Phytochemistry 1974 Vol 13 pp 99 to 102 Pergamon Press Printed in England

SEPARATION AND PROPERTIES OF TWO ORNITHINE CARBAMOYLTRANSFERASES FROM *PISUM SATIVUM* SEEDLINGS

SALWA EID, YOUSEF WALY and AHMED T ABDELAL*

Faculty of Agriculture, Ain Shams University, Cairo Egypt

(*Received* 24 *June* 1973 *Accepted* 20 *July* 1973)

Key Word Index—*Pisum sativum*, Leguminosae, pea, ornithine carbamoyltransferases

Abstract—Two ornithine carbamoyltransferases were separated from pea seedlings on DEAE-cellulose The two enzymes have different pH-activity curves Lineweaver–Burk plots for both enzymes are linear for carbamoyl phosphate and the Michaelis constants are of the same order of magnitude The plot for ornithine was linear for one enzyme but a concave down for the other indicating negative cooperativity The presence of two ornithine carbamoyltransferases is consistent with the presence of two pools for ornithine (one catabolic and the other anabolic) previously suggested to exist in plant materials

INTRODUCTION

ORNITHINE CARBAMOYLTRANSFERASE (carbamylphosphate L-ornithine carbamoyltransferase, E C 2 1 3 3) has been purified and characterized from different bacterial sources[1–5] as well as from mammalian liver [6,7] The presence of the enzyme in plants was also demonstrated[8–10] and its physiological role recently reviewed [11] In the course of the study on the regulation of arginine and uracil synthesis in pea seedlings, two ornithine carbamoyltransferases were detected The present paper reports the separation of these two enzymes and presents results concerning their properties and kinetic behaviour

RESULTS

Separation of two ornithine carbamoyltransferases

Solid ammonium sulphate was added to the extract slowly with stirring at 0° to give 40% saturation (240 g l^{-1}) The solution was allowed to stand for 30 min The precipitate obtained after centrifugation at 22000 *g* for 20 min contained no significant enzyme ac-

* Present address Department of Bacteriology University of California Davis CA 95616 U S A

[1] NAKAMURA, M and JONES, M E (1970) *Methods in Enzymology* (TABOR, H and TABOR, C W, eds), Vol 27A, pp 286–294, Academic Press, New York
[2] STALON, V, RAMOS, F, PIERARD, A and WIAME, J M (1967) *Biochim Biophys Acta* **139,** 91
[3] RAMOS, F, STALON, V, PIERARD, A and WIAME, J M (1967) *Biochim Biophys Acta* **139,** 98
[4] RODGER, P and NOVELLI, G D (1962) *Arch Biochem Biophys* **96,** 398
[5] RAVEL, J M, GRONA, M L, HUMPHREYS, J S and SHIVE, W (1959) *J Biol Chem* **234,** 1452
[6] BURNETT, G H and COHEN, P P (1957) *J Biol Chem* **229,** 337
[7] REICHARD, P (1957) *Acta Chem Scand* **11,** 523
[8] REIFER, I and KLECZKOWSKI, K Z (1960) *Z Naturforsch* **15b,** 431
[9] KATING, H (1963) *Biochem Z* **336,** 489
[10] WIELGOT, B MORAWSKA, G and REIFER, I (1965) *Acta Soc Botan Polon* **34,** 205
[11] OAKS A and BIDWELL R G S (1970) *Ann Rev Plant Physiol* **21,** 43

tivity and was discarded The supernatant was brought to 60% saturation by further addition of the salt (130 g l $^{-1}$) The precipitate obtained was centrifuged and dissolved in 0 1 M potassium phosphate buffer, pH 7 6 The solution was then dialysed against 0 02 M potassium phosphate buffer pH 7 6

The dialysed preparation was applied to a DEAE-cellulose column (2 × 40 cm) equilibrated with 0 02 M potassium phosphate buffer pH 7 6 Unadsorbed proteins were washed with the same buffer Proteins adsorbed to the column were then eluted by an increasing convex gradient of potassium phosphate buffer pH 7 6 The gradient was produced in a constant volume mixing flask containing 500 ml of 0 02 M buffer and fed from a reservoir containing 0 5 M buffer The eluate was collected in 2 5 ml fractions at a rate of 2 ml per min Representative fractions were tested for ornithine carbamoyltransferase activity

The activity was found in two portions The first was eluted between 0 09 and 0 13 M with the peak of activity at 0 10 M The second was eluted between 0 32 and 0 39 with the peak at 0 36 M The two peaks of activity eluted with the low and the high buffer molarities are referred to as **1** and **2** in the following sections Table 1 summarizes the separation procedure

