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DIFFERENCE BETWEEN URACILYLALANINE SYNTHASES AND CYSTEINE SYNTHASES IN *PISUM SATIVUM**

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Key Word Index—*Pisum sativum*; Leguminosae; uracilylalanine synthase; cysteine synthase; isoenzyme; enzyme purification; biosynthesis; heterocyclic β-substituted alanine; willardiine; isowillardiine; cysteine; *O*-acetyl-L-serine.

Abstract—Purification of cysteine synthase from seedlings of pea (*Pisum sativum*) reveals the presence of three forms of this enzyme, separated by chromatography on DEAE-Sephadex A-50, and also differences between the cysteine- and uracilylalanine-synthases. Isoenzymes A and B of pea cysteine synthase were purified about 1200-fold and had specific activities of 933 U/mg protein and 892 U/mg protein, respectively. Both isoenzymes were found to have the same M_r (52 000) and to dissociate into two identical subunits (M_r 26 000). The K_m value of isoenzyme A is 2.1 mM for *O*-acetyl-L-serine (OAS) and 36 μM for sulphide, while that of isoenzyme B is 2.3 mM for OAS and 38 μM for sulphide. None of the three isoenzymes from pea seedlings catalyses the formation of the uracilylalanines L-willardiine and L-isowillardiine from OAS and uracil, although isoenzyme A catalyses the formation of β-cyano-L-alanine, and isoenzyme C catalyses the formation of L-quisqualic acid and L-mimosine. Other significant differences occur in the substrate specificity of the three isoenzymes. Several properties, including the amino acid composition of the purified cysteine synthase isoenzymes, are also described.

INTRODUCTION

In our recent studies [1, 2], we have presented evidence that cysteine synthases purified from the leaves of *Spinacia oleracea* and *Quisqualis indica* var. *villosa* can also catalyse the formation of some heterocyclic β-substituted alanines in the presence of *O*-acetyl-L-serine (OAS, **1**) and suitable precursors. We recently also purified β-(pyrazol-1-yl)-L-alanine synthase from *Citrullus vulgaris* [3] and L-mimosine synthase from *Leucaena leucocephala* [4]. These results suggest that naturally occurring heterocyclic β-substituted alanines such as L-willardiine (**2**) and L-isowillardiine (**3**) are synthesized by a reaction mechanism comparable to the one that results in the formation of L-cysteine (**4**) and *S*-substituted L-cysteines.

Two isomeric uracilylalanines, L-willardiine (**2**) and L-isowillardiine (**3**) have been found in pea seedlings [5, 6], **2** is also present in seeds of some *Acacia* species [7], while **3** was found in the seeds of 42 out of 163 species of *Crotalaria* tested [8]. The biosynthesis of these compounds in pea seedlings was studied *in vivo* using labelled precursors [9–11], and *in vitro* using uracil and OAS as substrates [10, 11]. From our results we suggested that two enzymes are involved in the biosynthesis of the two isomeric uracilylalanines [11]. In a more recent study Ahmmad *et al.* [12] made a more detailed investigation on the partially purified uracilylalanine synthases from *P. sativum* cv. Meteor, they suggested that only one enzyme is responsible for the biosynthesis of both isomers.

In the course of our continuing study on the biosynthesis of this group of uncommon amino acids, we have now attempted the purification of cysteine synthase from *P. sativum* seedlings, which contain uracilylalanines (**2**, **3**) and high enzyme activities for the formation of these compounds, in order to make a detailed comparison with the uracilylalanine synthases [11, 12], heterocyclic β-substituted alanine synthases [3, 4] and cysteine synthases described before [1, 2].

