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Properties of Ornithine Carbamoyltransferase from *Pisum sativum* L.

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ABSTRACT

Some properties of ornithine carbamoyltransferase from chloroplasts isolated from leaves of *Pisum sativum* L. (cv Marzia) were compared with those of the enzyme partially purified (316-fold) from shoots of seedlings after 3 weeks of cultivation.

Both preparations showed a pH optimum at pH 8.3 and had the same affinity to ornithine (K_m = 1.2 millimolar) as well as to carbamoyl phosphate (K_m = 0.2 millimolar). The approximate molecular weight determined by gel sieving was 77,600.

A desalted ammonium sulfate precipitate from 14-day seedlings (inclusive roots and senescing cotyledons) was applied on a column of anion exchanger. The elution pattern showed one peak of ornithine carbamoyltransferase activity. This elution pattern was the same as observed for the enzyme from chloroplasts.

The results suggest the presence of one form of ornithine carbamoyltransferase in pea seedlings.

In higher plants, OCT[1] (EC 2.1.3.3.) is involved in the synthesis of citrulline. The enzyme has been localized in chloroplasts from pea leaves (23) and plastids from soybean cell suspension cultures (21) by means of density gradient studies.

Two isozymes of OCT have been found in sugarcane (8), pea seedlings (7), root nodules from black alder (13), and apple leaves (22). These isozymes show different elution patterns from an anion exchange column (7, 8, 13) and have different kinetic properties (7, 8, 13, 22). A cytoplasmic and a mitochondrial enzyme with mol wt of 79,000 and 224,000, respectively, were distinguished in sugarcane (8). It has been suggested that the two isozymes from pea seedlings (7) and sugarcane (8) have different physiological functions. An anabolic and a catabolic function have been proposed which are related to the synthesis and the degradation of citrulline, respectively. In root nodules of black alder, OCT is involved in the synthesis of citrulline. The function of the second enzyme is therefore not clear.

In the present study, we have investigated the properties of a partially purified OCT from pea shoots. These properties are compared with those of OCT from chloroplasts.

MATERIALS AND METHODS

Plant Material. Plants (*Pisum sativum* L. cv Marzia) were cultivated in a controlled environment with a daily regime of 12 h light (30 $w \cdot m^{-2}$; Philips TLF 40W/55) at 18°C, 12 h dark at 15°C. Unless stated otherwise, the seedlings were harvested after 3 weeks. The shoots were cut about 1 cm above the cotyledons and stored at −20°C. Freshly harvested leaves of seedlings after 3 weeks of cultivation were used for isolation of chloroplasts.

Enzyme Purification. Unless stated otherwise all steps were carried out at 4°C.

Protein Extraction. Proteins were extracted from 500 to 600 g (fresh weight) pea shoots along the lines described by Eid *et al.* (7). The dry powder obtained after acetone precipitation was kept at −20°C. Prior to use, the powder was extracted during 1 h in 1 L buffer containing 50 mM K-phosphate (pH 7.8) and 1 mM 2-mercaptoethanol. The extract was passed through a Perlon screen (mesh width 45 μm) and then centrifuged for 7 min at 20,000 *g*. This is the filtrate referred to as step 1 in Table I.

Ammonium Sulfate Precipitation. The supernatant fluid was brought to 40% saturation by solid $(NH_4)_2SO_4$. The supernatant obtained after centrifugation (20 min at 22,000 *g*) was brought to 65% $(NH_4)_2SO_4$ saturation and centrifuged again. The precipitate was dissolved in about 300 ml buffer containing 50 mM K-phosphate (pH 7.8), 50 mM L-orn, and 1 mM 2-mercaptoethanol.

Heat Treatment. The extract was heated under stirring to 61°C in a water bath. The solution was maintained at 61 to 62°C for 1 min, then cooled in ice and centrifuged (7 min at 20,000 *g*) to sediment the denaturated proteins. The supernatant was filtered through filter paper and then brought to 70% saturation by addition of solid $(NH_4)_2SO_4$. The precipitate obtained after centrifugation (20 min at 22,000 *g*) was resuspended in 40 ml buffer containing 50 mM K-phosphate (pH 7.8), 50 mM L-ornithine, 1 mM 2-mercaptoethanol, and $(NH_4)_2SO_4$ (70% saturation). Celite 545 (3 g) was added to this suspension.

