Phytochemistry, Vol 27, No 11, pp 3379–3383, 1988
Printed in Great Britain

0031–9422/88 $3.00+0.00
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PURIFICATION AND CHARACTERIZATION OF CYSTEINE SYNTHASE FROM *BRASSICA JUNCEA**

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(*Received* 10 *February* 1988)

Key Word Index—*Brassica juncea*; Cruciferae, cysteine synthase; enzyme purification; biosynthesis; substrate specificity; amino acid composition; heterocyclic β-substituted alanines; L-cysteine; *O*-acetyl-L-serine.

Abstract—Purification of cysteine synthase from the leaves of *Brassica juncea* reveals that this enzyme has an apparent M_r of 52000 and can be dissociated into identical subunits of M_r 26000, each of which contains one molecule of pyridoxal 5'-phosphate. The K_m value is 2.5 mM for *O*-acetyl-L-serine and 43 μM for sulphide. The synthase catalysed the formation of *S*-substituted L-cysteines and also heterocyclic β-substituted alanines, such as β-(pyrazol-l-yl)-L-alanine and β-(3-amino-1,2,4-triazol-l-yl)-L-alanine. Other significant differences were found between this enzyme and cysteine synthases from other sources. Several properties, including the amino acid composition of the purified cysteine synthase, are also described.

INTRODUCTION

Recently we have presented evidence that cysteine synthases purified from *Spinacia oleracea* [1], *Quisqualis indica* var. *villosa* [2], *Pisum sativum* [3] and *Citrullus vulgaris* [4] can catalyse the formation of some heterocyclic β-substituted alanines such as β-(pyrazol-l-yl)-L-alanine and L-quisqualic acid in the presence of *O*-acetyl-L-serine (OAS) and suitable precursors. We also demonstrated that some heterocyclic β-substituted alanine synthases could be regarded as isoenzymes of cysteine synthase [2, 4], while β-(pyrazol-l-yl)-L-alanine synthase from *C. vulgaris* [5] and L-mimosine synthase from *Leucaena leucocephala* [6] were found to have physicochemical properties similar to the above enzymes. These results suggest that naturally occurring heterocyclic β-substituted alanines are synthesized by a reaction mechanism comparable to the biosynthesis of cysteine, and that the enzymes catalysing these reactions form a group of physicochemically similar proteins that may be phylogenetically related.

During our continuing study of this group of enzymes, and of the biosynthesis of heterocyclic β-substituted alanines, we have now attempted the purification of cysteine synthase from *B. juncea* leaves, which do not contain heterocyclic β-substituted alanines, in order to make a detailed comparison with the enzymes previously described [1–6].

RESULTS

Purification

The initial extraction and purification of cysteine synthase from 7 kg fresh weight of *B. juncea* leaves was according to the previously described method [4]. The enzyme was prepared simultaneously with the β-(pyrazol-l-yl)-L-alanine 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, affinity chromatography on L-methionine-Sepharose 4B and preparative PAGE (Table 1).

The purified cysteine synthase activity was eluted at 70–80 mM K-Pi buffer on L-methionine-Sepharose 4B affinity chromatography (step 8), and the enzyme activity for β-(pyrazol-l-yl)-L-alanine synthase was completely overlapped with the peak of cysteine synthase.

The procedure outlined in Table 1 afforded *ca* 1300-fold purification of cysteine synthase with a specific activity of 410 U/mg protein and a yield of 5.8%, compared to the total cysteine synthase activity of the crude extract.

Properties of cysteine synthase

Some properties of the cysteine synthase are shown in Table 2. 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. [7]. Cysteine synthase activity was found invariably as a single peak, corresponding to an M_r of 52000. The purified enzyme was subjected to SDS-PAGE on 12% gels to determine its subunit structure, following the method of ref. [8]. A single band with an M_r of 26000 indicated that the enzyme was composed of two identical subunits and that it had been purified to apparent homogeneity.

The identification of bound pyridoxal 5'-phosphate (PLP) in the purified enzyme was demonstrated by direct spectrophotometric measurements [9]. The synthase has one molecule of PLP bound to each subunit, the same as the cysteine synthases from other sources [1, 4, 10].

