Phytochemistry, Vol. 28, No. 9, pp. 2285–2291, 1989.
Printed in Great Britain.

0031-9422/89 $3.00+0.00
Maxwell Pergamon Macmillan plc.

PURIFICATION AND PROPERTIES OF β-CYANO-L-ALANINE SYNTHASE FROM *VICIA ANGUSTIFOLIA**

FUMIO IKEGAMI, KYOKO TAKAYAMA, TAKASHI KURIHARA, SEIICHI HORIUCHI, CHIHO TAJIMA, RYOICHI SHIRAI† and ISAMU MURAKOSHI

Faculty of Pharmaceutical Sciences and †College of Arts and Sciences, Chiba University, Yayoi-cho 1-33, Chiba 260, Japan

(*Received in revised form* 6 *February* 1989)

Key Word Index—*Vicia angustifolia*; Leguminosae; β-cyano-L-alanine synthase; cysteine synthase; enzyme purification; substrate specificity; amino acid composition; β-cyano-L-alanine; L-cysteine; *O*-acetyl-L-serine; heterocyclic β-substituted alanines; distribution; higher plants.

Abstract—β-Cyano-L-alanine synthase was purified *ca* 1000-fold to homogeneity from the immature seeds of *Vicia angustifolia*. The purified enzyme has an apparent M_r of 54000 consisting of two identical subunits. The subunits contain one molecule of pyridoxal 5'-phosphate each. The K_m value is 3.6 mM for L-cysteine and 0.5 mM for cyanide. β-Cyano-L-alanine synthase from *V. angustifolia* also catalyses the formation of some *S*-substituted L-cysteines and some heterocyclic β-substituted alanines from L-cysteine or *O*-acetyl-L-serine as an additional catalytic activity. Significant differences were found between this enzyme and β-cyano-L-alanine synthases from other sources. The amino acid composition of the purified enzyme, and also the occurrence and distribution of β-cyano-L-alanine synthase and cysteine synthase activities in some higher plants are given.

INTRODUCTION

In our recent studies [1, 2], we described the purification and the properties of β-cyano-L-alanine (BCA) synthase from *Spinacia oleracea* and *Lathyrus latifolius* and we presented evidence that BCA synthase, as an additional catalytic activity, could also catalyse the formation of some β-substituted alanines and *S*-substituted L-cysteines in the presence of L-cysteine or *O*-acetyl-L-serine (OAS) and suitable precursors. We could also demonstrate that some cysteine synthases purified from plants [3–7] could catalyse the formation of β-substituted alanines, including BCA, in the presence of OAS and suitable precursors. These results suggested that both enzymes could play a similar role in the detoxification of endogenous molecules such as cyanide, hydrogen sulphide or pyrazole, thereby forming both primary and secondary metabolites like BCA, L-cysteine and the β-substituted alanines.

In the course of our continuing study of this group of pyridoxal 5'-phosphate (PLP)-containing enzymes, and of the biosynthesis of heterocyclic β-substituted alanines and BCA, we have now attempted the purification of BCA synthase from *Vicia angustifolia*, which contains BCA and vicianin as cyano compounds, in order to make a detailed comparison with the enzymes previously described [1, 2, 8–13]. We have also studied the occurrence and distribution of BCA synthase and cysteine synthase activities in both cyanogenic and non-cyanogenic plants in order to obtain some information about the metabolic detoxification of cyanide or sulphide, and the physiological role of these enzymes in a variety of higher plants. The intracellular distribution of both enzyme activities in some higher plants is also given.

*Parts of this work were reported at the 105th and 108th Annual Meetings of the Pharmaceutical Society of Japan at Kanazawa, 5 April 1985 (Abstracts p. 452) and at Hiroshima, 5 April 1988 (Abstracts p. 308), respectively.

RESULTS

Purification of BCA synthase

The methods described in our previous paper [1] were used for the extraction and purification of BCA synthase from 2.5 kg fresh weight of the immature seeds of *V. angustifolia*. The enzyme was prepared simultaneously with the cysteine synthase activity by a 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, hydrophobic chromatography on AH-Sepharose 4B, preparative PAGE and hydroxylapatite chromatography on HP-40 as summarized in Table 1.

