Phytochemistry, 1970, Vol. 9, pp. 959 to 964. Pergamon Press. Printed in England.

THREONINE DEAMINASE FROM PAUL'S SCARLET ROSE TISSUE CULTURES

D. K. DOUGALL

Academic Faculty of Microbial and Cellular Biology, The Ohio State University, U.S.A.

(*Received* 12 *September* 1969)

Abstract—The presence of threonine deaminase in cell-free extracts of Paul's Scarlet Rose tissue cultures has been demonstrated. The enzyme has a high requirement for potassium in the assay mixture and is inhibited by L-isoleucine.

INTRODUCTION

STUDIES of the effects of isoleucine added to the culture medium of Paul's Scarlet Rose cells on the biosynthesis of protein amino acids, gave results which were consistent with end-product inhibition of isoleucine biosynthesis.[1] Additional evidence that L-threonine deaminase (L-threonine hydro-lyase, deaminating; E.C 4.2.1.16) is the first enzyme of isoleucine biosynthesis in plant cells has been cited.[2] Threonine deaminase from plant cells has not yet been described, although the enzyme from animals[3] and a variety of microorganisms[4–10] have been studied extensively. The biosynthetic threonine deaminase of microorganisms was early recognized as an enzyme whose activity is regulated by end-product inhibition. Many of the above studies are directed toward understanding the mechanism of this regulation. In addition, the level of the enzyme in some microorganisms is regulated by repression and derepression.[9]

In this paper evidence for the presence of threonine deaminase in cells of Paul's Scarlet Rose is presented. A brief report of this work appeared previously.[11] The substrate specificity, inhibition by isoleucine and other properties of the enzyme in cell-free extracts, will be discussed. The results of attempts to alter the level of the enzyme in the tissue by the presence or absence of substrates and products in the medium will also be presented.

RESULTS

Linearity. The yield of α-ketobutyrate was linear with respect to enzyme concentration at 35° in the standard assay mixture up to 1 μmole of keto-acid produced. The production of α-ketobutyrate by the enzyme remained linear for at least 90 min at 35°.

[1] D. K. DOUGALL, *Plant Physiol.* **40**, 891 (1965).
[2] D. K. DOUGAL and M. M. FULTON, *Plant Physiol.* **42**, 941 (1967).
[3] J. S. NISHIMURA and D. M. GREENBERG, *J. Biol. Chem.* **236**, 2684 (1961).
[4] R. O. BURNS and M. H. ZARLENGO, *J. Biol. Chem.* **243**, 178 (1968).
[5] J. P. CHANGEAUX, *Bull. Soc. Chim. Biol.* **46**, 927 (1964).
[6] P. DATTA, *J. Biol. Chem.* **241**, 5836 (1966).
[7] C. LEITZMANN and R. W. BERNLOHR, *Biochim. Biophys. Acta* **151**, 449 (1968).
[8] H. RUBICHON-SCHULMAJSTER and P. T. MAGEE, *European J. Biochem.* **3**, 492 (1968).
[9] H. E. UMBARGER and B. BROWN, *J. Bact.* **71**, 443 (1956).
[10] H. R. WHITELY and M. TAHARA, *J. Biol. Chem.* **241**, 4881 (1966).
[11] D. K. DOUGALL, *Fed. Proc.* **25**, 748 (1966).

Buffers. The specific activity of the enzyme was lowered if any component was omitted from the extraction buffer. The presence of Polyclar AT during cell breakage also lowered the specific activity. Passage of the enzyme through Sephadex columns in the absence of K_2HPO_4, pyridoxal phosphate or dithiothreitol resulted in lowered specific activity of the enzyme.

Stabilization of the enzyme. The enzyme lost activity very rapidly at 0° unless maintained in the presence of isoleucine or potassium phosphate. Losses of activity occurred if extracts in 1·0 M K_2HPO_4 were stored at 0° for 24 hr. The loss of 75 per cent of the activity occurred in 24 hr when extracts were stored in 0·5 M K_2HPO_4. In 5×10^{-4} M L-isoleucine and 0·05 M PO_4 approximately half the activity was lost at the end of 6 days whereas in 10^{-3} M or higher concentrations of L-isoleucine, the activity was retained at 6 days. Complete stabilization also was found in 30 per cent glycerol at 6 days.

pH optimum. With tris HCl and 0·2 M K_2HPO_4 in the assay mixture the activity of the Sephadex-treated enzyme was optimal from pH 9·2 to 9·6 at 30°.

