incubated for 30 min at 30°. The reaction was terminated

by the addition of 0.2 ml of 1.5 M trichloroacetic acid. Precipitated protein was sedimented by centrifugation and discarded. 1 ml of supernatant was adjusted to pH 11.0 (thymol blue) with KOH (1 M) to facilitate conversion of *O*-acetylserine to *N*-acetylserine[3] and allowed to stand for 10 min at room temperature. This solution was passed through a sulfonic acid resin (H^+ form) column (6 cm × 0.9 cm), and the column washed with 20 ml of water. An aliquot (5 ml) of the material not retained (*N*-acetylserine) was added to 15 ml of the solution of BRAY[9] and the radioactivity assayed using a liquid scintillation counter. Identification of *O*-acetylserine as the product of the reaction and the stoichiometry of O to N shifting of the acetyl group has been reported[3].

O-Acetylserine sulfhydrylase. Reaction mixtures (1 ml) contained 60 μmoles HEPES buffer (pH 7.5), 0.05 μmole pyridoxal phosphate, 5 μmoles *O*-acetylserine,

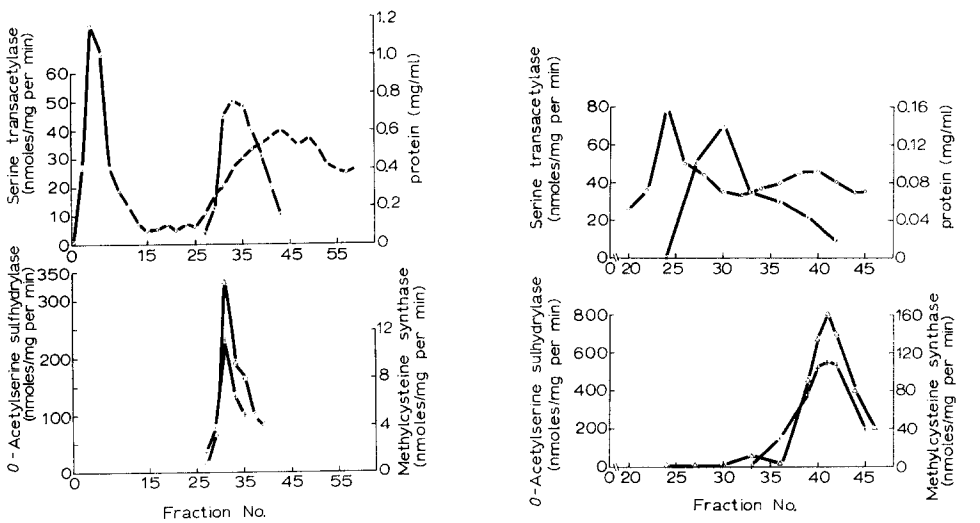

Fig. 1. Illustrates the fractionation of serine transacetylase and *O*-acetylserine sulfhydrylase from kidney bean on a DEAE-cellulose column. The column was washed for 15 tubes before starting the gradient; for additional details of the procedure see text. ○—○, protein in fractions; ×—×, serine acetylation; △—△, cysteine synthesis; +—+, methylcysteine synthesis.

Fig. 2. Illustrates the fractionation of serine transacetylase and *O*-acetylserine sulfhydrylase from kidney bean by Agarose gel filtration. The bed volume of the column was equivalent to 20 tubes, so that Tube 20 is the first tube to contain protein; for additional details of the procedure see text. ○—○, protein in fractions; ×—×, serine acetylation; △—△, cysteine synthesis; +—+, methylcysteine synthesis.

1 μmole $Na_2{}^{35}S$ (1 μC) and protein. When *S*-methylcysteine formation was measured, 0.025 μmole [^{14}C]methanethiol (1 μC) was included in the reaction mixture instead of $Na_2{}^{35}S$. Reaction mixtures were incubated in stoppered tubes for 30 min at 30°. The reaction was terminated by the addition of 0.2 ml of 1.5 M trichloroacetic acid; precipitated protein was sedimented by centrifugation and discarded. The supernatant was applied to a sulfonic acid resin (H^+ form) column (6 cm × 0.9 cm) and washed free of sulfide with 25 ml of distilled water. Cysteine was eluted with 3 M NH_4OH (15 ml) and the radioactivity measured on a 5-ml aliquot as above.

