Phytochemistry, Vol. 23, No. 5, pp. 973–977, 1984.
Printed in Great Britain.

0031-9422/84 $3.00 + 0.00

PURIFICATION AND CHARACTERIZATION OF β-(PYRAZOL-1-YL)-L-ALANINE SYNTHASE FROM *CITRULLUS VULGARIS**

ISAMU MURAKOSHI, FUMIO IKEGAMI, YASUKO HINUMA and YUKARI HANMA

Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho 1–33, Chiba, 260, Japan

(*Received* 23 *August* 1983)

Key Word Index—*Citrullus vulgaris*; Cucurbitaceae; watermelon; β-(pyrazol-1-yl)-L-alanine synthase; enzyme purification; *O*-acetyl-L-serine; β-(pyrazol-1-yl)-L-alanine.

Abstract—From seedlings of *Citrullus vulgaris* the enzyme β-(pyrazol-1-yl)-L-alanine synthase was purified 200-fold, when it showed electrophoretic homogeneity (MW 58 000) and could be dissociated into identical subunits (MW 32 000) each containing one molecule of pyridoxal 5'-phosphate. The K_m value was 2.5×10^{-3} M for *O*-acetyl-L-serine and 7.4×10^{-2} M for pyrazole. The enzyme did not catalyse the formation of related β-substituted alanines, such as L-mimosine and L-quisqualic acid, and significant differences were found between the β-(pyrazol-1-yl)-L-alanine synthase and β-substituted alanine synthases and cysteine synthase from other sources.

INTRODUCTION

β-(Pyrazol-1-yl)-L-alanine (BPA) was first isolated from the pressed juice by Shinano *et al.* [1] and the seeds of watermelon (*Citrullus vulgaris*) [2] and has subsequently been found in several other cucurbit plants [2]. Cucurbit seeds normally contain significant amounts of γ-L-glutamyl-BPA together with free BPA [3].

In *C. vulgaris* seedlings BPA has been shown to derive from pyrazole and *O*-acetyl-L-serine (OAS) by Murakoshi *et al.* [4]. In the course of our work on the enzyme systems from higher plants catalysing the formation of β-substituted alanines, we have demonstrated the enzymatic synthesis of a number of β-substituted alanines in cell-free extracts from several higher plants [4–11].

In order to enable more detailed studies of the biosynthesis of this group of non-protein amino acids we have attempted to purify these enzymes to homogeneity. In this paper the purification and the characterization of BPA-synthase from one-week-old seedlings of *C. vulgaris* is described.

RESULTS

Distribution of β-(pyrazol-1-yl)-L-alanine synthase in C. vulgaris *seedlings*

BPA-synthase activity was compared in extracts prepared from the dry seeds and from 2-, 5-, 7- and 10-day-old seedlings. The activity per mg-total protein present in the extracts increased progressively with increasing age of the seedlings over a 10-day growth period. The specific activity of BPA-synthase in extracts of 7-day-old seedlings (cotyledons removed) was about 15 times that of cotyledons extracts. Virtually no activity was found in extracts of the dry seeds.

Purification of β-(pyrazol-1-yl)-L-alanine synthase from C. vulgaris *seedlings*

The enzyme was prepared from 1.6 kg fr.wt of 6 to 7-day-old seedlings (cotyledons removed) using heat treatment, ammonium sulphate fractionation, gel filtration on Sephadex G-100 and ion-exchange chromatography on DEAE-cellulose or DEAE-Sephacel as summarized in Table 1. The enzyme activity was eluted at 0.04–0.06 M KCl on DEAE-cellulose (DE-52) chromatography (Fig. 1) and the BPA-synthase band obtained after DEAE-Sephacel chromatography had a constant specific activity across the band (Fig. 2). The above procedure gave a 200-fold purification of the enzyme with a specific activity of 355 U/mg protein and a yield of 3.9%.

Properties of the purified enzyme

The purified enzyme was sensitive to freezing even in presence of 20% glycerol. Freeze-thawing or lyophilization inactivated the enzyme rapidly. No enzyme activity was lost during storage for one month at 0–2°. The MW of the enzyme was determined by reference to standard curves obtained by gel filtration on a Sephadex G-100 column according to the method of Andrews [12] and was estimated to be $58\,000 \pm 2000$.