The protein demonstrating BCA synthase activity was separated from cysteine synthase activity after the AH-Sepharose 4B column was eluted with a concentration gradient of K-Pi buffer. The enzyme activity for BCA synthase was eluted at 80–105 mM and cysteine synthases eluted at 50–70 mM and 125–140 mM as shown in Fig. 1.

The complete procedure (Table 1) afforded an apparent purification of *ca* 1000-fold for BCA synthase with a specific activity of 120 U/mg protein and a yield of 0.5%, as compared to the total BCA synthase activity of the crude extract.

Properties of the purified BCA synthase

The M_r of the purified enzyme was estimated by analytical gel filtration using Sephadex G-100 (1.5 × 115 cm) according to the method of ref. [14]. BCA

Table 1. Summary of the purification of β-cyano-L-alanine synthase from *Vicia angustifolia*

Purification step	Total activity (units*)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Fold
1. Crude extract†	4450	36800	0.121	100	1
2. 55°-heated supernatant‡	4200	31400	0.134	94.4	1.1
3. Ammonium sulphate precipitate§	2680	11000	0.244	60.2	2.0
4. DEAE-Sephadex A-50 (160–220 mM)	2110	2300	0.92	47.4	7.6
5. Sephadex G-100 (peak fractions)	1520	430	3.50	34.2	28.9
6. AH-Sepharose 4B (80–105 mM)	580	48.5	12.0	13.0	99.2
7. Ultrogel AcA 44 (peak fractions)	360	20.5	17.5	8.1	145
8. Preparative PAGE	82	2.1	39.0	1.8	322
9. 2nd DEAE-Sephadex A-50 (100–130 mM)	70	1.5	46.7	1.6	386
10. Hydroxylapatite HP-40 (peak fractions)	24	0.2	120	0.5	992

*A unit of enzyme activity represents 1 μmol of product formed per min at 30°, in 50 mM Tris–HCl buffer, pH 9.
†Starting from 2.5 kg of the immature seeds of *Vicia angustifolia*.
‡55°, 1 min.
§35–60% saturation and desalted on Sephadex G-25.

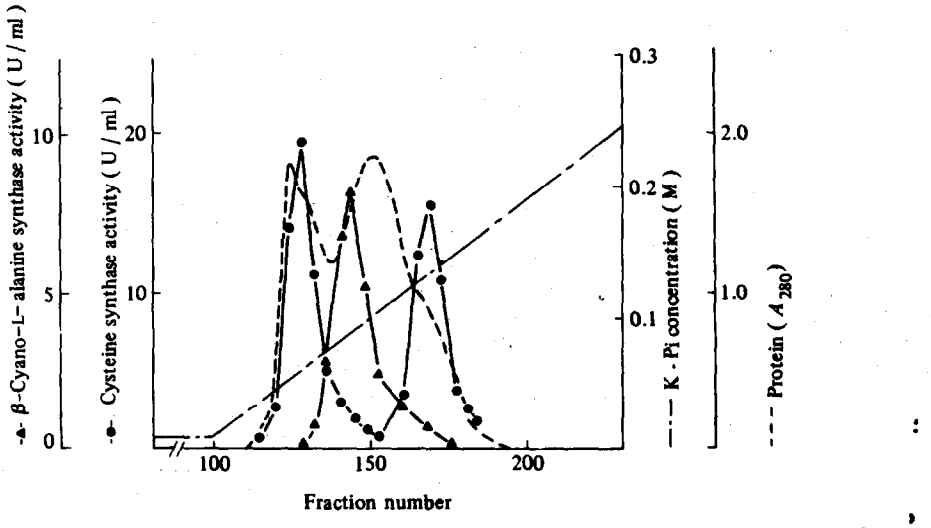

Fig. 1. Elution patterns of β-cyano-L-alanine synthase and cysteine synthase after the AH-Sepharose 4B column chromatography. β-Cyano-L-alanine synthase activity (—▲—), cysteine synthase activity (—●—) and protein (A_{280}, ---).

synthase activity was found invariably as a single peak, corresponding to an *Mr* of 54000. The purified enzyme was subjected to SDS-PAGE on 4–20% gradient gels to determine its subunit structure, following the method of ref. [15]. A single band with an M_r about one-half that of the intact enzyme indicated two identical subunits, and that it has been purified to apparent homogeneity.

