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D-ALANINE IN GERMINATING *PISUM SATIVUM* SEEDLINGS

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Abstract—Germinating pea seedlings (*Pisum sativum* var. Alaska) contain high concentrations of D-alanine, which occurs in the decotyledonized parts as the conjugates, *N*-malonyl-D-alanine and γ-L-glutamyl-D-alanine. By contrast, free alanine in pea seedlings is almost all L-isomer. During early stages of the germination, γ-L-glutamyl-D-alanine increased significantly and amounted to *ca.* 2·5 μmol/seedling at 8 days.

INTRODUCTION

WHILE studying the free and conjugated amino acids in pea seedlings, we found that the non-cationic fraction prepared from the ethanol extract of pea seedlings contained many ninhydrin-negative conjugated amino acids,[1] one of the most predominant being *N*-malonyl-D-alanine.[2]

On the other hand, in the course of configurational studies of the ninhydrin-positive peptides in pea seedlings we found that γ-glutamylalanine, which is extensively produced during the early growth of pea,[3] was constituted of L-glutamic acid and D-alanine.[4] To our knowledge, these are the first reports on the natural occurrence of D-alanine in higher plants.

Although many investigations on the metabolism of D-amino acids in higher plants have been already achieved, they have been done under the conditions of the exogenous administration of D-isomer of amino acids.[5–8]

Little work has appeared on the natural occurrence of D-amino acids in higher plants[9–12] and the physiological significance is still uncertain. In the present study, therefore, the

[1] SASAOKA, K., OGAWA, T. and FUKUDA, M. (1972) *Agr. Biol. Chem.* **36,** 383.
[2] OGAWA, T., FUKUDA, M. and SASAOKA, K. (1973) *Biochim. Biophys. Acta* **297,** 60.
[3] VIRTANEN, A. I. and BERG, A-M. (1954) *Acta Chem. Scand.* **8,** 1089.
[4] FUKUDA, M., OGAWA, T. and SASAOKA, K. (1973) *Biochim. Biophys. Acta* **304,** 363.
[5] ALDAG, R. W. and YOUNG, J. L. (1970) *Agronomy J.* **62,** 184.
[6] ALDAG, R. W. and YOUNG, J. L. (1970) *Planta* **95,** 187.
[7] POKORNY, M., MARČENKO, E. and KEGLEVIĆ, D. (1970) *Phytochemistry* **9,** 2175.
[8] LEDEŠIĆ, B., POKORNY, M. and KEGLEVIĆ, D. (1971) *Phytochemistry* **10,** 3085.
[9] ZENK, M. H. and SHERF, H. (1963) *Biochim. Biophys. Acta* **71,** 737.
[10] KLOSTERMAN, H. J. LAMOUREUX, G. L. and PARSONS, J. L. (1967) *Biochemistry* **6,** 170.
[11] GRAY, D. O. and FOWDEN, L. (1972) *Phytochemistry* **11,** 745.
[12] GRAY, D. O. (1972) *Phytochemistry* **11,** 751.

formation of D-alanine and its quantitative changes during the early stages of the germination were followed to obtain information about the metabolism of D-amino acids and their biochemical significance in higher plants.

RESULTS AND DISCUSSION

D-Alanine contents of the dark-grown pea seedlings (6-day-old) are given in Table 1. The large amounts of alanine in the acid hydrolyzate of the 75% ethanol extract of the decotyledonized pea seedlings decreased by treatment with D-amino acid oxidase. The D-isomer represented more than 80% of total alanine in the hydrolyzate. The free amino acid fraction obtained from the ethanol extract of decotyledonized pea seedlings showed only a small change in alanine content after treatment with D-amino acid oxidase, but alanine released after hydrolysis clearly disappeared during enzymatic oxidation. It is suggested that almost all the conjugated alanine in the cationic soluble-nitrogen fraction was D-isomer. Small amounts of D-alanine (*ca.* 0·05 μmol/seedling) occurred in free form.

TABLE 1. D-ALANINE CONTENTS IN DECOTYLEDONIZED *Pisum sativum* SEEDLINGS*

Fractions	Alanine (μmol/seedling)		% of D-isomer
	Before enzymatic oxidation	After enzymatic oxidation†	
75% Ethanol extract (hydrolyzate)	2·02	0·288	85·8
Amino acid fraction	0·252	0 202	20·0
Amino acid fraction (hydrolyzate)	1 17	0·198	83·1
Non-cationic fraction (hydrolyzate)	0·105	0·012	88·6

* 6-Day-old † D-Amino acid oxidase used.

On the other hand, the fraction not retained on a cationic exchange resin (Amberlite IR-120 B, H^+) also contained considerable amounts of conjugated D-alanine (0·1 μmol/seedling), which represented 90% of total alanine in this fraction.

