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ENZYMATIC SYNTHESIS OF ISOXAZOLINONE GLUCOSIDE BY EXTRACTS FROM *PISUM* AND *LATHYRUS* SEEDLINGS

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Key Word Index—*Lathyrus odoratus*; sweet pea; *Pisum sativum*; pea; Leguminosae; heterocyclic glucoside; isoxazolinone glucoside; UDP-glucose; biosynthesis.

Abstract—Enzymatic synthesis of 2-β-D-glucopyranosyl-3-isoxazolin-5-one is described, using isoxazolin-5-one and UDP-glucose as precursors and enzyme extracts of *Pisum sativum* and *Lathyrus odoratus*. *Lathyrus* extracts show a three-fold higher activity than those of *Pisum*.

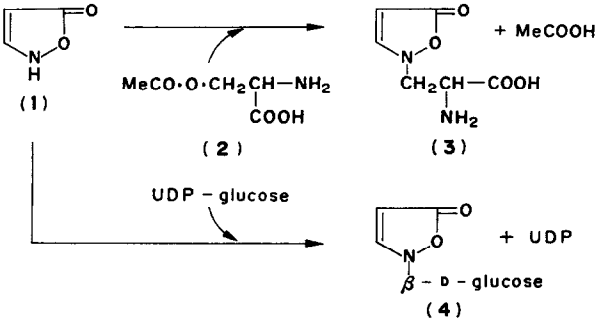
A heterocyclic glucoside 2-β-D-glucopyranosyl-3-isoxazolin-5-one (**4**) has recently been isolated in small quantities by Lambein *et al.* [1] and Van Rompuy *et al.* [2] from sweet pea (*Lathyrus odoratus*) seedlings together with other 3-isoxazolin-5-one derivatives such as β-(3-isoxazolin-5-on-2-yl)-alanine (**3**), 2-(2-cyanoethyl)-3-isoxazolin-5-one [2] and 2-carboxyl-methyl-3-isoxazolin-5-one [2].

In a recent report, Murakoshi *et al.* [4] have shown that β-(3-isoxazolin-5-on-2-yl)alanine (**3**) can be synthesized by an enzyme in pea seedlings from 3-isoxazolin-5-one (**1**) and *O*-acetylserine (**2**), and not serine itself, analogous to the synthesis of heterocyclic β-substituted alanines in higher plants [5–7].

This paper reports the presence and some properties of a condensing enzyme in *Pisum sativum* and *L. odoratus* seedlings that catalyzes the synthesis of 2-β-D-glucopyranosyl-3-isoxazolin-5-one (**4**) from 3-isoxazolin-5-one (**1**) and uridine-5′-diphosphoglucose (UDP-glucose) as shown in the scheme.

Enzyme preparations used in the investigation were crude extracts of *Pisum* and *Lathyrus* seedlings, from which low MW substances were removed by treatment with Sephadex G-25 as described in previous papers [4–8]. Unless otherwise specified, extracts prepared from *Pisum* seedlings were used.

The reaction product obtained from the enzyme experiments was characterized as **4** by its TLC behaviour on silica gel G in comparison with that of authentic natural 2-β-D-glucopyranosyl-3-isoxazolin-5-one [1, 2].



Isoxazolinone glucoside (**4**) was clearly separated from UDP-glucose and glucose when chromatograms were developed in solvents a and b (see Experimental). The glucoside (**4**) was not formed in reaction mixtures lacking either 3-isoxazolin-5-one (**1**) or UDP-glucose. The product was also not formed when the enzyme extract was pretreated at 100° for 15 min.

Further confirmation of the identity of the reaction product was obtained by measuring ^{14}C incorporation from UDP-glucose [U-^{14}C] provided

as a substrate into 2-β-D-glucopyranosyl-3-isoxazolin-5-one (**4**): after separation by TLC, radioactivity associated with anthrone-positive substances was measured using a gas-flow radiochromatogram scanner. The migration of the major radioactive spot corresponds with that of the natural product isolated from the intact plants.

