Note

Subcellular Distribution and Some Properties of Citrullinase in *Euglena gracilis* Z

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It is evident that in *Euglena gracilis* arginine acts as the important nitrogen reserve for growth.¹⁾ Accumulated arginine is rapidly metabolized for renewed cell growth when a carbon source is aquired, and the amino acid is catabolized by the so-called arginine dihydrolase pathway in which arginine deiminase and citrullinase participate.²⁾ In our preceeding paper²⁾ we reported the purification and some properties of arginine deiminase and discussed the regulation of arginine metabolism by the enzyme.

The occurrence of citrullinase has been reported in some bacteria³⁾ and *Tetrahymena*,⁴⁾ a protozoan, and the *Tetrahymena* enzyme has been partially purified, but no information is available on its subcellular location and further properties. This paper describes the subcellular distribution, purification, and some properties of citrullinase in *Euglena gracilis*.

E. gracilis Z was cultured for 5 days at 27°C under illumination (2500 lux) in glucose-glutamate medium.¹⁾ The activity of citrullinase was assayed by measuring

¹⁴CO₂ evolution from L-[carbamoyl-¹⁴C]-citrulline (0.1 μCi/mM) by the enzyme reaction described previously.¹⁾ Succinate semialdehyde dehydrogenase, a mitochondrial marker enzyme;⁵⁾ glucose-6-phosphatase, a microsomal marker enzyme;⁶⁾ ribulose-1,5-bisphosphate carboxylase, a chloroplast-marker enzyme;⁷⁾ and glutamate dehydrogenase (NADP-linked), a cytosolic marker enzyme,⁸⁾ were assayed by the cited methods.

Protein was determined by the method of Bradford.⁹⁾

The molecular weight of citrullinase was determined by the method of Andrews¹⁰⁾ using a column (1.5 × 60 cm) of Sephacryl S-200 and by SDS-polyacrylamide gel electrophoresis by the method of Weber and Osborn¹¹⁾ using an electrophoresis calibration kit as protein standards from Pharmacia Fine Chemicals.

A cell homogenate of *Euglena* was obtained by partial digestion of the pellicle followed by mild mechanical disruption,¹²⁾ and fractionated by differential centrifugation by the method of Tokunaga *et al.*⁸⁾ Table I shows the distribution of the citrullinase activity and activities of marker enzymes in the subcellular fractions from differential centrifugation of the homogenate of *E. gracilis*. Citrullinase was found only in the mitochondrial fraction, as judged from the distribution of succinate semialdehyde dehydrogenase. Sonication of the isolated mitochondrial fraction released the citrullinase as well as succinate semialdehyde dehydrogenase, a marker enzyme for the mitochondrial matrix.⁵⁾ That citrullinase is distributed in parallel with arginine deiminase as described previously²⁾ indicates that the arginine dihydrolase pathway functions in the mitochondria of *Euglena*.

Citrullinase was purified from *Euglena* cell-free extract. The crude extract containing 187.2 mg of protein was centrifuged at 100,000 × *g* for 1 hr and the supernatant was put on a DEAE-cellulose column (3 × 20 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The column was washed with the same buffer and then eluted

TABLE I. DISTRIBUTION OF CITRULLINASE AND MARKER ENZYMES IN SUBCELLULAR FRACTIONS OF *E. gracilis*

	Enzyme activity ^a			
	Cytosol	Mitochondria	Microsomes	Chloroplasts
Citrullinase	6.8 (2.7) ^b	84.2 (33.4) ^b	0.9 (0.4) ^b	5.1 (2.0) ^b
Glutamate dehydrogenase (NADP-linked)	94.1	0	0	0
Succinate semialdehyde dehydrogenase (NADP-linked)	5.1	86.9	0	3.5
Glucose-6-phosphatase	0	0	27.3	0
Ribulose-1,5-bisphosphate carboxylase	7.5	9.9	3.7	76.0

^a % against the activity in the crude extract.

^b Specific activity, nmol/mg protein/min.

with 300 ml of a linear concentration gradient (0 to 0.3 M) of potassium chloride – 10 mM phosphate buffer, pH 7.0. The enzyme activity was eluted at 0.2 M potassium chloride and the fractions were collected and concentrated by adding ammonium sulfate to 70% of saturation. The enzyme solution was chromatographed on a Sephacryl S-200 column (1 × 73 cm) equilibrated with potassium phosphate buffer, pH 7.0. The active fractions were combined and DEAE-cellulose column chromatography and Sephacryl S-200 gel filtration were repeated to purify it further. By these steps, citrullinase was purified 64-fold over the cell-free extract. The preparation exhibited only one protein band in SDS-polyacrylamide disc gel electrophoresis. This is the first case of purification of this citrullinase to homogeneity.

The molecular weight of this enzyme was calculated to be 94,000 by Sephacryl S-200 column chromatography and 31,000 by SDS-polyacrylamide gel electrophoresis, indicating that the citrullinase consists of 3 identical subunits. The enzyme reaction proceeded stoichiometrically to form NH_3 , CO_2 , and ornithine from citrulline by determining formed NH_3 and ornithine, and decreased citrulline with a Hitachi amino acid autoanalyzer, KLA-5, and evolved CO_2 by the method described above.

The enzyme was stable up to 40°C after treatment for 10 min, and retained 87% of its activity after treatment at 50°C. The activity was completely lost at 60°C. The maximum activity of the enzyme was obtained at 30°C and pH 7.0 to 7.2. The enzyme was stable in the range of 6.0 to 7.0 when treated at various pHs for 10 min at 50°C before the enzyme reaction. *Tetrahymena* enzyme is maximally active at pH 6.8.⁴⁾

Euglena enzyme obeyed Michaelis-Menten kinetics toward citrulline and the K_m value was 4.55 mM, while *Tetrahymena* enzyme has been reported not to follow normal Michaelis-Menten kinetics, giving 1.06 mM as the citrulline concentration at which it reached a half-maximal reaction rate, and it is inhibited by high concentrations of citrulline. From the Hill plots of the kinetics the n value was 1.4 ± 0.15 .⁴⁾ *Euglena* enzyme was strongly inhibited by sulfhydryl reagents such as mersalyl and HgCl_2 at 1 mM and the inhibition was removed by 2-mercaptoethanol, indicating that a sulfhydryl group in

the protein is concerned with the active center of the enzyme. ADP and AMP with and without addition of P_i (each 1 mM) in the presence of Mg^{2+} had no effect on the activity and other divalent cations like Co^{2+} , Mn^{2+} , Ca^{2+} , Ni^{2+} , and Fe^{2+} hardly affected the enzyme activity. Slade³⁾ and Slade *et al.*¹³⁾ reported, using a cell-free extract, that the citrullinases obtained from *Streptococcus* and *Pseudomonas* required AMP or ADP, P_i and Mg^{2+} for reaction. They did not report the occurrence of the reverse reaction of ornithine transcarbamylase or the existence of carbamate kinase to form ATP, CO_2 , and NH_3 via carbamoylphosphate. Effects of some arginine analogues containing guanidino group and nitrogenous compounds related to arginine metabolism on the enzymatic hydrolysis of citrulline were not shown to be different from arginine deiminase.²⁾

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