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PURIFICATION AND PROPERTIES OF CYSTEINE SYNTHASE FROM *SPINACIA OLERACEA**

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; spinach; cysteine synthase; β-substituted alanine synthase; enzyme purification; amino acid composition; *O*-acetyl-L-serine; cysteine; heterocyclic β-substituted alanines.

Abstract—Cysteine synthase was purified 3200-fold from *Spinacia oleracea* leaves. The purified enzyme has an apparent M_r of 60 000 ± 2000 and can be dissociated into identical subunits of M_r 32 000 ± 2000. The subunits contain one molecule of pyridoxal 5'-phosphate. The K_m value is 2.9 mM for *O*-acetyl-L-serine and 22 μM for sulphide. Cysteine synthase from *S. oleracea* catalysed the formation of β-(pyrazol-1-yl)-L-alanine, and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine, and significant differences were found between this enzyme and β-substituted alanine synthases and cysteine synthase from other sources. Amino acid composition of the purified enzyme was also determined.

INTRODUCTION

In our laboratory, a number of naturally occurring heterocyclic β-substituted alanines have been synthesized enzymatically *in vitro* with crude extracts from different plant sources [1–10]. Recently two such enzymes have been purified to homogeneity: β-(pyrazol-1-yl)-L-alanine (BPA) synthase from *Citrullus vulgaris* and L-mimosine synthase from *Leucaena leucocephala* [9, 10]. Both enzymes are specific for *O*-acetyl-L-serine (OAS) as a donor for the alanyl moiety but show marked differences in specificity for the heterocyclic substrates as an acceptor. The similarities between these two enzymes however suggested a common evolutionary origin of the cucurbit and the legume enzyme.

The biosynthesis of non-protein amino acids in plants may be due to the action of enzymes normally involved in the biosynthesis of protein amino acids that, during the course of evolution, have acquired a different specificity. In the case of the heterocyclic β-substituted alanines, it was first suggested that these natural compounds may arise by non-specific action of tryptophan synthase [11–13]. When OAS was recognized as the donor for the alanyl moiety, the enzymatic formation of heterocyclic β-substituted alanines was compared to the formation of *S*-methyl-L-cysteine from methanethiol and OAS as shown in Scheme 1. A number of *S*-substituted cysteines occurring in plants are also formed by the cysteine synthase catalysed reaction of OAS and thiol compounds [14–20]. This enzyme also catalyses the formation of selenocysteine in higher plants [21].

The group of enzymes producing heterocyclic β-substituted alanines generally establishes an N–C bond between the β-carbon of alanine and a ring-nitrogen. Occasionally a C–C bond is formed in a natural product [4] or an O–C bond is formed with a synthetic precursor *in vitro* [7].

To obtain a better understanding of the relationship between protein amino acid metabolism and the metabolism of this group of non-protein amino acids, we have attempted the purification of cysteine synthase from spinach (*Spinacia oleracea*), which does not contain heterocyclic β-substituted alanines, in order to make a detailed comparison with the purified BPA-synthase from *C. vulgaris* [9] and with the purified L-mimosine synthase from *L. leucocephala* [10].

In this paper the purification and properties of cysteine synthase from *S. oleracea* leaves are described in comparison with β-substituted alanine synthases and cysteine synthase from other sources.

