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THE BIOSYNTHETIC ORIGIN OF THE PYRAZOLE MOIETY OF β-PYRAZOL-1-YL-L-ALANINE

ERIC G. BROWN, KHAWOLA A. M. FLAYEH and JOHN R. GALLON

Department of Biochemistry, University College of Swansea, Singleton Park, Swansea SA2 8PP, Wales, U.K.

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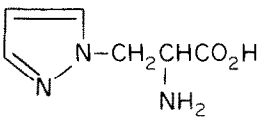
Key Word Index—*Cucumis sativus*; Cucurbitaceae; cucumber; pyrazole; β-pyrazol-1-yl-L-alanine; 1,3-diaminopropane; biosynthesis.

Abstract—Biosynthetic studies implicate 1,3-diaminopropane as a precursor of the pyrazole moiety of β-pyrazol-1-yl-L-alanine.

INTRODUCTION

β-Pyrazol-1-yl-L-alanine (**1**), an isomer of histidine, was first detected [1] in the juice of water melon (*Citrullus vulgaris*). Elucidation of its structure is described in refs. [2, 3]. This non-protein amino acid



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has since been found, together with the peptide γ-L-glutamyl-β-pyrazol-1-yl-L-alanine in seeds of many species of Cucurbitaceae [4]. The free amino acid is present to the extent of 1 g/kg of water melon seeds [3].

It has been shown that extracts of cucurbit seedlings form β-pyrazolylalanine from pyrazole and *O*-acetylserine and that the enzymic reaction requires pyridoxal 5-monophosphate as a cofactor [5, 6]. The present work concerns a preliminary study of the biosynthetic origin of the pyrazole ring of β-pyrazolylalanine. Pyrazole iself has been reported to occur in cucumber seeds [5, 7] and is the limiting factor in the synthesis of β-pyrazolylalanine by cucumber seedlings [3]. The biosynthesis of the pyrazole moiety is of biochemical interest in being one of the few known examples of the biological formation of a N—N covalent bond.

RESULTS AND DISCUSSION

Metabolism of pyrazole by germinating cucumber seeds

Cucumber seeds were allowed to imbibe a solution containing 3.6 μCi [3,4-^{14}C]pyrazole and germinated for 70 hr. An ethanolic extract was prepared and examined by two-dimensional PC using butan-1-ol:ammonia in one direction and butan-1-ol:acetic acid:water in the other (see Experimental). Radioactive metabolites were eluted and their radioactivity determined (Table 1). Comparison of their chromatographic behaviour in several different solvent systems with that of authentic samples of known pyrazole derivatives, identified two of the major radioactive spots (P_1 and P_3) as γ-glutamyl-β-pyrazol-1-yl-alanine and β-pyrazol-1-yl-alanine, respectively. High voltage electrophoresis confirmed this identification. Of the total radioactivity recovered, 94% was in β-pyrazolylalanine and 2.4% in the peptide, γ-glutamyl-β-pyrazolylalanine. The remaining 3.6% was distributed amongst the four unidentified metabolites, most of it in P_2 (Table 1).

Production of β-pyrazolylalanine and its peptides during growth

The β-pyrazolylalanine content of seeds and young seedlings of *Cucumis sativus* was determined at various stages of germination and growth. The results (Fig. 1) showed an increased content during

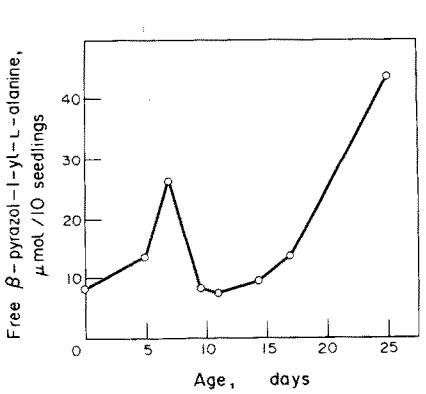

Fig. 1. Amount of free β-pyrazolylalanine present in cucumber seeds and seedlings during germination and growth. Mature dry seeds of *Cucumis sativus* were put to soak at zero time. The data shown are from a single experiment but the trends were reproducible in four separate experiments.

