Phytochemistry, Vol. 27, No. 3, pp. 697 701, 1988.
Printed in Great Britain.

0031 9422/88 $3.00+0.00

PURIFICATION AND CHARACTERIZATION OF CYSTEINE SYNTHASES FROM *CITRULLUS VULGARIS**

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(*Received* 29 *June* 1987)

Key Word Index—*Citrullus vulgaris*; Cucurbitaceae; watermelon; cysteine synthase; isoenzyme; enzyme purification; substrate specificity; amino acid composition; heterocyclic β-substituted alanine; β-(pyrazol-1-yl)-L-alanine; *O*-acetyl-L-serine.

Abstract—Two forms of cysteine synthase from *Citrullus vulgaris* seedlings were purified *ca* 2000-fold to homogeneity, respectively, using both conventional and affinity chromatographic methods. Both isoenzymes were found to have the same M_rs of 58 000, and SDS-PAGE showed that they contained two identical subunits with M_rs of 29 000, respectively. Amino acid analysis indicated that isoenzymes A and B have almost similar amino acid compositions, except for the number of cysteine and methionine residues. The K_m value of isoenzyme A is 2.6 mM for *O*-acetyl-L-serine (OAS) and 36 μM for sulphide, while that of isoenzyme B is 1.5 mM for OAS and 33 μM for sulphide. Data on the substrate specificity show that both isoenzymes catalyse the formation of β-(pyrazol-1-yl)-L-alanine and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine, although only isoenzyme A catalyses the formation of β-cyano-L-alanine. Several properties of the purified cysteine synthase isoenzymes are described.

INTRODUCTION

Previous work in our laboratory on cysteine synthase have shown that these enzymes purified from *Spinacia oleracea* [1], *Quisqualis indica* var. *villosa* [2] and *Pisum sativum* [3] can also catalyse the formation of some heterocyclic β-substituted alanines such as β-(pyrazol-1-yl)-L-alanine (BPA) and L-quisqualic acid in the presence of *O*-acetyl-L-serine (OAS) and suitable precursors. We have also recently described the 200-fold purification of BPA synthase from *Citrullus vulgaris* seedlings, and that the enzyme shows a high degree of specificity for the substrates [4].

In the course of our continuing study on the biosynthesis of this group of non-protein amino acids, we have therefore attempted the purification of cysteine synthase from *C. vulgaris* seedlings, which contain high BPA synthase activity, in order to make a detailed comparison with BPA synthase [4], L-mimosine synthase [5] and cysteine synthases described previously [1–3]. In this paper we describe the purification and characterization of the cysteine synthase isoenzymes from *C. vulgaris* seedlings and compare the enzyme with β-substituted alanine synthases and cysteine synthases from other sources. We also discuss the relationship between the purified cysteine synthase and BPA synthase present in the same plant.

RESULTS

Purification

Initially the extraction and purification of cysteine synthase from 8.2 kg fresh weight of 7- to 8-day-old seedlings (cotyledons removed) followed our previous methods [1–5]. The enzyme was prepared simultaneously with the BPA synthase activity by a procedure including heat treatment, ammonium sulphate fractionation, gel filtration on Sephadex G-100, ion-exchange chromatography on DEAE-Sephadex A-50, affinity chromatography on L-methionine-Sepharose 4B and preparative polyacrylamide gel electrophoresis (PAGE) as summarized in Table 1.