RESULTS

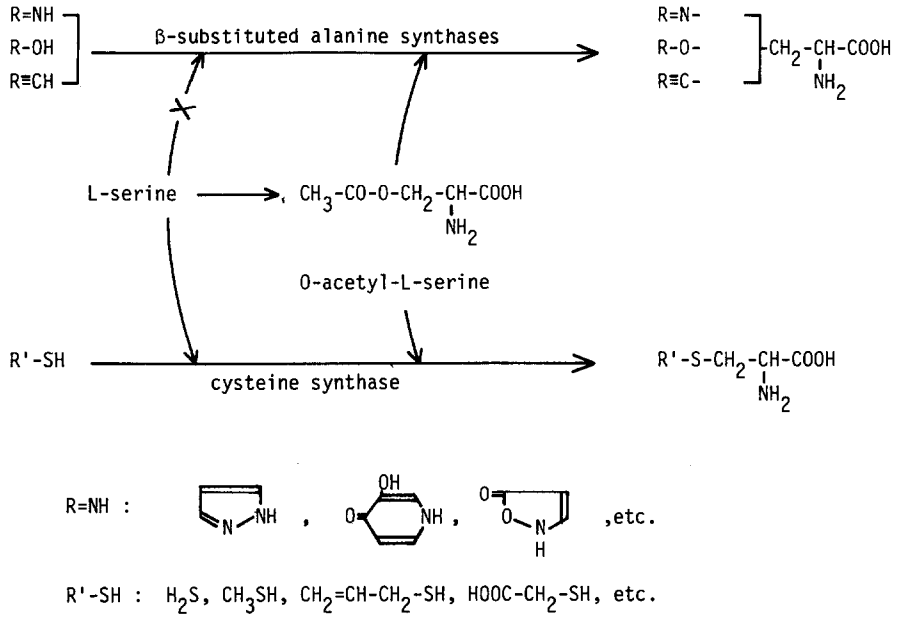
Purification of cysteine synthase from Spinacia oleracea *leaves*

The enzyme was prepared from 7.5 kg fr. wt of leaves by the procedure including heat treatment, ammonium sulphate fractionation, gel filtration on Sephadex G-100 or Ultrogel AcA 44, ion-exchange chromatography on DEAE-Sephadex A-50 or DEAE-cellulose, hydroxylapatite treatment and preparative polyacrylamide-gel electrophoresis as summarized in Table 1. The purified enzyme activity was eluted at 70–110 mM KCl on DEAE-cellulose (DE-52) chromatography (step 6) and cysteine synthase band obtained after DE-52 chromatography had a constant specific activity across the band. The above procedure afforded *ca* 3200-fold purification of cysteine synthase with a specific activity of 543 U/mg protein and a yield of 18.7%.

Properties of cysteine synthase in S. oleracea

The M_r of the purified enzyme from *S. oleracea* was determined by analytical gel filtration [22]. The column of

*Parts of this work were reported at the 104th Annual Meeting of the Pharmaceutical Society of Japan at Sendai, March 28, 1984 (Abstracts p. 121).



Scheme 1. Biosynthetic pathways for heterocyclic β-substituted alanines and *S*-substituted L-cysteines in higher plants.

Table 1. Summary of purification of cysteine synthase from *Spinacia oleracea*

Purification step	Total activity (units)*	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Fold
1. Crude extract†	37167	215340	0.17	100	1
2. 60°-heated supernatant‡	34947	85445	0.41	94.0	2.4
3. Ammonium sulphate precipitate§	33554	45927	0.73	90.3	4.3
4. DEAE-Sephadex A-50 (110–175 mM)	26713	4340	6.16	71.9	36.2
5. Sephadex G-100 (peak fractions)	20900	658	31.8	56.2	187
6. 1st DEAE-cellulose (70–110 mM)	17652	118	150	47.5	882
7. Ultrogel AcA 44 (peak fractions)	12358	70.2	176	33.2	1035
8. Hydroxylapatite (peak fractions)	9514	42.3	225	25.6	1324
9. Polyacrylamide-gel electrophoresis	7480	14.1	530	20.1	3118
10 2nd DEAE-cellulose (70–100 mM)	6956	12.8	543	18.7	3194

*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 7.5 kg of the fresh leaves of *Spinacia oleracea*.
‡60°, 2 min.
§35–70% saturation.

Sephadex G-100 (1.5 × 115 cm) was first calibrated with standard proteins; cysteine synthase activity was found invariably as a single peak, corresponding to an M_r of 60000 ± 2000. The purified enzyme was subjected to SDS-polyacrylamide-gel electrophoresis on 12% gels to determine its subunit structure, following the method of King and Laemmli [23]. A single band with an M_r of 32000 ± 2000 indicated two identical subunits.

The identification of bound pyridoxal 5'-phosphate (PLP) in the purified enzyme was demonstrated by direct spectrophotometric measurements [24]. Cysteine synthase in *S. oleracea* has one molecule of PLP bound to each subunit.