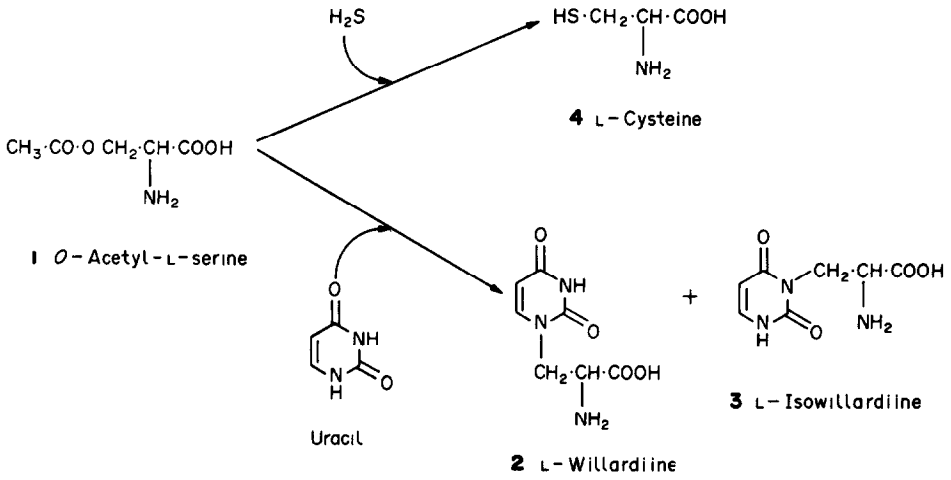
In this paper we describe the purification and the properties of cysteine synthase isoenzymes from *P. sativum* seedlings in comparison with β-substituted alanine synthases and cysteine synthases from other sources. Differences between the purified cysteine synthase and the uracilylalanine synthases present in the same seedlings will be discussed.

RESULTS

Occurrence of uracilylalanine synthases and cysteine synthases in pea

Higher levels of uracilylalanine synthase activities were present on a fresh weight basis in eight-day-old etiolated seedlings, but activities were negligible in the aerial parts and the roots of 10-day-old or 21-day-old green plants grown in a greenhouse. In the etiolated eight-day-old seedlings the specific activities of 0.22 mU/mg protein for willardiine synthase and of 0.41 mU/mg protein for isowillardiine synthase were within the range of previously reported values [11]. Cysteine synthase activities, on the other hand, were present in higher levels in these etiolated seedlings (0.304 U/mg protein) than in the aerial parts (0.122 U/mg protein) and the roots (0.066 U/mg protein) of 10-day-old pea plants.

*Parts of this work were reported at the 106th Annual Meeting of the Pharmaceutical Society of Japan at Chiba, 2 April 1986 (Abstracts, p. 183).



Scheme 1. Biosynthetic pathways for L-cysteine and uracilylalanines in higher plants.

Purification of the cysteine synthase isoenzymes and uracilylalanine synthases from pea seedlings

The methods described in the previous papers [1–4] were used for the extraction and purification of cysteine synthases from 3 kg fresh wt of the etiolated decotyledonised seedlings. The enzymes were prepared simultaneously with the uracilylalanines-forming enzyme activities by a procedure including heat treatment, ammonium sulphate fractionation, ion-exchange chromatography on DEAE-Sephadex A-50, gel filtration on Sephadex G-100, affinity chromatography on L-methionine-Sepharose 4B and preparative polyacrylamide gel electrophoresis (PAGE) as summarized in Table 1.

The proteins with cysteine synthase activity were completely separated into three peaks when the first DEAE-Sephadex A-50 column was eluted with a concentration gradient of K-Pi buffer. The enzyme activities for uracilylalanine synthase were incompletely resolved into two peaks, one of them exhibiting only isowillardiine synthase activity. Both these peaks partially overlapped with the narrower peak of cysteine synthase A eluting at 80–90 mM K-Pi. Cysteine synthase B eluted at 130–

