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ENZYMIC ALANYLATION OF AN ISOXAZOLINONE GLUCOSIDE BY LEGUME SEEDLING EXTRACTS

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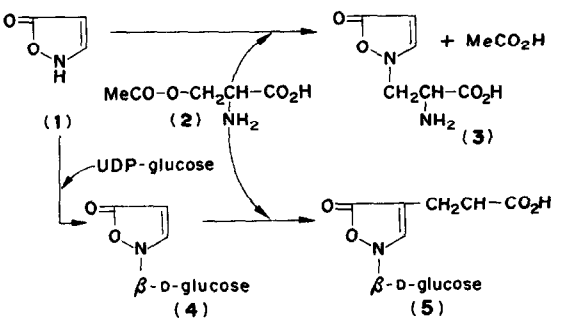
Key Word Index—*Lathyrus odoratus*; *Pisum sativum*; *Leucaena leucocephala*; Leguminosae; *Citrullus vulgaris*; Cucurbitaceae; heterocyclic glucoside; isoxazolinone glucoside; isoxazolinone amino acid; enzymology of biosynthesis.

Abstract—Enzymic synthesis of the natural product β-(2-β-D-glucopyranosyl-3-isoxazolin-5-on-4-yl) alanine is described, using the natural isoxazolinone glucoside and *O*-acetyl-L-serine as substrates and extracts from seedlings as enzyme preparations. *Lathyrus odoratus* extracts show a higher activity than those of *Pisum sativum*, *Citrullus vulgaris* and *Leucaena leucocephala*.

INTRODUCTION

Two UV-sensitive heterocyclic β-substituted alanines have been isolated by Lambein *et al.* from pea (*Pisum sativum*) seedlings [1,2]. The structures assigned on the basis of chemical and spectroscopic methods were β-(3-isoxazolin-5-on-2-yl)alanine (**3**) and β-(2-β-D-glucopyranosyl-3-isoxazolin-5-on-4-yl)alanine (**4**). Recently, they were isolated also from field pea (*Pisum arvense*), lentil (*Lens culinaris*) and sweet pea (*Lathyrus odoratus*) seedlings [3]. These amino acids are not present in the dry seeds, but they are rapidly formed during the germination.

In recent reports Murakoshi *et al.* [4–6] have described enzymic systems from plants which catalyze the synthesis of heterocyclic β-substituted alanines; serine is first converted to an *O*-acetyl derivative, which undergoes condensation with the appropriate heterocyclic compounds to yield the heterocyclic β-substituted alanines such as mimosine [4], β-pyrazolylalanine [4], quisqualic acid [5], and 3-amino-1,2,4-triazolylalanine [6]. By extension of these observations, Murakoshi *et al.* [7] have reported that crude extracts of *Pisum* seedlings catalyze the synthesis of β-(3-isoxazolin-5-on-2-yl)alanine (**3**) from *O-acetyl*-L-serine (**2**) and 3-isoxazolin-5-one (**1**) as shown in Scheme 1.



Scheme 1.

We have now further extended these observations and shown that β-(2-β-D-glucopyranosyl-3-isoxazolin-5-on-4-yl)alanine (**5**) may be synthesized from *O*-acetyl-L-serine (**2**) and 2-β-D-glucopyranosyl-3-isoxazolin-5-one (**4**) by an analogous reaction catalyzed by extracts of *Pisum* and *Lathyrus* seedlings as shown in Scheme 1; neither serine nor *O*-phosphoserine could serve as a donor of the alanyl-moiety. The isoxazolinone glucoside

(**4**) also has been found in *Lathyrus* seedlings by Lambein *et al.* [8] and Van Rompuy *et al.* [9], and synthesized enzymically by Murakoshi *et al.* [10].

RESULTS

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 previously [4–7,10]. The enzyme systems and the standard incubation mixtures capable of synthesizing the alanine derivative (**5**) are described in the Experimental. The reaction product formed in the radioisotopic and non-radioisotopic assays was characterized as **5** by chromatographic comparison with the authentic material. Paper chromatography in two systems showed the presence of a ninhydrin-positive compound with the same R_f as **5** (see Experimental). After ninhydrin spray, both the reaction product and authentic **5** gave a pale yellow brown color that subsequently turned violet. The reaction product was also identified as **5** by TLC on silica gel G by using anthrone–H_2SO_4 spray to visualize carbohydrates. Standard amino acid analysis also confirmed the identity: both the enzymic reaction product and an authentic sample of **5** eluted from the column at a position between taurine and *S*-methylcysteinesulfoxide.

In the radioisotopic assays *O*-acetyl-L-serine-[3-^{14}C], was provided as a substrate for the enzymic alanylation, and after incubation, the reaction mixture was analyzed. In the elution profiles of the amino acid analyzer, coincidence was found between peaks of ninhydrin-positive material and of radioactivity emerging at the same position as an authentic sample of **5**. Scanning of paper chromatograms also showed incorporation of the [^{14}C]-labeled substrate into **5**.

Some properties of the enzyme-dependent synthesis of β-(2-β-D-glucopyranosyl-3-isoxazolin-5-on-4-yl)alanine (**5**) were studied. Unless otherwise specified, enzyme preparations obtained from *Pisum* seedlings were used as the source of enzyme activity. The rate of synthesis of **5** was constant for at least 120 min but the rate then decreased. The optimum pH for the enzyme-dependent synthesis of **5** was 7·5 (0·1 M potassium phosphate buffer). The enzyme was active only in a narrow pH range (6·5–8·5).