The enzyme exhibited a single pH optimum at pH 8.3 although there was a rapid acetyl shift from O to N atoms in the substrate above *ca* pH 8.0. Lineweaver–Burk plots gave K_m values of 2.9 mM for OAS and 22 μM for sulphide. The K_m value for OAS was less than that determined for L-mimosine synthase from *L. leucocephala* [10] and for cysteine synthase from *Brassica chinensis* var. *Komatsuna* [17], while it was almost the same value with that determined for BPA-synthase from *C. vulgaris* [9].

The addition of pyridoxal and PLP had an inhibitory effect on the activity of this enzyme, the decrease being about 30% and 15% at 1 mM, respectively, while PLP requirements have been reported for BPA-synthase. The PLP-enzyme inhibitors, hydroxylamine and potassium cyanide at a concentration of 1 mM both caused 5–15% inhibition.

Substrate specificity

Under standard assay conditions, cysteine synthase in *S. oleracea* clearly appears to be specific for OAS as a donor for the alanyl moiety. Under identical conditions the activity was 2.2% and 0.52% in the presence of β-chloro-L-alanine and *O*-sulpho-L-serine as compared to OAS at 12.5 mM, respectively. No detectable activity was found with *O*-phospho-L-serine or with L-serine. The purified enzyme showed 0.78% activity in the presence of *O*-acetyl-D-serine.

The enzyme also showed distinct substrate specificity when a variety of thiol compounds or *N*-heterocyclic compounds were used as an acceptor for the thiol moiety or the alanyl moiety. Table 2 shows the relative activities of the purified enzyme with different substrates. Cysteine synthase in *S. oleracea* could not synthesize L-mimosine, L-quisqualic acid, L-willardiine or L-isowillardiine when suitable substrates were provided, while this enzyme synthesized *S*-substituted L-cysteines. The purified enzyme however could synthesize BPA, β-(3-amino-1,2,4-triazol-1-yl)-L-alanine and *O*-ureidoserine in low activities. The different heterocyclic substrates were tested at the pH found optimal for the alanylation of the respective substrates [2–10].

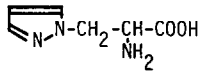
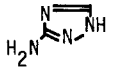
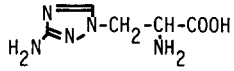
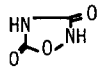
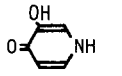
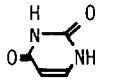
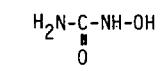
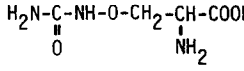
Amino acid composition

Data on the amino acid composition of cysteine synthase from *S. oleracea* leaves are presented in Table 3. The amino acids were determined after 24, 48 and 72 hr acid hydrolysis of the purified enzyme with a specific activity of 543 U/mg protein by using an automatic amino acid analyser. The data presented show that the enzyme contains a large amount of glutamic acid (57 residues), glycine (51 residues), as well as alanine (41 residues), valine (38 residues), isoleucine (36 residues), leucine (40 residues) and lysine (37 residues). The M_r calculated on the basis of the amino acid composition, is 61 100, which corresponds to the value found by gel filtration on a column of Sephadex G-100 (60 000 ± 2000). Cysteine synthase obtained from *S. oleracea* leaves was different in the amino acid composition with the enzyme purified from microorganisms [25, 26].

DISCUSSION

In previous work we have demonstrated that higher

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

Thiol compound and *N*-heterocyclic compound	Amino acid synthesized	Relative velocity of synthesis (%)
H_2S	L-cysteine	100
CH_3SH	*S*-methyl-L-cysteine	31.7
$CH_2{=}CH{-}CH_2{-}SH$	*S*-allyl-L-cysteine	17.6
$HOOC{-}CH_2{-}SH$	*S*-carboxymethyl-L-cysteine	2.5
		3.8
		0.97
	L-quisqualic acid	0
	L-mimosine	0
	L-willardiine	0
	L-isowillardiine	0
		9.7

The relative rates of synthesis were compared with that of L-cysteine. The reaction conditions are given in the Experimental and are as described before [9, 10].

Table 3. Amino acid composition of cysteine synthase purified from *S. oleracea*

Amino acids	Residues/ 60000 g
Asp	31
Thr	26*
Ser	34*
Glu	57
Pro	28
Gly	51
Ala	41
Val	38
Cyt	3
Met	11
Ile	36
Leu	40
Tyr	12
Phe	22
Trp	0
Lys	37
His	4
Arg	15
Total	486

*Extrapolated to zero time from data for 24, 48 and 72 hr hydrolysis.