Table 1. Summary of the purification of cysteine synthases from *Pisum sativum*

Purification step	Total activity (units*)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Fold
1. Crude extract†	6790	22300	0.304	—	—
2. 55°-Heated supernatant‡	7420	16300	0.455	—	—
3. Ammonium sulphate precipitate§	9040	11500	0.786	100	1
4. 1st Sephadex G-100 (peak fractions)	6310	1290	4.90	70	6.2
5. DEAE-Sephadex A-50					
Isoenzyme A (80–90 mM)	3050	26.0	117	33.7	148
Isoenzyme B (130–145 mM)	945	27.3	34.6	10.5	43.9
Isoenzyme C (185–195 mM)	65	8.5	7.6	0.7	9.7
6. 2nd Sephadex G-100 (peak fractions)					
Isoenzyme A	1800	11.4	158	19.9	201
Isoenzyme B	491	4.55	108	5.4	137
Isoenzyme C	42	3.02	13.8	0.46	18
7. Affinity chromatography					
Isoenzyme A (25–35 mM)	1560	7.41	211	17.3	268
Isoenzyme B (45–55 mM)	296	0.67	442	3.3	561
Isoenzyme C (65–75 mM)	12.2	0.23	53	0.14	67
8. Polyacrylamide-gel electrophoresis					
Isoenzyme A	625	0.67	933	6.7	1184
Isoenzyme B	107	0.12	892	1.2	1132
Isoenzyme C	5.4	0.08	68	0.06	86

*A unit of enzyme activity represents 1 μmol of product formed/min at 30°, in 50 mM K-Pi buffer, pH 8.0.
†Starting from 3 kg of fresh etiolated decotyledonised seedlings of *P. sativum*.
‡55°, 1 min.
§30–70% saturation and desalted on Sephadex G-25.

145 mM and cysteine synthase C at 185–195 mM K-Pi as shown in Fig. 1. The much broader peaks of uracilylalanine synthase eluted at K-Pi concentrations of 60–110 mM.

The complete procedure as shown in Table 1 afforded apparent purifications of *ca* 1200-fold for isoenzymes A and B and a 90-fold purification for isoenzyme C; with specific activities of 933 U/mg protein for A, 892 U/mg protein for B, 68 U/mg protein for C, and yields of 6.7%, 1.2% and 0.06% respectively, as compared to the total cysteine synthase activity of the ammonium sulphate precipitate desalted on Sephadex G-25.

Uracilylalanine synthases were only partially purified by the same procedure which was shortened until the DEAE-Sephadex A-50 chromatography step, indicating the presence of at least two proteins involved in the formation of uracilylalanines (Fig. 1). The purified cysteine synthase isoenzymes had no uracilylalanine synthase activity (see also Table 2).

Properties of the purified cysteine synthase isoenzymes

The M_rs of the purified enzymes from pea seedlings were estimated by analytical gel filtration using Sephadex G-100 (1.5 × 115 cm) according to the method of ref. [13]. Cysteine synthase activity of isoenzymes A, B and C was found invariably as a single peak, corresponding to M_r of 52 000. The purified isoenzymes A and B were subjected to