The purified enzyme had absorbance peaks 280 and 410 nm, typical for a PLP-enzyme. Direct spectrophotometric measurements [16] indicated that BCA synthase in *V. angustifolia* had one molecule of PLP bound to each subunit.

The pH optimum was found to be pH 9.4–9.5 with Tris–HCl buffer. Lineweaver–Burk plots gave K_m values of 3.6 mM for L-cysteine and 0.5 mM for cyanide. The K_m value for L-cysteine was higher than that determined for BCA synthases from other plants [1, 2, 9–11], but less than that of the enzyme from *Asparagus officinalis* [17]. The K_m value for CN^- was almost the same value as those for BCA synthases from both *L. latifolius* [2] and blue lupin [9], but again less than those of enzymes from spinach [1] and *A. officinalis* [17].

The addition of PLP had an accelerating effect on the activity of this enzyme, the increase being *ca* 15% at a concentration of 10 μM, but was inhibitory at higher concentration, 1 mM causing 30% inhibition. An activation of 20% by the addition of PLP has been reported for BCA synthases from spinach [1] and *L. latifolius* [2]. The enzyme was sensitive to PLP-enzyme inhibitors: sodium borohydride at concentrations of 10 μM and 1 mM caused 10 and 95% inhibition, respectively, while hydroxylamine had a less inhibitory effect, the decrease being 10 and 30% at 10 μM and 1 mM, respectively.

Substrate specificity

Under standard assay conditions, BCA synthase in *V. angustifolia* clearly appears to be specific for L-cysteine as

Table 2. Relative synthetic rates of β-substituted alanines and *S*-substituted cysteines from L-cysteine or *O*-acetyl-L-serine by β-cyano-L-alanine synthase purified from *V. angustifolia*

Substrate	Amino acid synthesized	Relative velocity of synthesis (%)	
		L-Cysteine	*O*-Acetyl-L-serine
NaCN	β-Cyano-L-alanine	100	4.2
Pyrazole	β-(pyrazol-1-yl)-L-Alanine	0.49	0.13
3-Amino-1,2,4-triazole	β-(3-amino-1,2,4-triazol-1-yl)-L-Alanine	3.1	0.07
Uracil	L-Willardiine	0	0
	L-Isowillardiine	0	0
3,5-Dioxo-1,2,4-oxadiazolidine	L-Quisqualic acid	0	0
3,4-Dihydroxypyridine	L-Mimosine	0	0
Hydroxyurea	*O*-Ureido-L-serine	0	0
H_2S	L-Cysteine	n.d.	7.2
MeSH	*S*-Methyl-L-cysteine	12.6	6.5
$CH_2=CH-CH_2-SH$	*S*-Allyl-L-cysteine	11.8	3.1
$HOOC-CH_2-SH$	*S*-Carboxymethyl-L-cysteine	0	0

The relative rates of synthesis were compared with that of β-cyano-L-alanine from L-cysteine and sodium cyanide.

a donor for the amino-substrate, an observation in line with previous findings [1, 2]. In the presence of β-chloro-L-alanine, OAS and L-cystine the activity was respectively 142, 4.2 and 4.7% under identical conditions. No detectable activity was found with *O*-phospho-L-serine, *O*-sulpho-L-serine or with L-serine. D-Cysteine or *O*-acetyl-D-serine also did not act as substrates.

The purified enzyme also showed distinct substrate specificity when a variety of thiol compounds or *N*-heterocyclic compounds were used as acceptors for the amino-substrates. The relative activities of the purified enzyme with different substrates are shown in Table 2. BCA synthase in *V. angustifolia* could synthesize *S*-substituted L-cysteines from both L-cysteine and OAS, except *S*-carboxymethyl-L-cysteine. The enzyme could also synthesize β-(pyrazol-1-yl)-L-alanine and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine in low activities, while this enzyme could not synthesize L-mimosine, L-quisqualic acid, L-willardiine, L-isowillardiine or *O*-ureido-L-serine. Thus, the specific activities of the purified BCA synthase towards a variety of substrates are different from those of BCA synthases from spinach [1], *L. latifolius* [2] and from other sources [8–13], and also from those of cysteine synthases from other sources [3–7]. The different substrates were tested under the same conditions as described previously [1, 2].