Table 1. Distribution of radioactivity amongst metabolites of [3,4-^{14}C]pyrazole in germinating cucumber seeds

^{14}C-Metabolite	Identity	$R_f \times 10^2$ Solvent 1	$R_f \times 10^2$ Solvent 2	Total incorporation (dpm $\times 10^{-2}$)
P_1	γ-L-Glutamyl-β-pyrazol-1-yl-L-alanine	5	9	169
P_2	—	5	15	93
P_3	β-Pyrazol-1-yl-L-alanine	16	27	6633
P_4	—	29	39	51
P_5	—	14	73	61
P_6	—	16	57	53

Solvent 1 was *n*-BuOH satd with NH_4OH (s.g. 0.88) and solvent 2 was *n*-BuOH–HOAc–H_2O (90:10:29). P_2, and P_4–P_6 were unidentified.

the first 7 days after imbibition had begun. This was followed during the next 2 days by a quantitatively comparable decrease and then a sharp rise in concentration which was still continuing at the end of the 25 day period examined. The initial increase in the β-pyrazolylalanine content of germinating seeds and seedlings has been previously observed [7] and attributed, at least in part, to the hydrolysis of γ-L-glutamyl-β-pyrazol-1-yl-alanine. As Table 2 shows, the β-pyrazolylalanine content of young fruits increased with weight but in the older, larger fruits the content had decreased substantially.

To assess the amount of β-pyrazolylalanine present as peptide in seeds, seedlings and fruits, the content of this amino acid in plant extracts was determined before and after acid hydrolysis. The results (Table 2) showed that in dry, mature seeds of *Cucumis sativus*, *ca* 72% of the total β-pyrazolylalanine is present as peptide. However, 7 days after being set to germinate, all the β-pyrazolylalanine peptide had disappeared with a concomitant increase in the content of the free amino acid. No peptide containing β-pyrazolylalanine could be detected throughout the subsequent 25 day period. Within the fruits, although no β-pyrazol-yl-alanine peptide was present initially, its concentration increased substantially with age and weight (Table 2). These observations confirm the earlier report [7] that the initial increase in β-pyrazolylalanine content (Fig. 1) during germination and growth of young seedlings is attributable to hydrolysis of β-pyrazolylalanine-containing peptides but they additionally show that the increase which begins later is due to *de novo* synthesis of free β-pyrazolylalanine. As there was a considerably higher total content of β-pyrazolylalanine (free + peptide) in the younger fruits than in the older larger ones (Table 2), it is apparent that the β-pyrazolylalanine is extensively metabolized to compounds

Table 2. Content of β-pyrazolylalanine in (a) growing seedlings and (b) developing fruit of *Cucumis sativus*

	Age of seedlings (days)	Free β-pyrazolylalanine (μmol/10 seedlings)	Peptide β-pyrazolylalanine (μmol/10 seedlings)	Total β-pyrazolylalanine (μmol/10 seedlings)
(a)	0	9.8	25	34.8
	7	26.7	0	26.7
	17	15.47	0	15.47
	25	42.4	0	42.4
	Wt of fruit (g)	**Free β-pyrazolylalanine (μmol/fruit)**	**Peptide β-pyrazolylalanine (μmol/fruit)**	**Total β-pyrazolylalanine (μmol/fruit)**
(b)	10.5	10.48	0	10.48
	16.8	26.31	9.47	35.78
	43.7	477.19	28.07	505.26
	287.0	93.80	51.58	145.38

These results represent a typical set of the analytical data obtained.

other than the peptides. The nature of these products is obscure but they may be identical to the unidentified compounds observed to be formed from [^{14}C]pyrazole in germinating seeds of *Cucumis sativus* (Table 1).