RESULTS AND DISCUSSION

Purification procedure

Serine transacetylase. $(NH_4)_2SO_4$ fractionation resulted in a partial separation of the serine transacetylase and *O*-acetylserine sulfhydrylase activities (Table 1). Refractionation of the 0–40% $(NH_4)_2SO_4$ fraction results in a relatively greater loss of *O*-acetylserine sulfhydrylase than serine acetylase activity. When the 0–40% $(NH_4)_2SO_4$ fraction was further fractionated on DEAE-cellulose column no additional separation of these two activities was obtained (Fig. 1), but a 4.6-fold purification of serine transacetylase was accomplished. However, gel filtration of the DEAE cellulose eluate on a agarose column resulted in complete separation of the activities (Fig. 2). Table I shows that serine transacetylase and *O*-acetylserine sulfhydrylase were purified 50-fold. Each enzyme was free of activity for the other.

TABLE I

PURIFICATION OF SERINE TRANSACETYLASE AND *O*-ACETYLSERINE SULFHYDRYLASE FROM KIDNEY BEAN

Assay conditions as described in text.

Purification step	*Specific activity (nmoles/ mg per min)*	*Total protein (mg)*	*Recovery (%)*	*Rel. purification*
Serine transacetylase				
Dialyzed homogenate	1.3	840	100	—
0–40% $(NH_4)_2SO_4$ satn.	6.1	144	80	5
0–40% $(NH_4)_2SO_4$ refractionated	10.4	72	69	8
50–80% $(NH_4)_2SO_4$ satn.	0	100	0	0
DEAE-cellulose eluate	46.2	10	42	37
Biogel A-0.5 m eluate	68.1	5	31	54
O-Acetylserine sulfhydrylase				
Dialyzed homogenate	15.8	500	100	—
0–40% $(NH_4)_2SO_4$ satn.	4.2	180	10	0
0–40% $(NH_4)_2SO_4$ refractionated	1.9	90	2	0
50–80% $(NH_4)_2SO_4$ satn.	31.3	152	60	2
DEAE-cellulose eluate	280.9	11	39	18
Biogel A-0.5 m eluate	892.3	2	23	56

In Salmonella, KREDICH *et al.*[2] reported that serine transacetylase and *O*-acetylserine sulfhydrylase activities are associated in a protein complex, cysteine synthase. The relative ease with which separation of these two activities was achieved using extracts from higher plants would suggest that either no cysteine synthetase complex exists in plants or that the complex is dissociated by the salt present during the extraction and purification of the protein.

O-Acetylserine sulfhydrylase. *O*-Acetylserine sulfhydrylase activity free of contaminating serine acetylase was present in the 50–80% $(NH_4)_2SO_4$ fraction (see ref. 6). This fraction was further purified by DEAE-cellulose chromatography and agarose gel filtration. In each case the peak of activity corresponded to that which was obtained for the *O*-acetylserine sulfhydrylase activity present in the 0–40% $(NH_4)_2SO_4$ fraction.

Serine transacetylase

Properties. Serine acetylation was linear with time (30 min) and enzyme concentration (50–200 μg of DEAE-cellulose eluate protein), when assayed using the standard incubation procedure. The enzyme had maximum activity at pH 8.25–8.5 in either HEPES, *N*,*N*-bis(2-hydroxyethyl)glycine or Tris buffers. The calculated K_m for serine in the transacetylase reaction is $6 \cdot 10^{-4}$ M and for acetyl-CoA is $2 \cdot 10^{-4}$ M (Figs. 3 and 4).

The enzyme lost 50% of its activity when stored for one week at 2°, but could be stored at −20° for 1 week without measurable loss. These figures refer to a protein concentration of 10 mg/ml, when the protein was diluted losses were substantially higher. For instance, protein eluted from the Biogel column (0.1 mg/ml) lost 50% of its activity overnight. The half life of the enzyme at 50° was 25 min and at 60° was 2 min.

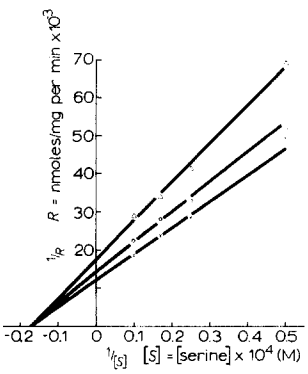

Fig. 3. Lineweaver–Burk plots of the rates of serine acetylation. Reaction conditions in text. Acetyl-CoA concentrations: ×—×, $1 \cdot 10^{-3}$ M, ○—○, $5 \cdot 10^{-4}$ M; △—△, $2.5 \cdot 10^{-4}$M.

