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Enzymatic Synthesis of Lupinic Acid, a Novel Metabolite of Zeatin in Higher Plants[1]

Lupinic acid(III), a metabolite of the phytohormone zeatin(I), was synthesized from O-acetyl-L-serine(II) and zeatin by an enzyme in higher plants. Some properties of the enzyme are described.

Keywords——*Lupinus luteus*; Leguminosae; biosynthesis; enzyme; phytohormone; amino acid; lupinic acid; zeatin; O-acetyl-L-serine

Lupinic acid(III) is one of the principal metabolites of the phytohormone zeatin(I) in *Lupinus* seedlings. The chemical structure has recently been assigned by Macleod, *et al.*[2] as β-(6-(4-hydroxy-3-methyl-but-*trans*-2-enylamino)purin-9-yl)alanine(III) and confirmed by comparing the natural compound with synthetic material in all measurable respects.

Murakoshi, *et al.* have recently described enzyme systems from higher plants which catalyze the synthesis of heterocyclic β-substituted alanines: serine is first converted to an O-acetyl derivative, and an alanyl-moiety derived from this activated intermediate then undergoes condensation with the appropriate heterocyclic compound to yield β-pyrazol-1-ylalanine,[3] mimosine,[3] quisqualic acid,[4] β-(2- β-D-glucopyranosyl-3-isoxazolin-5-on-4-yl)alanine,[5] β-(3-isoxazolin-5-on-2-yl)alanine[6] and 3-amino-1,2,4-triazol-1-ylalanine (a metabolite of 3-amino-1,2,4-triazole in plants)[7] in *Citrullus*, *Mimosa*, *Quisqualis*, *Pisum*, *Lathyrus*, and *Leucaena* seedlings.

We have now further extended these observations and shown that lupinic acid(III) may be synthesized from zeatin(I) and O-acetyl-L-serine (II) by an analogous reaction catalyzed by extracts of *Lupinus* seeds and seedlings as shown in Fig. 1. The lupinic acid synthetase

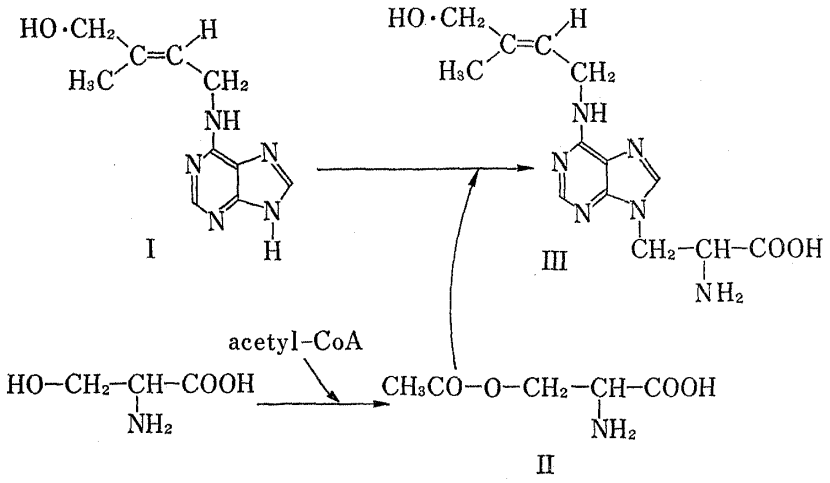

Fig. 1. Scheme for the Biosynthesis of Lupinic Acid (III) by Enzymes in *Lupinus* and *Citrullus* Seedlings