Table 3. Amino acid composition of β-cyano-L-alanine synthase purified from *V. angustifolia*

Amino acids	Numbers of residues/54 000 g*
Asp	36
Thr	26
Ser	48
Glu	48
Pro	28
Gly	66
Ala	42
Val	40
Cys	18
Met	12
Ile	26
Leu	44
Tyr	4
Phe	16
Trp	0
Lys	28
His	8
Arg	24
Total	514

*Results are expressed as residues/mol and are based on an M_r of 54 000. Values for Thr and Ser are extrapolated to zero-time 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. Determination of tryptophan was made by alkaline hydrolysis and methanesulphonate hydrolysis [3, 28].

Amino acid composition

The amino acid composition of the purified enzyme is given in Table 3. The data presented show that BCA synthase in *V. angustifolia*, like BCA synthases in spinach [1] and *L. latifolius* [2], does not contain tryptophan, while BCA synthase purified from blue lupin does contain tryptophan [10]. The results obtained in this study also indicate that the enzyme consists of 514 residues and contains large amounts of glycine (66 residues), serine (48 residues) and glutamic acid (48 residues). The amino acid composition of BCA synthase obtained is similar to those of BCA synthases purified from spinach and *L. latifolius*, but it differs in its content of cysteine and methionine residues; its composition is very different from the BCA synthase enzymes purified from blue lupin [10] or white lupin [11]. The M_r of BCA synthase, calculated on the basis of the amino acid composition, is 54 500, which corresponds to the value found by gel filtration on Sephadex G-100 (54 000).

Occurrence and distribution of BCA synthase and cysteine synthase activities

BCA synthase activity was determined in the crude cell-free extracts of 20 species, belonging to six plant families. Among the cyanogenic plants examined, only *Eriobotrya japonica* had no detectable activity of BCA synthase, while *Prunus persica, Lathyrus sylvestris* and *V. angustifolia* had the highest activity on a per mg protein basis (Table 4). The *Vicia* and *Lathyrus* species, which contain BCA as a normal constituent, have a relatively high activity of BCA synthase. The non-cyanogenic plants in general have a lower activity of BCA synthase, averaging less than half of the mean value of the BCA synthase activity in the cyanogenic plants. When the activity is calculated on a fresh weight basis, the difference in the mean value is much higher. However, no clear separation between cyanogenic and non-cyanogenic plants can be made on the basis of the BCA synthase activity, because the non-cyanogenic plants *Lathyrus japonicus, Lathyrus pratensis* and *Spinacia oleracea* have a higher BCA synthase activity (Table 5) than some cyanogenic plants (Table 4).

Cysteine synthase activity was detected in all the higher plants examined. Relatively high activities were found in the cyanogenic plant *L. sylvestris* (Table 4) and in the non-cyanogenic plant *L. pratensis* (Table 5). No significant difference in cysteine synthase activities was found between the two groups of higher plants.

The intracellular distribution of both enzyme activities was studied in several plants. The data obtained show that more than 60% of the BCA synthase activity is consistently present in the mitochondria fraction and more than 90% of the cysteine synthase activity is present in the cytosol fraction.

DISCUSSION

In this study we have purified BCA synthase from immature seeds of *V. angustifolia* to apparent homogeneity and a comparison has been made of its properties and substrate specificities with those of previously purified enzymes [1–7].

In order to assess these findings from the viewpoint of the cyanide and sulphide metabolism and from the viewpoint of the biosynthesis of β-substituted alanines in higher plants, we have studied the occurrence of BCA synthase and cysteine synthase activities in both cyanogenic and non-cyanogenic plants tested. As cyanogenic plants we consider those plants producing BCA in detectable amounts, apparently from CN^- and L-cysteine or OAS, in addition to the cyanophoric plants producing HCN when their tissues are crushed [18].

