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ENZYMATIC SYNTHESIS OF THE NEUROEXCITATORY AMINO ACID QUISQUALIC ACID BY CYSTEINE SYNTHASE*

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Key Word Index—*Quisqualis indica* var. *villosa*; Combretaceae; cysteine synthase; isoenzyme; enzyme purification; biosynthesis; heterocyclic β-substituted alanines; quisqualic acid; *O*-acetyl-L-serine; cysteine.

Abstract—Purification of cysteine synthase from the leaves of *Quisqualis indica* var. *villosa* reveals the presence of two forms of this enzyme, separated by chromatography on DEAE-Sephadex A-50. Isoenzyme A was purified 10 000-fold and had a specific activity of 10.8 U/mg protein. Isoenzyme B was purified 460-fold with a specific activity of 0.49 U/mg protein. Both isoenzymes have the same M_rs (58 000) and dissociate into identical subunits (M_r 29 000). The K_m value of isoenzyme A is 1.9 mM for *O*-acetyl-L-serine and 59 μM for sulphide, while that of isoenzyme B is 7.1 mM for *O*-acetyl-L-serine and 4.0 mM for 3,5-dioxo-1,2,4-oxadiazolidine. Both isoenzymes catalyse the formation of cysteine from *O*-acetyl-L-serine and hydrogen sulphide, but only isoenzyme B catalyses the formation of L-quisqualic acid. Other significant differences occur in the substrate specificity of the two isoenzymes. Some properties of the purified cysteine synthase isoenzymes are also described.

INTRODUCTION

The neuroexcitatory amino acid L-quisqualic acid, β-(3,5-dioxo-1,2,4-oxadiazolidin-2-yl)-L-alanine (QA), was isolated from the seeds of *Quisqualis indica* L. and *Q. indica* var. *villosa* Clarke [1]. The enantio-specific synthesis and the biomimetic synthesis of QA have recently been described [2, 3]. The QA-forming enzyme was partially purified from the leaves of *Q. indica* and from the seedlings of *Pisum sativum*, and its properties were studied [4].

In a previous paper [5], we presented evidence that cysteine synthase from spinach leaves can also catalyse the formation of a number of non-protein amino acids in the presence of *O*-acetyl-L-serine (OAS) and suitable precursors. These non-protein amino acids include *S*-substituted L-cysteines and heterocyclic β-substituted alanines such as β-(pyrazol-1-yl)-L-alanine (BPA) and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine. We recently also purified BPA-synthase from *Citrullus vulgaris* [6] and L-mimosine synthase from *Leucaena leucocephala* [7].

These results suggest that naturally occurring heterocyclic β-substituted alanines are synthesized by a reaction mechanism comparable to the one that results in the formation of cysteine, and that the physical properties of the enzymes involved are very similar to those of the known cysteine synthases from higher plants.

During our continuing study on the biosynthesis of this group of uncommon amino acids, we have now attempted the purification of the enzyme catalysing the formation of QA, and of the cysteine synthase from *Q. indica* var. *villosa*, in order to make a detailed comparison with the enzymes described before [5–7].

In this paper we describe the existence of an isoenzyme of cysteine synthase catalysing the formation of QA in the leaves of *Q. indica* var. *villosa* (Scheme 1).

RESULTS

Purification of the cysteine synthase isoenzymes from Quisqualis indica *var* villosa *leaves*

The methods described in the previous papers [5–7] were used for the extraction and purification of cysteine synthases from 1 kg fr. wt of leaves. The enzymes were prepared simultaneously with the QA-forming enzyme activity by a procedure including heat treatment, ammonium sulphate fractionation, ion-exchange chromatography on DEAE-Sephadex A-50, gel filtration on Sephadex G-100 or Ultrogel AcA 44 and preparative polyacrylamide gel electrophoresis (PAGE) as summarized in Table 1.

The proteins demonstrating cysteine synthase activity were completely separated into two peaks after a second chromatography on DEAE-Sephadex A-50 and elution with a concentration-gradient of K-Pi buffer. Isoenzyme B eluted at 170–180 mM and isoenzyme A at 200 mM, as shown in Fig. 1.