Fractionated Dissolving of the Ammonium Sulfate Precipitate. The protein and celite suspension was packed into a 1.6 × 40 cm column containing a bed of celite (about 3 cm) in the same buffer as used for the protein suspension. The column was washed with 100 ml of this buffer (volume = 30 ml/h); 10 ml fractions were collected. The proteins were eluted (volume = 30 ml/h) with a linear gradient between 257 ml of the equilibrating buffer at 70% $(NH_4)_2SO_4$ saturation and 300 ml buffer without $(NH_4)_2SO_4$. The OCT was eluted at about 45% $(NH_4)_2SO_4$ saturation. The active fractions were pooled and precipitated by adding $(NH_4)_2SO_4$ to 85% saturation. After centrifugation, the pellet was dissolved in a minimal volume of buffer containing 50 mM K-phosphate (pH 7.8) and 1 mM 2-mercaptoethanol and passed through a Sephadex G-25 column (2.6 × 20 cm) equilibrated with the same buffer.

Chromatography on DEAE-Sephadex A-50. The dialyzed protein solution was chromatographed on a column of DEAE-Sephadex A-50 (1.6 × 15 cm) that had been equlibrated with buffer containing 50 mM K-phosphate (pH 7.8) and 1 mM 2-mercaptoethanol. The column was washed with 80 ml of this buffer. The OCT was eluted (volume = 40 ml/h) with a linear gradient of NaCl (0–300 mM) in 150 ml starting buffer. Fractions of 5 ml were collected. The OCT activity was eluted at 100 mM

[1] Abbreviations: OCT, ornithine carbamoyltransferase; L-orn, L-ornithine; CP, carbamoyl phosphate.

NaCl. The active fractions were pooled and concentrated by ultrafiltration in an Amicon Diaflow equipment with a PM 110 membrane to a minimal volume.

Gelfiltration on Sephacryl S-200. The enzyme preparation was applied on a column (1.6 × 80 cm) of Sephacryl S-200 equilibrated with buffer containing 50 mM K-phosphate (pH 7.8), 1 mM 2-mercaptoethanol, and 0.02% NaN_3. The column was run at 35 ml/h; fractions of 2 ml were collected. The active fractions were pooled and concentrated by ultrafiltration in an Amicon Diaflow equipment with a PM 110 membrane; a minimal volume was dialyzed against buffer containing 0.1 M Tes (pH 8.2) and then stored at −20°C.

Disc PAGE. Gels (0.4 × 8 cm) containing 7.5% acrylamide were prepared according to Davis (described as gel system no. 1 in Reference 15). Proteins (14 μg/gel) were detected by staining with Coomassie Brilliant Blue R250 according to Chrambach *et al.* (4).

Mol Wt Determination. A column (1.6 × 80 cm) of Sephacryl S-200 equilibrated with buffer containing 50 mM K-phosphate (pH 7.8), 1 mM 2-mercaptoethanol, and 0.02% NaN_3 was used to determine the approximate mol wt by gel sieving. The column was calibrated by noting the elution volumes (v_e) of aldolase, bovine albumin, ovalbumin, chymotrypsinogen, and Cyt *c*. The void volume (v_o) was determined with blue dextran. The parameter K_{av} (10) was calculated according to the formula $K_{av} = (v_e - v_o)/(v_t - v_o)$ in which v_t represents the total bed volume.

Enzyme Fraction from Chloroplasts. Chloroplasts were purified from 8 g of pea leaves according to Mills and Joy (17). The chloroplasts were purified by washing through a layer containing a treated silica sol (Percoll). The recovery of Chl and marker enzymes in the chloroplast pellet as reported by Mills and Joy (17) has been confirmed in this study (data not shown). To obtain a clear preparation of proteins, a volume of 5 ml precooled acetone (−20°C) containing 10 mM 2-mercaptoethanol was added to the chloroplast pellet. After shaking (30 s) and centrifugation (10 min at 1000 *g*), the precipitate was washed once with the precooled acetone. The precipitate was dissolved in buffer containing 0.1 M Tes (pH 8.2) or 50 mM K-phosphate (pH 7.8) with 1 mM 2-mercaptoethanol and stored at −20°C. After thawing, the fraction was centrifuged to obtain a clear preparation.