1) This work was presented at the 20th Kanto Branch Meeting of the Pharmaceutical Society of Japan at Tokyo, November 27, 1976. (Meeting Abstracts, p. 13).
2) J.K. Macleod, R.E. Summons, C.W. Parker, and D.S. Letham, *J. Chem. Soc. Chem. Comm.* **1975**, 809.
3) I. Murakoshi, H. Kuramoto, J. Haginiwa, and L. Fowden, *Phytochem.*, **11**, 177 (1972).
4) I. Murakoshi, F. Kato, J. Haginiwa, and T.Takemoto, *Chem. Pharm. Bull.* (Tokyo), **22**, 473 (1974).
5) I. Murakoshi, F. Ikegami, F. Kato, J. Haginiwa, F. Lambein, L. Van Rompuy, and R. Van Parijs, *Phytochem.*, **14**, 1515 (1975).
6) I. Murakoshi, F. Kato, F. Haginiwa, and L. Fowden, *Chem. Pharm. Bull.* (Tokyo), **21**, 918 (1973).
7) I. Murakoshi, F. Kato, and J. Haginiwa, *Chem. Pharm. Bull.* (Tokyo), **22**, 480 (1974).

clearly appears to be specific for the O-acetyl-L-serine as a substrate: neither the O-acetyl-D-serine nor the esters of L-serine or L-serine could serve as a donor of the alanyl-moiety.

Crude enzyme preparations were obtained from the immature seeds, and from the hypocotyls of seedlings of *Lupinus luteus* grown in the dark for 5—6 days at 30°. Unless otherwise stated, the enzyme fractions were prepared from the immature seeds of *L. luteus*(soft-greenish) essentially as described in previous papers[3-7]. The enzyme fractions partially purified by ammonium sulphate precipitation, heat treatment, and desalting on a Sephadex G-25 (fine) column were used as the source of lupinic acid synthetase.

Reaction mixture used to demonstrate lupinic acid formation contained O-acetyl-L-serine or O-acetyl-L-serine-3- ^{14}C(1.36 μmoles, 0.14 μCi), zeatin (2.0 μmoles) and 0.4 ml of enzyme preparation (equivalent to 2 g the immature seeds fresh weight) in a final volume of 0.5 ml. Reaction mixtures were normally maintained at pH 7.3—7.4 by 0.1 M potassium phosphate buffer and incubated at 30° for appropriate periods. Reactions were terminated by the addition of 3 volume of ethanol and precipitated protein was removed by centrifugation. Aliquots of the residual supernatant were examined chromatographically for the presence of lupinic acid by paper chromatography in the following solvent systems: 1, butan-1-ol–acetic acid–water (8: 2: 3, by vol); 2, butan-1-ol–acetic acid–water (90: 10: 29, by vol); 3, pyridine–butan-1-ol–water (1: 1: 1, by vol); 4, propan-2-ol–10% NH_4OH (7: 3, by vol). These solvents indicated clearly the presence of a product, reacting positively with ninhydrin(purple colour), that was inseparable from added authentic lupinic acid. The *Rf* values for lupinic acid obtained in these solvents were 0.44, 0.29, 0.52 and 0.50, respectively, whilst 0-acetyl-L-serine exhibited the following *Rf* data; 0.37, 0.21, 0.43, respectively(0-acetyl-L-serine is rapidly converted to N-acetyl-L-serine in the solvent 4). Under the same conditions, L-serine moved at *Rf*'s of 0.24, 0.10, 0.28, and 0.42, respectively. The formation of lupinic acid was not detected in incubation mixtures from which zeatin or O-acetyl-L-serine was omitted, nor when the enzyme extracted was pretreated at 100° for 15 min.

The enzyme catalyzed formation of lupinic acid was also determined by carbon-14 incorporation from O-acetyl-L-serine-3-^{14}C as a substrate into lupinic acid. Radioactivity was associated with the ninhydrin positive product with the same *Rf* as lupinic acid. Further confirmation of the identity of the lupinic acid formed using an automatic amino acid analyzer was not possible because of the instability of lupinic acid under standard operating conditions (50 cm and 150 cm, 50°, 0.2 N sodium citrate buffer, pH 3.25).