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 enzyme. Means of duplicate analyses are given. The test conditions were as described in Experimental.

plants containing heterocyclic β-substituted alanines also contain enzymes catalysing the formation of these compounds from *O*-acetyl-L-serine and the suitable heterocycle [1–10]. The purification to near homogeneity of two such enzymes has been described: BPA-synthase from *Citrullus vulgaris* and L-mimosine synthase from *Leucaena leucocephala*, which have different biosynthetic capacities [9, 10]. The physicochemical similarities however suggest the possibility that these two proteins may have evolved from a common ancestor. In the present study we have purified *ca* 3200-fold an enzyme from spinach involved in the biosynthesis of protein amino acids which catalyses the formation of cysteine by a reaction comparable to the formation of heterocyclic β-substituted alanines as shown in Scheme 1.

The M_rs for plant cysteine synthases have been reported to be in the range of 62000–70000 [17–20]. The plant enzyme can be dissociated into two identical subunits, each with an M_r approximately one-half that of the intact enzyme [17, 18] and PLP is tightly bound to the enzyme [17]. The properties of the enzyme we have purified from *S. oleracea* leaves are almost the same as those of the cysteine synthase from *Brassica chinensis* var. *Komatsuna* [17], *Raphanus sativas* [18] and from spinach leaves by Schmidt [20].

Cysteine synthase from *S. oleracea* could catalyse the formation of *S*-alkyl-L-cysteines from OAS and corresponding thiol compounds, and also could synthesize β-substituted alanines, such as BPA, β-(3-amino-1,2,4-triazol-1-yl)-L-alanine and *O*-ureidoserine in low activities (Table 2). BPA-synthase from *C. vulgaris* could not synthesize *S*-methyl-L-cysteine [9].

Although there is ample proof that cysteine synthase from *S. oleracea* is different from BPA-synthase from *C. vulgaris* and from L-mimosine synthase from *L. leucocephala*, there is also a considerable similarity among those three enzymes in their physicochemical properties, the M_r of the subunits, the presence of PLP and perhaps the number of aromatic amino acids per subunit. Lacking further information on the amino acid composition or the sequence of these three proteins the phylogenetic relationship cannot be firmly assessed, while this study presents the first detailed data of the amino acid composition of cysteine synthase from higher plants (Table 3). It can however be suggested that the heterocyclic β-substituted alanine synthase may have arisen by gene duplication of the gene for cysteine synthase. This could enable the plant to ensure the synthesis of cysteine as a primary metabolite and to have a less restricted evolution of the second gene that could undergo mutations and adapt to novel substrates without jeopardizing the production of the primary metabolite.

EXPERIMENTAL

Materials. Spinacia oleracea L. var. 'Parade' was grown in the botanical garden of our university. Leaves were collected and then cooled for 1 hr at 0–4° before enzyme extraction. Sephadex G-100 and DEAE-Sephadex A-50 were purchased from Pharmacia. DEAE-cellulose (DE-52) and Ultrogel AcA 44 were obtained from Whatman and from LKB, respectively. All other chemicals used were of the highest commercial grade available.

Activity assay. The enzyme preparations obtained were dissolved in 50 mM K-Pi buffer, pH 8.0 (Buffer A). Substrate concns were 4–6 mM for sulphide and 12.5 mM for OAS. Incubation was at 30° for 10 min; the total reaction vol. was 1.0 ml, utilizing up to 0.2 ml of enzyme (corresponding to 2–400 μg of protein). Reactions were terminated by the addition of 0.2 ml of 7.5% TCA and the formation of L-cysteine was spectrophotometrically measured at 560 nm using an acid ninhydrin reagent according to the method of ref. [27]. The unit of enzyme activity used in this paper is equivalent to 1 μmol of L-cysteine produced per 1 min. Protein was determined by the method of ref. [28].