The above procedure afforded apparent purifications of 460- and 10 000-fold for isoenzymes B and A, respectively, with specific activities of 0.49 U/mg protein for B, 10.8 U/mg protein for A, and yields of 0.84 and 21.7%, respectively, compared to the cysteine synthase activity of the crude extract.

* Parts of this work were reported at the 103rd Annual Meeting of the Pharmaceutical Society of Japan at Sendai, 5 April 1983 (Abstracts, p. 210) and were also reported at the 28th Annual Meeting of Kanto Branch of the Pharmaceutical Society of Japan at Tokyo, 10 November 1984 (Abstracts, p. 74).

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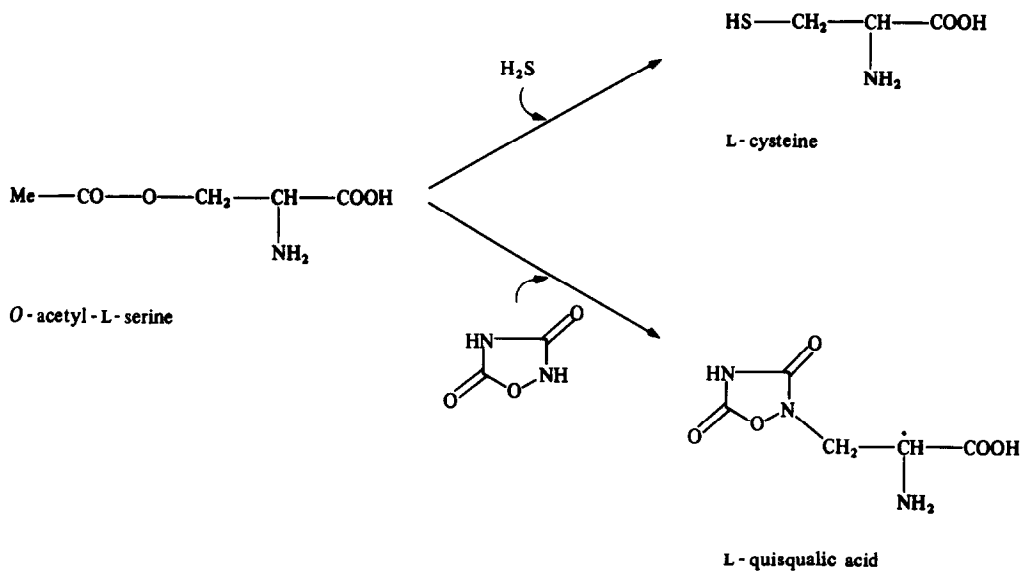

Scheme 1. Biosynthetic pathways for L-cysteine and L-quisqualic acid in higher plants.

Properties of the purified cysteine synthase isoenzymes

The M_rs of the purified enzymes from *Q. indica* var. *villosa* were estimated by analytical gel filtration using Sephadex G-100 (1.5 × 115 cm) according to the method of Andrews [8]. 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 sodium dodecyl sulphate (SDS) PAGE on 12% gels to determine their subunit structures, following the method of King and Laemmli [9]. This suggests that both enzymes are composed of two identical subunits, with the same M_rs of 29 000, and that they have been purified to apparent homogeneity.

The presence of pyridoxal 5'-phosphate (PLP) in both isoenzymes is suggested by their spectral data, as compared to cysteine synthase and heterocyclic β-substituted alanine synthases from other sources [5–7].

The enzymes exhibited a single pH optimum at pH 8.2–8.3, respectively, although there was a rapid acetyl shift from O to N atoms in OAS above *ca* pH 8.0.

The isoenzymes of cysteine synthase from *Q. indica* var. *villosa* display quite different relative activities, but their responses to OAS are essentially the same. Isoenzymes A and B have K_m values of 1.9 and 7.1 mM for OAS, respectively, but they show no indication of inhibition by OAS at concentrations up to 30 mM and their kinetics follow the standard Michaelis–Menten relationship. The

Table 1. Summary of the purification of cysteine synthases from *Quisqualis indica* var. *villosa*

