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DE NOVO SYNTHESIS OF D-ALANINE IN GERMINATING *PISUM SATIVUM* SEEDLINGS

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Key Word Index—*Pisum sativum*; Leguminosae; pea; biosynthesis; D-alanine; γ-L-glutamyl-D-alanine; *N*-malonyl-D-alanine.

Abstract—The amounts of D-alanine derivatives, γ-L-glutamyl-D-alanine and *N*-malonyl-D-alanine, increase rapidly during the early growth of pea seeds. Pyruvate-[1-^{14}C], L-alanine-[U-^{14}C], D-alanine-[U-^{14}C], L-alanine-[^{15}N] and $^{15}NH_4Cl$ were therefore fed to the seedlings and the incorporation investigated. Labelling results revealed that pea seedlings can utilize these exogenous compounds to form D-alanine and that labelled L-alanine is effectively converted to the D-enantiomer with retention of ^{14}C and, largely, ^{15}N label. Enzyme analyses *in vitro* provided additional evidence that the extract of pea seedlings catalyzes the direct conversion of L-alanine to D-alanine. The data suggest that the *de novo* synthesis of D-alanine in pea seedlings occurs by a racemase reaction.

INTRODUCTION

In previous studies [1–4], the authors identified a number of D-amino acids, such as D-alanine, D-α-amino-*n*-butyric acid, D-aspartic acid and D-glutamic acid in pea seedlings. Among them, D-alanine derivatives, γ-L-glutamyl-D-alanine (GA) and *N*-malonyl-D-alanine (MA), were found to be extensively produced during early germination [5]. D-alanine is the most widely reported D-amino acid in higher plants in the free or conjugated state [6]. However, its origin is still obscure, since it is not known whether it is actually synthesized *de novo*. For this reason the fate of labelled compounds fed to pea seedlings has been studied.

RESULTS AND DISCUSSION

Surface sterilized pea seeds were germinated for various periods of time in the dark to select the stage of growth at which the greatest accumulation of D-alanine derivatives occurs. The amounts of GA rapidly increased during the first 8 days of germination reaching a maximum, *ca* 3 μmol/seedling. MA also increased steadily during germination. The maximum production of D-alanine was observed between days 6 and 8 of seedling growth and was about 0.7 μmol/day/seedling. In contrast, free D-alanine was kept at low levels (*ca* 0.05 μmol/seedling) through the experimental periods. Therefore, the 6-day-old seedlings were used for the isotope experiments.

Pyruvate-[1-^{14}C], L-alanine-[U-^{14}C], D-alanine-[U-^{14}C] and $^{15}NH_4Cl$ were administered separately to the

Table 1. Utilization of $^{15}NH_3$ for D-alanine synthesis by pea seedlings

Amino acid	^{15}N incorporated (atom % excess)
Free L-alanine	2.95
Free D-alanine	2.65
Conjugated D-alanine*	1.14

$^{15}NH_4Cl$ was supplied to the seedlings at the concn of 10 mM (99.5 at % excess) for 24 hr.
* Degradation product obtained from GA.

Table 2. Utilization of ^{14}C label for synthesis of D-alanine by pea seedlings

Compound administered*	Specific activity (μCi/μmol)	^{14}C incorporated (dpm/μmol) Free		Conjugated†	
		L-alanine	D-alanine	D-alanine	L-glutamic acid
Pyruvate-[1-^{14}C]	14	2111	822	141	148
Pyruvate-[1-^{14}C] + L-alanine	14	—	—	131	196
Pyruvate-[1-^{14}C] + D-alanine	14	—	—	55	133
L-Alanine-[U-^{14}C]	168	—	—	6552	4490
D-Alanine-[U-^{14}C]	43	—	—	12776	783

* Labelled compounds were supplied at the amounts of 0.5 μCi/seedling for 5 hr. Unlabelled compounds were added at the concn of 2 mM. † Degradation products derived from GA.

