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PURIFICATION AND CHARACTERIZATION OF L-MIMOSINE SYNTHASE FROM *LEUCAENA LEUCOCEPHALA**

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Key Word Index—*Leucaena leucocephala*; Leguminosae; L-mimosine synthase; enzyme purification; *O*-acetyl-L-serine; 3,4-dihydroxypyridine, L-mimosine.

Abstract—L-Mimosine synthase has been isolated from *Leucaena leucocephala* seedlings and purified 280-fold by heat treatment, ammonium sulphate fractionation, gel filtration and ion-exchange chromatography. The enzyme was shown to be homogeneous by gel electrophoresis (MW 64 000 ± 2000) and to consist of two identical subunits with MWs of 32 000 ± 2000. The purified enzyme has a K_m value of 6.25×10^{-3} M for *O*-acetyl-L-serine and 5.0×10^{-3} M for 3,4-dihydroxypyridine. In these and other properties, the enzyme differs from β-(pyrazol-1-yl)-L-alanine synthase from *Citrullus vulgaris* seedlings.

INTRODUCTION

L-Mimosine, β-(3-hydroxy-4-pyridon-1-yl)-L-alanine, occurs in the legume species *Mimosa pudica* [1, 2] and *Leucaena leucocephala* [3]. It is known for its cytostatic effects against growing hair follicles and reproductive organs [4–8]. Murakoshi *et al.* [9] have studied the formation of L-mimosine from 3,4-dihydroxypyridine (DHP) and *O*-acetyl-L-serine (OAS) using cell-free extracts of *Leucaena* seedlings. Recent work [9–16] has shown OAS to play an important role as a key intermediate in the biosyntheses of β-substituted alanines in higher plants, and the β-substituted alanine synthases exhibit a clear specificity for OAS as a donor of the alanyl moiety. We have recently described the 200-fold purification of β-(pyrazol-1-yl)-L-alanine (BPA) synthase from *Citrullus vulgaris*, and that the enzyme shows a high degree of specificity for the substrates, OAS and pyrazole, which occur in *C. vulgaris* [17, 18] and therefore seem to be the natural ones for the enzyme; the formation of L-mimosine, L-willardiine and several other heterocyclic β-substituted alanines could not be observed when suitable substrates were provided [19].

In this paper we describe the purification of L-mimosine synthase from *L. leucocephala* seedlings, a second plant enzyme catalysing the formation of β-substituted alanines, and compare this enzyme with BPA-synthase from *C. vulgaris*.

RESULTS

Purification of L-*mimosine synthase from* Leucaena leucocephala *seedlings*

Initially the extraction and purification of L-mimosine synthase from *L. leucocephala* followed our previous method [19], i.e. the enzyme was prepared from 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, as summarized in Table 1. The L-mimosine synthase band obtained after the second DEAE-cellulose (DE-52) chromatography had a constant specific activity across the band. The protein from this band eluted from a calibrated Sephadex G-100 column essentially as a single species (see below). The above procedure gave a 280-fold purification of the enzyme with a specific activity of 189 U/mg protein and a yield of 1.5%.

Properties of L-*mimosine synthase*

The MW was determined by gel filtration of the purified enzyme according to the method of Andrews [20]. The column of Sephadex G-100 (2.6 × 42 cm) was first calibrated with standard proteins; L-mimosine synthase activity was found invariably as a single peak, corresponding to a MW of 64 000 ± 2000.

The purified enzyme was subjected to SDS-polyacrylamide-gel electrophoresis on 8% gels in the presence of 8 M urea to determine its subunit structure, following the method of Weber and Osborn [21]. A single band, MW 32 000 ± 2000, indicated two identical subunits.

The presence of bound pyridoxal 5′-phosphate in the purified enzyme was demonstrated by direct spectrophotometric measurements [22]: one molecule of pyridoxal 5′-phosphate is bound to each subunit (Table 2).

A plot of L-mimosine synthase activity against pH gave a bell-shaped curve with a maximum at pH 7.8 although there was a rapid acetyl shift from O to N atoms in the substrate above *ca* pH 8.0.

Lineweaver–Burk plots gave K_m values of 6.25×10^{-3} M for OAS and 5.0×10^{-3} M for DHP, as indicated in Table 2. With BPA-synthase from *C. vulgaris*, a K_m value of 2.5×10^{-3} M for OAS was determined [19].

Under standard assay conditions, L-mimosine synthase

*Parts of this work were reported at the 100th and the 101st Annual Meeting of the Pharmaceutical Society of Japan at Tokyo, 5 April 1980 (Abstracts, p. 236) and at Kumamoto, 2 April 1981 (Abstracts, p. 524) and were included in the dissertation presented by F. I. at Tokyo University, 1981 (in Japanese).