Substrate specificity. Substrate specificity was examined using Sephadex-treated enzyme and an assay mixture at pH 9·5 containing tris-HCl and 0·2 M K_2HPO_4. With L-serine (0·04 M) and DL-allo-cystathionine (0·02 M), the yield of keto-acid was 45 per cent and 20 per cent of that obtained with 0·04 M L-threonine. No keto-acid was produced when the following were present at 0·04 M in the assay mixture: D-serine, D- or L-methionine, D- or L-homoserine, D-threonine, DL-δ-hydroxy-α-amino-n-butyric acid, D- or L-cysteine, DL-α-amino butyric acid, L-ornithine, *O*-succinyl-DL-homoserine, DL-hexahomoserine. α-ketobutyrate was not destroyed when incubated with the Sephadex-treated enzyme at 35° for 1 hr.

At 0·02 M L-threonine or above the rate of the reaction was greatest. The concentration of L-threonine giving maximum rate was not affected by pH between 8·0 and 9·5.

TABLE 1. THE EFFECT OF INORGANIC SALTS ON THE ACTIVITY OF THREONINE DEAMINASE

Salt	Final concentration	Activity (%)*
—	—	0·0
K_2SO_4	0·1 M	100
KNO_2	0·2 M	2
KNO_3	0·2 M	12
K oxalate	0·1 M	99
K acetate	0·2 M	134
K_2HPO_4	0·1 M	130
Na_2HPO_4	0·1 M	10
KNa tartarate	0·2 M	104
KF	0·1 M	93
KCl	0·2 M	86
KCl	0·1 M	77
KBr	0·1 M	56
KI	0·1 M	8
RbCl	0·2 M	73
$(NH_4)_2SO_4$	0·1 M	77

* Activity is given relative to that found using K_2SO_4.

In all cases the crude enzyme preparation was passed through a column of Sephadex G-50 in a 0·01 M tris, pH 9·0, containing 10^{-2} M Na_2EDTA, 10^{-3} M dithiothreitol or 10^{-2} M β-mercaptoethanol, and 2×10^{-4} M pyridoxal phosphate.

Effects of inorganic ions on the reaction rate. The enzyme has an absolute requirement for a monovalent inorganic cation in the assay mixture. However, the role of this ion is not known, it may be stabilization of the enzyme or it may be activation. Table 1 shows the effects of various salts on the rate of the reaction. Potassium shows the highest activity with rubidium and ammonium ions being able to substitute in part. The results with sodium phosphate were variable ranging from 0 to 38 per cent in five experiments. The results from sodium potassium tartarate indicate that sodium is not inhibiting in the presence of potassium. Other ions tested at 1 mM in the assay mixture that would not replace K^+ were Al^{+++}, Ca^{++}, Co^{++}, Fe^{+++}, Hg^{++}, Mg^{++}, Ni^{++}, Sn^{++}, Sn^{++}, Zn^{++}, Cu^{++}.

Acetate and phosphate are the counter ions giving highest enzyme activity. Nitrate is completely inhibitory and the halides fall into a series of increasing inhibitory action which parallels atomic weight. The inhibition by nitrate increased with nitrate concentration when the potassium level was kept constant with potassium sulphate.

The effect of potassium concentration in the assay mixture was explored. When K_2SO_4 was used, the activity of the enzyme was still increasing at 0·4 N K^+ in the assay mixture. In the presence of 0·2 M Na_2HPO_4, increase in the potassium concentration by the addition of K_2SO_4 above 0·12 M did not increase the activity of the enzyme. This suggests activation of the enzyme by phosphate. Double reciprocal plots of the data from the effects of K_2SO_4, KCl or K_2HPO_4 on the enzyme activity were not linear indicating complex kinetics.

TABLE 2. THE ACTIVITY OF THREONINE DEAMINASE IN THE PRESENCE OF L-ISOLEUCINE

L-Isoleucine conc. (M)	pH 8·0	pH 9·0	pH 9·5
		Activity*	
10^{-5}	99 ± 2 (2)	100 ± 2 (2)	103 ± 3 (4)
10^{-4}	6 ± 3 (3)	58 ± 15 (4)	87 ± 5 (3)
10^{-3}	4	3 ± 1 (4)	11 ± 8 (3)

* As a per cent of that in the absence of isoleucine.

The number of observations used to give the mean ± average deviation from the mean is given in parentheses.

TABLE 3. EFFECT OF L-VALINE ON THREONINE DEAMINASE ACTIVITY IN THE PRESENCE AND ABSENCE OF L-ISOLEUCINE

L-Valine conc. (M)	L-Isoleucine conc. 0	L-Isoleucine conc. 5×10^{-4} M
0	100*	11
10^{-5}	80	13
10^{-4}	85	10
10^{-3}	98	20
5×10^{-3}	95	57
2×10^{-2}	80	67

* Data given as a per cent of the activity in absence of either amino acid.