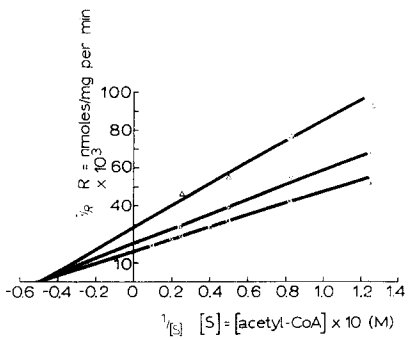

Fig. 4. Lineweaver–Burk plots of the rates of serine acetylation. Reaction conditions in text. Serine concentrations: ×—×, $1 \cdot 10^{-3}$ M; ○—○, $5 \cdot 10^{-4}$ M; △—△, $2.5 \cdot 10^{-4}$ M.

Specificity. Serine transacetylase was apparently specific for serine and did not catalyze the acetylation of homoserine or threonine. The enzyme did not catalyze exchange of the acetyl group from *O*-acetylserine or *O*-acetylhomoserine to serine. By contrast, Neurospora homoserine transacetylase catalyzes an exchange between homoserine and *O*-acetylhomoserine[10].

Inhibitors. The effect of various compounds on serine transacetylase activity is shown in Table II.

The enzyme was inhibited by *p*-chloromercuribenzoate and *N*-ethylmaleimide, compounds known to react with sulfhydryl groups. The enzyme was partially inhibited by hydroxylamine, probably due to a lowering of the acetyl-CoA level by formation of a hydroxamate. The inhibition by isoniazid was less than might be expected if the enzyme required pyridoxal phosphate as a cofactor, and no pyridoxal phosphate stimulation of the enzyme could be demonstrated.

Since serine transacetylase has been implicated in the methionine biosynthetic pathway, the effect of various sulfur-containing amino acids on activity was investi-

TABLE II

INHIBITORS OF SERINE TRANSACETYLASE ACTIVITY

Assay conditions as described in text.

Inhibitor	*Concn. (mM)*	*Inhibition (%)*
p-Chloromercuribenzoate	1	74.9
N-Ethylmaleimide	1	86.7
Hydroxylamine	1	38.6
Isoniazid	1	6.5
CoA	2.5	88.0
	0.5	70.0
Dithioerythritol	10	5.0
L-Methionine	10	19.5
	5.0	7.8
L-Homocysteine	10	24.7
	5.0	20.4
L-Cysteine	1	65.0
	0.5	44.0
D-Cysteine	10	48.2
	1	3.1

gated. Methionine was only slightly inhibitory (20% at relatively high concentrations, $1 \cdot 10^{-2}$ M). It is noteworthy that in *Escherichia coli* high concentrations of methionine are required to inhibit *O*-succinylhomoserine synthetase, but in the presence of *S*-adenosylmethionine lower concentrations are effective[11]. No synergistic interaction between L-cysteine ($2 \cdot 10^{-4}$ M), *S*-adenosylmethionine ($1 \cdot 10^{-3}$ M) or methionine ($1 \cdot 10^{-3}$ M) was observed, when tested as inhibitors of serine acetylation. L-Cysteine was a more effective inhibitor than L-homocysteine. In bacteria KREDICH AND TOMKINS[1] demonstrated a 50% inhibition of serine transacetylase activity at a cysteine concentration of $1.1 \cdot 10^{-6}$ M. By contrast, the higher plant enzyme was not inhibited by cysteine at concentrations below $5 \cdot 10^{-5}$ M. When cysteine replaced serine in the incubation mixture there was a chemical reaction between acetyl-CoA and cysteine with a half maximal rate at $2 \cdot 10^{-3}$ M. One of the products of this reaction was positively identified by paper chromatography as *N*-acetylcysteine (probably formed with *S*-acetylcysteine as intermediate). The inhibition by high levels ($5 \cdot 10^{-3}$ M) of D-cysteine, L-cysteine and L-homocysteine may be principally due to removal of acetyl-CoA in a non-enzymatic reaction. Inhibition of the enzyme by L-cysteine at concentrations ($5 \cdot 10^{-4}$ M) at which D-cysteine has no affect clearly indicates an interaction of L-cysteine with the enzyme; however, a kinetic analysis of this inhibition is complicated by the presence of the above mentioned chemical reaction. It has been noted that *O*-acetylserine and L-cysteine react non-enzymatically to form *N*-acetylcysteine[12]; this reaction might also be significant at the concentrations of cysteine used in the present experiments.

