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BIOSYNTHESIS OF β-(1,2,4-TRIAZOL-1-YL)ALANINE IN HIGHER PLANTS

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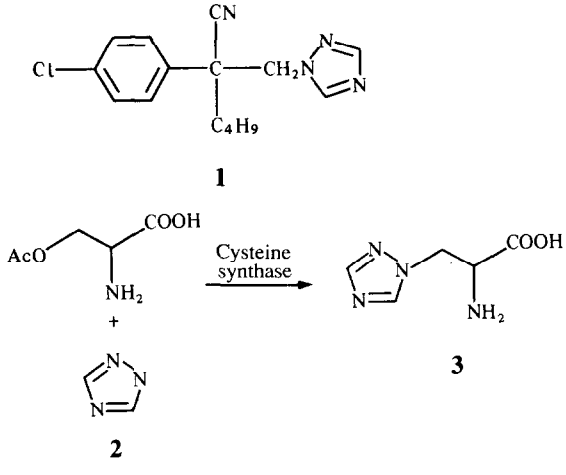
Key Word Index—*Pisum sativum*; Leguminosae; cysteine synthase; biosynthesis; metabolite; fungicide; Myclobutanil; β-(1,2,4-triazol-1-yl) alanine; 1,2,4-triazole; *O*-acetyl-L-serine.

Abstract—β-(1,2,4-triazol-1-yl)Alanine, an important metabolite of the triazole-based fungicide Myclobutanil, was shown to be derived from *O*-acetyl-L-serine and 1,2,4-triazole by cysteine synthase in higher plants. Some properties of this enzyme in the biosynthesis of β-(1,2,4-triazol-1-yl)alanine are described.

INTRODUCTION

The triazole-based fungicide Myclobutanil (**1**) is degraded to 1,2,4-triazole (**2**) in soil. An important metabolite of **1** in plants is β-(1,2,4-triazol-1-yl)alanine (**3**). The biosynthesis of **3** from **1** was proposed to occur by production of **2** in soil which was then converted to **3** in plants. During our continuing study on the biosynthesis of heterocyclic β-substituted alanines in higher plants, we have demonstrated the formation of a number of β-substituted alanines such as L-quisqualic acid [1], β-(pyrazol-1-yl)-L-alanine [2] and others [3–5] by some cysteine synthases prepared from several higher plants. In this paper we describe the biosynthetic pathway for the formation of **3** from *O*-acetyl-L-serine (OAS) and 1,2,4-triazole (**2**) by cysteine synthases in higher plants as shown in Scheme 1.



Scheme 1. Biosynthetic pathway for β-(1,2,4-triazol-1-yl)alanine (**3**) in higher plants.

* Parts of this work were reported at the 33rd Annual Meeting of Kanto Branch of the Pharmaceutical Society of Japan at Tokyo, 11 November 1989 (Abstracts, p. 61).

RESULTS AND DISCUSSION

The enzyme cysteine synthase was purified mainly from the seedlings (cotyledons removed) of pea (*Pisum sativum*), and the cysteine synthase isoenzyme B was used directly as the source of enzyme activity for the formation of **3**.

Under standard assay conditions, the enzyme purified from pea seedlings clearly formed **3**. The purified enzyme showed low activity in the presence of *O*-acetyl-D-serine, while no enzyme activity was detectable when OAS was substituted by *O*-phospho-L-serine or L-serine.

The optimum pH for the enzymatic formation of **3** was 8.4–8.5 using 50 mM Tris–HCl buffer. The synthase activity for **3** was dependent upon the concentration of 1,2,4-triazole (**2**) used. A relatively low final concentration of **2** (*ca* 70 mM) was sufficient to give a maximum rate of **3** formation in the presence of a fixed concentration (12.5 mM) of OAS. Higher concentrations of **2** progressively inhibit the enzymatic formation of **3**. The Lineweaver–Burk plot gave a K_m value of 125 mM for **2**.

The addition of pyridoxal 5′-phosphate (PLP) to the reaction mixtures at 1 mM increased the rate of **3** formation by *ca* 50%; addition of a higher concentration (10 mM) caused *ca* 10% inhibition of the synthase activity. In the presence of the PLP-enzyme inhibitors NH_2OH or $NaBH_4$ at 1 mM, the enzyme activity was inhibited 25 or 90%, respectively.