Assay of OCT. Composition of the reaction mixture was based on the method of Nakamura and Jones (18) and contained 60 mM Tes (adjusted with NaOH to pH 8.2), 2.5 mM L-orn, 10 mM dilithium CP, and 50 μl enzyme preparation in a final volume of 1 ml. The citrulline formed was determined colorimetrically (3). A unit of enzyme was taken as that amount which would catalyze the formation of 1 μmol citrulline per min at 25°C under standard assay conditions.

Protein. Protein was determined by the method of Lowry *et al.* (12).

Chemicals. Celite 545 (washed with acid) was purchased from John Mansville. Percoll, Sephadex G-25, DEAE-Sephadex A-50, Sephacryl S-200, blue dextran, and aldolase were obtained from Pharmacia. Bovine albumin, ovalbumin, chymotrypsinogen, and Cyt *c* were obtained from Serva. L-Orn and Tris were from BDH. CP was purchased from Boehringer Mannheim GMBH.

RESULTS

Enzyme Purification. Table I summarizes a representative preparation of OCT from pea shoots. A typical elution chromatograph obtained after application the protein and celite suspension on a column with celite is shown in Figure 1. The purified enzyme hardly lost any activity when it was stored at −20°C in buffer containing 50 mM K-phosphate (pH 7.8) and 1 mM 2-mercaptoethanol for at least 1 month.

The homogeneity of the 316-fold purified preparation was investigated by means of disc PAGE (7.5%). Four bands were visible after electrophoresis (14 μg protein/gel) and staining with Coomassie Brilliant Blue R250. The purity of the enzyme preparation can probably be enhanced when less steep gradients are used with the column of celite and the column of DEAE-Sephadex A-50.

Intracellular Localization of OCT. The enzyme cosedimented with the chloroplast markers Chl and NADP-linked glyceraldehyde-3-P dehydrogenase (data not shown). This agrees with the results of Taylor and Stewart (23) on the localization of OCT in pea leaves.

pH Optimum. Assay of the partially purified enzyme and the enzyme fraction from chloroplasts at different pH values resulted in the same pH curve (Fig. 2). The pH optimum was at pH 8.3. Assay in 60 mM Tris-HCl at pH 8.2 resulted in a loss (80%) of activity.

Affinity to Ornithine and CP. When OCT activity was measured at pH 8.2 with varying ornithine concentrations (up to 10 mM), no substrate inhibition was observed. Affinity for ornithine was determined by plotting the velocities as a function of substrate concentration according to the Michaelis-Menten equation (data not shown). A K_m of 1.2 mM was calculated for both the partially purified enzyme preparation from shoots and the enzyme preparation from chloroplasts.

The CP concentration was varied up to 10 mM at an ornithine concentration of 2.5 mM. A K_m of 0.2 mM for CP was calculated for both enzymes preparations. The correlation coefficients of the double reciprocal plots used for the calculation of both K_m values still exceeded 0.995.

Phosphate is a competitive inhibitor with respect to CP. A K_i of 1.1 to 1.3 mM was calculated for the purified enzyme and for the enzyme directly isolated from the chloroplasts.

Table I. *Summary of OCT Purification from Shoots of P. sativum L.*

Purification Step	Total Volume	Total Protein	Total Activity	Yield	Specific Activity	Purification
	ml	*mg*	*units*	*%*	*units/mg protein*	*fold*
1. Filtrate	840	2153	1105	100	0.5	
2. Ammonium sulfate 40–65%	287	844	907	82	1.1	2
3. Heat treatment, 70% $(NH_4)_2SO_4$ precipitate of the supernatant	40.5	104	655	59	6.3	13
4. Celite, 85% $(NH_4)_2SO_4$ precipitate of the eluate	4.3	44	505	46	11.5	23
5. DEAE-Sephadex A-50, after ultrafiltration	1.7	4.3	222	20	51.6	103
6. Sephacryl S-200, after ultrafiltration	2.3	0.5	84	7.6	157.8	316