Conjugated alanine in the cationic amino acid fraction was present almost exclusively as γ-L-glutamyl-D-alanine because the increase in alanine after acid hydrolysis of the amino acid fraction was equivalent to the amount of γ-L-glutamyl-D-alanine disappearing. Almost 90% of alanine conjugates in the non-cationic fraction was *N*-malonyl-D-alanine (see Ref. 2). These facts suggest that the D-alanine in pea seedlings occurs as two conjugates, γ-L-glutamyl-D-alanine and *N*-malonyl-D-alanine.

In cotyledons of the germinating pea, trace amounts of conjugated D-alanine were found, which was assumed to be a contaminant from roots or shoots; no detectable free D-alanine was found in cotyledons.

Thus, to estimate the changes in D-alanine contents of the germinating pea seedlings, γ-L-glutamyl-D-alanine in the amino acid fraction and the ninhydrin-negative conjugated D-alanine (mainly *N*-malonyl-D-alanine) in the non-cationic fraction were assayed by an automatic amino acid analyzer.

These D-alanine conjugates were found in both roots and shoots. The amounts of γ-L-glutamyl-D-alanine increased significantly during the first 8 days of the germination reaching a maximum, *ca.* 2·5 μmol/seedling and thereafter declining rapidly. The D-alanine conjugate

in the non-cationic fraction also increased during germination. The decrease in γ-L-glutamyl-D-alanine between the 8th and 12th day of seedling growth was about 10 times larger than the increase in the amount of *N*-malonyl-D-alanine formed in the same period. However, no increase in the concentration of free D-alanine was observed, and so further metabolism to unknown compounds presumably occurred.

In most reports on the metabolism of D-amino acids administered to higher plants, *N*-malonyl conjugates have been considered as characteristic metabolites.[7,8,13] The evidence in this paper supports the *de novo* syntheses of *N*-malonyl D-amino acids in higher plants, as described in the case of *N*-malonyl-D-tryptophan.[9] However, in the case of pea seedlings, D-alanine is extensively converted to its γ-L-glutamyl peptide, the amount of which is about ten times greater than that of *N*-malonyl derivative.

Recently, Aldag and Young[6] reported that some of free alanine in the extract of corn roots disappeared following treatment with D-amino acid oxidase. This observation and the findings reported in this paper suggest the possibility that D-alanine may have a widespread occurrence in higher plants.

EXPERIMENTAL

Materials. Crystalline D-amino acid oxidase and lactate dehydrogenase and other reagents used in the present experiments were as described previously.[2,4]

Plant materials. Pisum sativum var. Alaska were sterilized with 0·1% Osvan and were germinated in the dark at 25° for given periods as described previously.[14]

Fractionation of plant material. Cotyledons and other parts (roots and shoots) were extracted with 75% EtOH. These extracts were separated into non-cationic and cationic amino acid fractions by using Amberlite IR-120 B (H^+ form) resin columns.

Determination of γ-L-glutamyl-D-alanine and N-malonyl-D-alanine. γ-L-Glutamyl-D-alanine and other free amino acids were determined with an automatic amino acid analyzer, Yanagimoto LC-5S, using the following conditions: Bio-Rad Aminex A-4 resin (0·9 × 90 cm), sodium citrate buffer, 0·2 M, pH 3·25 and 4·25, at 52°, flow rate 100 ml/hr. The elution volumes of γ-L-glutamyl-D-alanine and free alanine under above conditions are 42·5 and 118 ml, respectively. *N*-Malonyl-D-alanine was determined by the following procedures: the non-cationic fraction was applied to a column of Dowex 1 × 4 (100–200 mesh, acetate form) and then the column was washed with 5 M HOAc. The desired compound eluted with 6·5 M HCO_2H from the column was concentrated and hydrolyzed with 6 M HCl. The hydrolyzate was treated with D-amino acid oxidase. The decrease of alanine contents by the enzymatic oxidation was assumed to be the amounts of *N*-malonyl-D-alanine as defined in the previous paper.[2]

Determination of D-alanine. The enzymatic determination was carried out with D-amino acid oxidase, and formation of pyruvate was detected by the lactate dehydrogenase system as described previously.[2] After the enzymatic reaction, the amino acid analysis of the reaction mixture was carried out to confirm the disappearance of alanine. The configuration of alanine was also confirmed chromatographically according to the method of Manning and Moore.[15]

[13] Rosa, N. and Neish, A. C. (1968) *Can. J. Biochem.* **46**, 797.
[14] Sasaoka, K. (1958) *Mem. Res. Inst. Food Sci. Kyoto Univ.* **14**, 42.
[15] Manning, J. M. and Moore, S. (1968) *J. Biol. Chem.* **243**, 5591.