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AN AMINOTRANSFERASE SPECIFIC FOR THE D-ENANTIOMORPH OF METHIONINE

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Abstract—An enzyme was isolated from germinating peanut seed and shown to be an aminotransferase specific for the D-enantiomorph of methionine. The keto acid of methionine, α-keto-γ-methylthiobutyrate, was isolated from the reaction mixture and identified. Of the keto acids tested pyruvic acid was the most effective acceptor for the amino group of methionine. A small amount of enzyme was isolated which gave only one band on disc gel electrophoresis.

INTRODUCTION

AN AMINOTRANSFERASE specific for D-methionine has been isolated and partially purified from germinating peanut seed (*Arachia hypogea* L. cv. Starr). Aminotransferases in plants have been known for sometime[1] but in so far as we are aware, none have been previously reported to be specific for D-methionine.

Both enantiomeric forms of amino acids are found in some living organisms, the most clearly defined role for the D-isomers is in bacterial cell walls.[2] A role for D-amino acids in higher plants has yet to be defined although it has recently been shown[3] that several D-amino acids, including D-methionine, are readily absorbed and apparently metabolized within hours by maize and ryegrass. This observation suggests, of course, that pathways exist for their utilization.

Enzymes specific for D-amino acids have been known for many years, the classic example being D-amino acid oxidases of animal tissues.[4] Thus, whereas finding a plant enzyme specific for the transamination of D-methionine was not expected, it is certainly not without precedence in nature.[5,6]

The expected keto analog of methionine, α-keto-γ-methylthiobutyrate (KMB) and alanine (formed as the amino group acceptor) were found in the mixture after the reaction. KMB is readily broken down by a non-enzymatic free-radical type mechanism in the presence of light and flavin into ethylene, CO_2, formic acid, methyldisulfide and ammonia.

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[3] ALDAG, R. W., YOUNG, J. L. and YAMAMATO, M. (1971) *Phytochemistry* **10,** 267
[4] KREBS, H. A. (1935) *Biochem. J.* **29,** 1620.
[5] MEISTER, A. (1955) in *Advances in Enzymology* (FORD, F. F., ed.), Vol. 16, p. 206, Interscience, New York.
[6] SALLACH, H. J. and FAHEIN, L. A. (1969) in *Metabolic Pathways* (GREENBURG, D. M., ed.), 3rd Edn, Vol. III, p. 2, Academic Press, New York.

The ethylene production is equivalent to the amount of KMB formed and was used to measure the reaction rate. Aminotransferases for both enantiomeric forms of methionine were present in the crude extract, but only the enzyme active for the D-form was found in the purified fractions.

RESULTS AND DISCUSSION

Production of ethylene by the crude extract (supernatant from centrifugation at 33 000 g of homogenized embryos) was shown to be due to enzymatic activity and was not attributable to the slower non-enzymatic reaction previously reported.[7] The extract was essential for ethylene production; activity was lost when the extract was boiled, and increasing the amount of extract resulted in a proportional increase in ethylene production. Moreover, increasing the incubation period gave a linear increase in KMB as represented by ethylene production. Other tissue fractions (mitochondrial, lipid, microbodies and cell debris) showed no activity. Light had no significant effect upon the transamination reaction, and there was no ethylene formed in the dark, indicating that an intermediate was being produced which was converted to ethylene upon addition of FMN in the presence of light. The presence of reducing agents (cysteine, β-mercaptoethanol or dithiothreitol) during extraction was essential for maintaining activity. The reducing agents inhibited the assay at the levels necessary to protect the enzyme, a situation also noted with other aminotransferases,[8] but the dilution produced by the assay medium decreased the concentration of the reducing agent sufficiently so that the reaction was not markedly inhibited. Of the three α-keto acids tested (pyruvic, oxalacetic and α-ketoglutaric), pyruvic proved to be the most effective acceptor of the amino group from methionine in the purified extract. Pyridoxal phosphate was added to the extraction buffers to stabilize the enzyme, and it was found to be required in the assay for maximum activity.

TABLE 1. PURIFICATION OF A METHIONINE AMINOTRANSFERASE FROM *Arachis hypogea* EMBRYOS

Fraction	Vol. (ml)	Enzyme activity total (units)	Protein total (g)	Specific activity (nl/g/hr)	Recovery (%)	Purification
Crude	15·4	342	3·90	88	100	1·0
35–50% $(NH_4)_2SO_4$	14·5	643	0·55	1167	188	13·3
CPS*	12·5	640	0·29	2200	187	25·0
45–70% Acetone precipitate	5·7	182	0·02	10 061	53	114·3

Extraction employed 0·1 M phosphate buffer with 0·2 mM pyridoxal phosphate, 10 mM EDTA and 15 mM β-mercaptoethanol. The contents of each reaction flask are given in Experimental.
* Cold precipitate supernatant.

With the crude extract, both D- and L-methionine were effective substrates for transamination. The D-enantiomorph was ca. 50% more active than the L-form, and the sum of their activities equaled that of equimolar amounts of DL-methionine. There are several possible

[7] YANG, S. F., KU, H. S. and PRATT, H. K. (1967) *J. Biol. Chem.* **242,** 5247.
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explanations for these results. First, two aminotransferases could be present, one specific for each isomer. A racemase for methionine such as found in certain microorganisms[9,10] could account for the results by converting D- to L-methionine. It has also been observed that some enzymes have the ability to utilize both enantomeric forms of a substrate.[2] Subsequent work has revealed that the ability of the extract to use L-methionine was lost in ammonium sulfate fractionation.