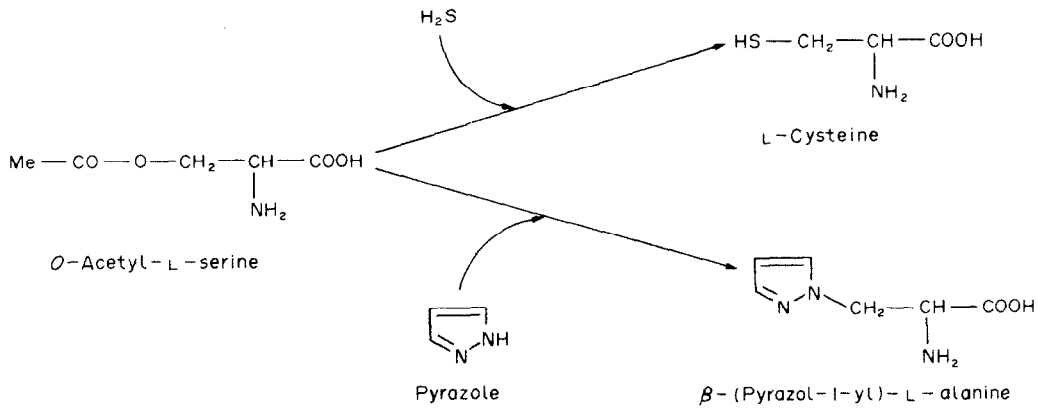
Two peaks exhibiting cysteine synthase activity were separated completely after the first DEAE-Sephadex A-50 column was eluted with a concentration gradient of K-Pi buffer. Cysteine synthase A eluted at 70–90 mM and cysteine synthase B at 170–200 mM, respectively, and the enzyme activities for BPA synthase were completely overlapped with both peaks of cysteine synthase (Fig. 1).

The complete procedure as shown in Table 1 afforded apparent purifications of *ca* 2000-fold for isoenzymes A and B; with sp act of 649 U/mg protein for A, 691 U/mg protein for B, and yields of 12.4 and 1.1%, respectively, compared to the total cysteine synthase activity of the crude extract.

Properties of isoenzymes

The M_rs of the purified enzymes from *C. vulgaris* seedlings were estimated by analytical gel filtration using Sephadex G-100 (1.5 × 115 cm) according to the method of ref. [6]. Cysteine synthase activity of isoenzymes A and B was found invariably as a single peak, corresponding to M_rs of 58 000. The purified isoenzymes were subjected to SDS-PAGE on 12% gels to determine their subunit structures, following the method of ref. [7]. This suggests that both enzymes are composed of two identical subunits, with a M_r of 29 000, and that they have been purified to apparent homogeneity. The identification of bound pyridoxal 5′-phosphate (PLP) in the purified enzymes was

* Parts of this work were reported at the 30th Annual Meeting of Kanto Branch of the Pharmaceutical Society of Japan at Niigata, 8 November 1986 (Abstracts, p. 50).



Scheme 1. Biosynthetic pathways for L-cysteine and β-(pyrazol-1-yl)-L-alanine in higher plants.

Table 1. Summary of the purification of cysteine synthases from *Citrullus vulgaris*

Purification step	Total activity (units*)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Fold
1. Crude extract†	10090	30860	0.327	100	1
2. 55°-Heated supernatant‡	9312	26120	0.357	92.3	1.1
3. Ammonium sulphate precipitate§	6891	3646	1.89	68.3	5.8
4. 1st Sephadex G-100 (peak fractions)	4903	477	10.3	48.6	31.5
5. DEAE-Sephadex A-50					
Isoenzyme A (70–90 mM)	3268	34.7	94.2	32.4	288
Isoenzyme B (170–200 mM)	452	8.5	53.2	4.5	163
6. 2nd Sephadex G-100 (peak fractions)					
Isoenzyme A	2451	20.3	121	24.3	370
Isoenzyme B	310	4.6	67.4	3.1	206
7. Affinity chromatography					
Isoenzyme A (20–35 mM)	1797	11.3	159	17.8	486
Isoenzyme B (40–55 mM)	237	1.5	158	2.4	483
8. Polyacrylamide-gel electrophoresis					
Isoenzyme A	1246	1.9	649	12.4	1985
Isoenzyme B	110.5	0.2	691	1.1	2113

*A unit of enzyme activity represents 1 μmol of product formed per min at 30°, in 50 mM K-Pi buffer (pH 8).

†Starting from 8.2 kg of fresh etiolated seedlings of *Citrullus vulgaris* (cotyledons removed).

‡55°, 1 min.

§40–60% saturation.

demonstrated by direct spectrophotometric measurements [8]. Both cysteine synthase isoenzymes in *C. vulgaris* have one molecule of PLP bound to each subunit similar to the cysteine synthases and heterocyclic β-substituted alanine synthases from other sources [1, 4, 5, 9].