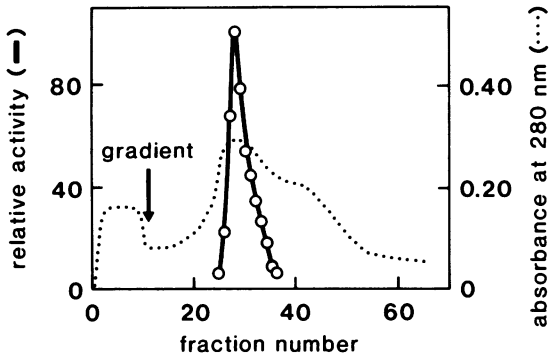

FIG. 1. Chromatography of OCT from *Pisum sativum* L. on a column of Celite 545 (1.6 × 10 cm). Proteins were eluted with a linear gradient (70–0% $(NH_4)_2SO_4$ in 557 ml). Fractions of 10 ml were collected at a flow rate of 30 ml/h. 100% represents 72 units/fraction.

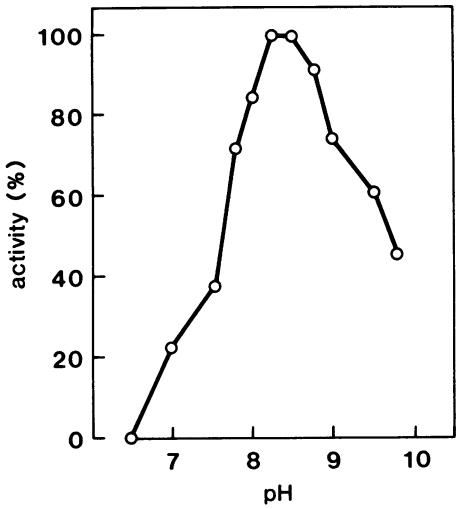

FIG. 2. Dependance of enzyme activity on pH. The standard reaction mixture was buffered with Mes (pH 6.5–7.0), Tes (pH 7.0–8.0), and diethanolamine-acetate (pH 8.0–10.0). 100% represents 0.05 units/ml enzyme preparation.

Estimated Mol Wt. The approximate mol wt of OCT was determined with the partially purified enzyme after the ion-exchange purification step and with the enzyme fraction from chloroplasts. The OCT activity eluted from the Sephacryl S-200 column as a sharp, symmetrically shaped peak. The approximate mol wt of OCT (after three runs of each preparation) amounted to 77,600 (Fig. 3). Some loss (about 40%) was observed regardless of the amount of applied activity.

Ion-Exchange Chromatography. To investigate the presence of isozymes, an experiment (Table II; Fig. 4) with 14-d seedlings (inclusive roots and senescing cotyledons) was carried out along the lines described by Eid *et al.* (7) but on a smaller scale. One peak of OCT activity was observed, which eluted from the column when a concentration of 100 mM NaCl was reached. This enzyme is probably the first enzyme (Table III) eluted in the experiment of Eid *et al.* (7).

The elution pattern of OCT was the same as that shown in Figure 4 when a partially purified preparation after the celite step (Table I) or the enzyme fraction from chloroplasts was applied on a DEAE-Sephadex A-50 column.

DISCUSSION

The enzyme preparation from chloroplasts and the partially purified OCT from pea shoots have the same kinetic properties,

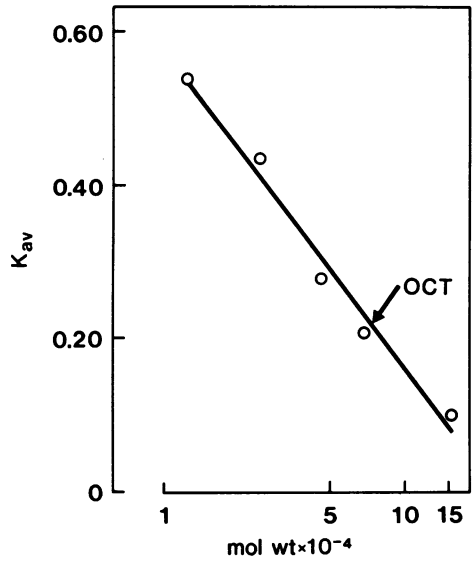

FIG. 3. Estimation of mol wt by gel filtration. Standard proteins (O) and OCT (↓) were eluted from a Sephacryl S-200 column (1.6 × 80 cm) with buffer containing 50 mM K-phosphate (pH 7.8), 1 mM 2-mercaptoethanol, and 0.02% NaN_3. Fractions of 1.7 ml were collected. The elution volume of each standard protein was determined by observing *A* at 280 nm. The standard proteins used were aldolase (158,000), bovine albumin (67,000), ovalbumin (45,000), chymotrypsinogen (25,000), and Cyt *c* (12,300). For calculation of K_{av}, see text.