Purification of cysteine synthase from S. oleracea *leaves.* All operations were carried out at 0–4°. Cysteine synthase was prepared from 7.5 kg of fresh leaves by the same procedure up to ammonium sulphate fractionation as previously described [9, 10]. The protein precipitating between 35 and 70% saturation was collected and dissolved in 30 mM K-Pi buffer, pH 8.0 containing 10 mM 2-mercaptoethanol and 0.5 mM EDTA (Buffer B). The resulting solns dialysed against Buffer B were applied on the DEAE-Sephadex A-50 column (4.6 × 15 cm) pre-equilibrated with Buffer B and then eluted with a linear gradient of increasing K-Pi concns (30–200 mM) in the same buffer. Fractions with significant synthase activity (110–175 mM K-Pi fractions) were pooled and were concd by $(NH_4)_2SO_4$ precipitation. The step 4 fraction was applied on a column (4.6 × 90 cm) of Sephadex G-100 pre-equilibrated with Buffer B containing 100 mM KCl. The eluate was collected in 16 ml fractions and the pooled active fractions were dialysed overnight against Buffer B. The dialysed fraction was then applied on a column (3.0 × 15 cm) of DEAE-cellulose (DE-52) pre-equilibrated with Buffer B. The column was then washed extensively with Buffer B containing 30 mM KCl and was eluted with a linear KCl-gradient (30–200 mM) in Buffer B. The enzyme activity was eluted at

70–110 mM KCl, and was concd by Immersible CX-10 (Millipore). The step 6 fraction was applied on a column (2.0 × 90 cm) of Ultrogel AcA 44 pre-equilibrated with Buffer B containing 50 mM KCl. The eluate was collected in 3 ml fractions and the pooled active fractions were concd by Immersible CX-10. The step 8 fraction was applied on a column (2.0 × 1.2 cm) of hydroxylapatite pre-equilibrated with, and eluted by, 10 mM K-Pi buffer, pH 8.0, containing 10 mM KCl and 4 mM 2-mercaptoethanol. The eluate was collected in 2 ml fractions and the pooled active fractions were concd by Immersible CX-10. The resulting soln was then subjected to the preparative polyacrylamide-gel electrophoresis on 7.5% gels at pH 8.3 (Tris–glycine buffer). Cysteine synthase activity obtained from gel slices was finally applied on a column (1.2 × 11 cm) of DE-52 pre-equilibrated in Buffer B containing 30 mM KCl and then eluted with a linear KCl-gradient (30–200 mM) in Buffer B. The highly purified enzyme fraction (70–100 mM KCl fractions) was a yellow soln and was stable for at least three months at 0°. This enzyme fraction was used in all further expts.

Properties of cysteine synthase. This was performed as described previously [9].

Identification of heterocyclic β-substituted alanines and S-substituted L-*cysteines as reaction products.* The normal reaction mixtures contained 12.5 mM of OAS, 70 mM of thiol compounds or *N*-heterocyclic compounds and 200 μl of enzyme preparation (corresponding to 4 μg of protein), in a final vol. of 0.4 ml of Buffer A. Incubation was at 30° for 30 min and the reactions were terminated by the addition of 30 μl of 1 N KOH. The resulting soln acidified with 15 μl of 6 N HCl was examined for the formation of heterocyclic β-substituted alanines and *S*-substituted L-cysteines by using an automatic amino acid analyser (Hitachi 835-10) under standard operating conditions (2.6 × 25 cm column, 33–68°, Li-citrate buffer system pH 3.0–7.0, flow rate 0.275 ml/min).

Determination of amino acid composition. The purified enzyme (1.10 mg) was hydrolysed in 2 ml of 6 N HCl in a sealed tube at 110° for 24, 48 and 72 hr using Reacti-Therm Heating Module (Pierce). After hydrolysis and evaporation *in vacuo*, 2.0 ml of 0.02 N HCl was added and then the sample (25 μl) was analysed on a Hitachi amino acid analyser 835-10. Determination of tryptophan was made by the general method described in ref. [29] and also made by alkaline hydrolysis: the enzyme (0.55 mg) was hydrolysed in 1 ml of 2.5 N NaOH containing 0.375% starch in a sealed tube at 110° for 24 hr. After hydrolysis and evaporation *in vacuo*, 1.0 ml of 0.02 N HCl was added and then the sample (100 μl) was analysed. Duplicate analyses were carried out and then mean of the results were used to determine the amino acid composition of cysteine synthase purified from *S. oleracea*.

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