Table 1. Summary of purification of L-mimosine synthase from *Leucaena leucocephala*

Purification step	Volume (ml)	Total activity (units)*	Total protein (mg)	Sp. act. (units/mg protein)	Yield (%)
1. Crude extract†	1550	8840	13100	0.675	100
2. 55°-heated supernatant‡	1530	8570	12300	0.695	96.9
3. Ammonium sulphate precipitate§	45	2070	650	3.18	23.4
4. 1st Sephadex G-100 (peak fractions)	85	1075	89	12.1	12.2
5. 1st DEAE-cellulose (0.1–0.14 M)	28	895	16 5	54.2	10.1
6. 2nd Sephadex G-100 (peak fractions)	14	374	2.5	150	4.2
7. 2nd DEAE-cellulose (0.1–0.11 M)	6	132	0 7	189	1.5

*One unit of enzyme activity represents 1 nmol of product formed per min at 28°, in 0.03 M KPi buffer, pH 7.8.
†Starting from 420 g of the fresh seedlings of *Leucaena leucocephala* (31.2 mg protein/g seedlings).
‡55°, 2 min.
§40–60% saturation.

appears to be specific for OAS as a substrate: *O*-phospho-L-serine or L-serine could not serve as donor molecules. However, *O*-succinyl-L-serine and *O*-sulpho-L-serine could donate an alanyl group but only to the extent of 60 and 32% respectively of that of OAS at 12.5 mM.

Table 3 shows the relative activities of the purified enzyme for various heterocyclic acceptor substrates. The formation of L-mimosine, β-(pyrazol-1-yl)-L-alanine, β-(3-amino-1,2,4-triazol-1-yl)-L-alanine and *O*-ureidoserine was catalysed, but that of L-quisqualic acid and the uracilylalanines L-willardiine and L-isowillardiine was not. DHP was by far the best substrate. In order to avoid critical differences in tautomeric forms of potential acceptor substrates, a comparison of the activities was made at the respective pH optima shown in Table 3.

DISCUSSION

L-Mimosine synthase has been purified 280-fold from *L. leucocephala* seedlings. This is the second plant enzyme catalysing the formation of β-substituted alanines that has been purified to near-homogeneity, enabling critical comparison of its properties with that of BPA-synthase from *C. vulgaris*.

Both enzymes show a clear preference for OAS as the alanyl donor, but the K_m value determined for L-mimosine synthase is 2.5 times higher than that for BPA-synthase. The two enzymes, however, show marked differences in specificity for the heterocyclic acceptor substrate. L-Mimosine synthase can synthesize BPA to the extent of 81.8% of L-mimosine formation, but BPA-synthase could not synthesize L-mimosine in detectable amounts. Neither enzymes synthesized L-quisqualic acid, L-willardiine or L-isowillardiine when appropriate substrates were provided.

In most non-protein heterocyclic β-substituted alanines, the alanyl moiety is linked to the ring by an N–C bond, β-(2-β-D-glucopyranosyl-3-isoxazolin-5-on-4-yl)alanine being a rare exception [13]. In contrast, the protein amino acids phenylalanine, tyrosine and tryptophan have the alanyl moiety linked to the ring-system by a C–C bond. Furthermore, the pathways involved in the biosynthesis of these protein amino acids [23–25] are different from those leading to the non-protein β-substituted alanines, and the enzymes involved are also quite distinct. The presence of non-protein β-substituted alanines in higher plants then does not represent an error of primary metabolism, nor a lack of specificity in the enzymes implicated in protein amino acid biosynthesis.

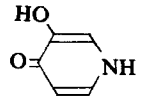
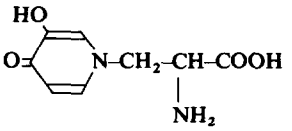
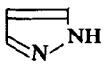
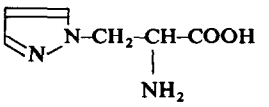
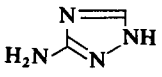
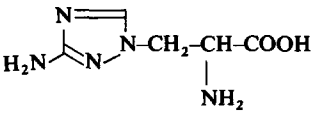
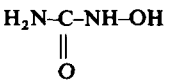
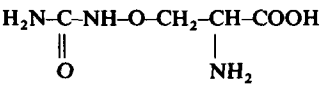
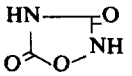
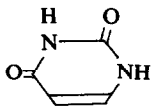
The similarity in the physicochemical properties between enzymes from a cucurbit and a legume, and the similarity in their quaternary structures consisting of two

Table 2. Summary of the physicochemical properties and kinetics of L-mimosine synthase prepared from the seedlings of *Leucaena leucocephala*

Property	*L. leucocephala*
$E^{1\%}_{1\,cm}$ at 280 nm	13 3
Absorption maxima (pH 7.8)	280, 410 nm
$A_{260\,nm}/A_{280\,nm}$ (pH 7 8)	0 67
MW (Sephadex G-100 filtration)	64000 ± 2000
MW of subunit (SDS-disc gel electrophoresis)	32000 ± 2000
No. of subunits	2
Pyridoxal 5′-phosphate bound to the enzyme	2 moles/mole enzyme
K_m for *O*-acetyl-L-serine*	6.25×10^{-3} M
K_m for 3,4-dihydroxypyridine*	5.0×10^{-3} M

*K_m values were determined from Lineweaver–Burk plots and optical properties were measured by direct spectrophotometric measurements as described previously [19].