Polyacrylamide gel electrophoresis using 0.1% sodium dodecylsulphate [13] showed a single band with a MW of $32\,000 \pm 2000$. Electrophoresis on gels containing 8 M urea again exhibited a single band indicating that the enzyme consists of identical subunits. Thus the native enzyme appears to consist of two subunits, with the same MW.

The identification of bound pyridoxal 5'-phosphate in the purified enzyme was demonstrated by direct spectrophotometric measurements [14]. BPA-synthase has one

*Parts of this work were reported at the 100th and the 101st Annual Meeting of the Pharmaceutical Society of Japan at Tokyo, April 5, 1980 (Abstracts p. 236) and at Kumamoto, April 2, 1981 (Abstracts p. 523) and were included in the dissertation presented by F.I. at Tokyo University 1981 (in Japanese).

Table 1. Summary of purification of β-(pyrazol-1-yl)-L-alanine synthase from *Citrullus vulgaris*

Purification step	Volume (ml)	Total activity (units)*	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)
1. Crude extract†	2260	10000	5830	1.72	100
2. 55°-heated supernatant‡	2240	7070	3290	2.15	70.5
3. Ammonium sulfate precipitate§	45	4200	340	12.4	42
4. 1st Sephadex G-100 (peak fractions)	70	2440	56	43.6	24.3
5. 1st DEAE-cellulose (0.04–0.06 M)	35	1490	10.5	142	14.9
6. 2nd Sephadex G-100 (peak fractions)	14	470	1.8	261	4.7
7. DEAE-Sephacel (0.08–0.09 M)	6	390	1.1	355	3.9

*A unit of enzyme activity represents 1 nmol of product formed/min at 28°, in 0.03 M K-Pi buffer, pH 7.4.
†Starting from 1.6 kg of the fresh seedlings of *Citrullus vulgaris* (3.6 mg protein/g seedlings).
‡55°, 2 min.
§40–60% saturation.

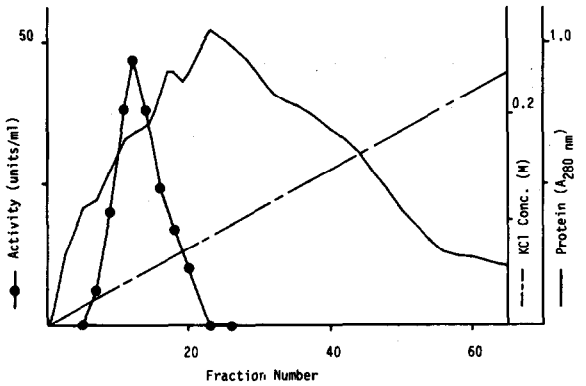


Fig. 1. DEAE-cellulose column chromatography of BPA-synthase from *Citrullus vulgaris*. A column (1.3 × 12 cm) was equilibrated with Buffer A before chromatography. The column was washed with the same buffer and then eluted with a linear 0–0.35 M KCl gradient (– – –) in the same buffer, 5 ml fractions were assayed for BPA-synthase (—●—) and for protein (——) as shown in Experimental.

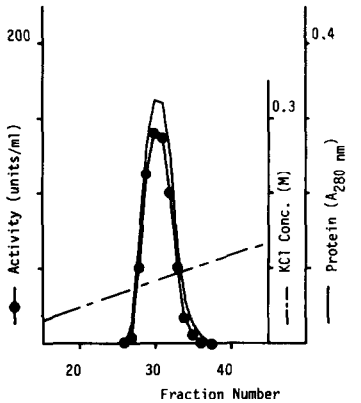


Fig. 2. DEAE-Sephacel column chromatography of BPA-synthase obtained by Sephadex G-100 column. The dialysed step 6 fraction was applied on a column (1.3 × 6 cm) that had been equilibrated with Buffer B. The column was washed with the same buffer and then eluted with a linear KCl-gradient (0–0.35 M) in the same buffer (– – –), 2 ml fractions were assayed for BPA-synthase (—●—) and for protein (——) as shown in Experimental.

molecule of pyridoxal 5'-phosphate bound to each subunit (Table 2). The enzyme exhibited a single pH optimum at pH 7.3–7.4. It lost half of its activity below pH 6.5 and above pH 8.5.

