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Enzymic Syntheses of 3-Hydroxy-5-phenylisoxazole Glucosides, Metabolites of O,O-Diethyl O-(5-Phenyl-3-isoxazolyl)-phosphorothioate in Higher Plants[1]

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Enzyme preparations obtained from *Citrullus*, *Pisum* and *Leucaena* seedlings catalyzed the glucosylation of 3-hydroxy-5-phenylisoxazole (II) by UDP-glucose to yield 3-β-D-glucopyranosyloxy-5-phenylisoxazole (III) and 2-β-D-glucopyranosyl-5-phenyl-4-isoxazolin-3-one (IV).

Some other properties of the enzyme(s) are described.

The enzyme preparations also catalyzed the hydrolysis of III into II and D-glucose, but an analogous reaction for the hydrolysis of IV could not be demonstrated at pH values in the range 4.5 to 6.5.

Many derivatives of 3-isoxazolyl phosphate were synthesized and tested for the insecticidal activity and the oral toxicity of the compounds in mice: it was found by Sampei, *et al.*[3] that especially in O,O-diethyl O-(5-phenyl-3-isoxazolyl)phosphorothioate, mammalian toxicity substantially reduced with little reduction of insecticidal activity.

Studies with carbon-14 labelled O,O-diethyl O-(5-phenyl-3-isoxazolyl)phosphorothioate (I) or 3-hydroxy-5-phenylisoxazole (II) revealed that they were rapidly transformed into 3-β-D-glucopyranosyloxy-5-phenylisoxazole (III) and 2-β-D-glucopyranosyl-5-phenyl-4-isoxazolin-3-one (IV) in higher plants such as cabbage and pea.[4]

The present study is mainly concerned with the enzyme-dependent syntheses of 3-β-D-glucopyranosyloxy-5-phenylisoxazole (III) and 2-β-D-glucopyranosyl-5-phenyl-4-isoxazolin-3-one (IV) from 3-hydroxy-5-phenylisoxazole (II) and uridine-5'-diphosphoglucose(UDP-glucose) catalyzed by extracts from watermelon(*Citrullus vulgaris*), pea(*Pisum sativum*) and *Leucaena leucocephala* seedlings, as shown in Figure 1.

The procedures adapted for the preparation of the enzyme extract, the incubation of reaction mixtures and the analysis of reaction products were essentially the same as described for the enzymic syntheses of glucosyl-metabolites of 3-hydroxy-5-methylisoxazole by *Pisum*, *Citrullus* and *Leucaena* seedling extracts by Murakoshi, *et al.*[5]

Enzyme preparations obtained from *Citrullus* seedlings had greater activity for the rate of formation of IV besides III than those of other plant sources, so that *Citrullus* extracts were used as the source of enzyme for the reaction unless otherwise specified.

The reaction products obtained in these enzymic experiments were characterized as III and IV by their thin-layer chromatographic comparisons on Silica gel G with authentic materials, isolated from plants supplied with I or II, in two solvent systems. Further confir-

1) This work was presented at the 94th Annual Meeting of the Pharmaceutical Society of Japan at Sendai, April 4, 1974 (Meeting Papers, II. p. 218).
2) Location: *a) Yayoi-cho, Chiba*; *b) Hiromachi-1-chome, Shinagawa-ku, Tokyo.*
3) N. Sampei, K. Tomita, H. Tsuji, T. Yanai, H. Oka, and T. Yamamoto, *Ann. Sankyo Res. Lab.* (Tokyo), **22**, 221 (1970).
4) M. Ando, M. Nakagawa, Y. Iwasaki, and K. Tomita, *Agr. Biol. Chem.*, "in press."
5) I. Murakoshi, F. Ikegami, F. Kato, K. Tomita, S. Kamimura, and J. Haginiwa, *Chem. Pharm. Bull.* (Tokyo), **22**, 2048 (1974).