Purification step	Total activity (units)*	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Purification factor (-fold)
1. Crude extract†	21.4	20 100	0.00107	100	1
2. 60°-heated supernatant‡	21.0	17 200	0.00122	98.1	1.14
3. Ammonium sulphate precipitate§	20.3	8 210	0.00247	94.9	2.31
4. 1st DEAE-Sephadex A-50 (170–250 mM)	19.4	377	0.0539	90.7	50.4
5. Sephadex G-100 (peak fractions)	17.1	57.1	0.299	79.9	279
6. 2nd DEAE-Sephadex A-50					
Isoenzyme A (200 mM)	9.40	3.98	2.36	43.9	2210
Isoenzyme B (170–180 mM)	0.42	7.56	0.056	2.0	33
7. Ultrogel AcA 44 (peak fractions)					
Isoenzyme A	6.65	1.30	5.12	31.1	4790
Isoenzyme B	0.29	1.97	0.147	1.4	137
8. Polyacrylamide-gel electrophoresis					
Isoenzyme A	4.65	0.483	10.8	21.7	10 100
Isoenzyme B	0.18	0.367	0.49	0.84	458

*A unit of enzyme activity represents 1 μmol of product formed/min at 30°, in 50 mM K-Pi buffer, pH 8.
†Starting from 1 kg of fresh leaves of *Q. indica* var. *villosa*.
‡60°, 1 min.
§20–50% saturation.

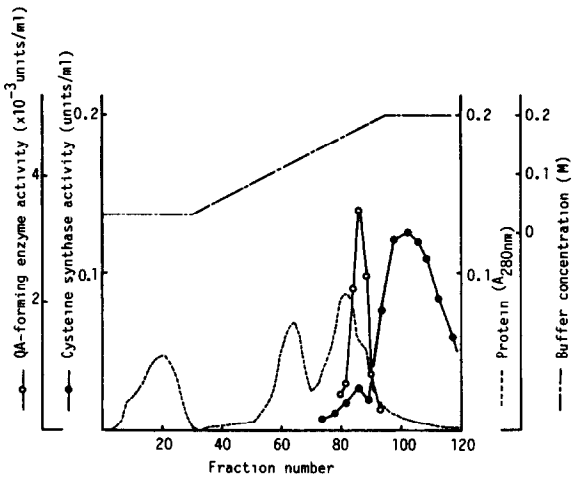

Fig. 1. Elution patterns of isoenzymes A and B of cysteine synthase after the second DEAE-Sephadex A-50 column chromatography. QA-forming enzyme activity (○——○), cysteine synthase activity (●——●) and protein (A_{280}, – – –) were monitored as shown in the Experimental.

$K_{m,OAS}$ of isoenzyme A is less than that determined for cysteine synthase from spinach [5] and for isoenzyme A of cysteine synthase from *Phaseolus vulgaris* [10], while it is very close to the values determined for cysteine synthase from *Raphanus sativus* [11] and for isoenzyme B of cysteine synthase from *P. vulgaris* [10]. The $K_{m,OAS}$ of isoenzyme B, on the other hand, has almost the same value as that determined for L-mimosine synthase from *L. leucocephala* [7].

The response of isoenzyme A to sulphide concentrations below 0.2 mM was examined and a K_m value of 59 μM was found. This value is higher than that determined for cysteine synthase from spinach [5]. Furthermore, the response of isoenzyme B to 3,5-dioxo-1,2,4-oxadiazolidine concentrations was examined and a K_m value of 4.0 mM was found.

The formation of QA by isoenzyme B is enhanced by PLP, the increase being maximal (50%) at 6 μM, although the addition of PLP and pyridoxal had no effect on the primary activity of these enzymes. Similar requirements have been reported for BPA-synthase [5] and for uracilyl-alanine synthases from *P. sativum* [12].

Substrate specificity

Under standard assay conditions, the cysteine synthase isoenzymes in *Q. indica* var. *villosa* clearly appear to be specific for OAS as a donor of the alanyl moiety. In isoenzyme A, the activity was 11% in the presence of β-chloro-L-alanine in comparison with the activity with OAS at 12.5 mM under identical conditions. No detectable activity was found with *O*-phospho-L-serine, *O*-sulpho-L-serine or L-serine. Purified isoenzyme A showed no activity in the presence of *O*-acetyl-D-serine.

Isoenzymes A and B 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. Isoenzyme A could not synthesize QA, L-willardiine, L-isowillardiine or *O*-ureido-L-serine when suitable substrates were provided, while this enzyme synthesized *S*-substituted L-cysteines. However, isoenzyme A could synthesize BPA, β-(3-amino-1,2,4-triazol-1-yl)-L-alanine and β-cyano-L-alanine at low rates.

