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Enzymic Synthesis of 3-Amino-1,2,4-triazol-1-ylalanine, A Metabolite of 3-Amino-1,2,4-triazole in Plants

3-Amino-1,2,4-triazol-1-ylalanine(III) is the major metabolic product of 3-amino-1,2,4-triazole(I) in plants.[1-4] III appeared in almost all the extracts of plants treated with I. Similarly, Frisch, *et al.*[5] reported that when a range of heterocyclic compounds possessing an NH= group such as pyrazoles, triazoles, tetrazoles and indole were supplied to seedlings the corresponding β-substituted alanines were synthesized even through the synthesized compounds were not normally present in the seedlings used.

We have recently reported that crude extracts of plants catalyze the synthesis of the β-substituted alanines mimosine,[6] β-pyrazol-1-ylalanine,[6] 2-alanyl-3-isoxazolin-5-one[7] and quisqualic acid[8] (2-alanyl-3,5-dioxo-1,2,4-oxadiazolidine[9]) by condensation of the appropriate heterocyclic compound with an alanyl moiety arising from O-acetylserine.

In this paper we report an analogous reaction catalyzed by extracts of pea (*Pisum sativum*), watermelon (*Citrullus vulgaris*) and *Leucaena leucocephala* for the synthesis of III from I and O-acetylserine (II); neither serine nor O-phosphoserine served as a donor of the alanyl fragment.

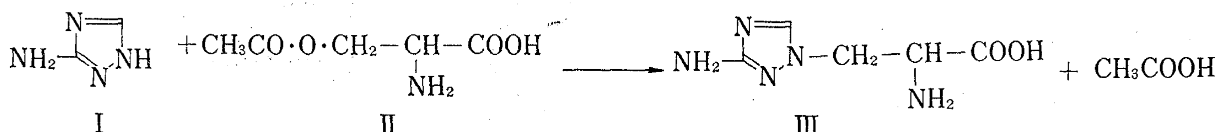

Fig. 1

Seedlings of pea, watermelon and *L. leucocephala* were grown in the dark for 3—4 days at 30°. Unless otherwise stated, enzyme extracts were prepared from *Pisum* seedlings as described in previous papers.[7,8] The protein-containing solution, free from low mol. wt. substances, was used as the source of enzyme activity.

Reaction mixtures used to demonstrate the formation of III were conducted at 30° for 90 min in a final volume of 0.4 ml and contained O-acetylserine (5 μmoles), 3-amino-1,2,4-triazole (30 μmoles) and 0.2 ml of enzyme solution which contained the protein from 0.4 g fresh weight of seedlings; reaction mixtures were normally maintained at pH 7.8 by 0.1 M potassium phosphate buffer. Reactions were terminated by the addition of 3 volumes of ethanol and precipitated protein was removed by centrifugation. Aliquots of the supernatant solution were examined chromatographically for the presence of III. The formation of III was not detected in incubation mixtures from which I or II was omitted, nor when the enzyme extracted was pretreated at 100°. The formation of III in terminated reaction mixtures was examined by paper chromatographic comparison with authentic material using the following solvent systems: 1, propan-2-ol–formic acid–water (80: 4: 20, v/v); 2, 2-methyl-propan-2-ol–

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formic acid–water (70: 15: 15, v/v). Both solvents indicated the formation of a product which reacted with ninhydrin, and ran with the same *Rf* as III; *Rf*'s for III determined for these solvents were 0.07 and 0.13, respectively, whilst II had the following *Rf*'s: 0.37 and 0.54, respectively. Under the same conditions, serine moved at *Rf*'s of 0.23 and 0.39, respectively. Authentic III was isolated from seedlings supplied with I as described by Massini[4] and Frisch, *et al.*[5] and also prepared by chemical synthesis from 3-acetamido-1,2,4-triazole and α-acetamidoacrylic acid by a modification of the method of Murakoshi, *et al.*[10]

The enzyme catalyzed formation of III was also determined by carbon-14 incorporation from O-acetylserine-3-^{14}C. After incubation radioactivity was associated with a ninhydrin-positive substance which chromatographed with the same *Rf* as III; radioactivity on the chromatograms was monitored as previously described.[6–8]

The identity of the reaction product as III was confirmed using an automatic amino acid analyzer (Shibata model AA-500, Tokyo); under standard operating conditions,[6–8] both III and the incubation product eluted from the column at approx. 501 min (250 ml), *i.e.*, at a position slightly overlapping with alanine.

Some properties of the enzyme-dependent synthesis of III were studied: the product was quantitatively estimated by the method of Atfield, *et al.*[11] Addition of pyridoxal phosphate had no effect on the formation of III. The optimum pH for the formation of III was 7.8 although the effect of pH on enzyme activity could be confused by a rearrangement of II to form N-acetylserine. In all subsequent experiments the pH was adjusted to 7.8. The optimum concentration of I for enzyme-catalyzed formation of III was 75 mM (the concentration of II was 12.5 mM). The rate of formation of III was constant for at least 60 min but the rate then decreased until at 100 min when no further synthesis was detected. The amount of III formed after 100 min represented a yield of approx. 2—3% with respect to the initial amount of II. The property of crude extracts to catalyze the formation of III decreased by approx. 40% when the extracts were stored at 0° for 25 hr.

Enzyme preparations from *Citrullus* and *Leucaena* also catalyzed the synthesis of III as described for *Pisum* extracts: the initial rate of formation of III catalyzed by enzyme preparations from *Citrullus* and *Leucaena* was less than that catalyzed by *Pisum* seedlings extracts but, whereas the synthesis of III in incubation mixtures containing *Pisum* enzyme closed after 1 hr, mixtures containing *Citrullus* and *Leucaena* enzyme continued to synthesize III for at least 7 hr (7—8% yield).

III was also synthesized non-enzymatically in about 8% yield in the presence of pyridoxal phosphate: the synthesis was performed at 55° for 5 hr in a reaction mixture containing I (30 μmoles), II or serine (5 μmoles) and pyridoxal phosphate (100 μg) in 0.5 ml of 0.1N acetate buffer, pH 5.0.

A more detailed investigation of the enzyme from *Pisum*, *Citrullus* and *Leucaena* responsible for the production of III is in progress in our laboratory.

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