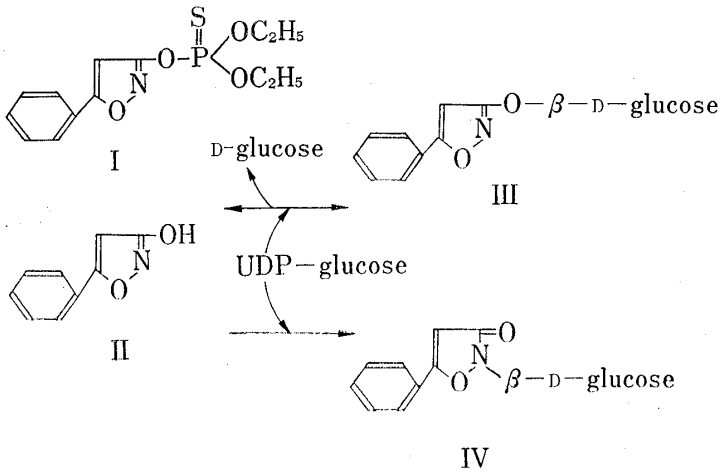

Fig. 1. Scheme for the Biosyntheses of 3-β-D-Glucopyranosyloxy-5-phenylisoxazole (III) and 2-β-D-Glucopyranosyl-5-phenyl-4-isoxazolin-3-one (IV), and for the Hydrolysis of 3-β-D-Glucopyranosyloxy-5-phenylisoxazole (III) by Enzymes in *Citrullus*, *Pisum* and *Leucaena* Seedlings

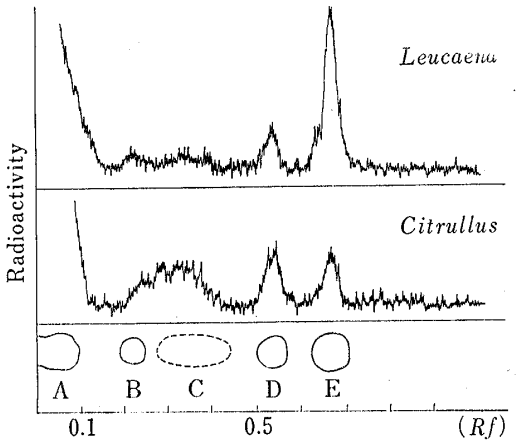

Fig. 2. The Profile of Carbon-14 Incorporation from UDP-(U-^{14}C)-glucose into III and IV by an Enzyme(s) in *Leucaena* and *Citrullus* Seedlings

TLCs were developed in solvent 1: marker spots of A, B, C, D, and E are UDP-glucose, D-glucose, unknown, IV and III, respectively.

mation of the identity of the reaction products as III and IV was obtained by measuring carbon-14 incorporation from UDP-(U-^{14}C)-glucose provided as substrate into III and IV: after separation on thin-layer chromatography (TLC), radioactivity associated with anthrone-positive substances was measured. The migration of the major radioactive spots corresponded with those of the authentic samples, as shown in Figure 2.

III and IV were not formed in reaction mixtures lacking II or UDP-glucose, nor were the products formed when the enzyme extract was pretreated at 100° for 15 min.

Some properties of the enzyme-dependent syntheses of III and IV were studied. The optimum pH values for the formation of III and IV were 8.0, using 0.1M potassium phosphate buffer. The enzyme(s) was active only a narrow pH range (Fig. 3). The amounts of III and IV formed from II and UDP-glucose were proportional to time for at least 80 min and 100 min, respectively, but the rates then decreased: the rate of the formation of III reached a maximum value at about 120 min, whilst other enzyme(s) in incubation mixtures gradually degraded the 3-β-D-glucopyranosyloxy-5-phenylisoxazole (III) initially produced, but the incubation mixtures continued to synthesize IV for at least 5 hr tested, as shown in Figure 4. The optimum concentrations of UDP-glucose for the enzyme-catalyzed formations of III and IV were about 250 mM (the concentration of II was 14.3 mM), as shown in Figure 5. The enzyme exhibited reasonable stability when stored at 0° and, after 26 hr, exhibited about 70—73% of the activity associated with a fresh-prepared extract. The formations of III and IV were not dependent upon added magnesium ion.

Enzyme preparations from *Leucaena* and *Pisum* also catalyzed the syntheses of III and IV as described for *Citrullus* extracts: the specific activity of the enzyme preparations from *Leucaena* was approximately 7—8 fold greater than that catalyzed by *Citrullus* and *Pisum* seedling extracts but, whereas the synthesis of III in incubation mixtures containing *Citrullus* enzyme closed after 120 min, mixtures containing *Pisum* and *Leucaena* enzyme continued to synthesize III for at least 5 hr tested, as shown in Figure 6.

The glucosyltransferase did not catalyze the syntheses of III and IV when D-glucose, D-glucose-1-phosphate, cellobiose and 2′-deoxythymidine-5′-phosphoglucose were tested as glucose donors in lieu of UDP-glucose.