Table II. *Ion-Exchange Chromatography with a Desalted 40 to 65% Ammonium Sulfate Precipitate from Pea Seedlings*

After washing twenty 14-d seedlings (fresh wt 24 g, inclusive roots and senescing cotyledons), the filtrate was prepared along the lines described by Eid *et al.* (7).

Step	Total Activity	Yield
	units	%
Filtrate	15.7	100
Precipitate 40–65%	10.6	67.5
Sephadex G-25	9.4	59.9
DEAE-Sephadex A-50	7.2	45.9

the same mol wt, and show the same elution patterns from a DEAE-Sephadex A-50 column. Therefore, a second isozyme with other properties located outside the chloroplast seems unlikely. The results of our experiment with pea seedlings carried out according to Eid *et al.* (7) also indicated the presence of one OCT. However, the similarity observed in the elution patterns when isozymes of OCT were separated after extraction from pea seedlings (7), sugarcane (8), and root nodules from black alder (13) makes it difficult to neglect the existence of isozymes. In all these studies, about 80% of the activity was eluted at a low ionic strength (E_1, Table III), whereas the remaining activity was eluted at a high ionic strength (E_2, Table III).

The mitochondrial location as demonstrated for the second enzyme (E_2) from sugarcane (8) is not convincing since plastids may have been cosedimented. An anabolic and a catabolic function were suggested for the mitochondrial and cytoplasmic enzyme, respectively (8). By referring to different ornithine pools in plants, an anabolic and a catabolic function were also proposed with pea seedlings as an explanation for the existence of two isozymes (7).

However, a catabolic function of OCT in higher plants seems unlikely because of the following arguments. OCT is located in the chloroplast. The chloroplast is involved in the biosynthesis of many amino acids (16) which makes a catabolic function of

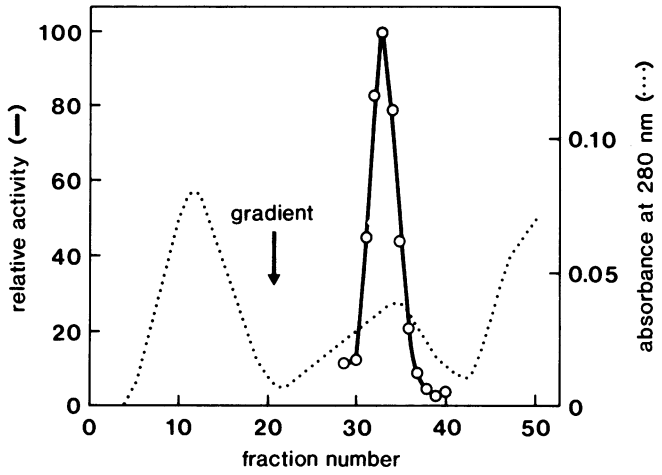

FIG. 4. Chromatography of OCT on a DEAE-Sephadex A-50 column (0.9 × 14 cm) after application of a desalted ammonium sulfate precipitate from intact pea seedlings (inclusive roots and senescing cotyledons). Proteins were eluted with a linear gradient (0–600 mM NaCl in 64 ml). Fractions of 2.1 ml were collected at a flow rate of 20 ml/h. 100% represents 1.8 units/fraction.

OCT in pea leaves unlikely. Conversion of L-orn occurs in the chloroplast as well as in the mitochondria. However, in the latter organelle, L-orn is not derived from citrulline but from the degradation of arginine by arginase activity (6, 24). Thus, a catabolic function of OCT in this organelle seems unlikely. A mitochondrial location of anabolic OCT as suggested for sugarcane has been demonstrated cytochemically in the root nodules of black alder (20). This somewhat surprising result indicates differences between higher plants which are not yet understood.