Since only small amounts of **4** were available, the effect of the concentration of **4** on the formation of **5** was not studied in detail.

The enzyme was reasonably stable. When stored at 0° for 25 hr the remaining activity was about 75% of the activity associated with a freshly prepared extract. Addition of pyridoxal-5′-phosphate up to 25 μg/ml to the reaction mixture caused neither stimulation nor inhibition of **5** formation.

Enzyme preparations from other plant species were examined for their ability to catalyze the formation of **5** from *O*-acetyl-L-serine and the natural isoxazolinone glucoside (**4**). The specific activity of enzyme preparations from *Lathyrus* seedlings was approximately 1·5-fold greater than the enzyme preparations from *Pisum* seedlings. Enzyme preparations [4] from watermelon (*Citrullis vulgaris*) and *Leucaena leucocephala*, catalyzing the formation of β-pyrazolylalanine and mimosine, also catalyzed the synthesis of **5** as described for *Pisum* and *Lathyrus* seedling extracts. The initial rates of formation of **5** by *Citrullus* and *Leucaena* enzyme preparations were only slightly lower than those obtained with *Pisum* seedling extracts.

For three natural occuring isoxazolinone derivatives the enzymic synthesis has been described starting from the free isoxazolinone ring (**1**). While this heterocyclic ring could be formed by chemical interaction of hydroxylamine with nucleic acids, [11,12] no biosynthetic pathway has been reported.

The question whether or not the alanylation at the N-2 position of the free isoxazolinone ring [7] and the alanylation at the C-4 position of the isoxazolinone glucoside involves the same enzyme system is currently under investigation.

EXPERIMENTAL

Labeled chemicals. *O*-acetyl-L-serine-[3-^{14}C] was synthesized from L-serine-[3-^{14}C] [13]. The product was diluted with unlabeled *O*-acetyl-L-serine to obtain the sp. act. as used in the different incubation mixtures.

Plant materials. Pea (*Pisum sativum*), watermelon (*Citrullus vulgaris*) and *Leuceana leucocephala* seedlings had grown in moistened vermiculite in the dark for 3–4 days at 30°, and sweet pea (*Lathyrus 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 preparations. All operations were carried out at about 0°. Seedlings were macerated in 0·2 M K–Pi buffer, pH 7·5, containing 0·5% (w/v) 2-mercaptoethanol (3 ml/10 g seedlings). The extract was pressed through fine muslin and centrifuged at 25000 *g* for 30 min. The supernatant was applied to a column of Sephadex G-25 (fine) equilibrated with 0·1 M K–Pi buffer, pH 7·5, and the same buffer was used to elute the protein fraction and to remove the low MW substances as described in previous papers [4–7,10]. The eluted protein fraction was used immediately as the source of enzyme activity.

Reaction mixtures. The normal reaction mixtures contained 2-β-D-glucopyranosyl-3-isoxazolin-5-one (10 μmol), *O*-acetyl-L-serine or *O*-acetyl-L-serine-[3-^{14}C] (5 μmol, 0·5 μCi) and 0·2 ml of enzyme preparation in a final volume of 0·4 ml. The pH of the incubation mixtures was normally adjusted to pH 7·5 with 0·1 M K–Pi buffer. The mixtures were incubated at 30° and the reaction was terminated by addition of 3 vol. EtOH. The pptd protein was removed by centrifugation, and the supernatant solution was examined chromatographically for the presence of **5**. Occasionally, *O*-acetyl-L-serine was replaced by L-serine or *O*-phosphoserine as a donor of the alanyl moiety. Pyridoxal-5′-phosphate (5–25 μg/ml) was added to certain reaction mixtures.

Assay of β-(2-β-D-glucopyranosyl-3-isoxazolin-5-on-4-yl)-alanine (**5**) *formation.* The formation of **5** was demonstrated by subjecting the supernant solutions to chromatography, using ninhydrin and anthrone–H_2SO_4 as chromogenic reagents. The product formed was identical with the authentic material (**5**). Paper chromatographic procedures were carried out in: (1) *iso*PrOH–HCOOH–H_2O (20:1:5, by vol); (2) *iso*-PrOH–H_2O (7:3, v/v). R_f values for **5**, *O*-acetyl-L-serine and L-serine in solvent (1) were 0·17, 0·47 and 0·33 respectively and in solvent (2) 0·23, 0·50 and 0·37 respectively. The reaction product was also characteristized as **5** by TLC on Si gel G (Merck type 60) with PhOH–H_2O–EtOH (3:1:1, by wt) by using anthrone–H_2SO_4 for carbohydrates. R_f values of **5**, **4** and D-glucose were 0·11, 0·49 and 0·27, respectively.

Further confirmation of the identity of the reaction product as **5** was obtained using AAA under standard operating conditions (150 cm column 50°, 0·2 N NaCi buffer, pH 3·25, flow rate 0·5 ml/min), **5** was eluted from the column at about 162 min (83–84 ml). between the peaks due to taurine and S-methylcysteinesulfoxide. Radioactivity associated with each amino acid peak was recorded with a Packard monitoring flow system coupled to a ratemeter. When paper chromatograms were developed in solvent (1), radioactivity associated with each ninhydrin-positive substance on the chromatograms was determined using a gas-flow 4π radiochromatogram scanner.

Quantitative determinations of **5** was also made using the cadmium–ninhydrin reagent and the general method described by Atfield and Morris [14] as reported in the previous papers [4–7].

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