Data were averaged from four experiments. In one pair, valine concentrations above 10^{-4} M were tested; in the other pair, concentrations below 5×10^{-3} M were used. pH 9·5.

TABLE 4. THE GROWTH AND ENZYMATIC ACTIVITY OF TISSUE GROWN IN THE PRESENCE OF COMPOUNDS METABOLICALLY RELATED TO THREONINE

Supplements to medium	Conc. (mM)									
Glutamine	1·3			1·3	1·3	1·3	1·3	1·3	1·3	1·3
Glutamic acid (NH_4^+)		3·6	3·6							
Methionine sulfone			1·0							
Threonine				2·0	1·0					
Isoleucine						1·0	1·0	0·5	1·0	
Valine							0·5	0·5		
Leucine							0·5			
Calcium pantothenate							0·21			
All protein amino acids except ileu									1·0 ea.	1·0 ea.
Specific activity (μmole α-ketobutyrate/hr/mg protein at 35°	13·7 + 2·2 (8)	17·6 ± 4·0 (2)	9·4 ± 3·3 (2)	11·2 ± 1·9 (2)	12·5 ± 2·2 (2)	10·3 ± 1·5 (8)	9·2 ± 2·5 (4)	13·1 ± 1·9 (2)	19·2 ± 0·2 (2)	18·0 ± 0·9 (4)
Protein (mg/50 ml medium)	42 ± 12 (8)	37 ± 30 (2)	11 ± 3 (2)	25 ± 16 (2)	50 ± 9 (2)	32 ± 17 (8)	36 ± 23 (4)	50 ± 8 (2)	67 ± 39 (2)	76 ± 28 (4)

Number of determinations used to give mean ± average deviation from the mean is shown in parentheses. Tissue was grown for 4–6 days in the medium.

TABLE 5. THE EFFECT OF GLUCOSE AND COMPOUNDS METABOLICALLY RELATED TO THREONINE ON THE GROWTH AND THREONINE DEAMINASE ACTIVITY IN ROSE TISSUE CULTURES

Supplements to medium	Conc. (mM)									
Glucose	111			111		111				
Threonine				1·0						
Isoleucine						1·0	1·0	0·5	1·0	
Valine								0·5	0·5	
Leucine									0·5	
Calcium pantothenate									0·21	
Specific activity (μmole α-ketobutyrate/mg protein/hr	6·8 ± 1·8 (2)	2·5 ± 0·4 (6)	8·0 ± 2·1 (2)	4·1 ± 1·6 (4)	7·0 ± 3·1 (2)	3·6 ± 0·4 (4)	3·6 ± 1·4 (2)	2·9 ± 0·3 (2)		
Protein (mg/50 ml medium)	70 ± 7 (2)	5·1 (6)	76 ± 4 (2)	6·0 (4)	74 ± 11 (2)	6 ± 2 (4)	4·5 ± 0·5 (2)	4·5 ± 0·5 (2)		

The number of determinations combined to give the mean ± average deviation is given in parentheses. The culture conditions and assay methods are given in the text. Culture medium PN-25 was modified only as required by the above table.

Inhibition by L-*isoleucine and reversal by* L-*valine.* The enzyme is inhibited by isoleucine as shown in Table 2. At 10^{-5} M or below there is no inhibition or activation of the enzyme. The degree of inhibition at a fixed concentration of isoleucine decreases with increasing pH. The effect of L-valine on the activity of the enzyme at pH 9·5 is shown in Table 3. There seems to be a small inhibition (*ca.* 10 per cent) by valine at all concentrations when the assay is carried out in the presence of saturating L-threonine and K_2HPO_4. The interaction of L-isoleucine and L-valine on the activity of the enzyme is also shown in Table 3. L-Valine partially reverses L-isoleucine inhibition at the higher concentrations tested.

Effects of growth conditions on the specific activity of threonine deaminase. The effects on the specific activity of threonine deaminase of supplementing the culture medium with compounds which are metabolically related to threonine are shown in Table 4. In addition, the effects of some mixtures of amino acids is shown. Methionine sulphone was included, because it increases the specific activity of glutamine synthetase in this tissue (unpublished observations). The effects of some of these compounds in the presence and absence of glucose is shown in Table 5. Treatments which might be expected to lead to substrate derepression or product repression gave no marked changes in specific activity of the enzyme. Omission of glucose from the culture medium led to a decrease of the specific activity of the threonine deaminase. When all or most of the protein amino acids were provided in the medium, an increase in specific activity of the enzyme occurred. These results show that the cells respond by changing composition only when there are gross changes made in the composition of their culture medium.