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

*Parts of this work were reported at the 107th Annual Meeting of the Pharmaceutical Society of Japan in Kyoto, 4 April 1987 (Abstracts, p. 359) and were also reported at the Symposium on Amines in Plants of the Phytochemical Society of Europe in London, 21 December 1987 (Poster Session).

Table 1 Summary of the purification of cysteine synthase from *B. juncea*

Purification step	Total activity (units*)	Specific activity (units/mg protein)	Yield (%)	Purification (fold)
1 Crude extract†	31400	0 31	100	1
2 60°-heated supernatant‡	31390	0 54	99 9	1 74
3 Ammonium sulphate precipitate§	31230	0 98	99 4	3 16
4 1st DEAE-Sephadex A-50 (115–150 mM)	19050	27 2	60 7	87 7
5 Sephadex G-100 (peak fractions)	14290	98 5	45 5	318
6 2nd DEAE-Sephadex A-50 (50–60 mM)	9140	155	29 1	500
7 Ultrogel AcA 44 (peak fractions)	8110	213	25 8	687
8. Methionine-Sepharose 4B (70–80 mM)	6853	292	21 8	942
9 Polyacrylamide-gel electrophoresis	2296	401	7.31	1294
10 3rd DEAE-Sephadex A-50 (60–70 mM)	1830	410	5 83	1323

*A unit of enzyme activity represents 1 μmol of product formed per min at 30°, in 50 mM K-Pi buffer, pH 8 0
†Starting from 7 kg of the fresh leaves of *B juncea*
‡60°, 1 min.
§30–70% saturation and desalted on Sephadex G-25

Table 2 Summary of the physicochemical properties and kinetics of *B juncea* cysteine synthase

Property	
Absorption maxima (pH 8 0)	280, 410 nm
A_{410}/A_{280} (pH 8 0)	0 33
A_{260}/A_{280} (pH 8 0)	0 66
M_r (Sephadex G-100 filtration)	52 000
M_r of subunit (SDS-PAGE)	26 000
Number of subunits	2
Pyridoxal 5'-phosphate bound to the enzyme	2 mol/mol enzyme
K_m for *O*-acetyl-L-serine*	2 5 mM
K_m for H_2S*	0 043 mM

*K_m values were determined from Lineweaver–Burk plots

Cysteine synthase from *B juncea* has a K_m value of 2.5 mM for OAS, but it shows no indication of inhibition by OAS at concentrations up to 25 mM The K_m value for OAS is almost the same as the one determined for both isoenzyme A of cysteine synthase [4] and β-(pyrazol-1-yl)-L-alanine synthase [5] from *C vulgaris*, and is also very close to the values determined for cysteine synthase from spinach [1] and *Raphanus sativus* [11], and for isoenzyme B of cysteine synthase from pea [3] and *Phaseolus vulgaris* [12], 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* [10] and for L-mimosine synthase from *L leucocephala* [6]

The response of the purified enzyme to sulphide concentrations below 0.2 mM was examined and a K_m value of 43 μM was found. This value is higher than the one determined for cysteine synthase from spinach [1], *P sativum* [3] and *C vulgaris* [4], and for isoenzyme B of cysteine synthase from *P. vulgaris* [12], but it is less than that determined for isoenzyme A of cysteine synthase from *Q indica* var *villosa* [2].

The addition of PLP had no effect on the primary activity of this enzyme like the cysteine synthase isoenzymes from *C vulgaris* [4]. The PLP-enzyme inhibitors, hydroxylamine and potassium cyanide both at a concentration of 1 mM caused 5–15% inhibition

Substrate specificity

Under standard assay conditions, cysteine synthase from *B juncea* clearly appears to be specific for OAS as a donor of the alanyl moiety. No detectable activity was found when OAS is replaced by *O*-phospho-L-serine or L-serine, an observation in line with previous findings [1–6], while the activity was 5 8% in the presence of β-chloro-L-alanine under identical conditions The purified enzyme showed no activity in the presence of *O*-acetyl-D-serine.