The enzymes exhibited a single pH optimum at pH 8, although there was a rapid acetyl shift from *O* to *N* atoms in OAS above *ca* pH 8.

The isoenzymes of cysteine synthase from *C. vulgaris* display somewhat different relative activities, but their responses to OAS are essentially the same. Isoenzymes A and B have K_m values of 2.6 and 1.5 mM for OAS, respectively, but they show no indication of inhibition by OAS at concentrations up to 20 mM. The $K_{m,OAS}$ of isoenzyme A has almost the same value as that determined for BPA synthase from the same plant [4] and is also very close to the values determined for cysteine synthase from spinach [1] and for isoenzyme B of cysteine synthases from both pea [3] and *Phaseolus vulgaris* [10], while it is less than that determined for isoenzyme B of cysteine synthase from *Q. indica* var. *villosa* [2], cysteine synthase from *Brassica chinensis* var. *Komatsuna* [9] and for L-mimosine synthase from *Leucaena leucocephala* [5]. The $K_{m,OAS}$ of isoenzyme B, on the other hand, is less than that determined for cysteine synthase from all other sources [1–3, 9–11].

The response of isoenzymes A and B to sulphide concentrations below 0.2 mM was examined and K_m values of 36 and 33 μM were found, respectively. These values are higher than that determined for cysteine synthase from spinach [1], but are almost the same as

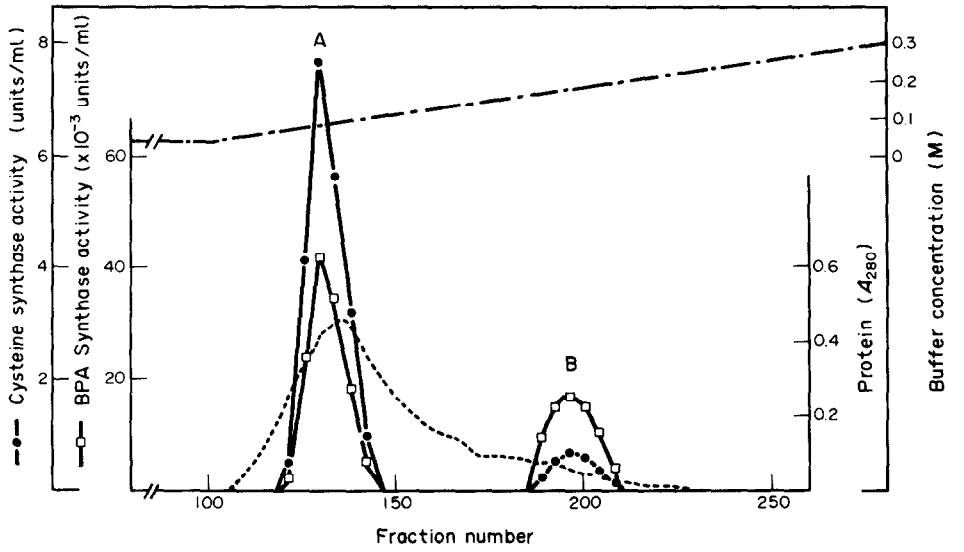


Fig. 1. Elution patterns of isoenzymes A and B of cysteine synthase after the first DEAE-Sephadex A-50 column chromatography. Cysteine synthase activity (●——●), BPA synthase activity (□——□) and protein (A_{280}, - - -) were monitored as shown in the Experimental.

those determined for isoenzymes A and B of cysteine synthase from pea [3] and for isoenzyme B of cysteine synthase from *P. vulgaris* [10].

The addition of PLP had no effect on the primary activity of these enzymes, while PLP requirements have been reported for BPA synthase from the same plant [4] and for uracilylalanine synthases from pea [12]. The PLP-enzyme inhibitors, hydroxylamine and potassium cyanide at a concentration of 1 mM both caused 5–10% inhibition.