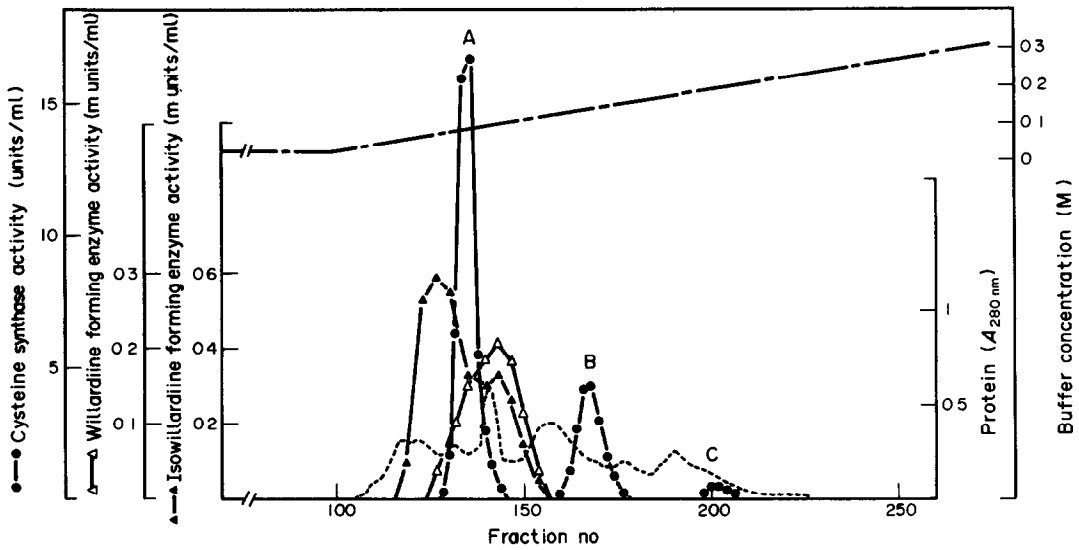


Fig. 1. Elution patterns of cysteine synthases and uracilylalanine synthases after the first DEAE-Sephadex A-50 column chromatography. Cysteine synthase activity (●—●), willardiine-forming enzyme activity (Δ—Δ), isowillardiine-forming enzyme activity (▲—▲) and protein (A_{280},---) were monitored as shown in the Experimental.

Table 2. Relative synthetic rates of *S*-substituted L-cysteines and β-substituted alanines by cysteine synthase isoenzymes purified from *Pisum sativum*

Thiol compound and *N*-heterocyclic compound	Amino acid synthesized	Relative velocity of synthesis (%)		
		Isoenzyme A	Isoenzyme B	Isoenzyme C
H_2S	L-Cysteine	100	95.6	7.3
MeSH	*S*-Methyl-L-cysteine	4.0	1.7	n.d.
$CH_2=CH-CH_2-SH$	*S*-Allyl-L-cysteine	2.6	6.8	n.d.
$HOOC-CH_2-SH$	*S*-Carboxymethyl-L-cysteine	2.3	1.6	n.d.
Uracil	L-Willardiine	0	0	0
	L-Isowillardiine	0	0	0
Pyrazole	β-(Pyrazol-1-yl)-L-alanine	1.33	0.52	0.001
3-Amino-1,2,4-triazole	β-(3-Amino-1,2,4-triazol-1-yl)-L-alanine	0.74	0.62	0.001
3,5-Dioxo-1,2,4-oxadiazolidine	L-Quisqualic acid	0	0	1.16
3,4-Dihydroxypyridine	L-Mimosine	0	0	0.02
Hydroxyurea	*O*-Ureido-L-serine	0	0	0
NaCN	β-Cyano-L-alanine	22.1	0	0

The relative rates of synthesis were compared with that of L-cysteine by isoenzyme A. The reaction conditions are given in the Experimental and are as described before [1–4].

n.d., not determined.

sodium dodecyl sulphate (SDS)-PAGE on 12% gels to determine their subunit structures, following the method of ref. [14]. This suggests that both enzymes are composed of two identical subunits, with a M_r of 26 000, and that they have been purified to apparent homogeneity, while that of isoenzyme C has not been determined because of a low yield.

The presence of pyridoxal 5'-phosphate (PLP) in both isoenzymes A and B is suggested by their spectral data, as compared to cysteine synthase and heterocyclic β-substituted alanine synthases from other sources [1–4].

The enzymes exhibited a single pH optimum at pH 8.0, respectively, although there was a rapid acetyl shift from *O* and *N* atoms in OAS above *ca* pH 8.0.

The isoenzymes of cysteine synthase from pea display quite different relative activities, but their responses to OAS are essentially the same. Isoenzymes A and B have K_m values of 2.1 and 2.3 mM for OAS, respectively, but they are not inhibited by OAS at concentrations up to 25 mM. The K_ms for OAS of isoenzymes A and B are less than that determined for cysteine synthase from spinach [1] and for isoenzyme B of cysteine synthase from *Q. indica* var. *villosa* [2], while they are very close to the values determined for cysteine synthase from *Raphanus sativus* [15] and for isoenzyme A of cysteine synthase from *Q. indica* var. *villosa* [2]. The K_m for OAS of isoenzyme B has the same value as that determined for isoenzyme B of cysteine synthase from *Phaseolus vulgaris* [16].