Crude enzyme preparations of *Citrullus*, *Pisum* and *Leucaena* catalyzed the hydrolysis of III into II and D-glucose (Fig. 1), thereby implying a second enzyme system. The optimum pH for this reaction was 6.0 in 0.1M potassium acetate buffer. However the analogous reac-

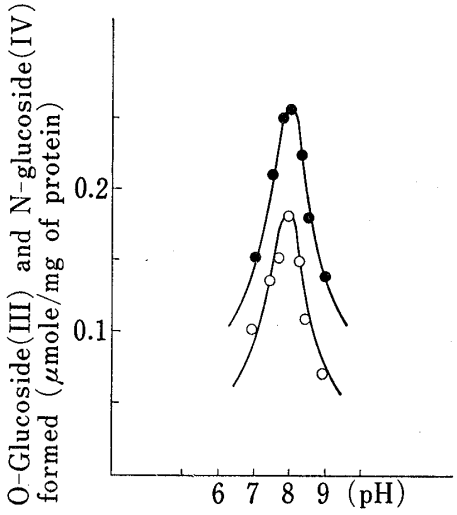

Fig. 3. Effect of pH on the Syntheses of III (Closed Circles) and IV (Open Circles) Catalyzed by Enzyme(s) in *Citrullus* Seedlings

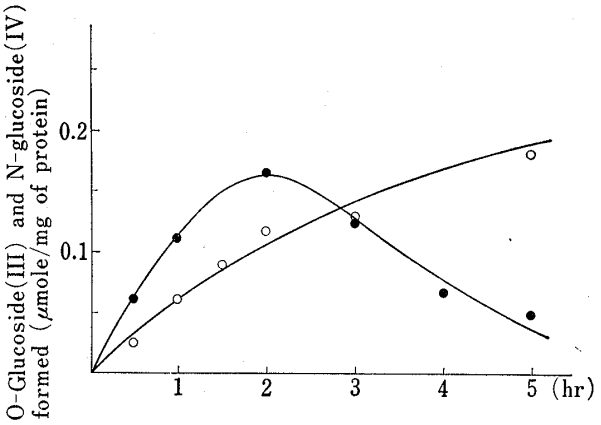

Fig. 4. Time Courses for the Syntheses of III (Closed Circles) and IV (Open Circles) Catalyzed by an Enzyme(s) in *Citrullus* Seedlings

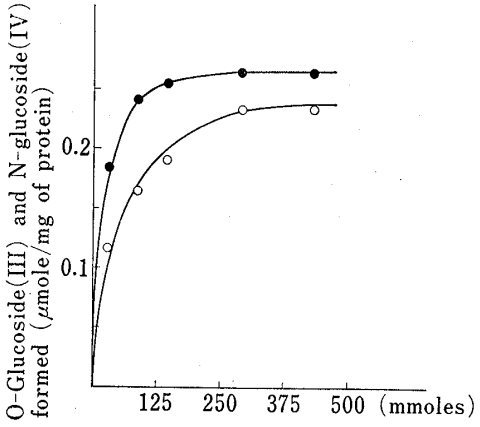

Fig. 5. Effect of UDP-glucose Concentration upon the Formations of III (Closed Circles) and IV (Open Circles) Catalyzed by an Enzyme(s) in *Citrullus* Seedlings

The concentration of II was 14.3 mM.

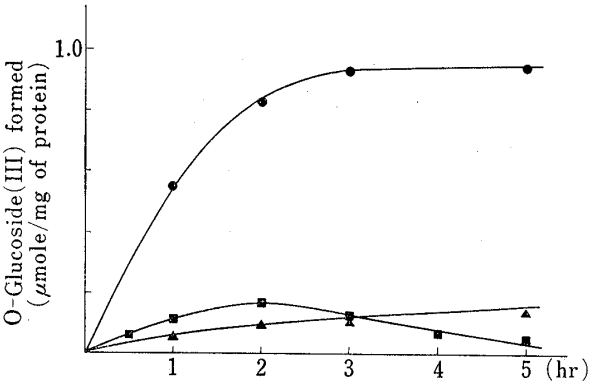

Fig. 6. Comparative Time Courses for the Synthesis of III Catalyzed by an Enzyme(s) in *Pisum* (Closed Triangles), *Citrullus* (Closed Squares), and *Leucaena* (Closed Circles) Seedlings

tion for the hydrolysis of IV could not be demonstrated, as well as that of 2-β-D-glucopyranosyl-5-methyl-4-isoxazolin-3-one in a previous paper.[5]

A more detailed investigation of the synthetase(s) from *Citrullus*, *Pisum* and *Leucaena* responsible for the production of III and IV is in progress in our laboratories.

Experimental

Plant Materials——Watermelon (*Citrullus vulgaris*) seedlings were grown in moistened vermiculite in the dark for 3—4 days ar 30°, pea (*Pisum sativum*) and *Leucaena leucocephala* seedlings for 4—5 days at 30°. After harvest, the testas were removed and the seedlings were cooled at 0° for 30 min before extraction.