Table 3. Utilization of L-alanine-[U-^{14}C] and L-alanine-^{15}N for D-alanine synthesis by pea seedlings. The two isotopically labelled compounds were combined to yield a soln which contained 28 μmol of L-alanine per ml (7.14×10^5 dpm/μmol. 99.3 atom % excess) and then supplied to the seedlings for 24 hr

Amino acid	^{14}C incorporated (dpm/μmol)	Dilution	^{15}N incorporated (atom % excess)	Dilution
D-alanine*	1501	1 : 48	0.99	1 : 100

* Degradation product derived from GA.

seedlings to see if D-alanine was formed from them. As shown in Table 1, germinating pea can utilize the exogeneous nitrogen to form D-alanine. The ^{15}N label was effectively incorporated into free D-alanine as well as free L-alanine, while the apparent incorporation into the D-alanine moiety of GA was about 50% of that found in free D-alanine. These data suggested that active synthesis of D-alanine and accumulation of GA proceeded in the 6-day-old seedlings. The utilization of radioactive carbon to form D-alanine is shown in Table 2. Pyruvate-[1-^{14}C] was effectively incorporated into free and conjugated D-alanine. After Van Slyke decarboxylation with ninhydrin[6], 95% of the ^{14}C in D-alanine was recovered as CO_2, demonstrating that all the label was retained in C-1 of D-alanine. The incorporation of L-alanine-[U-^{14}C] into D-alanine was much larger than that obtained with pyruvate-[1-^{14}C]. The addition of unlabelled L-alanine to the medium of pyruvate-[1-^{14}C] reduced the incorporation of ^{14}C into the D-alanine moiety of GA, whereas the radioactivity of the L-glutamic acid part was not affected. Such isotope competition was more effectively observed when unlabelled D-alanine was added to the medium containing labelled pyruvate. This and the result obtained with D-alanine-[U-^{14}C] indicated a direct incorporation of D-alanine into GA, after its formation from L-alanine. Additional evidence for the direct formation of D-alanine from L-alanine was obtained by supplying L-alanine-[U-^{14}C] and L-alanine-[^{15}N] simultaneously to the seedlings. As shown in Table 3, the retention of ^{15}N isotope in the D-alanine moiety of GA amounted to *ca* 50% of ^{14}C label. If the reaction proceeded through an intermediate, such as pyruvate, the D-alanine formed would possess a carbon skeleton derived largely from L-alanine, but would contain only a small amoung of L-alanine nitrogen. This, therefore, strongly suggested that the formation of D-alanine from L-alanine involved the retention of the ^{15}N-amino group of the latter. In order to examine this further, enzyme analyses were carried out *in vitro*. An extract of the decotyledonized 6-day-old pea seedlings, free of endogenous low MW substances, catalyzed the direct conversion of L-alanine to its D-enantiomer. Thus a racemase occurs in germinating pea seedlings. In conclusion, both the tracer and the enzyme analyses indicate that there is *de novo* synthesis of D-alanine in pea via a racemase reaction. The occurrence of a similar system in a higher plant has been reported in tryptophan metabolism in the cell cultures of the tobacco plant [8].

EXPERIMENTAL

Plant material. Seeds of pea (*Pisum sativum* L. cv Alaska) were sterilized with Osvan (0.01%) and germinated in the dark on moist filter paper at 25° as described previously [9]. 10 seedlings (decotyledonized, 6-day-old) were placed in a small vessel containing the aq. soln of the appropriate labelled compounds and were incubated for given periods under the same conditions.

Determination of amino acids At the end of the incubation, the roots were washed thoroughly with H_2O and then the seedlings were homogenized with 75% EtOH and extracted at 4°. Determination of D-alanine and its conjugates in the extract was performed by methods described earlier [5]. Separation of the isomer of alanine was carried out by using a chromatographic technique (Aminex A-4, 0.9 × 70 cm column, 0.2 M Na-citrate buffer, pH 4.25 [1]) after a derivatization to the respective L-leucyl dipeptides according to the method of ref. [10]. Identity of amino acids was confirmed by an automatic amino acid analyzer, Yanagimoto LC-5S.

Labelled compounds and radioactive assay. The isotopes were obtained with following specifications: Na pyruvate-[1-^{14}C] (14 mCi/mmol), D-alanine-[U-^{14}C] (43 mCi/mmol), L-alanine-[U-^{14}C] (168 mCi/mmol), L-alanine-^{15}N (99.5 at. %) and $^{15}NH_4Cl$ (99.5 at %). The ^{14}C radioactivity was determined by a liquid scinillation spectrometer, Aloka LCS-602 equipped with an automatic external standardization system. ^{15}N analysis was performed at Japan Isotopic Center, the Institute of Physical and Chemical Research, Tokyo, Japan

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