The response of isoenzymes A and B to sulphide concentrations below 0.2 mM was examined and K_m values of 36 and 38 μM were found, respectively. These values are higher than that determined for cysteine synthase from spinach [1], but are almost the same value as that determined for isoenzyme B of cysteine synthase from *P. vulgaris* [16].

Substrate specificity

Under standard assay conditions, the cysteine synthase isoenzymes from pea seedlings clearly appear to be specific for OAS as a donor of the alanyl moiety. When OAS is substituted by β-chloro-L-alanine, the activities of isoenzymes A and B were only 3.2 and 8% respectively of the activity with OAS, under otherwise identical conditions. No activity could be detected when OAS is substituted by *O*-phospho-L-serine, *O*-sulpho-L-serine or L-serine. The purified enzymes also showed no activity in the presence of *O*-acetyl-D-serine.

The cysteine synthase isoenzymes also showed a distinct substrate specificity when a variety of thiol compounds or *N*-heterocyclic compounds were used as an acceptor for the alanyl moiety. Table 2 shows the relative activities of the purified enzymes with different substrates. None of the isoenzymes could catalyse the formation of L-willardiine (**2**), L-isowillardiine (**3**) or *O*-ureido-L-serine when suitable substrates were provided. When pyrazole or 3-amino-1,2,4-triazole were given as substrates, the corresponding heterocyclic β-substituted alanines were synthesized by the three isoenzymes to a greatly varying degree: isoenzyme A being respectively 150 and 20% more active than B, but about 10^3 times more active than C. On the other hand, isoenzyme C is the only one capable of catalysing the formation of L-quisqualic acid and L-mimosine. The isoenzymes A and B differ in their activities to synthesize different *S*-substituted L-cysteines and also in the fact that only A shows a considerable β-cyano-L-alanine synthase activity.

The specific activities of isoenzymes A, B and C towards a variety of substrates are also different from those of the cysteine synthase isoenzymes from *Q. indica* var. *villosa* [2] and from other sources [1, 15–17]. The different substrates were tested under the same conditions as described previously [1].

Amino acid composition

The amino acid compositions of the purified isoenzymes A and B are given in Table 3. These can be compared with the amino acid compositions of cysteine synthases purified from spinach [1], from *C. vulgaris* (unpublished results) and from microorganisms [18, 19]. Only the enzyme from yeast contains tryptophan. The number of cysteine and methionine residues is invariably 1 and 5 respectively for the enzymes from microorganisms, the plant enzymes contain 18–22 *S*-containing amino acids except isoenzyme B from pea seedlings which contains 48. The plant enzymes and the yeast enzyme all have 28–32 structurally important proline residues, again excepting the isoenzyme B from pea seedlings which contains 68, the *Salmonella typhimurium* isoenzymes contain 16–17 proline residues.

The M_rs of isoenzymes A and B, calculated from the amino acid compositions, are both 51 900, which agrees with the values estimated by gel filtration on Sephadex G-100 (52 000).

DISCUSSION

In the preceding paper [2], we reported the presence of two isoenzymes of cysteine synthase in *Q. indica* var. *villosa* and the role of one of them in the biosynthesis of L-quisqualic acid. In the present study we have purified three cysteine synthases from etiolated pea seedlings to apparent homogeneity and a comparison has been made of their properties and substrate specificities with those of the previously purified enzymes [1–4].

It appears that the three purified enzymes show no uracilylalanine synthase activity. They could be separated by gradient elution with K-Pi buffer from a DEAE-Sephadex A-50 column. During the same procedure a peak with isowillardiine synthase activity and a peak with both willardiine and isowillardiine synthase activities were found. These uracilylalanine synthase enzymes from pea seedlings were not further purified in this study, but it seems clear that they are not the same proteins as the cysteine synthases.

The three enzymes purified in this study have very similar physicochemical properties: they have the same M_rs of 52 000 and consist of identical subunits of M_rs 26 000, they have the same pH optimum of 8.0 and they contain pyridoxal 5'-phosphate. Although the specific activities of the purified enzymes are different we consider that they can be regarded as isoenzymes.

