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Enzymic Synthesis of 3-Hydroxy-5-methylisoxazole Glucosides, Metabolites of 3-Hydroxy-5-methylisoxazole in Higher Plants[1]

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Crude extracts prepared from *Citrullus*, *Pisum* and *Leucaena* seedlings catalyze the glucosylation of 3-hydroxy-5-methylisoxazole (I) by uridine-5′-diphosphoglucose to yield 3-β-D-glucopyranosyloxy-5-methylisoxazole (II). The enzyme(s) from *Citrullus* also catalyzed the synthesis of 2-β-D-glucopyranosyl-5-methylisoxazolin-3-one (III), although at a very slow rate. The optimum pH of the reaction was 7.5. Some other properties of the enzyme(s) are also described.

Extracts of *Citrullus* also catalyzed the hydrolysis of II into I and D-glucose. The optimum pH for this reaction was 5.5. An analogous reaction for the hydrolysis of III could not be demonstrated.

3-Hydoxy-5-methylisoxazole (I) is an effective fungicide against the damping-off organisms of higher plants and also acts as a plant growth-regulator.[3–6] When ^{14}C-labelled 3-hydroxy-5-methylisoxazole was supplied to plants it was rapidly transformed into 3-β-D-glucopyranosyloxy-5-methylisoxazole (II) and 2-β-D-glucopyranosyl-5-methyl-4-isoxazolin-3-one (III).[7]

The present study is mainly concerned with the enzyme-dependent syntheses of 3-β-D-glucopyranosyloxy-5-methylisoxazole (II) and 2-β-D-glucopyranosyl-5-methylisoxazolin-3-one (III) from 3-hydroxyl-5-methylisoxazole (I) and uridine-5′-diphosphoglucose (UDP-glucose) catalyzed by extracts from watermelon (*Citrullus vulgaris*), *Leucaena leucocephala* and pea (*Pisum sativum*) seedlings.

Enzyme preparations used in the investigation were crude extracts of *Citrullus*, *Leucaena* and *Pisum* seedlings, from which low molecular weight substances were removed by treatment with Sephadex G-25 (fine) as described in previous papers.[8–12] Unless otherwise specified, extracts prepared from *Citrullus* seedlings were used as the source of enzyme.

The normal reaction mixture used to demonstrate the enzyme-dependent syntheses of II and III is described in the experimental section. An analogous radioisotopic method is also described.

The reaction products formed in the normal and radioisotopic assays were characterized as II and III by chromatographic comparison with authentic natural materials in a thin-layer of silica gel G in solvents 1, 2 and 3. II and III were not formed if I or UDP-glucose were

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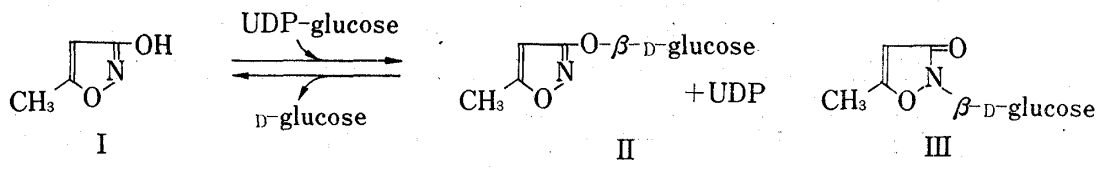

Fig. 1. Scheme for the Biosyntheses of II and III, and Its Hydrolysis by Enzymes in *Citrullus*, *Pisum*, and *Leucaena* Seedlings

omitted from the reaction mixture. Similarly, II and III were not formed when the enzyme extract was pretreated at 100° for 15 min.

Some properties of the enzyme-dependent syntheses of II and III using *Citrullus* extracts were studied: the products were quantitatively estimated by the anthrone method for carbohydrates described by Morris[13] and Scott, *et al.*[14] The optimum pH for the formation of II was 7.5, using 0.1M potassium phosphate buffer. The amount of II formed from I and UDP-glucose was proportional to time for at least 90 min; the formation of II continued for a further 4.5 hr although the rate of formation decreased (Fig. 2). The optimum concentration of UDP-glucose for the synthesis of II was about 290 mM (Fig. 3). Under the same conditions, the enzyme(s) prepared from *Citrullus* seedlings also catalyzed the formation of III but the rate of formation was only 3—4% of the rate for the formation of II. The glucosyltransferase did not catalyze the synthesis of II when D-glucose, D-glucose-1-phosphate and cellobiose were tested as glucose donors in lieu of UDP-glucose. Enzyme activity slowly decreased during storage at 0° in the dark; after 26 hr the activity decreased by 23%.

Enzyme preparations from *Pisum* and *Leucaena* also catalyzed the synthesis of II as described for *Citrullus* extracts, but the formation of III could not be detected. The initial rate of formation of II catalyzed by enzyme preparations from *Leucaena* was less than that catalyzed by *Citrullus* extracts but the rate of formation of II catalyzed by *Pisum* extracts was slightly greater than for *Citrullus* during a time course of 120 min (Fig. 2). The formation of glucosides was not dependent upon added magnesium ion.