Some properties of the enzyme-dependent synthesis of 2-β-D-glucopyranosyl-3-isoxazolin-5-one (**4**) were studied. The time course of **4** formation was proportional to time for at least 90 min; the rate then decreased. The optimum pH for the enzyme dependent synthesis of **4** was 7·5, using 0·1 M potassium phosphate buffer; the enzyme was active only in the pH range 6–9. The isoxazolinone glucoside synthetase activity was dependent upon the concentration of UDP-glucose used but a concentration of UDP-glucose of 125 M was sufficient to give maximum rates.

The enzyme was unstable and in two experiments crude plant extracts were stored at 0° for 25 hr. The residual enzyme activity was 60 and 65%, respectively, of the initially assayed activity. The formation of **4** was not influenced by added Mg^{2+}.

Enzyme preparations from 4 to 5-day-old etiolated seedlings of *L. odoratus* also catalyzed the synthesis of **4** as described for *Pisum* extracts: the specific activity of extracts from *Lathyrus* was *ca* three-fold greater than those of extracts from *Pisum*.

The glucosyltransferase did not catalyze the synthesis of **4** when D-glucose, D-glucose-1-phosphate, cellobiose and 2′-deoxythymidine-5′-diphosphoglucose were tested as glucose donors in place of UDP-glucose.

Crude extracts of *Pisum* and *Lathyrus* also did not catalyze the hydrolysis of 2-β-D-glucopyranosyl-3-isoxazolin-5-one (**4**) into 3-isoxazolin-5-one (**1**) and D-glucose at pH values in the range 4·5–6·5.

EXPERIMENTAL

Plant materials. P. sativum seedlings were grown in moistened vermiculite in the dark for 4 days at 30°, and *L. odoratus* seedlings for 5–6 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 4–5°. Enzyme extracts were prepared by grinding 5 g of seedlings with a little quartz sand and 1·5 ml of 0·2 M KPi buffer, pH 7·5, containing 0·5% 2-mercapto-ethanol. The mixture was filtered through a fine muslin and the homogenate was centrifuged at 25000 *g* for 30 min at 0°. The supernatant was passed through a Sephadex G-25 (fine) column equilibrated with 0·1 M KPi buffer, pH 7·5, and the same buffer was used to elute the protein fraction (enzyme preparation) as described in previous papers [4–8]. A portion of the protein eluate was used immediately in the following experiments as the source of enzyme activity.

Reaction mixtures. The normal reaction mixtures contained 3-isoxazolin-5-one (5 μmol), UDP-glucose or UDP-glucose-[U-^{14}C] (30 μmol, 0·5 μCi) and 0·2 ml of enzyme preparation in a final vol. of 0·4 ml. The pH of the incubation mixture was normally 7·5 using 0·1 M KPi buffer. The mixture was incubated at 30° and the reaction terminated, usually after 2 hr, by the addition of three vol of 99% EtOH. Precipitated protein was removed by centrifuging, and the supernatant was examined chromatographically for the presence of 2-β-D-glucopyranosyl-3-isoxazolin-5-one. Occasionally, UDP-glucose was replaced by D-glucose, D-glucose-1-phosphate, cellobiose and 2′-deoxythymidine-5′-diphosphoglucose as glucose donors.

Assay of 2-β-D-glucopyranosyl-3-isoxazolin-5-one (**4**) *formation.* The formation of **4** in the residual supernatant could be followed by TLC on Si gel G and detection, using anthrone–H_2SO_4 reagent or I_2 vapor. It was shown to be identical with the authentic material by co-chromatography in the following solvent systems: a, pHOH–H_2O–EtOH (3:1:1, by wt); b, *n*-BuOH–HOAc–H_2O (12:3:5); c, *iso* PrOH–H_2O (8:2). The R_f values for **4** obtained in these solvents were 0·49, 0·51, and 0·79, respectively, whilst UDP-glucose had the following R_f values: 0·05, 0·08, and 0·66 respectively. In the same solvents, glucose had R_f values of 0·27, 0·43, and 0·77, so that formation of **4** was established most conclusively by using solvents a and b. Further confirmation of the identity of the reaction product was obtained by measuring ^{14}C incorporation from UDP-glucose-[U-^{14}C] into **4**, using a gas-flow radiochromatogram scanner. Quantitative determinations of **4** were also made using the anthrone–H_2SO_4 reagent and the general method for carbohydrates described by Morris [9], and Scott *et al.* [10], after eluting the **4**-band from the Si gel with H_2O.

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