The specific activities of isoenzymes A and B are almost the same, but that of isoenzyme C is about 14-fold lower than that of isoenzyme A. The K_m for OAS of both isoenzyme A (2.1 mM) and isoenzyme B (2.3 mM) are within the range of 2.1–6.2 mM reported for cysteine synthases from other sources [1, 15–17].

Among the substrates studied so far (Table 2), isoenzymes A and B catalyse the formation of *S*-substituted

Table 3. Amino acid compositions of cysteine synthases purified from *Pisum sativum*, *Citrullus vulgaris* and *Spinacia oleracea*

Amino acids	*Pisum sativum* Cysteine synthase		*Citrullus vulgaris* Cysteine synthase		*Spinacia oleracea* Cysteine synthase
	Isoenzyme A	Isoenzyme B	Isoenzyme A	Isoenzyme B	
	Residues/52 000 g*		Residues/58 000 g†		Residues/60 000 g‡
Asp	40	28	42	42	36
Thr	30	12	32	28	28
Ser	36	42	44	52	38
Glu	50	38	56	54	66
Pro	30	68	30	28	32
Gly	52	72	50	56	60
Ala	48	34	46	44	48
Val	36	30	40	36	42
Cys	16	44	4	14	6
Met	6	4	14	4	14
Ile	26	24	34	26	40
Leu	44	34	46	44	46
Tyr	2	2	18	18	14
Phe	22	14	22	24	26
Trp	0	0	0	0	0
Lys	28	18	28	24	42
His	6	6	10	12	4
Arg	22	36	22	32	18
Total	494	506	538	538	560

*Results are expressed as residues/mol and are based on an M_r of 52 000.
Values for Thr and Ser are extrapolated to zero-time hydrolysis.
†Unpublished results.
‡This result was recalculated based on ref. [1].
The numbers of residues of amino acids were calculated based on the results of analyses after 24, 48 and 72 hr acid hydrolysis of native enzymes. Means of duplicate analyses are given. Determination of tryptophan was made by alkaline hydrolysis.

L-cysteines from OAS and the corresponding thiol compounds. Isoenzyme B especially shows a high activity for 2-propene-1-thiol. The three isoenzymes catalyse the formation of two or more different heterocyclic β-substituted L-alanines. Only isoenzyme C catalyses the formation of L-quisqualic acid and L-mimosine. Together with the uracilylalanine synthases it thus appears that etiolated pea seedlings contain at least five different proteins capable of catalysing the formation of β-substituted L-alanines with M_rs of 50 000–52 000 and with similar physico-chemical properties, they exhibit very different substrate specificities with regard to the alanine-acceptor but with the same specificity towards OAS as alanine donor. Moreover, isoenzyme A also catalyses the formation of β-cyano-L-alanine from OAS and CN^-. This group of enzymes thus can play a role in the detoxification of endogenous- or eventually exogenous-toxic molecules such as cyanide, hydrogen sulphide or pyrazole, thereby forming secondary metabolites like the β-substituted alanines. Eventually these latter compounds may have a role as allelochemicals.

The amino acid compositions of cysteine synthases A and B are rather different, especially in the number of proline residues and cysteine residues. The differences are much greater than among the cysteine synthase isoenzymes from *Citrullus vulgaris* (unpublished results) or from *Salmonella typhimurium* [18]. When the known amino acid compositions of cysteine synthases are compared by a mathematical method [20] it is suggested that isoenzyme A seems to be closer to cysteine synthases from other plant sources than the isoenzyme B from pea seedlings. This makes it less likely that these isoenzymes might have originated from a recent gene duplication.

EXPERIMENTAL

Materials. Seedlings of *Pisum sativum* cv. Alderman were used in this work. Seeds were supplied by Sakata Seed Corporation (Japan). They were sown in moistened vermiculite and grown in the dark for 8 days at 28–30°, and 10 days or 21 days old plants were grown in a greenhouse of our medicinal plant gardens. After harvest, the cotyledons were removed, and the seedlings, aerial parts and roots were cooled for 1 hr at 0–4° before enzyme extraction. Sephadex G-25 and G-100, DEAE-Sephadex A-50 and Sepharose 4B were purchased from Pharmacia. L-Methionine-Sepharose 4B was prepared in our laboratory according to a modified method of ref. [21]. All other chemicals used were of the highest commercial grade available.