The enzyme exhibited typical Michaelis–Menten kinetics and a Lineweaver–Burk plot gave K_m values of 2.5 × 10^{-3} M for OAS and 7.4 × 10^{-2} M for pyrazole. The K_m value for OAS was less than that determined for L-mimosine synthase from *Leucaena leucocephala* [15]. The addition of pyridoxal 5'-phosphate enhanced the alanylating rate, the increase being maximal (40%) at 0.25 mM. Similar requirements have been reported for tryptophanases and β-tyrosinases from microorganisms [14, 16, 17].

The pyridoxal 5'-phosphate inhibitors, hydroxylamine and potassium cyanide at a concentration of 50 mM and 10 mM respectively almost completely inhibited the enzyme activity in a fashion analogous to multifunctional pyridoxal enzymes.

Substrate specificity

Under standard assay conditions the enzyme showed the highest activity in the presence of OAS as a donor for the alanyl moiety. Under identical conditions the activity was 16.4% in the presence of *O*-succinyl-L-serine and 2.5% in the presence of *O*-sulpho-L-serine as compared to OAS. No detectable activity was found with *O*-phospho-L-serine or with L-serine.

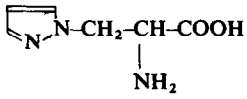
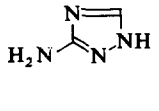
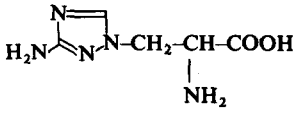
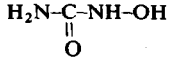
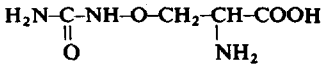
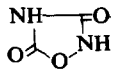
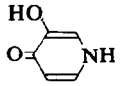
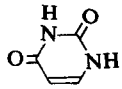
The enzyme also shows distinct substrate specificity when a variety of *N*-heterocyclic compounds were used as an acceptor for the alanyl group. Table 3 shows the relative activities of the purified enzyme with different substrates. The activity was only 10.6% in the presence of 3-amino-1,2,4-triazole and 8.7% in the presence of *N*-hydroxyurea as compared to pyrazole. BPA-synthase could not synthesize L-mimosine, L-quisqualic acid, L-willardiine or L-isowillardiine when suitable substrates were provided. BPA-synthase also could not synthesize *S*-methyl-L-cysteine [23]. The different heterocyclic sub-

Table 2. Summary of the physicochemical properties and kinetics of *Citrullus vulgaris* β-(pyrazol-1-yl)-L-alanine synthase

Property	*Citrullus vulgaris*
$E^{1\%}_{1cm}$ at 280 nm	13.6
Absorption maxima (pH 7.4)	280, 410 nm
A_{260nm}/A_{280nm} (pH 7.4)	0.59
A_{410nm}/A_{280nm} (pH 7.4)	0.074
MW (Sephadex G-100 filtration)	58 000 ± 2000
MW of subunit (SDS-disc gel electrophoresis)	32 000 ± 2000
Number of subunits	2
Pyridoxal 5'-phosphate bound to the enzyme	2 moles/mole enzyme
K_m for *O*-acetyl-L-serine*	2.5×10^{-3} M
K_m for pyrazole*	7.4×10^{-2} M

*K_m values were determined from Lineweaver–Burk plots.