The enzyme also showed a distinct substrate specificity

Table 3 Relative synthetic rates of *S*-substituted L-cysteines and *β*-substituted alanines by cysteine synthase purified from *B. juncea*

Thiol compound and *N*-heterocyclic compound	Amino acid synthesized	Relative rate of synthesis (%)
H_2S	L-Cysteine	100
MeSH	*S*-Methyl-L-cysteine	0.52
CH_2=CH–CH_2–SH	*S*-Allyl-L-cysteine	18.2
HOOC–CH_2–SH	*S*-Carboxymethyl-L-cysteine	6.1
Pyrazole (*O*-acetyl-L-serine)	*β*-(pyrazol-1-yl)-L-Alanine	1 31
(L-cysteine)		1.0
3-Amino-1,2,4-triazole	*β*-(3-amino-1,2,4-triazol-1-yl)-L-Alanine	1 12
3,5-Dioxo-1,2,4-oxadiazolidine	L-Quisqualic acid	0
3,4-Dihydroxypyridine	L-Mimosine	0
Uracil	L-Willardiine	0
	L-Isowillardiine	0
Zeatin	L-Lupinic acid	0
6-Benzylaminopurine	*β*-(6-benzylaminopurin-9-yl)-L-Alanine	0
Hydroxyurea	*O*-Ureido-L-serine	0.04
NaCN (*O*-acetyl-L-serine)	*β*-Cyano-L-alanine	12 3
(L-cysteine)		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 [1, 2]

when a variety of thiol compounds or *N*-heterocyclic compounds were used as an acceptor for the alanyl moiety. The relative activities of the purified enzyme with different substrates are shown in Table 3 Cysteine synthase from *B. juncea* could synthesize *S*-substituted L-cysteines and also some heterocyclic *β*-substituted alanines such as *β*-(pyrazol-l-yl)-L-alanine and *β*-(3-amino-1,2,4-triazol-l-yl)-L-alanine, but could not catalyse the production of L-quisqualic acid, L-willardiine, L-isowillardiine, L-mimosine, L-lupinic acid or *β*-(6-benzylaminopurin-9-yl)-L-alanine, when suitable substrates were provided. The purified enzyme could synthesize *β*-cyano-L-alanine from OAS (not L-cysteine) and CN^- at 12.3% of the cysteine synthase activity, and also synthesize *O*-ureido-L-serine with low yield. When OAS is replaced by L-cysteine, the enzyme could synthesize *β*-(pyrazol-l-yl)-L-alanine at 1% of the primary activity. Thus, the specific activities of cysteine synthase in *B. juncea* towards a variety of substrates are also different from those of cysteine synthases previously purified from other sources [1–4]. The different substrates were tested under the same conditions as described previously [1].

Amino acid composition

The amino acid composition of the purified enzyme is given in Table 4. The data presented show that the enzyme consists of 496 residues and contains a large amount of glutamic acid (62 residues), glycine (58 residues) and alanine (54 residues). The enzyme contains the same number of cysteine and methionine residues as cysteine synthase from spinach [1]. The plant cysteine synthases contain 18 to 22 *S*-containing amino acids except isoenzyme B from pea [3, 4], while the number of cysteine and methionine residues is invariably 1 and 5 respectively for the enzymes from microorganisms [13, 14].

The M_r calculated from the amino acid composition, is

Table 4 Amino acid composition of cysteine synthase purified from *B juncea*

Amino acids	Residues/mol*
Asp	26
Thr	28
Ser	30
Glu	62
Pro	26
Gly	58
Ala	54
Val	32
Cys	6
Met	14
Ile	42
Leu	42
Tyr	10
Phe	14
Trp	0
Lys	32
His	4
Arg	16
Total	496

*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

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 as described before [1, 3]. Means of duplicate analyses are given Determination of tryptophan was made by alkaline hydrolysis.