A 13-fold increase in specific activity resulting from fractionation with ammonium sulfate, was partially ascribable to an apparent increase in total enzyme units (Table 1). Upon removal of the cold precipitate supernatant (CPS), there was a 15% loss in protein and a 2-fold increase in activity. Acetone fractionation resulted in a large degree of purification, mainly due to removal of much inactive protein. A high MW for the enzyme was indicated by the fact that the active fraction initially was eluted in the void volume of the Sephadex G200 column. Aggregate formation was suspected but not supported by the data, as the activity increased in the remaining soluble fraction. Electrophoresis of the first two fractions in Table 1 showed nine bands and the acetone fraction had one less major band, whereas the fractions from the Sephadex G200 column gave only one band.

The mechanism for transamination would lead to the formation of one mole of alanine and KMB each for each mole of methionine utilized. Initial experiments failed to confirm this, and further investigation revealed that when continued for a long period (8 hr), the light reaction was inhibited by the large amounts of methionine and limited by the amount of FMN available. When data were corrected for inhibition and limited FMN, approximately a 1:1 ratio was found (Table 2), supporting the proposed mechanism for an aminotransferase reaction.

TABLE 2. MOLAR RATIOS OF ALANINE, KMB AND ETHYLENE PRODUCED

Reaction conditions	μmol product/3 flask/hr		
	Alanine*	KMB†	Ethylene
Light	0·120	—	0·148
Dark	0·205	0·137	—

* Cold precipitate supernatant fraction.
† $(NH_4)_2SO_4$ fraction.
The contents of each reaction flask are given in Experimental.

The above results indicate that an aminotransferase specific for D-methionine is present in germinating peanut embryos. The expected substrates for transamination are required and utilized, the anticipated intermediates were isolated, and cofactors generally recognized as necessary for transamination were required. The possibility of a racemase being present cannot definitely be ruled out as the purity of the enzyme is based on a single criterion, disc gel electrophoresis. The specificity for D-methionine in the purified fractions excludes the possibility that a transaminase is present which can use both enantiomers of methionine.

[9] KALLIO, R. E. and LARSON, A. D. (1955) in *Amino Acid Metabolism* (MCELROY, W. D. and GLASS, H. B., eds.), p. 624, John Hopkins Press, Baltimore.
[10] SHOCKMAN, G. D. and TOENNIES, G. (1954) *Arch. Biochem. Biophys.* **50**, 9.

EXPERIMENTAL

Tissue preparation and enzymatic assays. Peanuts were germinated and homogenates of the embryos (cotyledons removed) were prepared as previously described.[11] The reaction mixture contained 0·4 ml extract, 3 μmol DL-methionine, 6 μmol pyruvate, 0·2 μmol pyridoxal-5′-phosphate and 1 ml 0·1 M phosphate buffer, pH 7·8 in a vol. constant for each experiment in a 25 ml Erlenmeyer flask. After 2 hr incubation in the dark at 31°, followed by addition of 10 nmol of FMN and 10 min under a light bank (6964 lx), a 1 ml sample was withdrawn from the gas phase of each flask and ethylene determined by GLC on an active alumina column with a FID. Ethylene production was used as an assay for KMB formation since the amount of ethylene formed is proportional to the amount of KMB present[7,12,13] under the assay conditions used. One unit of enzyme activity was defined as the quantity of enzyme that produced 1 nanoliter of ethylene per hr of dark reaction, while specific activity was defined as enzyme units per protein.

Purification of extract. The crude extract was fractionated as shown in Table 1. Upon standing in the ice bath a precipitate formed in the ammonium sulfate supernatant fluid. When removed by centrifugation, the remaining supernatant liquid (cold precipitate supernatant) was used for acetone fractionation. The supernatants from both ammonium sulfate and acetone fractions were dialyzed against buffer (3 and 6 hr, respectively). Supernatant liquid from the acetone fractionation was placed on a Sephadex G200 column and 0·5 ml fractions collected and read at 280 nm.

Protein, KMB and alanine analysis. Protein fractions were assayed for ethylene producing activity, and protein determined by the Lowry method.[14] The homogeneity of each fraction was determined by disc gel electrophoresis,[15] the gels being stained with Coomassie Brilliant Blue.[16] Alanine and KMB from the assay media were quantitatively determined, following incubation periods of 8 hr, to allow for production of quantities large enough for detection. After ethylene analysis, extracts from duplicate flasks were combined with the deionized H_2O rinses and desalted by the procedure of Dreze *et al.*[17] with the following modifications. Protein was precipitated with TCA (12·5% final concentration), and the TCA removed by diethyl ether. The sample was desalted on an ion exchange column, reduced to dryness and washed 3×, then dissolved in 0·5 ml 1% HCl. A 0·2 ml aliquot was applied to an amino acid analyzer, and the amount of alanine determined. After removal of protein from the dark-incubated samples as described above, dinitrophenylhydrazone derivatives of the α-keto acids present in the assay media were made,[18] the samples were extracted with ethyl acetate–dioxane (8:1) and 100 μl of each sample streaked on Silica gel thin layer plates. Two solvent systems were used for elution: *n*-butanol:acetic acid (19:1)[19] to move all hydrazones from the origin, and light petrol–ethyl formate–propionic acid (26:14:3),[20] to complete separation of hydrazones. Bands containing KMB hydrazones were removed and eluted in 3% sodium bicarbonate, read at 380 nm, and the amount of KMB in each determined from a standard curve.

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