Table 3. Relative synthetic rates of β-substituted alanines from *N*-heterocyclic compounds and *O*-acetyl-L-serine by β-(pyrazol-1-yl)-L-alanine synthase

N-Heterocyclic compound	Amino acid synthesized	Relative velocity of synthesis (%)	Optimum pH
		100	7.4 [4]
		10.6	7.8 [7]
		8.7	7.5 [11]
	L-quisqualic acid	0	7.4 [6]
	L-mimosine	0	7.8 [4]
	L-willardiine L-isowillardiine	0	7.8 [10]
Me-SH	*S*-methylcysteine	0	7.8 [23]

The reaction mixtures containing 2.5 mM of OAS, 12.5–75 mM of *N*-heterocyclic compounds and 7 μg of enzyme in 0.4 ml of 0.03 M K-Pi buffer were incubated at each optimal pH for 30 min at 28°. The reaction mixtures were applied on paper chromatography and on an automatic amino acid analyser and then were assayed as described before [4–11]. The relative rates of synthesis were compared with that of BPA. Methylmercaptan was examined in comparison with cysteine synthase [23].

strates were tested at the pH found optimal for alanylation of the respective substrates to avoid critical differences in the tautomeric conformation of the heterocycle [4–11]. L-Mimosine synthase is similar to BPA-synthase in its reaction pattern [15].

DISCUSSION

In the present investigation a seven-step purification procedure has been developed to achieve purification to electrophoretic homogeneity of BPA-synthase from *C.*

vulgaris seedlings. While this enzymatic activity had been described before in crude extracts of the same plant species [4, 18], this study presents the first detailed account of the extensive purification of an enzyme catalysing the formation of a heterocyclic β-substituted alanine from higher plants.

The progressive increase of enzyme activity during the seedling growth is commensurate with enhanced concentrations of BPA [19]. This observation together with the fact that the enzyme is barely detectable in dry seeds suggests that it is synthesized *de novo* during development. However, the possibility remains that the enzyme protein may still be present in the seeds either as inactive precursor or as subunits.

Among the substrates studied so far (Table 3), the enzyme has a pronounced preference for OAS and pyrazole, the activity with other substrates being at least ten times smaller and often below the limit of detection. The K_m value of 2.5×10^{-3} M found for OAS is distinctly different from the K_m value of 6.25×10^{-3} M reported for L-mimosine synthase from *L. leucocephala* under similar conditions [15]. Furthermore, BPA-synthase does not show substrate inhibition like L-willardiine and L-isowillardiine synthase from *Pisum sativum* [10].

These findings emphasize that BPA-synthase from *C. vulgaris* is one of a group of enzymes from higher plants catalyzing analogous substitution reactions but with markedly different substrate specificities.

EXPERIMENTAL

Materials. C. vulgaris seeds were purchased from local nurseries, and were sown in moistened vermiculite and grown without added nutrients in the dark for 6–7 days at 26–28° unless otherwise stated. Seedlings were harvested, washed and the cotyledons were removed; they were then cooled for 30 min at 0–4° before enzyme extraction. Sephadex G-100 and DEAE-Sephacel were purchased from Pharmacia. DEAE-Cellulose (DE-52) and polyvinylpolypyrrolidone (PVPP) were obtained from Whatman and from Sigma, respectively. All other chemicals used were of the highest commercial grade available.

Activity assay. The routine assay was based on that described before [4]. The enzyme was dissolved in 0.03 M K-Pi buffer, pH 7.4 (Buffer A). Substrate concns were 75 mM for pyrazole and 2.5 mM for OAS. Incubation was at 28° for 30 min; the total reaction vol. was 0.4 ml, utilizing up to 0.2 ml of enzyme (corresponding to 7–100 μg of protein). Reactions were terminated by the addition of 0.02 ml of 10% NH_4OH and the formation of BPA measured by the general method described in ref. [20]. The unit of enzyme activity used in the present paper is equivalent to 1 nmol of BPA produced per 1 min. Protein was determined by the method of ref. [21].

Distribution of BPA synthase in C. vulgaris *seedlings.* The seedlings were harvested at specified stages and the cotyledons were removed. These or dry seeds were washed and cell-free extracts prepared by grinding with 0.1 M K-Pi buffer, pH 7.5 (1 ml/g fresh tissue) containing 10 mM 2-mercaptoethanol in a chilled mortar and pestle. The homogenates were centrifuged at 25000 *g* for 30 min at 0–2°. The supernatant was applied on a column of Sephadex G-25 (fine) to separate the protein from low MW substances and the activities in the solns were assayed.