Isoenzyme B, on the other hand, synthesized QA, BPA and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine, although this enzyme also could not synthesize L-willardiine, L-isowillardiine, *O*-ureido-L-serine or β-cyano-L-alanine. The different heterocyclic substrates were tested under the same conditions described previously [5]. Thus, both isoenzyme A and B show different substrate specificities as compared with cysteine synthase from spinach [5] and from other sources [10, 11, 13].

DISCUSSION

Several different types of cysteine synthase have been isolated from various higher plants [5, 10, 11, 13], and isoenzymes of cysteine synthase have been partially purified from seedlings of two *Phaseolus* species [10]. In

Table 2. Relative synthetic rates of *S*-substituted L-cysteines and β-substituted alanines by cysteine synthase isoenzymes purified from *Quisqualis indica* var. *villosa*

Thiol compound and *N*-heterocyclic compound	Amino acid synthesized	Relative velocity of synthesis (%)	
		Isoenzyme A	Isoenzyme B
H_2S	L-Cysteine	100	4.53
MeSH	*S*-Methyl-L-cysteine	7.7	n.d.
CH_2=CH–CH_2–SH	*S*-Allyl-L-cysteine	4.0	n.d.
HOOC–CH_2–SH	*S*-Carboxymethyl-L-cysteine	0	n.d.
3,5-Dioxo-1,2,4-oxadiazolidine	L-Quisqualic acid	0	0.015
Pyrazole	β-(Pyrazol-1-yl)-L-alanine	1.2	0.02
3-Amino-1,2,4-triazole	β-(3-Amino-1,2,4-triazol-1-yl)-L-alanine	0.59	0.022
Uracil	L-Willardiine	0	0
	L-Isowillardiine	0	0
Hydroxyurea	*O*-Ureido-L-serine	0	0
NaCN	β-Cyano-L-alanine	1.58	0

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 [5–7]. n.d., not determined.

the present study two cysteine synthases were purified to apparent homogeneity from the leaves of *Q. indica* var. *villosa*, and it is suggested that one of these enzymes is an isoenzyme catalysing the formation of QA.

Although this enzymatic activity has been described before in crude extracts of the same plant species [4], this study presents the first purification of this enzyme and its identification as an isoenzyme of cysteine synthase. Two enzymes with cysteine synthase activity as the major catalytic activity were isolated; both had similar physical properties with identical M_rs and consisted of two identical subunits. Only after a second passage through a DEAE-Sephadex A-50 column was separation achieved. We consider the two enzymes to be isoenzymes although the kinetic parameters differ considerably.

The specific activity of isoenzyme A is about 20-fold higher than that of isoenzyme B. The $K_{m,OAS}$ of both isoenzyme A (1.9 mM) and isoenzyme B (7.1 mM) fall just outside the range of 2.1–6.2 mM reported for cysteine synthases from other sources [5, 10, 11, 13]. The apparent purification factor of isoenzyme B is depressed due to the presence of the 20 times more active isoenzyme A. When the ratio of the activities or the ratio of the yields of the two enzymes are considered, we can roughly estimate that the purification factors of both isoenzymes are around 10^4.

Among the substrates studied so far (Table 2), the cysteine synthase isoenzymes catalyse the formation of *S*-substituted L-cysteines from OAS and the corresponding thiol compounds. Both isoenzymes catalyse the formation of some β-substituted alanines in low yields: BPA and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine. Only isoenzyme B catalyses the formation of QA. On the other hand, only isoenzyme A catalyses the formation of β-cyano-L-alanine from OAS and CN^-. β-Cyano-L-alanine (BCA) is the first intermediate in the process of cyanide assimilation in higher plants [14]. Cyanide is formed naturally, together with ethylene, from 1-aminocyclopropane-1-carboxylic acid (ACC) in higher plants and is detoxified by BCA-synthase, which combines it with cysteine to form BCA and hydrogen sulphide [15]. This latter enzyme is also a PLP-enzyme but with a reported M_r of around 50 000 and no subunits [16]. Interestingly, some cyanide-resistant bacteria also contain BCA-synthase activity which is ascribed to a secondary function of cysteine synthase. At least one true BCA-synthase, utilizing cysteine, occurs in the cyanide-producing bacterium *Chromobacterium violaceum*. This last enzyme has two identical subunits, each containing one molecule of PLP [17], analogous to the cysteine synthases from higher plants [5] and to the heterocyclic β-substituted alanine synthases [6, 7].