Activity assays. This was performed as described previously [1, 2]. The formation of L-cysteine was measured spectrophotometrically according to the method of ref. [22]. The formation of L-willardiine (2) and L-isowillardiine (3) was determined by using an automatic amino acid analyser (Hitachi 835–10) as described before [1]. The unit of enzyme activity used in this

paper was equivalent to 1 μmol of L-cysteine or uracilylalanines (**2, 3**) produced per min. Protein was determined by the method of ref. [23].

Enzyme preparations for uracilylalanine synthase assay and cysteine synthase assay. This was carried out at 0–4°. The crude enzyme preparations were obtained after centrifugation (15 000 g, 15 min) and passing through a Sephadex G-25 (fine) column as described before [11].

Purification of cysteine synthase isoenzymes from pea seedlings. All steps were carried out at 0–4°. Cysteine synthases were prepared from 3 kg of fresh etiolated seedlings (cotyledons removed), essentially as before [1, 2]. The 30–70% saturated $(NH_4)_2SO_4$ fraction was collected and dissolved in 30 mM K-Pi buffer, pH 8, containing 10 mM 2-mercaptoethanol and 0.5 mM EDTA (buffer A). The resulting solns were repeatedly applied to a column (8.0 × 40 cm) of Sephadex G-25 (fine) pre-equilibrated with buffer A. The protein fraction was concentrated by $(NH_4)_2SO_4$ precipitation and then applied to the first Sephadex G-100 column (4.6 × 90 cm) pre-equilibrated with the same buffer. The active fractions of step 4 were pooled and then applied to the first DEAE-Sephadex A-50 column (3 × 17 cm) pre-equilibrated with buffer A. The column was washed extensively with buffer A and the enzymes eluted with a linear gradient of K-Pi (30–300 mM) in the same buffer. Cysteine synthase activities were eluted at 80–90 mM, 130–145 mM and 185–195 mM K-Pi buffer, respectively, and were concentrated by $(NH_4)_2SO_4$ precipitation. The first active fractions (80–90 mM K-Pi fractions), second active fractions (135–145 mM K-Pi fractions) and third active fractions (185–195 mM K-Pi fractions) were individually applied to a column (2 × 95 cm) of Sephadex G-100 pre-equilibrated with buffer A. The eluates were collected in 2 ml fractions, and three series of active fractions were pooled and concentrated by Immersible CX-10 (Millipore). The resulting solns were then applied to a column (1.2 × 3 cm) of L-methionine-Sepharose 4B pre-equilibrated in 10 mM K-Pi buffer, pH 8, containing 10 mM 2-mercaptoethanol and 0.5 mM EDTA and the enzyme eluted with a linear gradient of K-Pi (10–150 mM) in the same buffer. Cysteine synthase isoenzyme activities were eluted at 25–35 mM for isoenzyme A, 45–55 mM for isoenzyme B and 65–75 mM for isoenzyme C, respectively, and were concd by Immersible CX-10. The resulting solns were individually subjected to prep. PAGE on 7.5% gels at pH 8.3 (Tris–glycine buffer). Cysteine synthase fractions obtained from gel slices were finally applied to a column (1.2 × 2.5 cm) of DEAE-Sephadex A-50 pre-equilibrated in buffer A containing 30 mM KCl. The highly purified enzyme fractions were used as isoenzymes A, B and C in all further expts.

Properties of cysteine synthase isoenzymes were studied by the methods of ref. [3].

Identification of heterocyclic β-substituted alanines and S-substituted L-cysteines as reaction products was performed as described previously [1, 2], using 0.4 μg of the purified enzymes.

Determination of amino acid compositions was also performed as described previously [1], using 0.01 mg of the purified enzymes.

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