Purification of BPA synthase from C. vulgaris *seedlings.* All operations were carried out at 0–4°. *Step* 1. 1.6 kg of fresh 6 to 7-day-old seedlings (cotyledons removed) were homogenized in 2 l. of 0.1 M K-Pi buffer, pH 7.5, containing 0.25 M sucrose and 10 mM 2-mercaptoethanol with insoluble polyvinylpolypyrrolidone (100 g). Homogenization was performed in an electric mixer at top speed for 2 min. The homogenate was pressed through nylon cloth and the filtrate centrifuged at 15000 *g* for 30 min. This supernatant was designated as crude extract. *Step* 2. The crude extract was placed in a water bath at 80° and stirred vigorously until the temp. of the preparation reached 55°, it was then kept in the water bath for an additional 1 min and gently swirled by hand. At the end of this time it was immediately chilled to 4° with ice water, and then centrifuged at 10000 *g* for 20 min. *Step* 3. The step 2 supernatant was subjected to $(NH_4)_2SO_4$ fractionation and the protein precipitating between 40 and 60% saturation was collected and dissolved in Buffer A. In this buffer, enzyme activity was stable for at least 7 days at 4°. *Step* 4. *Sephadex G-100 gel filtration.* The resulting solns were then applied on the Sephadex G-100 column (2.6 × 80 cm) pre-equilibrated with Buffer A and eluted with the same buffer. Fractions (5 ml) were collected and assayed for activity and protein, measured by *A* at 280 nm [22]. The elution profile of BPA-synthase showed a single activity peak and fractions containing high activity were concd by $(NH_4)_2SO_4$ precipitation and dialysed overnight against Buffer A. *Step* 5. *Column chromatography on DEAE-cellulose.* The dialysed step 4 fraction was applied on a column (1.3 × 12 cm) of DE-52 pre-equilibrated with Buffer A. The column was washed with 75 ml of Buffer A and then proteins were eluted with a linear gradient containing 0–0.35 M KCl in Buffer A. Fractions with significant synthase activity (0.04–0.06 M KCl fractions) were pooled and were concd by $(NH_4)_2SO_4$ precipitation. *Step* 6. *2nd Sephadex G-100 gel filtration.* The step 5 fraction was applied on a column (2.6 × 42 cm) of Sephadex G-100 pre-equilibrated with Buffer A. The eluate was collected in 2 ml fractions and the pooled active fractions were dialysed overnight against 0.01 M K-Pi buffer, pH 7.4 (Buffer B). *Step* 7. *Column chromatography on DEAE-Sephacel.* The dialysed fraction was then applied on a column (1.3 × 6 cm) of DEAE-Sephacel pre-equilibrated with Buffer B. The column was then washed extensively with the same buffer and was eluted with a linear KCl-gradient (0–0.35 M) in Buffer B. The enzyme activity was eluted at 0.08–0.09 M KCl, and was dialysed overnight against a large vol. of Buffer A. The purified enzyme fraction was a yellow soln and was stable for at least one month at 0–2°. This enzyme fraction was used in all further expts.

Spectrophotometric measurements of BPA synthase. The absorption spectrum of BPA-synthase was measured at 200–600 nm by using an automatic spectrophotometer (Hitachi). The protein concn was 1.65 mg per ml of Buffer A for BPA-synthase. The identification of bound pyridoxal 5′-phosphate in the purified enzyme was determined by measuring the *A* at 410 nm in comparison with that of standard pyridoxal 5′-phosphate: bound pyridoxal 5′-phosphate concn was 2.1 molecules per 1 molecule of BPA-synthase.

MW determination by gel filtration. The MW of the purified enzyme was determined by gel filtration chromatography using Sephadex G-100 in a 2.6 × 42 cm column [12]. The gel was equilibrated with Buffer A, which contained 0.1 M NaCl. The enzyme was detected by assaying fractions for activity. The following standard marker proteins were used: chymotrypsinogen A (MW 25000), ovalbumin (MW 45000) and bovine serum albumin (MW 68000). The column void vol. was determined in a separate run by using Blue Dextran 2000.