When the physicochemical properties of the purified enzyme from *V. angustifolia* and BCA synthases from spinach [1] and *L. latifolius* [2] are compared, they are found to be almost the same, even the amino acid compositions are similar in several aspects. This enzyme may belong to a second class which may contain the enzymes from spinach and *L. latifolius* and may also contain the structurally similar BCA synthase from *Chromobacterium violaceum* [13] as previously described [2]. This second class of enzyme also has some structural similarity with cysteine synthases in higher plants, responsible for the formation of some heterocyclic β-substituted alanines [3–7].

In *V. angustifolia* BCA synthase and cysteine synthases can be completely separated by the gradient elution with K-Pi buffer from the AH-Sepharose 4B column (Fig. 1).

Among the substrates studied so far (Table 2), BCA synthase from *V. angustifolia* could catalyse the formation of *S*-substituted L-cysteines from either L-cysteine or OAS as amino-substrates and corresponding co-substrates. The purified enzyme can also catalyse the synthesis of some heterocyclic β-substituted alanines such as β-(pyrazol-1-yl)-L-alanine and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine in low activities, but could not catalyse the formation of L-mimosine, L-quisqualic acid, L-willardiine, L-isowillardiine or *O*-ureido-L-serine, while the *L. latifolius* enzyme could synthesize L-quisqualic acid and *O*-ureido-L-serine. These observations are in line with our previous findings on BCA synthases and cysteine synthases in higher plants [1–7].

The amino acid compositions of BCA synthases from *V. angustifolia, L. latifolius* and from spinach are similar: these three enzymes contain similar amounts of proline (28–32 residues), alanine (42–44 residues), valine (40–42 residues) and lysine (28–32 residues), and rather large amounts of glutamic acid (48–64 residues) and glycine (58–66 residues). Greater differences are found in the numbers of cysteine, methionine and tyrosine residues, but the similarities among these enzymes are much greater than between the BCA synthases from blue lupin [10] and white lupin [11]. When the known amino acid compositions of the purified enzymes are compared by a mathematical method [19], it is suggested that the BCA synthases from *V. angustifolia, L. latifolius* and from spinach are closely related, and also that they are related to cysteine synthases from other plant sources, except the isoenzyme B from pea seedlings [5]. All these findings concerning plant BCA synthases and cysteine synthases suggest that all these enzymes may have a common ancestor and perhaps BCA synthase may have arisen by mutation within the gene for cysteine synthase during the course of evolution.

The observations in this study together with our previous findings [1–7] make it desirable to obtain more information regarding an important detoxification function for BCA synthase and cysteine synthase, and suggest that both enzymes may play a similar role as multifunctional enzymes 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 in higher plants. A similar metabolic detoxification by alanyl-substitution of unnatural heterocyclic chemicals has been described before in plants [20, 21].

BCA synthase activities were observed to be present in both cyanogenic and non-cyanogenic plants tested, except in the leaves of *E. japonica* (Tables 4, 5). Because of the differences in the protein content among the species, the data are presented on the basis of protein and fresh weight. The BCA synthase activity found in cyanogenic plants was higher than the activities found in non-cyanogenic plants. This enzyme activity was relatively high in *P. persica*, which contains cyanogenic glucosides prunasin and amygdalin, while the concentration of BCA is negligible in this plant. Moreover, no detectable activity of BCA synthase was found in *E. japonica* belonging to the same family. High activities of this enzyme were also detected in *Lathyrus* and *Vicia* species, in which BCA or its γ-glutamyl derivative were found. In *Lathyrus* plants

Table 4. β-Cyano-L-alanine synthase and cysteine synthase activities in cyanogenic plants

Plants	Part used	Cyano compounds	β-Cyano-L-alanine synthase activity*		Cysteine synthase activity*	
			U/mg protein	U/g fresh wt	U/mg protein	U/g fresh wt
Leguminosae						
Vicia angustifolia	aerial parts	Vicianin	0.072	3.23	0.24	10.9
	immature seeds	β-Cyano-L-alanine	0.121	1.78	0.53	7.8
Lathyrus sylvestris	aerial parts	γ-Glutamyl-β-cyano-L-alanine	0.118	1.87	0.95	15.0
Lathyrus latifolius	aerial parts	γ-Glutamyl-β-cyano-L-alanine	0.032	2.04	0.17	10.6
Trifolium repens	aerial parts	Linamarin Lotaustralin	0.027	2.29	0.47	21.7
Pisum sativum	aerial parts	Unknown	0.024	1.51	0.23	15.7
Rosaceae						
Prunus persica	leaves	Prunasin	0.154	1.09	0.48	3.35
Eriobotrya japonica	leaves	Amygdalin	0	0	0.02	0.15
Linaceae						
Linum usitatissimum	greenish seedlings	Linamarin Linustralin	0.027	0.65	0.41	9.74