Cysteine synthase isoenzyme A purified from pea and cysteine synthases purified from other plant species were also examined for their ability to catalyse the formation of **3**. Isoenzyme A in pea could synthesize **3** in the same manner as isoenzyme B (Table 1). Cysteine synthases in pea could thus synthesize some heterocyclic β-substituted alanines such as **3**, in line with our previous findings [1–5]. The activity of **3** formation could also be detected in the enzyme preparations obtained from the fresh leaves of *Lathyrus latifolius* and *Leucaena leucocephala*.

From the above results and from our earlier works [1–5], it can be suggested that the plant cysteine synthases also catalyse the formation of **3** from **2** and OAS in the detoxification role of this enzyme in higher plants. A similar metabolic detoxification by alanyl-substitution of a soil-fungicide has been described before in plants [6].

Table 1. Relative synthetic rates of β-substituted alanines by cysteine synthases purified from *Pisum sativum*

Substrate	Amino acid synthesized	Cysteine synthase	
		Isoenzyme A	Isoenzyme B
H_2S	L-Cysteine	100*	100
1,2,4-Triazole	β-(1,2,4-Triazol-1-yl)-L-alanine	5.81	3.13
3-Amino-1,2,4-triazole	β-(3-Amino-1,2,4-triazol-1-yl)-L-alanine	0.84	0.95
Pyrazole	β-(Pyrazol-1-yl)-L-alanine	0.60	0.93
3,5-Dioxo-1,2,4-oxadiazolidine	L-Quisqualic acid	0	0
3,4-Dihydroxypyridine	L-Mimosine	0	0
Uracil	L-Willardiine	0	0
	L-Isowillardiine	0	0
Hydroxyurea	*O*-Ureido-L-serine	0.05	0.04
NaCN	β-Cyano-L-alanine	22.08	27.81

*The relative rates of synthesis (%) were compared with that of L-cysteine formed by each enzyme, respectively. The reaction conditions are as described before [1–5].

EXPERIMENTAL

Materials. The seedlings of pea (*Pisum sativum*) were grown in moistened vermiculite in the dark for 5–6 days at 26–28°. Seedlings (cotyledons removed) were cooled for 1 hr at 0–4° before enzyme extraction. 1,2,4-Triazole was obtained from Aldrich. All other chemicals used were of the highest commercial grade available.

Purification of cysteine synthases. All operations were carried out at 0–4°. Cysteine synthases were prepared from the seedlings or young leaves by methods described in our previous papers [1–5].

Activity assay for **3** *formation.* Reaction mixtures used to demonstrate the formation of **3** contained 12.5 mM of OAS, 70 mM of **2** and 0.2 ml enzyme preparation containing 2–4 μg of the protein, and were incubated at 30° in a final vol. of 0.4 ml. The pH of the incubation mixtures was normally adjusted to pH 8.4 by 50 mM Tris–HCl buffer. Reactions were terminated by the addition of 30 μl of 1 M KOH. The resulting soln acidified with 15 μl of 6 M HCl was examined for the formation of **3**. The unit of enzyme activity used in this paper is equivalent to 1 μmol of L-cysteine or **3** produced per min. Protein was determined by a dye-binding method [7].

Identification of the reaction product as **3**. The formation of **3** was demonstrated by using an automatic amino acid analyser (Hitachi 835-10): under standard operating conditions (2.6 × 250 mm column, 33–68°, Li-citrate buffer system, pH 3.0–7.0, flow rate 0.275 ml min^{-1}), **3** eluted at *ca* 46 min from the column. The presence of **3** in final reaction mixtures was also established by TLC comparison with authentic material using the following solvent system: $CHCl_3$–MeOH–15 M NH_4OH (65:28:8). These methods indicated clearly the formation of a product, reacting positively with ninhydrin reagent, that was inseparable from added authentic **3**. The product was not formed in reaction mixtures lacking OAS or **2**, nor was it formed when the enzyme preparation was pretreated at 100° for 15 min. Quantitative determination of **3** was also made using an amino acid analyser.

Properties of cysteine synthase isoenzymes on the biosynthesis of **3** were studied by the methods of refs [1–5].

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