SDS-polyacrylamide-gel electrophoresis (with and without 8 M *urea).* The purified enzyme was subjected to dodecylsulphate electrophoresis on 8% gels at pH 7.2 (0.1 M Na-Pi buffer) according to the methods of ref. [13] and the running time was 2 hr at 4 mA/gel. Gels were stained with Coomassie Brilliant Blue R-250, followed by destaining in HOAc–MeOH–H_2O (1:5:5). The enzyme concn was 10–15 μg/gel and protein standards were

α-lactalbumin (MW 14 400), trypsin inhibitor (MW 20 100), carbonic anhydrase (MW 30 000), ovalbumin (MW 45 000) and bovine serum albumin (MW 68 000) and bromophenol blue was marker.

Kinetics and other properties. The pH optimum, curves relating enzyme activity to pyrazole and OAS concns, etc. were determined under standard assay conditions by using 100 μl samples of purified enzyme (equivalent to 7 μg of protein).

Acknowledgements—We are grateful to Professor L. Fowden, Harpenden, England, for his encouragement and to Dr. F. Lambein, State University of Ghent, Belgium, for reading the manuscript.

REFERENCES

1. Shinano, S. and Kaya, T. (1957) *J. Agric. Chem. Soc. Japan* **31**, 759.
2. Noe, F. F. and Fowden, L. (1960) *Biochem. J.* **77**, 543.
3. Dunnill, P. M. and Fowden, L. (1965) *Phytochemistry* **4**, 935, 937.
4. Murakoshi, I., Kuramoto, H., Haginiwa, J. and Fowden, L. (1972) *Phytochemistry* **11**, 177.
5. Murakoshi, I., Kato, F., Haginiwa, J. and Fowden, L. (1973) *Chem. Pharm. Bull.* **21**, 918.
6. Murakoshi, I., Kato, F., Haginiwa, J. and Takemoto, T. (1974) *Chem. Pharm. Bull.* **22**, 473.
7. Murakoshi, I., Kato, F. and Haginiwa, J. (1974) *Chem. Pharm. Bull.* **22**, 480.
8. Murakoshi, I., Ikegami, F., Kato, F., Haginiwa, J., Lambein, F., Van Rompuy, L. and Van Parijs, R. (1975) *Phytochemistry* **14**, 1515.
9. Murakoshi, I., Ikegami, F., Ookawa, N., Haginiwa, J. and Letham, D. S. (1977) *Chem. Pharm. Bull.* **25**, 520.
10. Murakoshi, I., Ikegami, F., Ookawa, N., Ariki, T., Haginiwa, J., Kuo, Y. -H. and Lambein, F. (1978) *Phytochemistry* **17**, 1571.
11. Murakoshi, I., Ikegami, F., Harada, K. and Haginiwa, J. (1978) *Chem. Pharm. Bull.* **26**, 1942.
12. Andrews, P. (1965) *Biochem. J.* **96**, 595.
13. Weber, K. and Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406.
14. Kumagai, H., Yamada, H., Matsui, H., Ohkishi, H. and Ogatá, K. (1970) *J. Biol. Chem.* **245**, 1773.
15. Murakoshi, I., Ikegami, F., Hinuma, Y. and Hanma, Y. (1984) *Phytochemistry* (in preparation).
16. Morino, Y. and Snell, E. E. (1967) *J. Biol. Chem.* **242**, 2800.
17. Yoshida, H., Utagawa, T., Kumagai, H. and Yamada, H. (1974) *Agric. Biol. Chem.* **38**, 2065.
18. Dunnill, P. M. and Fowden, L. (1963) *J. Exp. Botany* **14**, 237.
19. Dunnill, P. M. and Fowden, L. (1965) *Phytochemistry* **4**, 933.
20. Atfield, G. N. and Morris, C. J. O. R. (1961) *Biochem. J.* **81**, 606.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
22. Layne, E. (1957) *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds) Vol. 3, p. 452. Academic Press, New York.
23. Smith, I. K. and Thompson, J. F. (1971) *Biochim. Biophys. Acta* **227**, 288.