* The enzyme activity was determined at least twice for each plant, and the mean values are given. A unit of enzyme activity used (U) is equivalent to 1 μmol of β-cyano-L-alanine or L-cysteine produced per min, respectively.

Table 5. β-Cyano-L-alanine synthase and cysteine synthase activities in non-cyanogenic plants

Plants	Part used	β-Cyano-L-alanine synthase activity*		Cysteine synthase activity*	
		U/mg protein	U/g fresh wt	U/mg protein	U/g fresh wt
Leguminosae					
Vicia hirsuta	aerial parts	0.018	0.76	0.33	13.8
Lathyrus japonicus	greenish seedlings	0.082	0.78	0.40	3.79
Lathyrus odoratus	greenish seedlings	0.012	0.13	0.15	1.59
Lathyrus pratensis	aerial parts	0.064	0.54	1.0	8.65
Vigna radiata	greenish seedlings	0.009	0.09	0.24	2.50
Medicago sativa	greenish seedlings	0.017	0.20	0.36	4.46
Trifolium pratense	aerial parts	0.024	0.74	0.58	17.7
Leucaena leucocephala	aerial parts	0.012	0.15	0.12	1.46
	etiolated seedlings	0.019	0.20	0.78	8.22
Chenopodiaceae					
Spinacia oleracea	aerial parts	0.042	0.57	0.13	1.77
Cucurbitaceae					
Citrullus vulgaris	greenish seedlings	0.032	0.52	0.68	6.96
Rosaceae					
Fragaria ananassa	aerial parts	0.020	0.23	0.32	3.77
Umbelliferae					
Conium maculatum	greenish seedlings	0.015	0.38	0.23	6.06

*The enzyme activity was determined at least twice for each plant, and the mean values are given. A unit of enzyme activity used (U) is equivalent to 1 μmol of β-cyano-L-alanine or L-cysteine produced per min, respectively.

examined, *L. japonicus* and *L. pratensis* contain relatively high enzyme activities of BCA synthase, while these two species do not belong to cyanogenic plants among the genus *Lathyrus*. Cysteine synthase activity, on the other hand, was found to occur commonly in all the higher plants examined. Since some cysteine synthases catalysed the formation of BCA from OAS and CN^- as an additional catalytic activity [3–7], it seems likely that cysteine synthase in some plants containing little or no BCA synthase activity might have the function of cyanide detoxification like the BCA synthase.

When the intracellular distribution of both enzyme activities are studied in several plants (results not given) it was found that BCA synthase is mainly a mitochondrial enzyme, while cysteine synthase is mainly a cytosol enzyme. However, other results suggest that BCA synthase activities in plants might be present in both mitochondrial and cytosol fractions, because it has been reported that BCA synthase from *A. officinalis* [17] and *Elodea canadensis* [22] were present in the cytosol fraction. The presence of BCA synthase activity in the cytosol fraction may also be due to an additional activity of cysteine synthase enzymes in the cytosol, or alternatively from chloroplast enzymes released from broken chloroplasts during the experiments. Further work is needed to have a better understanding of the intracellular localization of the enzymes.

The higher BCA synthase activity in some cyanogenic plants can be explained by the turnover of the cyanogenic glucosides present [18]. The released HCN is then probably detoxified by BCA synthase.

EXPERIMENTAL

Materials. All plants examined were grown in the medicinal plant gardens of our University and the seedlings were grown in moistened vermiculite in the dark or in the light for 6–8 days at 26–28°. The aerial parts and seedlings were cooled for 30 min at 0–4° before enzyme extraction. After harvest, immature seeds were collected and also cooled for 1 hr at 0–4° before extraction. Sephadex G-25 and G-100, DEAE-Sephadex A-50, AH-Sepharose 4B and Ultrogel AcA 44 were purchased from Pharmacia-LKB. Hydroxylapatite HP-40 was obtained from Pentax. SDS-PAGE Plate 4/20 was obtained from Daiichi Pure Chemicals. All other chemicals used were of the highest commercial grade available.

