Communications to the Editor

[Chem. Pharm. Bull. 31(5)1777—1779(1983)]

BIOSYNTHESIS OF β-(6-BENZYLAMINOPURIN-9-YL)ALANINE, A METABOLITE OF CYTOKININ 6-BENZYLAMINOPURINE IN HIGHER PLANTS

Isamu Murakoshi, Chiharu Koide, Fumio Ikegami,* and Kikuko Nasu

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

β-(6-Benzylaminopurin-9-yl)alanine (3), a metabolite of the cytokinin 6-benzylaminopurine (2), was confirmed to be derived from O-acetyl-L-serine (1) and 2 by an enzyme in higher plants. Some properties of the enzyme are described.

KEYWORDS —— *Lupinus luteus* ; Leguminosae ; biosynthesis ; enzyme ; cytokinin ; amino acid ; β-(6-benzylaminopurin-9-yl)alanine ; 6-benzylaminopurine ; O-acetyl-L-serine

β-(6-Benzylaminopurin-9-yl)alanine (3) has recently been found as one of the principal metabolites of cytokinin 6-benzylaminopurine (2) in the seedlings of beans (*Phaseolus vulgaris*) by D.S. Letham *et al.*.[1] 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 quisqualic acid,[2] willardiine, isowillardiine,[3] lupinic acid[4] and others[5,6] by enzymes prepared from several higher plants.

This communication presents the biosynthetic pathway for the formation of 3 from 2 and O-acetyl-L-serine (1) by an enzyme in higher plants as shown in Chart 1.

The enzyme preparations were predominantly obtained from the immature seeds and the seedlings (cotyledons removed) of *Lupinus luteus* grown in the dark for 5-7 days at 26-28°C. Unless otherwise stated, enzyme fractions were prepared from the immature seeds of *L. luteus* (soft-greenish) essentially by methods described in previous papers[2-6] : the enzyme preparation, partially purified by heat treatment, $(NH_4)_2SO_4$ fractionation and desalting on Sephadex G-25 (fine) column, was used directly as the source of enzyme activity.

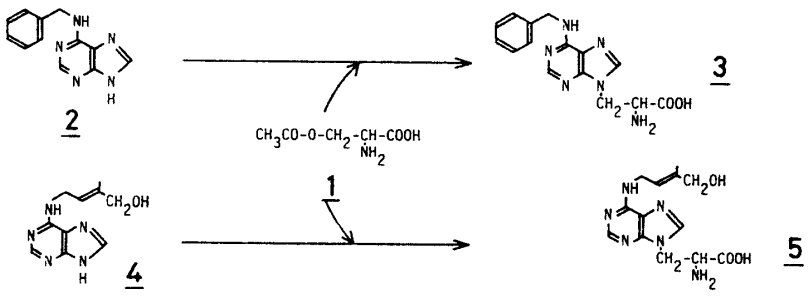

Chart 1. Scheme for the Biosyntheses of β-(6-Benzylaminopurin-9-yl)alanine (3) and Lupinic acid (5)[4] by Enzyme(s) in Higher Plants

Reaction mixtures used to demonstrate the formation of 3 contained 12.5 mM of 1, 5 mM of 2 and 0.2 ml enzyme preparation containing 0.75-1 mg of the protein and were incubated at 28°C in a final volume of 0.4 ml. The pH of the incubation mixtures was normally adjusted to pH 8.0 by 0.05 M K-Pi buffer. Reactions were terminated by the addition of 3 volumes of ethanol. The protein precipitated was removed by centrifugation and the resulting supernatant was examined chromatographically for the presence of 3 by HPLC : under standard assay conditions (3 x 500 mm, LiChrosorb SI 100 column, 270 nm, flow rate 1 ml/min), 3 eluted at about 7 min from the column in the following solvent system : 25% MeOH-Et_2O—25% NH_4OH—H_2O (100: 4: 3, by vol.). The presence of 3 in final reaction mixtures was also established by paper chromatographic comparison with authentic material, [7] using the following solvent systems : 1, n-butanol—acetic acid—H_2O (90: 10: 29, by vol.); 2, n-butanol—28% NH_4OH—H_2O (6: 1: 2, by vol. upper phase). This method 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 2 or 1 , nor was it formed when the enzyme preparation was pretreated at 100°C for 15 min.

Further confirmation of the identity of the reaction product as 3 was obtained by using an automatic amino acid analyzer (Hitachi 835-10, Tokyo). Under standard operating conditions (2.6 x 250 mm column, 33-68°C, Li-citrate buffer system, pH 3.0-7.0, flow rate 0.275 ml/min), 3 eluted at about 196 min from the column. Quantitative determination of 3 was also made using HPLC.