Our data presented in this paper and other results mentioned above indicate that the isoenzymes of cysteine synthase have the potential to catalyse analogous reactions with appropriate sulphydryl or imino analogues. At the same time they can play a role in the detoxification of endogenous molecules such as cyanide or hydrogen sulphide and form secondary metabolites like the β-substituted alanines, which may be useful to the plant as allelochemicals.

Although further information on the amino acid composition and sequence and on the intracellular localization of these isoenzymes was not obtained in this study, evidence for the presence of two isoenzymes of cysteine synthase and the role of one of them in the biosynthesis of the neuroexcitatory amino acid QA is presented. The additional activity of isoenzyme A as BCA-synthase may represent an alternative pathway for the detoxification of cyanide resulting in the formation of β-cyano-L-alanine.

In our previous study [5] we suggested that the heterocyclic β-substituted alanine synthase may have arisen by duplication of the gene for cysteine synthase, ensuring the production of the primary metabolite by one isoenzyme and allowing the other isoenzyme to adapt to new potential substrates during the course of evolution. The present study provides further proof of the low specificity exhibited by these isoenzymes to a variety of substrates.

EXPERIMENTAL

Materials. Leaves of *Quisqualis indica* L. var. *villosa* Clarke were obtained from plants grown in a greenhouse of our medicinal plant garden and then cooled for 1 hr at 0–4° before enzyme extraction. Sephadex G-100 and DEAE-Sephadex A-50 were purchased from Pharmacia. Ultrogel AcA 44 was obtained from LKB. All other chemicals used were of the highest commercial grade available.

Activity assays. This was performed as described previously [5]. The formation of L-cysteine was measured spectrophotometrically according to the method of ref. [18]. The unit of enzyme activity used in this paper was equivalent to 1 μmol of L-cysteine produced per min. Protein was determined by the method of ref. [19].

Purification of cysteine synthase isoenzymes from Q. indica *var.* villosa *leaves.* All steps were carried out at 0–4°. Cysteine synthases were prepared from 1 kg of fresh leaves, essentially as before [5]. The 20–50% 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 applied to a column (8.0 × 40 cm) of Sephadex G-25 pre-equilibrated with buffer A and the protein fraction was then applied to the first DEAE-Sephadex A-50 column (3 × 17 cm) pre-equilibrated with the same buffer. The active fractions of step 4 were pooled and concentrated by $(NH_4)_2SO_4$ precipitation and then applied to a column (4.6 × 90 cm) of Sephadex G-100 pre-equilibrated with buffer A. The eluate was collected in 4 ml fractions and the pooled active fractions were then applied to a second column (2 × 8 cm) of DEAE-Sephadex A-50 pre-equilibrated with buffer A. The column was then washed extensively with buffer A and the enzymes eluted with a linear gradient of K-Pi (30–200 mM) in the same buffer. Cysteine synthase activities were eluted at 170–180 mM and 200 mM K-Pi buffer, respectively, and were concentrated by Immersible CX-10 (Millipore). The first active fractions (170–180 mM K-Pi fractions) and second active fractions (200 mM K-Pi fractions) were individually applied to a column (2.0 × 95 cm) of Ultrogel AcA 44 pre-equilibrated with buffer A containing 50 mM KCl. The eluates were collected in 2 ml fractions, and two series of active fractions were pooled and concentrated by Immersible CX-10. The resulting solns were then 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 × 10 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 and B in all further expts.

Properties of cysteine synthase isoenzymes were studied by the methods of ref. [6].

Identification of heterocyclic β-substituted alanines and S-substituted L-*cysteines as reaction products* was also performed

as described previously [5], except that the formation of β-cyano-L-alanine was measured spectrophotometrically at 640 nm using a ninhydrin reagent after subjecting the reaction solns to TLC in pyridine–*n*-BuOH–H_2O (1:1:1).

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