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

N-Heterocyclic compound	Amino acid synthesized	Relative velocity of synthesis (%)	Optimal pH
		100	7.8 [9]
		81.8	7.4 [9, 19]
		30	7.8 [12]
		12.7	7.5 [16]
	L-Quisqualic acid	0	7.4 [11]
	L-Willardiine L-Isowillardiine	0	7.8 [15]

The reaction mixtures containing 12.5 mM of OAS, 12.5–125 mM of *N*-heterocyclic compounds and 17.5 μg of enzyme in 0.4 ml of 0.03 M KPi buffer were incubated at each optimal pH for 30 min at 28°. The reaction mixtures were separated by paper chromatography and automatic amino acid analyser and were then assayed as described before [9–16] The relative rates of synthesis were compared with that of L-mimosine.

identical subunits (MW 32000 ± 2000) each bearing a pyridoxal 5'-phosphate molecule indicate that the two enzymes may have a common evolutionary origin.

EXPERIMENTAL

Materials. Seeds of *L. leucocephala* were germinated as previously described [19]. Seedlings were harvested, washed and the cotyledons removed; they were then cooled for 30 min at 0–4° before enzyme extraction.

Enzyme activities Routine assays were conducted as described previously [9]. The enzyme was dissolved in 0.03 M KPi buffer, pH 7.8 (buffer A). Substrate concns were 12.5 mM for DHP and 12.5 mM for OAS. Incubation was at 28° for 30 min; the total reaction vol. was 0.4 ml, utilizing up to 0.2 ml enzyme soln (corresponding to 17.5–200 μg protein). Reactions were terminated by the addition of 0.02 ml 10% NH_4OH, and L-mimosine formation was determined with an automatic amino acid analyser (Shibata model AA-500, Tokyo) using 0.2 N Na citrate buffer (pH 4.25) on an intermediate column (1.0 × 50 cm). The unit of enzyme activity used in this paper is equivalent to 1 nmol of L-mimosine produced per min. Protein was determined by the method of ref. [26].

Homogenization and preliminary enzyme purification. All operations were carried out at 0–4°. Seedlings (420 g, minus cotyledons) were homogenized for 90 sec in 1500 ml 0.1 M KPi buffer, pH 7.5, containing 0.25 M sucrose and 10 mM 2-mercaptoethanol. Polyvinylpolypyrrolidone (50 g) was added to this mixture. The homogenate was pressed through nylon cloth and the filtrate centrifuged at 15000 *g* for 30 min. The supernatant was immersed in a water bath at 80° and stirred vigorously until the temp. of the soln reached 55°. It was then immediately chilled to 4° with ice water, and centrifuged at 10000 *g* for 20 min and the pellet discarded. The supernatants were subjected to $(NH_4)_2SO_4$ fractionation, and a 40–60% saturated $(NH_4)_2SO_4$ fraction was resuspended in buffer A. In this buffer, enzyme activity was stable for at least 7 days at 0–4°.

Sephadex G-100 gel filtration. The heat- and $(NH_4)_2SO_4$-treated enzyme preparation (45 ml) was applied to a column of Sephadex G-100 (2.6 × 80 cm) pre-equilibrated with buffer A, and was eluted by the same buffer. Fractions (5 ml) were collected and assayed for activity and protein, measured by *A* at 280 nm [27]. The elution profile of L-mimosine synthase showed a single activity peak and fractions (40–53) comprising this peak were concd by $(NH_4)_2SO_4$ precipitation and dialysed overnight against buffer A.

DEAE-cellulose chromatography. DEAE-cellulose (DE-52) was equilibrated with buffer A in a column measuring 1.3 × 12 cm and the enzyme preparation from the Sephadex G-100 column was applied. The column was washed with 75 ml buffer A and then proteins were eluted with a linear gradient containing 0–0.5 M KCl in buffer A. Fractions with significant synthase activity (0.1–0.14 M KCl fractions) were pooled and concd by membrane filtration.

Second Sephadex G-100 gel filtration. The enzyme preparation from the DE-52 column was applied to a column (2.6 × 42 cm) of Sephadex G-100 pre-equilibrated with, and eluted by, buffer A. The eluate was collected in 2 ml fractions and the pooled active fractions (14 ml) were dialysed overnight against buffer A.

Second DEAE-cellulose chromatography. DE-52 was equilibrated with buffer A in a column (1.3 × 6 cm) and the dialysed enzyme preparation from the second Sephadex G-100 column was applied. The column was washed with buffer A and was eluted with a linear gradient containing 0–0.5 M KCl in buffer A. L-Mimosine synthase activity was eluted at 0.1–0.11 M KCl in buffer A and the pooled active fractions were dialysed overnight against buffer A. The purified enzyme fraction was a pale yellow soln and was used immediately in all further expts.

Properties of L-mimosine synthase. This was performed as described previously [19].

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