Chemicals——3-Hydroxy-5-phenylisoxazole (II) was synthesized by the method of Tomita, *et al.*[6] 3-β-D-Glucopynosyoxy-5-phenylisoxazole (III) and 2-β-D-glucopyranosyl-5-phenyl-4-isoxazolin-3-one (IV)

6) K. Tomita, M. Nagano, T. Yanai, H. Oka, T. Murakami, and N. Sampei, *Ann. Sankyo Res. Lab.* (Tokyo), **22**, 215 (1970).

were obtained from the sources of plants treated with O,O-diethyl O-(5-phenyl-3-isoxazolyl)phosphorothioate (I) or 3-hydroxy-5-phenylisoxazole (II) as described by Sampei, *et al.*[4]

Enzyme Preparations——All operations were carried out at about 0—4°. Enzyme preparations were obtained from *Citrullus*, *Pisum* and *Leucaena* seedlings, from which low mol. wt. substances were removed by treatment with Sephadex G-25 (fine) as described in previous papers.[5,7-11] A portion of the protein eluate was employed immediately in the following experiments as the source of enzyme activity.

Reaction Mixtures——The normal reaction mixtures contained 3-hydroxy-5-phenylisoxazole (5 μmoles), UDP-glucose or UDP-(U-^{14}C)-glucose (50 μmoles, 0.5 μCi) and 0.2 ml of enzyme preparation in a final volume of 0.35 ml. The pH of the incubation mixtures was normally adjusted to pH 8.0 with 0.1 M potassium phosphate buffer. Incubations were conducted at 30° and terminated by addition of 3 volume of ethanol. Precipitated protein was removed by centrifugation, and the supernatant solution was examined chromatographically for the presence of 3-β-D-glucopyranosyloxy-5-phenylisoxazole (III) and 2-β-D-glucopyranosyl-5-phenyl-4-isoxazolin-3-one (IV).

Occasionally, UDP-glucose was replaced by D-glucose, D-glucose-1-phosphate, cellobiose and 2′-deoxythymidine-5′-diphosphoglucose as glucose donors. Unless otherwise specified, enzyme preparations from *Citrullus* seedlings were used as the source of enzyme activity.

Assay of 3-β-D-Glucopyranosyloxy-5-phenylisoxazole (III) and 2-β-D-Glucopyranosyl-5-phenyl-4-isoxazolin-3-one (IV) Formations——The formations of III and IV were demonstrated by subjecting the terminated incubation mixtures to TLC on silica gel G (Merck, Type 60): the compounds were detected with anthrone–H_2SO_4 reagent or iodine vapor as chromogenic reagents. The reaction products co-chromatographed with authentic III and IV in the following solvent systems: 1, ethyl acetate–butan-2-one–formic acid–water (5: 3: 1: 1, by vol); 2, benzene–methanol–dioxane–acetic acid (90: 30: 25: 10, by vol). The *Rf* values for III obtained in these solvents were 0.67 and 0.41, respectively, whilst II exhibited the following *Rf* data; 0.95 and 0.76, respectively. Under the same conditions, UDP-glucose moved at *Rf*'s of 0.08 and 0.03, whilst D-glucose gave the following *Rf* data; 0.22 and 0.12, respectively. *Rf* values determined for IV in solvent systems 1 and 2 were 0.54 and 0.31, respectively. Solvent 1 unequivocably resolved III and IV from II, UDP-glucose and D-glucose.

The formations of III and IV were also determined by measuring the incorporation of radioactivity into these compounds from UDP-glucose in reaction mixtures which were otherwise as described above. Labelled compounds were separated by TLC on silica gel G and radioactivity associated with anthrone-positive substances was measured using a gas-flow radiochromatogram scanner. The migration of the major radioactive spots corresponded with those of authentic III and IV, isolated from intact plants.

The formations of III and IV were quantitatively estimated by eluting the compounds from the TLC plates with water, and by using the anthrone–H_2SO_4 reagent and the general method described by Morris[12] and Scott, *et al.*[13]

Hydrolysis of III into II and D-Glucose by an Enzyme in *Citrullus* Seedlings——The reaction mixture contained 1 μmole of III in 0.1 ml of 0.1 M potassium acetate buffer, pH 6.0, and 0.2 ml of enzyme preparation from *Citrullus* seedlings. The reaction was conducted at 30° for 2 hr. At the end of the incubation, III was quantitatively degraded into II and D-glucose. The optimum pH for the hydrolytic enzyme in *Citrullus* seedlings was 6.0, using 0.1 M potassium acetate buffer. The formation of D-glucose was determined by a specific β-D-glucose oxidase system which was coupled to O-dianisidine as a chromogenic reagent as described in previous papers.[11,14] In some experiments, III was replaced by IV.

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