Some properties of the enzyme-dependent synthesis of lupinic acid were studied: the product was quantitatively estimated according to the ninhydrin-cadmium method by Atfield, *et al.*[8] The rate of formation of lupinic acid was constant for at least 120 min but the rate then decreased until at 360 min no further synthesis was detected: the amount of lupinic acid formed after 300 min represented a yield of approx. 35—36% with respect to the initial amount of O-acetyl-L-serine as shown in Fig. 2. When the enzyme preparations were stored at 0° for 24—25 hr, the residual enzyme activity was 58—60% of the activity initially assayed. The optimum pH of lupinic acid synthetase was 7.3—7.4 using 0.1 M potassium phosphate buffer: the enzyme was active only over a narrow pH range. The effect of concentration of zeatin on the enzyme-catalyzed formation of lupinic acid was not assessed under substrate-saturating conditions because of the problems of solubility and expense. The addition of exogenous pyridoxal phosphate up to 5—10 μg/ml to the reaction mixture did not affect the formation of lupinic acid but a higher concentration (100 μg/ml) caused 40—45% inhibition of the synthetase activity: maximum reaction rates were observed in a mixture containing no added pyridoxal phosphate. The addition of hydroxylamine and cyanide (100 μg/ml) to reaction mixtures gave a complete inhibition of lupinic acid synthetase activity.

8) G.N. Atfield and C.J.O.R. Morris, *Biochem. J.*, **81**, 606 (1961).

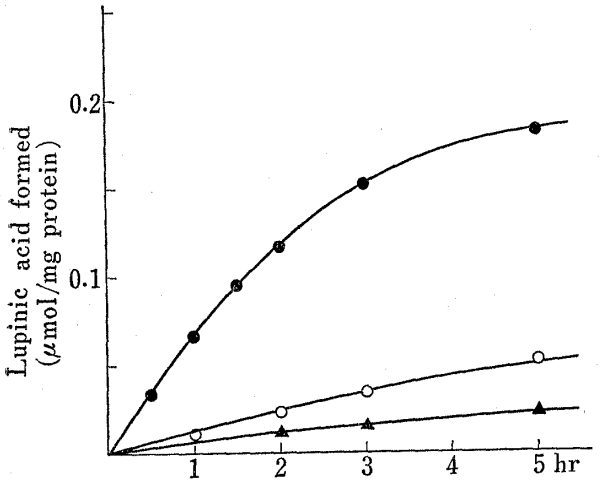

Fig. 2. Comparative Specific Activity for the Lupinic Acid Formation by an Enzyme in the Immature Seeds of *Lupinus luteus* (—●—), and in the Seedlings of *Lupinus luteus* (—○—) and *Citrullus vulgaris* (—▲—)

The Seedlings were grown in the dark for 5—6 days at 30°.

Enzyme preparations from other plant species were examined for their ability to catalyze the formation of lupinic acid from zeatin and O-acetyl-L-serine. The specific activity of enzyme preparations from the immature *Lupinus* seeds was approximately 4 and 8 fold greater than those of the hypocotyls of *Lupinus* seedlings and the whole seedlings of watermelon (*Citrullus vulgaris*), respectively, grown in the dark for 5—6 days at 30°. Enzyme preparations from the etiolated seedlings of *Leucaena leucocephala* and pea (*Pisum sativum*) did not catalyze the synthesis of lupinic acid although analogous reactions for the syntheses of mimosine[3] and β-(3-isoxazolin-5-on-2-yl)-alanine[7] *etc.* have been described in *Leucaena* and *Pisum* seedlings. This suggests that the enzyme from different plant species which catalyze the synthesis of β-substituted alanines from O-acetyl-L-serine have different substrate specificities. The activity of lupinic acid synthetase in the enzyme preparations from 4—6 day-old etiolated seedlings of maize (*Zea mays*) and spinach(*Spinacia oleracea*), and from the immature seeds of maize which are known as a rich source of zeatin derivatives was negligible.

A more detailed study of the enzyme catalyzing the condensation reaction to form lupinic acid is in progress in our laboratories.

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