52 000, which corresponds to the value estimated by gel filtration on Sephadex G-100 (52 000)

DISCUSSION

In this work, we have purified cysteine synthase from *B juncea* leaves to apparent homogeneity and a comparison has been made of its properties and substrate specificities with those of the previously purified enzymes [1–6] from the viewpoint of the biosynthesis of heterocyclic β-substituted alanines in higher plants

The M_r of the purified enzyme from *B. juncea* was 52 000 consisting of two identical subunits of M_r 26 000, while cysteine synthase from *B. chinensis* var *Komatsuna* has an M_r of 62 000 and consists of subunits of M_r 31 000 [10] The physicochemical properties of the enzyme we have purified from *B juncea* leaves are almost the same as those of cysteine synthases from other plant sources· the M_rs for plant cysteine synthases have been reported to be in the range 52 000–70 000, they can be dissociated into two identical subunits of M_rs approximately half that of the intact enzyme, they contain 2 mol of PLP and they have almost the same pH optimum of 8 0 [1–5, 10–12] The K_m value for OAS of this enzyme (2 5 mM) is also within the range of 1.5–7 1 mM reported for plant cysteine synthases

Among the substrates studied thus far (Table 3), cysteine synthase from *B juncea* could catalyse the formation of *S*-substituted L-cysteines from OAS and the corresponding thiol compounds This enzyme shows a higher affinity for thioglycollic acid than other cysteine synthases and this may be related to the presence of *S*-substituted L-cysteines such as *S*-carboxymethyl-L-cysteine in some cruciferous plants [15]. The purified enzyme could catalyse the formation of heterocyclic β-substituted alanines such as β-(pyrazol-l-yl)-L-alanine and β-(3-amino-1,2,4-triazol-l-yl)-L-alanine in low yields, but could not catalyse the formation of L-quisqualic acid, L-willardine, L-isowillardine or L-mimosine These observations are in line with our previous findings [1–4] The enzyme from *B juncea* was unable to catalyse the formation of L-lupinic acid and β-(6-benzylaminopurin-9-yl)-L-alanine, which are the metabolites of the cytokinins zeatin and 6-benzylaminopurine in higher plants [16] Moreover, the purified enzyme catalysed the formation of β-cyano-L-alanine from OAS and CN^- as an additional catalytic activity, this activity is different from the biosynthesis of β-cyano-L-alanine from L-cysteine and CN^- as catalysed by the 'true' β-cyano-L-alanine synthases from microorganisms and some higher plants [4, 17, 18]

All our findings concerning plant cysteine synthases suggest that this group of enzymes 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 an ecological role as allelochemicals This is in agreement with earlier statements by Fowden and Bell, proposing that some non-protein amino acids may have arisen by a modification of common pathways for the biosynthesis of the protein amino acids [19, 20] This modification can thus be effected by a change in substrate specificity of the enzymes involved

When the known amino acid compositions of cysteine synthases are compared by a mathematical method [21], it is suggested that cysteine synthase from *B juncea* seems to be close to cysteine synthases from other plant sources, except the isoenzyme B from pea seedlings [3] This makes it likely that a phylogenetic relationship exists among those enzymes

EXPERIMENTAL

Materials Leaves of *Brassica juncea* Czern et Coss were used in this work Seeds were supplied by the Sakata Seed Corporation (Japan) and they were grown in our medicinal plant gardens After harvest, leaves were collected and 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 Ultrogel AcA 44 was obtained from LKB L-Methionine-Sepharose 4B was prepared in our laboratory according to a modified method of ref [22] 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 [23] The formation of β-(pyrazol-l-yl)-l-alanine 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 is equivalent to 1 μmol of L-cysteine produced per min Protein was determined by the method of ref [24]