Different subunit configurations of OCT from one source have recently been demonstrated in an enzyme preparation from *Bacillus subtilis* (19), which is thought to contain an anabolic OCT. The relative amounts of these configurations (dimer, tetramer, and hexamer) differed from preparation to preparation. Only a dimer was found when 2-mercaptoethanol was added to the sucrose gradient used. It can therefore be concluded that one has to be careful to ascribe physiological functions to isozymes of OCT when they are found in enzyme preparations.

The mol wt of enzymes listed in Table III have been determined by means of gel chromatography or polyacrylamide gradient gel electrophoresis (black alder). Notwithstanding the large differences, a certain relationship can be pointed out. The mol wt of the subunit of pea OCT is 37,000 (5), which resembles the mol wt of the subunits from toad liver (36,400) and rat liver (36,800) measured via the same procedure (5). The mol wt of the OCT subunit from bacteria, yeast, and ox liver have been listed by Legrain *et al.* (11). More recently, data for chicken liver (25) and *B. subtilis* (19) have been published. All mol wt are in the range from 35,000 to 44,000, which means that a striking similarity exists between the different species. From the mol wt of the pea subunit (5), the mol wt of dimers, trimers, tetramers, and hexamers can be calculated as 74,000, 111,000, 148,000, and 222,000, respectively. Comparing these mol wt with the mol wt of enzymes in Table III, several configurations can be roughly distinguished. Evidence is present for bacteria, yeast, and mammalian tissue (11) that the native (anabolic) OCT is a trimer with a mol wt between 105,000 and 150,000. It may be clear from the data up to now that such evidence does not exist for higher plants. A systematic approach by determination of mol wt after the same procedure for storage of tissue and extraction of enzymes may help to find out whether the differences observed (Table III) result from the varying experimental procedures used.

Compared with the K_m values for L-orn, the K_m values for CP (Table III) vary rather widely. The high K_m for CP measured for pea OCT by Eid *et al.* (7) results from the assay in K-phosphate.

Table III. *Properties of OCT from Higher Plants and a Blue-Green Alga*

Source	Isozymes	pH Optimum	K_m for L-Orn	K_m for CP	Mol Wt	Reference No.
			mM			
P. sativum L. cv Marzia		8.3	1.2	0.2	77,600	this report
Pisum[a]		8.4	4.2[b]	0.91[b]		9
Pisum					105,000	26
P. sativum L.	E_1[c]	9.0		3.9		7
	E_2	7.0	4.7	6.4		
Saccharum sp., var H50-7209	E_1	7.5	3.11	0.12	79,000	8
	E_2	7.5–8.5	0.5	0.11	224,000	
Alnus glutinosa L. Gaertn	E_1	7.8	5.1	4.7		13
	E_2	8.4	2.6	2.4		
Alnus glutinosa L. Gaertn	E_1	7.8	10.0	1.1–2.8	200,000	14
Pyrus malus L.	E_1	7.8	1.22	1.9		22
	E_2	8.6	4.8	6.0		
Daucus carota L. var Nantes		7.5	3.3	0.009	158,000	1
Triticum aestivum L.					121,000	1
Nostoc muscorum Kützing (blue-green alga)		9.5	2.5	0.7	75,000	2

[a] It was not stated whether *P. sativum* or *P. arvense* was used.

[b] Values derived from data represented in substrate saturation curves.

[c] E_1 and E_2 were separated by their different elution patterns from a column of anion-exchanger with the exception of E_1 and E_2 from apple leaves, which were distinguished by their different pH optima.

Phosphate is a competitive inhibitor of OCT with respect to CP, as has been demonstrated for OCT from carrot cells (1) and a blue-green alga (2) and could also been confirmed in this study. We believe that the large differences in the K_m for CP between species may result from contamination with phosphate, possibly present in the solution of the rather labile CP. A relatively high K_m observed for CP might also result from phosphatase activity present in enzyme preparations which leads to a decreased availability of CP.

Although several authors use Tris as assay buffer, we observed 80% decrease in activity when Tris-HCl (60 mM, pH 8.2) was used. This phenomenon has also been observed (60% loss of activity in 0.1 M Tris-HCl at pH 7.5–8.5) with OCT from *B. subtilis* (19).

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