TABLE 1 SEPARATION OF ORNITHINE TRANSCARBAMYLASES FROM *Pisum sativum* SEEDLINGS

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Sp act (units mg)	Recovery (%)
Extract	850	46750	95200	2	100
Ammonium sulfate (40–60% saturation)	26	6180	68080	11	71
DEAE-cellulose					
enzyme **1**	62	96	28272	295	36
enzyme **2**	40	52	5920	114	

Properties of the separated enzymes

The effect of pH on initial velocities of the two enzymes was determined The results are presented in Fig 1 It is evident that the two enzymes have different pH optima Ornithine carbamoyltransferase **1** has a sharp pH optimum at 9 whereas the enzyme **2** has a broader pH optimum with maximal activity at pH 7 0

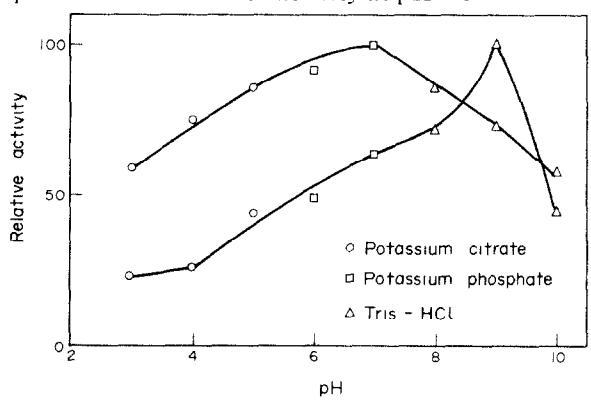

FIG 1 DEPENDENCE OF PEA SEEDLINGS ORNITHINE CARBAMOYLTRANSFERASES ON pH Final buffer molarity was 0 1 M

Preliminary studies on the two ornithine carbamoyltransferases from pea seedlings indicate a lack of specific metal ion requirement Addition of Mg^{2+}, Co^{2+}, Ni^{2+}, K^{+}, or Na^{+} at 1×10^{-3} M had no effect on enzyme activity Furthermore, inclusion of neutral K–EDTA at a concentration of 5×10^{-4} M in the dialysis buffer or the reaction mixture had no effect on the activity

The presence of mercaptoethanol (2×10^{-3} M) in the dialysis buffer or the reaction mixture did not affect the activities of either enzyme **1** or **2**, implying that free sulfhydryl groups are not essential for activity

Kinetic studies

The effects of varying the concentrations of ornithine and carbamyl phosphate on the activity of ornithine carbamoyltransferases **1** and **2** were studied The Lineweaver–Burk plots for carbamyl phosphate for the two enzymes were linear The values for Michaelis constants for carbamyl phosphate for the two enzymes are of the same order of magnitude ($3\,9 \times 10^{-3}$ and $6\,4 \times 10^{-3}$ M for **1** and **2** respectively) These values are also of the same order of magnitude as those reported for the enzymes from *Streptococcus lactis*[5] ($3\,7 \times 10^{-3}$ M) and from rat liver[6] (3×10^{-3} M) They are, however, considerably higher than those reported for *Streptococcus faecalis*[1] ($3\,6 \times 10^{-5}$ M) and *Escherichia coli*[4]

The plots for ornithine revealed an interesting difference between the two ornithine carbamoyltransferases of pea seedlings The double reciprocal plot for enzyme **2** was linear The Michaelis constant for that enzyme was calculated to be $4\,7 \times 10^{-3}$ M which is of the same order of magnitude as the bacterial and liver enzymes [1–7] The plot for enzyme **1** was not linear but a concave down One of the possible explanations of such a plot is the presence of negative cooperativity in the interactions of the sites for ornithine [12] Cleland has suggested[13] that negative cooperativity permits the apparent Michaelis constant to be adjusted to the physiological concentration, thus the enzyme always operates in the region of proportional control

DISCUSSION

The differences observed in pH optima and in kinetic properties of the two ornithine carbamoyltransferase activities separated on DEAE from pea seedling extracts suggest that they are two distinct enzymes

The occurrence of a catabolic and an anabolic ornithine carbamoyltransferase in *Pseudomonas fluorescens* was demonstrated by Stalon *et al*[2] The two enzymes were distinguished by their activities as a function of pH and by the regulation of their synthesis One enzyme was found to be subject to repression whereas the level of the other enzyme increased in the presence of arginine In contrast to the results presented here, Ramos *et al*[3] reported that the reciprocal plots for ornithine were linear for the two enzymes and the Michaelis constants were of the same order of magnitude It should be pointed out, however, that the highest ornithine concentration shown in their plots was 5×10^{-3} M for one enzyme and 1×10^{-2} M for the other [3] In this concentration range, our plots are linear for both enzymes

The separation of two ornithine carbamoyltransferases from *Bacillus licheniformis* was also reported [14] One of the two enzymes was repressible and the other inducible Unlike