Examination of possible precursors of pyrazole

In order to screen some possible precursors of pyrazole, the incorporation of [3,4-^{14}C]pyrazole into pyrazolylalanine and its peptides by excised shoots of 23-day-old cucumber seedlings was assessed in the presence and absence of the suspected precursor. The shoots were washed and extracted 24 hr after being exposed to the [^{14}C]pyrazole. Free β-pyrazolylalanine, together with its peptides, was isolated and identified by chromatography and high voltage electrophoresis. The radioactivity of the β-pyrazolylalanine and its peptides was determined in each case. The results (Table 3) show that, with the exception of β-aminopropionitrile, the putative precursors examined all caused a significant decrease both in the total radioactivity incorporated into β-pyrazolylalanine and in its specific activity. The most significant effect obtained (specific radioactivity of free β-pyrazolylalanine at 40% of control value) was with 1,3-diaminopropane. The fall in specific activity of the β-pyrazolylalanine synthesized, relative to that of the control, could be interpreted as either inhibition of the synthesis of radioactive β-pyrazolylalanine, or as donation to the synthetic process of unlabelled precursors of the pyrazole moiety. That the latter is the explanation of the effect with 1,3-diaminopropane was indicated by an experiment in which pyrazole (3 μmol), [3-^{14}C]L-serine (0.102 μCi, 0.3 μmol), pyridoxal 5-monophosphate (0.011 μmol) and 0.1 ml of an enzymic extract of 23-day-old cucumber shoots (see Experimental) were incubated in the presence and absence of 1,3-diaminopropane (1.434 μmol, pH 6.8). The total radioactivity of the isolated β-pyrazolylalanine from the incubation in the absence of 1,3-diaminopropane was 6684 dpm and that from the second incubate, which contained 1,3-diaminopropane, was 6707 dpm.

Using excised shoots, comparison was made of the amount of radioactivity incorporated into β-pyrazolylalanine from [^{14}C]pyrazole with that incorporated from [^{14}C]1,3-diaminopropane. With [3,4-^{14}C]pyrazole, 191 364 dpm were incorporated/μCi supplied. The corresponding figure for [1,3-^{14}C]1,3-diaminopropane was 28 664 dpm incorporated into β-pyrazolylalanine/μCi supplied. This means that pyrazole is 6.6-fold more efficient than 1,3-diaminopropane as a precursor of β-pyrazolylalanine, a finding compatible with the suggestion that 1,3-diaminopropane is a precursor of pyrazole for the synthesis of β-pyrazolylalanine.

To examine this further, [1,3-^{14}C]diaminopropane was synthesized and supplied to excised shoots of cucumber seedlings (23-day-old) as before. One batch of 16 shoots was allowed to take up 10 μCi of the radioactive 1,3-diaminopropane (sp. act. 29 μCi/nmol). After 24 hr, the shoots were extracted with ethanol and the extract chromatographed. Radioactive spots were located and eluted for further examination. Of the total radioactivity recovered (7 253 333 dpm), 71% was in unmetabolized 1,3-diaminopropane and 4% in β-pyrazolyalanine. The remainder (1 783 111 dpm) was distributed between six other, unidentified, compounds. On the basis of its chromatographic behaviour, one of these was identified as the γ-glutamyl peptide of β-pyrazolylalanine. It contained a total of 817 222 dpm which represents 11% of the radioactivity recovered.

Table 3. The effect of possible precursors of pyrazole on the incorporation of [3,4-^{14}C]pyrazole into β-pyrazol-1-yl-L-alanine and its peptides by *Cucumis sativus* (cucumber)

Experiment no.	Precursor supplied with [3,4-^{14}C]pyrazole	Radioactivity in β-pyrazolylalanine: Total radioactivity (dpm × 10^{-2})		Specific radioactivity (dpm × 10^{-2}/μmol)			
		Free	Bound*	Free		Bound*	
1	None (control)	4401	473	370	(100)	45	(100)
	1,3-Diaminobutyric acid	2841	321	268	(72.4)	45	(100)
	Asparagine	2741	311	292	(78.9)	41	(91.1)
2	None (control)	5186	209	316	(100)	8	(100)
	1,3-Diaminopropane	1912	112	126	(39.9)	4	(50)
	β-Cyanoalanine	2729	142	163	(51.6)	4	(50)
3	None (control)	3502		203	(100)		
	Spermidine	2050		124	(61.0)		
	β-Aminopropionitrile fumarate	3429		206	(100)		
	Fumarate	3409		205	(100)		

[3,4-^{14}C]Pyrazole (2.3 μCi, sp. act. 134.26 μCi/mmol) was dissolved in 2 ml of distilled water or a solution of the suspected precursor (pH 5.5). Batches of 20 excised shoots, from 23-day-old seedlings, were each supplied with a separate precursor solution. Shoots were extracted after 24 hr.

The figures in parentheses show the specific radioactivity as a percentage of the radioactivity incorporated into the respective control.

*β-Pyrazolylalanine present as peptide.