Substrate specificity

Under standard assay conditions, the cysteine synthase isoenzymes from *C. vulgaris* seedlings clearly appear to be specific for OAS as a donor of the alanyl moiety. No detectable activity was found when OAS is substituted by *O*-phospho-L-serine, *O*-sulpho-L-serine, β-chloro-L-alanine or L-serine, an observation in line with previous findings [1–5]. 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. The activity of isoenzyme B for sulphide was 107% that of isoenzyme A and isoenzymes A and B differ in their ability to synthesize different *S*-substituted L-cysteines. Isoenzymes A and B could synthesize BPA at 1.88 and 2.13% of the rate of cysteine synthesis, respectively; they also could effect the synthesis of β-(3-amino1,2,4-triazol-1-yl)-L-alanine, but could not catalyse the production of L-quisqualic acid, L-willardiine, L-isowillardiine or L-mimosine when suitable substrates were provided. Only isoenzyme A could synthesize β-cyano-L-alanine at 35.1% from OAS (not L-cysteine) and CN^-, and also synthesize *O*-ureido-L-serine in low activity.

Thus, the sp act of isoenzymes A and B towards a variety of substrates are also different from those of the cysteine synthase isoenzymes from *Q. indica* var. *villosa* [2] and *P. sativum* [3]. 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. The data presented show that both isoenzymes consist of the same 538 residues and isoenzyme A contains a large amount of glutamic acid (56 residues) and glycine (50 residues), while isoenzyme B contains a large amount of glycine (56 residues), glutamic acid (54 residues) and serine (52 residues). The isoenzymes A and B also differ in their content of cysteine and methionine residues. Cysteine synthases from plants contain 18 to 22 *S*-containing amino acids except isoenzyme B from pea [1, 3], while the number of cysteine and methionine residues is invariably one and five, respectively, for the enzymes from microorganisms [13, 14].

The M_rs of isoenzymes A and B, calculated from the amino acid compositions, are 58 000 and 57 900, respectively, which correspond to the values estimated by gel filtration on Sephadex G-100 (58 000).

DISCUSSION

In the present study we have purified two cysteine synthases from *C. vulgaris* seedlings to apparent homogeneity and a comparison has been made of their properties and substrate specificities with those of the previously purified enzymes [1–5].

It appears that the two purified enzymes show BPA synthase activity. They could be separated by gradient elution with K-Pi buffer from a DEAE-Sephadex A-50 column. During the same procedure peaks with BPA synthase activity were completely overlapped with that of cysteine synthase activity.

The two isoenzymes purified in this study have very similar physicochemical properties: they have the same

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

Thiol compound and *N*-heterocyclic compound	Amino acid synthesized	Relative velocity of synthesis (%)	
		Isoenzyme A	Isoenzyme B
H_2S	L-Cysteine	100	107
MeSH	*S*-methyl-L-cysteine	21.5	77.0
CH_2=CH-CH_2-SH	*S*-Allyl-L-cysteine	0.46	6.16
HOOC-CH_2-SH	*S*-Carboxymethyl-L-cysteine	3.46	3.01
Pyrazole	*β*-(Pyrazol-1-yl)-L-alanine	1.88	2.13
3-Amino-1,2,4-triazole	*β*-(3-Amino-1,2,4-triazol-1-yl)-L-alanine	0.98	1.40
3,5-Dioxo-1,2,4-oxadiazolidine	L-Quisqualic acid	0	0
3,4-Dihydroxypyridine	L-Mimosine	0	0
Uracil	L-Willardiine	0	0
	L-Isowillardiine	0	0
Hydroxyurea	*O*-Ureido-L-serine	0.17	0
NaCN	*β*-Cyano-L-alanine	35.1	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 [1–5].

Table 3. Amino acid compositions of cysteine synthase isoenzymes purified from *Citrullus vulgaris*

Amino acids	Cysteine synthase	
	Isoenzyme A	Isoenzyme B
	Residues/58 000 g*	
Asp	42	42
Thr	32	28
Ser	44	52
Glu	56	54
Pro	30	28
Gly	50	56
Ala	46	44
Val	40	36
Cys	4	14
Met	14	4
Ile	34	26
Leu	46	44
Tyr	18	18
Phe	22	24
Trp	0	0
Lys	28	24
His	10	12
Arg	22	32
Total	538	538

*Results are expressed as residues/mol and are based on an M_r of 58 000. Values for Thr and Ser are extrapolated to zero-time hydrolysis.

The numbers of residues of amino acids were calculated as described previously [1, 3].