Activity assays. This was performed as described previously [1]. The formation of L-cysteine was measured spectrophotometrically according to the method of ref. [23]. The unit of enzyme activity was equivalent to 1 μmol of BCA or L-cysteine produced per min at 30° in 50 mM Tris–HCl buffer, pH 9. Protein was determined by the method of ref. [24].

Enzyme preparations for BCA synthase and cysteine synthase activities assays. This was carried out at 0–4°. K-Pi (200 mM, pH 8) containing 0.5 mM EDTA, 10 mM 2-mercaptoethanol and 0.25 M sucrose was used as the extracting buffer, 2 ml of the buffer was used per 1 g of fresh wt of plant materials. The crude enzyme preparations were obtained after centrifugation (1000 *g*, 10 min) and passing through a Sephadex G-25 (fine) column pre-equilibrated with 30 mM K-Pi buffer, pH 8, containing 0.5 mM EDTA and 10 mM 2-mercaptoethanol (buffer A) as described before [25]. Preparation of the intracellular fractions was carried out by a modified method of ref. [26].

Purification of BCA synthase from the immature seeds of V. angustifolia. All operations were carried out at 0–4°. BCA synthase was prepared from 2.5 kg of fresh immature seeds, essentially as before [1–7]. The 35–60% saturated $(NH_4)_2SO_4$ fraction was collected and dissolved in 30 mM K-Pi buffer, pH 8, containing 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 30 mM KCl and 10 μM PLP (buffer B). The resulting solns were repeatedly applied to a column (8 × 25 cm) of Sephadex G-25 (fine) pre-equilibrated with buffer B. The protein fraction ob-

tained was applied to the first DEAE-Sephadex A-50 column (3 × 15 cm) pre-equilibrated with buffer B. The column was washed extensively with buffer B and the enzymes eluted with a linear gradient of KCl (30–500 mM) in the same buffer. BCA synthase activity was eluted at 160–220 mM KCl and was concd by $(NH_4)_2SO_4$ pptn. The resulting soln was applied to a column (4.6 × 90 cm) of Sephadex G-100 pre-equilibrated with buffer B. The active fraction was collected and dialysed against buffer A and then applied to a column (1 × 8 cm) of AH-Sepharose 4B pre-equilibrated with buffer A. The column was washed with buffer A and the enzymes eluted with a linear gradient of K-Pi (30–300 mM) in the same buffer. BCA synthase activity was eluted at 80–105 mM and two cysteine synthase activities were eluted at 50–70 mM and 125–140 mM K-Pi buffer, respectively, and BCA synthase active fraction (80–105 mM K-Pi fractions) was concd by $(NH_4)_2SO_4$ pptn. The active fraction was applied to a column (3 × 100 cm) of Ultrogel AcA 44 pre-equilibrated with buffer B. The eluates were collected and active fractions were concd by Immersible CX-10 (Millipore). The resulting soln was subjected to prep. PAGE on 7.5% gel at pH 8.3 (Tris–glycine buffer). BCA synthase fraction obtained from gel slices was applied to a column (1 × 1.5 cm) of DEAE-Sephadex A-50 pre-equilibrated with buffer A and then eluted with a linear gradient of KCl (0–300 mM) in buffer A. The active fraction (100–130 mM KCl fractions) was concd by Immersible CX-10 and then equilibrated in 10 mM K-Pi buffer, pH 8, containing 10 mM 2-mercaptoethanol and 30 mM KCl. The resulting soln was finally applied to a column (1.0 × 1.0 cm) of hydroxylapatite HP-40 pre-equilibrated with the same buffer. The highly purified enzyme fraction was concentrated by Immersible CX-10 and this enzyme preparation in 50 mM Tris–HCl buffer, pH 9, was used in all further expts.

Properties of BCA synthase were studied by the methods of ref. [27].