β-Pyrazolylalanine synthesis from 1,3-diaminopropane in cell-free extracts

Following the procedure of Dunnill and Fowden [5], an enzymic extract was prepared from 23-day-old cucumber seedlings and shown to be active in synthesizing β-pyrazolylalanine from pyrazole and serine. Using this extract and replacing pyrazole by [1,3-^{14}C]1,3-diaminopropane, (sp. act. 29.07 μCi/mmol), it was possible to detect formation of radioactive β-pyrazolylalanine (total radioactivity 2220 dpm). The reaction mixture consisted of enzymic extract (0.1 ml), pyridoxal phosphate (0.011 μmol), serine (0.3 μmol), and [1,3-^{14}C]-1,3-diaminopropane (0.114μCi, 3.92 μmol), all in a total volume of 0.13 ml at a final pH of 6.4.

Insufficient [^{14}C]diaminopropane was available for further examination of the incorporation but a similar enzymic system was used in an indirect approach. This employed non-radioactive 1,3-diaminopropane, with [1,2,3-^{14}C]*O*-acetylserine in place of the non-radioactive serine. Pyrazole was not supplied. The results (Table 4) show that 1,3-diaminopropane increases the incorporation of radioactive *O*-acetylserine into β-pyrazolylalanine. This is as would be expected if the previous conclusion, that 1,3-diaminopropane is a precursor of the pyrazole moeity of β-pyrazolylalanine, is valid. Incorporation of radioactive *O*-acetylserine in the absence of added 1,3-diaminopropane (Table 4) is attributable to presence in the crude enzymic extract of endogenous pyrazole [5]. Although, using the pentacyanoaminoferrate reagent [8], we could not detect pyrazole in these extracts, this procedure may not be sensitive enough to detect trace amounts which are sufficient to permit limited synthesis of [^{14}C]β-pyrazolylalanine.

The foregoing results implicate 1,3-diaminopropane as a precursor of the pyrazole moiety of β-pyrazolylalanine. Furthermore, that cucumber extracts contain 1,3-diaminopropane has recently been demonstrated by TLC and by GC (Flayeh, K. A. M., unpublished observation). The simplest explanation of the present findings is that 1,3-diaminopropane is first cyclized to pyrazole before being incorporated into β-pyrazolylalanine. This explanation is supported by analogy with the biosynthesis of other heterocyclic β-substituted alanines such as willardiine [9], isowillardiine [9] and mimosine [6] where the appropriate heterocyclic base is directly involved. Further support is given by the observation that pyrazole behaves as a limiting factor in the synthesis of β-pyrazolylalanine by non-dialysed extracts of cucumber seedlings [5]. Nevertheless, the work described does not exclude the possibility that the alanine side-chain is attached to one of the nitrogen atoms of 1,3-diaminopropane before the latter is cyclized.

Table 4. Formation of β-pyrazolylalanine from [1,2,3-^{14}C]*O*-acetylserine and 1,3-diaminopropane by enzymic extracts from cucumber seedlings

Additions to extract	Total radioactivity incorporated into β-pyrazolylalanine (dpm ± s.d.)
[^{14}C]*O*-Acetylserine (control)	2686 ± 453
[^{14}C]*O*-Acetylserine + 1,3-diaminopropane (10 mM)	4237 ± 241
[^{14}C]*O*-Acetylserine + 1,3-diaminopropane (100 mM)	6122 ± 404

The same amount of radioactivity (1082 × 10^3 dpm) was added in each treatment.

Results are the mean of four incubations.

EXPERIMENTAL

Materials. Seeds of *Cucumis sativus* cv Ridge Perfection, obtained from Asmer Seeds Ltd., Leicester, were germinated in moist vermiculite and seedlings grown at 25° under a lighting régime of 16 hr light (6 klx)/8 hr dark. Pyrazole, 2,4-diaminobutyric acid, β-cyano-L-alanine, β-aminopropionitrile fumarate, and spermidine trihydrochloride were purchased from Sigma (London) Chemical Co. Ltd., London. Diazald and 1,3-diaminopropane dihydrochloride were obtained from Aldrich Chemical Co. Ltd., Gillingham. A sample of β-pyrazol-1-yl-L-alanine was kindly provided by Dr. L. Fowden, FRS, Rothamsted Experimental Station, Harpenden. [U-^{14}C]Acetylene, [3-^{14}C]L-serine and [1,3-^{14}C]-1,3-dibromopropane were purchased from the Radiochemical Centre, Amersham.