The enzyme was also inhibited by CoA, a product of the reaction.

O-Acetylserine sulfhydrylase

Properties. Certain technical problems made it difficult to obtain meaningful data of the properties of this enzyme. Optimal activity of the enzyme was observed over a broad pH range, 7.5–8.5. Enzyme activity is, however, restricted to a limited

pH range because at acid pH, volatilization of sulfide occurs and at alkaline pH *O*-acetylserine is converted to *N*-acetylserine, which is not a substrate in the reaction. Similar problems also make valid kinetic measurements difficult. Experiments with compounds such as *p*-chloromercuribenzoate or sulfhydryl compounds such as L-cysteine or dithioerythritol are complicated by the reaction of these compounds with sulfide.

O-Acetylserine sulfhydrylase activity is linear with time in only a limited enzyme concentration range. For instance, at 30° enzymatic activity was linear for 30 min at protein concentrations of 0.4–2 μg of DEAE-cellulose eluted protein and at 19° for 20 min with 3.3–10 μg protein.

The enzyme lost 75% of its activity when stored for 1 week at 2° or frozen at −20°. In contrast to this relative cold lability, the enzyme lost no activity when heated at 50° for 25 min and only 20% of the activity was lost by heating for 20 min at 60°.

Attempts to demonstrate a pyridoxal phosphate requirement were unsuccessful. The enzyme was incubated for 10 min at 30° with $1 \cdot 10^{-3}$ M hydroxylamine and passed through a Sephadex column before addition of the substrates. No enzymatic activity was lost under these conditions and there was no stimulation of activity in the presence of $1 \cdot 10^{-4}$ M pyridoxal phosphate. At higher concentrations of hydroxylamine ($1 \cdot 10^{-2}$ M) there was a 75% inhibition of enzyme activity, but this was not restored by preincubating with $1 \cdot 10^{-4}$ M pyridoxal phosphate. KREDICH AND TOMKINS[1] were unable to identify pyridoxal phosphate as a cofactor in *O*-acetylserine sulfhydrylase isolated from Salmonella, although the enzyme contained a prominent absorption peak at 412 nm which was decreased by the addition of *O*-acetylserine to the enzyme[12].

Specificity. 50-fold purified *O*-acetylserine sulfhydrylase from beans did not catalyze transfer of sulfide to *O*-acetylhomoserine, which agrees with the observation of GIOVANELLI AND MUDD[6] using spinach extracts. However, the enzyme was able to catalyze the synthesis of methylcysteine from *O*-acetylserine and methylmercaptan, and the peaks of activity for cysteine and methylcysteine synthesis corresponded on DEAE-cellulose chromatography (Fig. 1) and agarose gel filtration (Fig. 2). This lack of specificity with regard to the sulfhydryl compound acceptor has been reported in bacteria[12] and suggested in higher plants[6,7].

General discussion

The results presented in this paper indicate that higher plants form cysteine in the same manner as bacteria, *i.e.* from *O*-acetylserine and sulfide. Conclusive proof that this is the normal pathway is lacking, because appropriate mutants are not available (*cf.* bacteria ref. 1). However, in higher plants cysteine biosynthesis is apparently not controlled by end-product inhibition as has been demonstrated in bacteria[1]. Despite a similarity of K_m's in the two systems, the concentration of cysteine required to obtain 50% inhibition of serine acetylase is 500-fold higher using the plant enzyme. ELLIS[13] recently demonstrated that higher plant ATP-sulfurylase is not subject to the same control mechanisms that have been demonstrated in lower organisms. A conclusion is that sulfur metabolism in higher plants is probably controlled by mechanisms other than those which are commonly demonstrated in bacteria.

The evidence presented here shows that *in vitro* *S*-methylcysteine synthesis from *O*-acetylserine and methylmercaptan is catalyzed by *O*-acetylserine sulfhydrylase. However, there is doubt that this reaction provides the normal pathway of methylcysteine synthesis *in vivo* since labeled cysteine and methyl labeled methionine are good precursors of labeled methylcysteine[14]. The question of whether methylcysteine is formed by methylation of cysteine or by thiomethylation of *O*-acetylserine in higher plants has been critically discussed by GRANROTH[15].

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