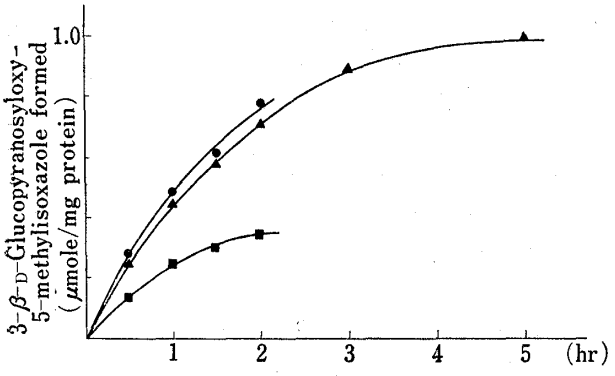

Fig. 2. The Comparative Time Courses for the Synthesis of 3-β-D-Glucopyranosyloxy-5-methylisoxazole

Catalyzed by extracts of *Pisum* (●—●), *Citrullus* (▲—▲) and *Leucaena* (■—■) seedlings: incubation conditions were as described in the text.

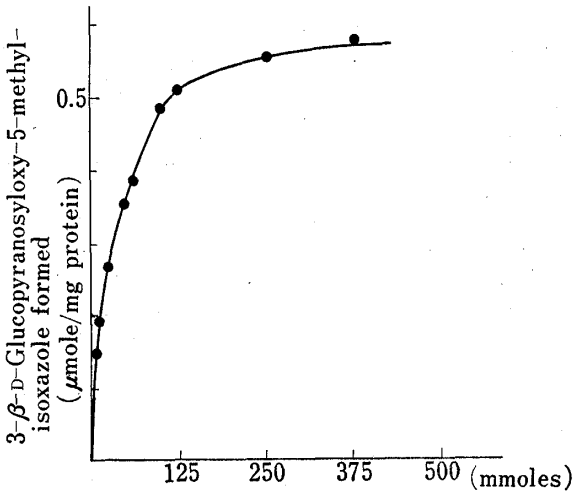

Fig. 3. Effect of UDP-Glucose Concentration on the Formation of 3-β-D-Glucopyranosyloxy-5-methylisoxazole catalyzed by Extract of *Citrullus* seedlings

Incubation concentrations were as specified in the tex except that the amount of UDP-glucose was replaced with the concentrations specified.

13) D.L. Morris, *Science*, **107**, 254 (1948).
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Crude extracts of *Citrullus*, *Leucaena* and *Pisum* catalyzed the hydrolysis of II into I and D-glucose (Fig. 1) thereby implying a second enzyme system. The optimum pH for this reaction was 5.5 in 0.1 M potassium acetate buffer. However the analogous reaction for the hydrolysis of III could not be demonstrated.

At present, it is not certain whether the formation of II and III *in vivo* and *vitro* is catalyzed by the same enzyme and a more thorough examination using purified enzymes is being undertaken in these laboratories in an attempt to answer this question.

Experimental

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

Enzyme Preparation——All operations were carried out at about 0°. Seedlings were macerated in 0.1 M potassium phosphate buffer, pH 7.5, containing 1% (v/v) 2-mercaptoethanol (3 ml/10 g of tissue). The extract was expressed through fine muslin and centrifuged at 25000 g for 30 min. The supernatant solution was applied to a column of Sephadex G-25 (fine) equilibrated with 0.1 M potassium phosphate buffer, pH 7.5, and the same buffer was used to elute the protein fraction (enzyme) free from low mol. wt. substances as described in previous papers.[8-12] The eluted protein was used as the source of enzyme activity.

Reaction Mixtures——The normal reaction mixtures contained 3-hydroxy-5-methylisoxazole (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; extracts from *Citrullus* were used as the source of enzyme unless otherwise specified. The pH of the incubation mixture was normally adjusted to pH 7.5 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-methylisoxazole (II) and 2-β-D-glucopyranosyl-5-methylisoxazolin-3-one (III). In some experiments, UDP-glucose was replaced by D-glucose, D-glucose-1-phosphate and cellobiose as glucose donors.

Assay of 3-β-D-Glucopyranosyloxy-5-methylisoxazole (II) and 2-β-D-Glucopyranosyl-5-methylisoxazolin-3-one (III) formation——The formation of II and III was demonstrated by subjecting the terminated incubation mixture to thin-layer chromatography (TLC) on silica gel (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 II and III in the following solvent systems: 1, butan-1-ol–acetic acid–water (8: 2: 3, by vol.); 2, ethyl acetate–pyridine–water (10: 4: 3, by vol.); 3, ethyl acetate–butan-2-one–formic acid–water (5: 3: 1: 1, by vol.). The *Rf* values for II obtained in these solvents were 0.52, 0.60 and 0.63, respectively, whilst I had the following *Rf* data; 0.81, 0.73 and 0.95, respectively. Under the same conditions, UDP-glucose moved at *Rf*'s of 0.06, 0.05 and 0.06, whilst D-glucose gave the following *Rf* data; 0.28, 0.29 and 0.22, respectively. *Rf* values determined for III in solvent systems 1, 2 and 3 were 0.35, 0.39 and 0.33, respectively. Solvents 1 and 3 clearly resolved II and III from I, UDP-glucose and D-glucose.

The formation of II and III was also determined by measuring the incorporation of radioactivity into these compounds from UDP-(U-^{14}C)-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 the migration of authentic II and III isolated from intact plants.

The formation of II and III was quantitatively estimated by eluting the compounds from the TLC plates with water and using the anthrone-H_2SO_4 reagent and the general method described by Morris[13] and Scott, *et al.*[14]

Hydrolysis of II into I and D-Glucose by *Citrullus* Seedling Extracts——The reaction mixture contained 2 μmoles of II in 0.1 ml of 0.1 M acetate buffer, pH 5.5, 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, II was quantitatively degraded into I and D-glucose. The optimum pH for the hydrolytic enzyme in *Citrullus* seedlings was 5.5, using 0.1 M acetate buffer. The formation of D-glucose was determined by the same method as described in a previous paper.[15] In some experiments, II was replaced by III.

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