Preparation and identification of pyrazol-1-yl-L-alanine. To prepare this amino acid, use was made of the pyridoxal model system described in [5]. A soln of pyrazole (1.47 mmol), L-serine (0.19 mmol), pyridoxal 5'-monophosphate (37 μmol) and $Al_2(SO_4)_3$ (7.9 μmol), in 0.1 M sodium acetate buffer, pH 4.7 (final vol. 3 ml) was heated at 60° for 20 hr. Pyrazolylalanine was separated, purified and authenticated with a known sample, using sequential PC in (1) butan-1-ol saturated with 3 M-ammonia, (2) butan-1-ol–HOAc–H_2O (90:10:29), (3) aq. PhOH (750 g/l) in the presence of an NH_3 vapour phase, (4) ammonia (17 M)–aq. soln of EDTA (1.26 g/l)–propan-1-ol–propan-2-ol–butan-1-ol–2-methylpropanoic acid (10:95:35:7.5:7.5:250), and followed by high voltage electrophoresis on Whatman 3 MM paper using an electrode potential of 3 kV (60 V/cm) and a formate–acetate buffer (pH 2).

Pyrazolylalanine in plant extracts was identified in a similar way using ninhydrin (0.1% in Me_2CO; w/v) as the locating agent.

Extraction and identification of γ-L-glutamyl-β-pyrazol-1-yl-L-alanine. The method used for extracting the peptide was essentially that described in [3]. The extract was chromatographed on Whatman No. 1 paper in solvent 1 in which the peptide migrates with R_{Leu} 0.16. The band was eluted and re-chromatographed in solvent 3. Subsequently, the peptide (R_f 0.53) was eluted and re-chromatographed in solvents 1 and 2, then subjected to high voltage electrophoresis at pH 2 as described for pyrazolylalanine. The identity was confirmed by acid hydrolysis [5] followed by PC and high voltage electrophoresis.

Determination of the concn of pyrazole derivatives. Pyrazole concn was determined by reaction with trisodium pentacyanoaminoferrate and HNO_2 followed by measurement of A_{458} of the product [8]. Pyrazolylalanine concn was measured by the ninhydrin procedure of Lee and Takahashi [10]. After chromatographic separation from free β-pyrazolylalanine, the concn of individual peptides of β-

pyrazolylalanine was determined using acid hydrolysis and chromatographic/electrophoretic separation of the component amino acid, by the ninhydrin method [10].

Measurement of radioactivity. Radioactivity was measured by liquid scintillation counting using a dioxane-based scintillant containing 2,5-diphenyloxazole, 1,4-bis-(5-phenyloxazole-2-yl)benzene and naphthalene. Each sample was counted twice for 20 min and automatically corrected for background.

Synthesis of radioactive precursors. [3,4-^{14}C]Pyrazole was synthesized from diazomethane (10 mmol) and [U-^{14}C]acetylene (500 μCi, sp. act. 119 mCi/mmol) by adapting the method of Pechmann [11] to a semi-micro scale. Diazomethane was generated from Diazald [12]. The purity of the radioactive pyrazole obtained was checked by TLC on Kieselgel G using ethylacetate as the solvent, and by 2-D PC on Whatman No. 1 paper using butan-1-ol satd with H_2O followed in the second dimension by solvent 2 described above. Pyrazole was located by use of the trisodium pentacyanoaminoferrate reagent [13] and radioactivity was located in a radiochromatogram scanner. Sp. act. of the purified pyrazole was 134.26 μCi/mmol.

Synthesis of [1,3-^{14}C]-1,3-diaminopropane was from [1,3-^{14}C]-1,3-dibromopropane (500 μCi, sp. act. 8.9 mCi/mmol) using the method described in [14]; minor modifications were made to adapt the method to a semi-micro scale. The identity and radiochemical purity of the synthesized compound was checked by thin-layer electrophoresis [15] and by 2-D PC as described above for pyrazole. The sp. act. of the synthesized [1,3-^{14}C]-1,3-diaminopropane was 29.07 μCi/mmol.

Enzymic preparation. The enzymic extract used for the precursor studies was obtained from cucumber seedlings (3–4 weeks old). Using a pre-cooled (4°) mortar and pestle, the seedlings were macerated with a mixture of K_2HPO_4/KH_2PO_4 [5]. The macerate was strained through muslin and centrifuged at 20 000 *g* for 20 min. After removal of a thin surface layer of lipid, the supernatant was decanted and used directly as the enzyme preparation.

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