Purification of cysteine synthase from B juncea leaves All operations were carried out at 0–4 Cysteine synthase was prepared from 7 kg of fresh leaves essentially as before [1–5] 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 obtained was applied to the first DEAE-Sephadex A-50 column (4 × 16 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 activity was eluted at 115–150 mM K-Pi buffer and was concentrated by $(NH_4)_2SO_4$ precipitation The resulting soln was applied to a column (4 6 × 90 cm) of Sephadex G-100 pre-equilibrated with buffer A The active fraction was collected and concentrated by Immersible CX-10 (Millipore) and again applied to the second DEAE-Sephadex A-50 column (1 5 × 3 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 (30–200 mM) in the same buffer Cysteine synthase activity was eluted at 50–60 mM K-Pi buffer, and was concentrated by Immersible CX-10 The active fraction was applied to a column (3 × 100 cm) of Ultrogel AcA 44 pre-equilibrated with buffer A, and was concentrated by Immersible CX-10 The resulting soln was applied to a column (1 2 × 3 cm) of L-methionine-Sepharose 4B pre-equilibrated in 10 mM K-Pi buffer, pH 8, containing 4 mM 2-mercaptoethanol and 0 2 mM EDTA and the enzymes eluted with a linear gradient of K-Pi (10–150 mM) in the same buffer Cysteine synthase activity was eluted at 70–80 mM K-Pi and was concentrated by Immersible CX-10 The resulting soln was then subjected to preparative PAGE on 7 5% gels at pH 8 3 (Tris-glycine buffer) Cysteine synthase activity obtained from gel slices was finally applied to a column (1 2 × 3 cm) of DEAE-Sephadex A 50 pre equilibrated in buffer A and then eluted with a linear gradient of K-Pi (30–200 mM) in buffer A The highly purified enzyme fraction (60–70 mM K-Pi fractions) was a yellow soln and was stable for at least 3 months at 0 This enzyme preparation in 50 mM K-Pi buffer, pH 8, was used in all further expts

Properties of cysteine synthase were studied by the methods of refs [1, 5]

Identification of heterocyclic β-substituted alanines and S-substituted L-*cysteines as reaction products* was performed as described previously [1, 2]. Identification of L-lupinic acid and β-(6-benzylaminopurin-9-yl)-L-alanine was established by HPLC as described before [16]

Determination of amino acid composition was also performed as described previously [1], using 0·05–0·1 mg of the purified enzyme

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

REFERENCES

1. Murakoshi, I, Ikegami, F and Kaneko, M (1985) *Phytochemistry* **24**, 1907
2 Murakoshi, I, Kaneko, M, Koide, C and Ikegami, F. (1986) *Phytochemistry* **25**, 2759
3 Ikegami, F, Kaneko, M, Lambein, F, Kuo, Y.-H. and Murakoshi, I (1987) *Phytochemistry* **26**, 2699
4 Ikegami, F, Kaneko, M, Kamiyama, H and Murakoshi, I. (1988) *Phytochemistry* **27**, 697
5. Murakoshi, I., Ikegami, F., Hinuma, Y. and Hanma, Y (1984) *Phytochemistry* **23**, 973
6. Murakoshi, I., Ikegami, F, Hinuma, Y. and Hanma, Y. (1984) *Phytochemistry* **23**, 1905.
7. Andrews, P. (1965) *Biochem J.* **96**, 595.
8 King, J and Laemmli, U. K. (1971) *J. Mol Biol.* **62**, 465
9. Kumagai, H., Yamada, H., Matsui, H., Ohkishi, H. and Ogata, K (1970) *J Biol. Chem* **245**, 1773
10. Masada, M., Fukushima, K. and Tamura, G (1975) *J. Biochem* **77**, 1107.
11. Tamura, G., Iwasawa, T., Masada, M. and Fukushima, K. (1976) *Agric. Biol. Chem.* **40**, 637.
12. Bertagnolli, B. L and Wedding, R. T. (1977) *Plant Physiol.* **60**, 115
13. Kredish, N. M., Becker, M. A. and Tomkins, G. M. (1969) *J. Biol. Chem.* **244**, 2428
14. Yamagata, S. (1976) *J. Biochem.* **80**, 787
15. Buziassy, C. and Mazelis, M. (1964) *Biochim. Biophys. Acta* **86**, 185
16 Murakoshi, I., Koide, C., Ikegami, F. and Nasu, K. (1983) *Chem. Pharm. Bull* **31**, 1777.
17 Ikegami, F., Takayama, K., Tajima, C. and Murakoshi, I. (1988) *Phytochemistry* **27**, 2011
18. Manning, K. (1986) *Planta* **168**, 61.
19. Bell, E. A (1976) *FEBS Letters* **64**, 29.
20. Fowden, L. and Lea, P J. (1979) *Adv. Enzymol.* **50**, 117.
21. Chernoff, H. (1973) *J. Am Stat. Assoc.* **68**, 361.
22. Matsumoto, I. and Seno, N. (1982) *J. Chromatogr.* **239**, 747.
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.