M_rs of 58 000 and consist of subunits of M_rs 29 000, they have the same pH optimum of 8 and they contain PLP. From these results and also the fact that the sp act of the purified isoenzymes A and B are almost the same, we consider that they are truly isoenzymes. Moreover, when the physicochemical properties of these purified enzymes are compared with those of BPA synthase purified before [4], they are almost the same. We also consider that the isoenzyme A of cysteine synthase from *C. vulgaris* seedlings also functions as a BPA synthase.

Among the substrates studied so far (Table 2), isoenzyme B exhibits higher activities with thiol compounds than does isoenzyme A. The two isoenzymes catalyse the formation of BPA and *β*-(3-amino-1,2,4-triazol-1-yl)-L-

alanine, while they cannot synthesize L-quisqualic acid, L-willardiine, L-isowillardiine or L-mimosine. Only isoenzyme A catalyses the formation of β-cyano-L-alanine, as well as *O*-ureido-L-serine. 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, which may have a role as allelochemicals.

The amino acid compositions of cysteine synthases A and B are very similar, except in the number of cysteine and methionine residues. When the known amino acid compositions of cysteine synthases are compared by a mathematical method [15], it would appear that both isoenzymes are close to cysteine synthases from other plant sources, except the isoenzyme B from pea seedlings [3]. This observation makes it likely that these isoenzymes might have originated from a recent gene duplication and also that the phylogenetic relationship among these enzymes can be firmly assessed, after obtaining the amino acid sequences.

Although information is lacking on the amino acid composition of BPA synthase purified previously, it appears likely that isoenzyme A of cysteine synthase and BPA synthase purified from *C. vulgaris* seedlings are identical, and that cysteine synthases may have a role in the biosynthesis of BPA. All these findings concerning plant cysteine synthase suggest that the enzyme can play a role in the detoxification of endogenous molecules such as pyrazole or cyanide in higher plants without jeopardizing the synthesis of L-cysteine as a primary metabolite.

EXPERIMENTAL

Materials. Citrullus vulgaris seeds were purchased from local nurseries, sown in moistened vermiculite and grown in the dark for 7–8 days at 26–28°. Seedlings were harvested, washed and the cotyledons removed; they were then 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 lab according to a modified method of ref. [16]. All other chemicals used were of the highest commercial grade available.

Activity assays. The routine assay of cysteine synthase activity was based on that described previously [1], according to the method of ref. [17]. The formation of BPA was determined using an automatic amino acid analyser as described before [1]. The unit of enzyme activity used in this paper is equivalent to 1 μmol of L-cysteine or BPA produced per min. Protein was determined by the method of ref. [18].

Purification of isoenzymes. All operations were carried out at 0–4°. Cysteine synthases were prepared from 8.2 kg of fresh etiolated seedlings (cotyledons removed), essentially as before [1–3]. The 40–60% satd $(NH_4)_2SO_4$ 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 then applied to the first Sephadex G-100 column (4.6 × 90 cm) pre-equilibrated with buffer A. The active fractions of step 4 were pooled and then applied to the first DEAE-Sephadex A-50 column (2 × 12.5 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 70–90 mM and 170–200 mM K-Pi buffer, respectively, and were concd by Immersible CX-10 (Millipore). The first and second active 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 two series of active fractions were pooled and concd by Immersible CX-10. 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 enzymes eluted with a linear gradient of K-Pi (10–150 mM) in the same buffer. Cysteine synthase isoenzymes activities were eluted at 20–35 mM for isoenzyme A and 40–55 mM for B, 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 cm) of DEAE-Sephadex A-50 pre-equilibrated in buffer A and then eluted with a linear gradient of K-Pi (30–300 mM) in buffer A. The highly purified enzyme fractions were used as isoenzymes A and B in all further expts.

Properties of cysteine synthase isoenzymes were studied by the methods of ref. [4].

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

Determination of amino acid compositions was performed as described previously [1], using 0.05–0.1 mg of the purified enzymes.

Acknowledgements—We are grateful to Sir L. Fowden, Rothamsted Experimental Station, England and to Dr F. Lambein, State University of Ghent, Belgium, for their valuable discussions in the preparation of this manuscript.

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