Identification of heterocyclic β-substituted alanines and S-substituted L-*cysteines as reaction products* was performed as described in ref. [3].

Determination of amino acid composition was also performed as described in ref. [3]. This was also achieved by the method of ref. [28].

Acknowledgement—We are grateful to Dr F. Lambein (State University of Ghent, Belgium) for his valuable discussions and suggestions in the preparation of this manuscript.

REFERENCES

1. Ikegami, F., Takayama, K., Tajima, C. and Murakoshi, I. (1988) *Phytochemistry* **27**, 2011.
2. Ikegami, F., Takayama, K. and Murakoshi, I. (1988) *Phytochemistry* **27**, 3385.
3. Murakoshi, I., Ikegami, F. and Kaneko, M. (1985) *Phytochemistry* **24**, 1907.
4. Murakoshi, I., Kaneko, M., Koide, C. and Ikegami, F. (1986) *Phytochemistry* **25**, 2759.
5. Ikegami, F., Kaneko, M., Lambein, F., Kuo, Y.-H. and Murakoshi, I. (1987) *Phytochemistry* **26**, 2699.
6. Ikegami, F., Kaneko, M., Kamiyama, H. and Murakoshi, I. (1988) *Phytochemistry* **27**, 697.
7. Ikegami, F., Kaneko, M., Kobori, M. and Murakoshi, I. (1988) *Phytochemistry* **27**, 3379.
8. Hendrickson, H. R. and Conn, E. E. (1969) *J. Biol. Chem.* **244**, 2632.
9. Akopyan, T. N., Braunstein, A. E. and Goryachenkova, E. V. (1975) *Proc. Natl Acad. Sci. U.S.A.* **72**, 1617.
10. Akopyan, T. N. and Goryachenkova, E. V. (1976) *Biokhimiya* **41**, 906.
11. Galoyan, S. M., Tolosa, E. A., Willhardt, I. G. and Goryachenkova, E. V. (1981) *Biokhimiya* **46**, 1855.
12. Leonova, T. G., Goryachenkova, E. V. and Strogonov, B. P. (1983) *Fiziologiya Rastenii* **30**, 130.
13. Macadam, A. M. and Knowles, C. J. (1984) *Biochim. Biophys. Acta* **786**, 123.
14. Andrews, P. (1965) *Biochem. J.* **96**, 595.
15. King, J. and Laemmli, U. K. (1971) *J. Mol. Biol.* **62**, 465.
16. Kumagai, H., Yamada, H., Matsui, H., Ohkishi, H. and Ogata, K. (1970) *J. Biol. Chem.* **245**, 1773.
17. Cooney, D. A., Jayaram, H. N., Swengros, S. G., Alter, S. C. and Levine, M. (1980) *Int. J. Biochem.* **11**, 69.
18. Conn, E. E. (1980) *Encyclopedia of Plant Physiology*, New Series (Bell, E. A. and Charlwood, B. V., eds), Vol. 8, p. 461. Springer, Berlin.
19. Chernoff, H. (1973) *J. Am. Stat. Assoc.* **68**, 361.
20. Massini, P. (1963) *Acta Bot. Neerl.* **12**, 64.
21. Murakoshi, I., Ikegami, F., Nishimura, T. and Tomita, K. (1985) *Phytochemistry* **24**, 1693.
22. Timoteeva, S. S., Kraeva, V. Z., Tolosa, E. A. and Velikodvorskaya, V. V. (1984) *Fiziologiya Rastenii* **31**, 462.
23. Gaitonde, M. K. (1967) *Biochem. J.* **104**, 627.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
25. Murakoshi, I., Ikegami, F., Ookawa, N., Ariki, T., Haginiwa, J., Kuo, Y.-H. and Lambein, F. (1978) *Phytochemistry* **17**, 1571.
26. Hogeboom, G. H. (1955) *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds), Vol. 1, p. 16. Academic Press, New York.
27. Murakoshi, I., Ikegami, F., Hinuma, Y. and Hanma, Y. (1984) *Phytochemistry* **23**, 973.
28. Simpson, R. J., Neuberger, M. R. and Liu, T.-Y. (1976) *J. Biol. Chem.* **251**, 1936.