The β-(6-benzylaminopurin-9-yl)alanine (3) synthase clearly appears to be specific for O-acetyl-L-serine (1) as a donor for the alanyl-moiety under standard assay conditions. No enzyme activity was detectable when 1 was substituted by O-acetyl-D-serine, O-phospho-L-serine or L-serine. The optimum pH for the enzymatic formation of 3 was 8.0-8.3 using 0.05 M K-Pi buffer. The synthase activity for 3 was dependent upon the concentration of 1 used. A relatively low final concentration of 1 at around 10 mM was sufficient to give the maximum rate of 3 formation, but higher concentrations of 1 progressively inhibited the enzymatic formation of 3 . The Lineweaver-Burk plot gave Km value of 4.5 mM for 1 . The addition of exogenous pyridoxal 5'-phosphate to the reaction mixtures at about 0.23 mM increased the rate of 3 formation about 25 %, but a higher concentration of 2.3 mM caused 15 % inhibition of the synthase activity. Similar requirements have been reported for β-(pyrazol-1-yl)-L-alanine synthase, [6] willardiine and isowillardiine synthases. [3] In the presence of NH_2OH or KCN at a concentration of 50 mM or 2 mM, respectively, the enzyme activity was inhibited more than 50 %.

Enzyme preparations from other plant species were also examined for their ability to catalyze the formation of 3 . The activity of 3 formation could be detected in the enzyme preparations obtained from the seedlings of *L. luteus* and *Pisum sativum* and from the fresh leaves of *Spinacia oleracea*. The most active enzyme preparations for 3 formation were obtained from the immature seeds of *L. luteus*, which was a rich source of cytokinins. [8] The specific activity of enzyme preparations obtained from the immature seeds of *L. luteus* was approximately 20-fold greater than that from the same plant seedlings as shown in Fig. 1 . The activity in enzyme preparations from watermelon (*Citrullus vulgaris*), *Leucaena leucocephala* and maize (*Zea mays*) seedlings was negligible, while watermelon and

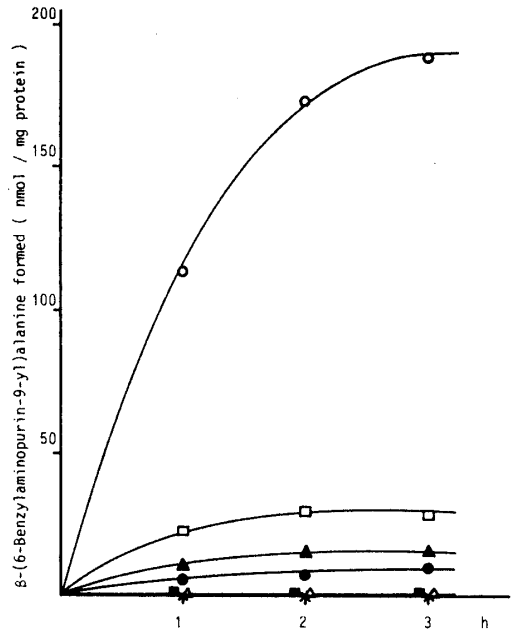

Fig. 1. Comparative Specific Activity for the Formation of β-(6-Benzylaminopurin-9-yl)alanine (3) by an Enzyme in the Immature Seeds of *Lupinus luteus* (○), and in the Seedlings of *L. luteus* (●), *Pisum sativum* (□), *Citrullus vulgaris* (■), *Leucaena leucocephala* (△) and *Zea mays* (X), and in the Leaves of *Spinacia oleracea* (▲)

L. leucocephala contain high enzyme activities for the formation of β-(pyrazol-1-yl)-L-alanine and L-mimosine, respectively.[6]

From the above results and from earlier work in this laboratory, it can be suggested that the enzymes catalyzing the formation of β-substituted alanines show markedly differences in specificity for the heterocyclic substrate as acceptor when prepared from different plant species and are different enzymes.

At present, it is not certain which enzymes are involved in 3 formation, but presumably 3 is derived from 1 and 2 by the same enzyme with lupinic acid (5) formation (Chart 1).

A more detailed investigation on the enzymes from higher plants catalyzing the formation of 3 and other β-substituted alanines is in progress in our laboratory.

REFERENCES AND NOTES

1) D.S. Letham, R.E. Summons, C.W. Parker, and J.K. MacLeod, *Planta*, 146, 71(1979).
2) I. Murakoshi, F. Kato, J. Haginiwa, and T. Takemoto, *Chem.Pharm.Bull.*, 22, 473 (1974).
3) I. Murakoshi, F. Ikegami, N. Ookawa, T. Ariki, J. Haginiwa, Y.-H. Kuo, and F. Lambein, *Phytochemistry*, 17, 1571 (1978).
4) I. Murakoshi, F. Ikegami, N. Ookawa, J. Haginiwa, and D.S. Letham, *Chem.Pharm. Bull.*, 25, 520 (1977).
5) I. Murakoshi, F. Ikegami, K. Harada, and J. Haginiwa, *Chem.Pharm.Bull.*, 26, 1942 (1978).
6) I. Murakoshi, F. Ikegami, Y. Hinuma, and Y. Hanma, Parts of this work were reported at the 101st Annual Meeting of the Pharmaceutical Society of Japan at Kumamoto, April 2, 1981 (Abstracts p. 523-4).
7) 3 was easily synthesized from 1 and 2 by a modification of the PLP-catalyzed chemical reactions, reported by I. Murakoshi *et al.* at the 98th Annual Meeting of the Pharmaceutical Society of Japan at Okayama, April 5, 1978 (Abstracts p. 346), etc..
8) F. Skoog, and D.J. Armstrong, *A.Rev.Plant Physiol.*, 21, 359 (1970).

(Received February 26, 1983)