DISCUSSION

The data presented show that threonine deaminase is present in extracts of Paul's Scarlet Rose tissue. The enzyme is similar in many respects to the threonine deaminases from other sources. For example, it is inhibited by L-isoleucine,[4–6] and this inhibition is reversed by L-valine.[6] It is stabilized by high concentration of potassium phosphate;[5,10] increasing pH decreases the degree of L-isoleucine inhibition.[4,6]

The enzyme is also different from any of those previously described, although it is most similar to that from *Rhodopseudomonas spheroides*[6] differing only in pH optimum and the effect of pH on L-isoleucine inhibition and to that from *E. coli*[9] differing in pH optimum and the effect of potassium phosphate on the activity. Threonine deaminase from Paul's Scarlet Rose seems to be unique in its requirement for a monovalent cation in the assay mixture which is best satisfied by K^+. These differences show that the threonine deaminase from Paul's Scarlet Rose is not identical with that from other sources.

The ability to demonstrate the presence of threonine deaminase in cell-free extracts from Paul's Scarlet Rose and its inhibition by L-isoleucine is further evidence to that presented by Dougall and Fulton[2] for α-ketobutyrate as an intermediate in L-isoleucine biosynthesis.

MATERIALS AND METHODS

Actinomycin D was generously provided by Dr. Arnold Demain of Merck, Sharpe & Dohme. The sources of many of the chemicals have been described.[2] The remainder were the best commercially available grade.

Cultivation of Tissue

Stock cultures of Paul's Scarlet Rose tissue were maintained on medium PN-25 as described by Dougall.[1] Inocula for experiments were suspension cultures of tissue obtained either by growing callus tissue in liquid

medium or by subculture. To start an experiment, portions of 7-day-old cultures were diluted 1:25 with 50 ml of the experimental medium. Glucose replaced sucrose in all experimental media. If a reduced nitrogen source is not specified it was ammonium glutamate (3·4 mM). The basal medium for any experiment is PN-25 with the compound(s) being varied omitted. At the termination of a growth experiment each flask was checked for sterility by streaking a loopful of culture on slopes of potato dextrose agar (Difco) and nutrient agar (Difco). The slopes were incubated at 30° for 2–3 weeks. Results from any flasks were discarded if the corresponding slopes showed the presence of bacteria or fungi.

Enzyme Extraction

Tissue was separated from the medium by filtration through a Rapid-Flo milk-filter disk and weighed. All subsequent operations were done at 0–4°. The tissue was subject to sonic oscillation with a Bronwill Biosonic at full power for 30 sec/g fr. wt. in 1 ml/g fr. wt. of buffer at pH 8·0 containing 2×10^{-2}M K phosphate. 2×10^{-3}M EDTA, 2×10^{-2}M β mercapto ethanol or 2×10^{-3}M dithiothreitol, 4×10^{-4}M pyridoxal phosphate and 10^{-3} M L-isoleucine. The homogenate was centrifuged at 27,000 g for 10 min and a portion of the supernatant passed through a column of Sephadex G-50 in buffer at pH 9·0 containing M K_2HPO_4, 10^{-3} M EDTA, 10^{-2} M β-mercaptoethanol or 10^{-3} M dithiothreitol and 2×10^{-4} M pyridoxal phosphate. The Sephadex eluates were assayed as soon as possible.

The assay mixture (0·5 ml) contained 0·1 M tris-HCl, 0·04 M L-threonine, 0·2 M K_2HPO_4, 2×10^{-4} M EDTA, 4×10^{-5} M pyridoxal phosphate and either 2×10^{-3} M β-mercaptoethanol or 2×10^{-4} M dithiothreitol and enzyme. The incubation was terminated by the addition of 0·05 ml of 50% TCA and the keto-acid determined by the method of Friedman and Haugen[12] except that KOH was substituted for NaOH to avoid sodium phosphate crystallization from the mixtures. Absorptivity was measured at 515 nm using a Gilford Model 300 spectrophotometer.

Acknowledgements—The skilful assistance of Miss Jane Hertel in this study is gratefully acknowledged. Supported by The U.S. Atomic Energy Commission Contract No. AT(11-1)-1403. AEC Document No. 100-1403-9.

[12] T. E. FRIEDEMANN and G. E. HAUGEN, *J. Biol. Chem.* **147**, 415 (1943).