[12] LEVITZKI, A and KOSHLAND, D E Jr (1969) *Proc Nat Acad Sci U S* **62**, 1121
[13] CLELAND, W W (1970) *The Enzymes* (BOYER, P D ed) Vol 2 pp 57 3rd Edn Academic Press New York
[14] BERNLOHR R W (1966) *Science* **152**, 87

the two enzymes reported in this work and those of *Pseudomonas*,[2,3] the enzymes from *Bacillus* had the same pH optimum No kinetic studies were reported for the *Bacillus* enzymes

In plants, there is some evidence for the presence of two ornithine pools Labeled arginine was converted to proline[15,16] and this conversion was more evident in cotyledons of germinating peas which actively metabolize arginine[17] The enzyme ornithine-ketoacid aminotransferase which converts ornithine to glutamine-γ-semialdehyde, has also been detected in plants[11] This enzyme was shown by Davis and Mora[18] to be principally a catabolic enzyme involved in arginine degradation in *Neurospora* Furthermore Mazelis[19] showed recently that ornithine-α-ketoglutaric aminotransferase of peanuts increased considerably during germination Based on the labeling studies and on the presence of ornithine-ketoacid aminotransferase in plants, Oaks and Bidwell[11] suggested the presence of two ornithine pools in plant materials a pool contributing carbon to arginine and one contributing carbon to proline or glutamic acid Thus exogenous arginine could be converted to proline via the sequence arginine → citrulline → ornithine → glutamine-γ-semialdehyde → Δ'-pyrroline-5-carboxylate → proline The presence of two such ornithine pools was shown more conclusively in studies with wild type and auxotrophic mutants of *Neurospora*[18,20,21]

The possible existence of two ornithine pools in plants[11,15,17] together with the separation of two ornithine carbamoyltransferases reported here suggest that in pea seedlings as in *Pseudomonas*[2,3] and *B licheniformis*,[14] the enzymes play different roles

EXPERIMENTAL

Preparation of extracts from pea seedlings Pea seeds (var Little Marvel) were obtained from the Egyptian Ministry of Agriculture The seeds were planted 13 mm deep and grown for 15 days in a greenhouse The seedlings were washed with H_2O and frozen at −20 for 2 hr The seedlings were homogenized (3 × 20 sec) in a Waring blendor (osterizer) with acetone (10 ml g) precooled to −20 and containing 10 mM mercaptoethanol The slurry was filtered and the cake resuspended in acetone-mercaptoethanol at −20 The suspension was rehomogenized (3 × 20 sec) and the filter cake dried at room temp for 3 hr The powder was stored at −20 Prior to use the powder was extracted in cold 0 1 M potassium phosphate buffer pH 7 6 for 30 min followed by centrifugation at 22 000 *g* for 30 min

Assay of ornithine carbamoyltransferase The enzyme was assayed at 30 by measurement of citrulline formed[22] The reaction mixture (final vol 2 ml) contained dilithium carbamyl phosphate (20 μmol) ornithine (20 μmol) K phosphate buffer (200 μmol pH 7 6) and the enzyme The reaction was started by the addition of the enzyme Aliquots (0 5 ml) were withdrawn at intervals of time and mixed with 2 5 ml of the H_2SO_4-diacetyl monoxime-diphenylamine sulfonate mixture[22] The tubes were kept in ice until the determination of citrulline was completed The sampling times were chosen so that a linear formation of citrulline was observed during the whole period Blanks containing the assay mixture but no enzyme and assay mixture without ornithine were routinely made The extracts were dialysed against 0 02 M K phosphate buffer before assay Specific activity is expressed as μmol of citrulline formed per hr per mg of protein Protein was determined[23] using crystalline bovine serum albumin as the standard

Materials DEAE-cellulose was purchased from Bio-Rad Laboratories (Richmond California) All other materials were obtained from British Drug House Co (Poole England)

[15] Naylor A W (1959) *Symp Soc Exp Bot* **13**, 193
[16] Boulter D and Barber J T (1963) *New Phytologist* **62**, 301
[17] Jones V M and Boulter D (1968) *New Phytologist* **67**, 925
[18] Davis R H and Mora J (1968) *J Bact* **96** 383
[19] Mazelis M (1969) *Phytochemistry* **8**, 801
[20] Vogel H J and Bonner D M (1954) *Proc Nat Acad Sci U S* **40**, 688
[21] Vogel R H and Kopac M J (1959) *Biochim Biophys Acta* **36**, 505
[22] Gerhart, J C and Pardee A B (1962) *J Biol Chem* **237**, 891
[23] Lowry O H Rosebrough N J Farr A L and Randall R J (1951) *J Biol Chem* **193**, 265