CRYSTALLINE ARGININE RACEMASE

Takamitsu Yorifuji and Koichi Ogata
Department of Agricultural Chemistry, Kyoto University,
Kyoto, Japan

and

Kenji Soda*
Institute for Chemical Research, Kyoto University, Uji,
Kyoto-Fu, Japan

Received February 10, 1969

Summary. Arginine racemase has been purified from the extract of *Pseudomonas graveolens* approximately 2,300-fold by a procedure including column chromatography on DEAE-cellulose, DEAE-Sephadex and Sephadex G-150. The purified enzyme was crystallized. The crystalline enzyme is homogeneous upon analysis by ultracentrifugation and its molecular weight is 167,000. The enzyme exhibits absorption maxima at 280 mμ and 420 mμ, and contains firmly bound pyridoxal 5'-phosphate.

Several amino acid racemases have been partially or highly purified; lysine racemase by Ichihara *et al.*(1960), glutamate racemase by Glaser (1960) and Tanaka *et al.*(1961), alanine racemase by Diven *et al.*(1964), Free *et al.* (1968) and Rosso *et al.*(1968), and proline racemase by Cardinale and Abeles (1968).

Arginine racemase was discovered in the extract of *Ps. graveolens* by Soda, Yorifuji and Ogata (1967). The present communication describes the purification, crystallization of this enzyme and some of its properties.

Purification and Crystallization.

Ps. graveolens IFO 3460 was grown in a medium composed of 1.0% peptone, 0.2% yeast extract, 0.05% L-arginine·HCl, 0.5% NaCl and 0.1% K_2HPO_4 (pH 7.2). The cultures were grown at 30° for about 24 hr under aeration. The cells were harvested by centrifugation and washed twice with 0.85% sodium chloride.

*To whom requests for reprints should be addressed.

Step 1. All subsequent operations were performed at 0-5°. The washed cells (about 2,000 g wet wt.) were suspended in 5 liters of 5 mM Tris-HCl buffer, pH 8.0, and disrupted in 250 ml portions by treatment for 20 min in a 19-kc sonic disintegrator. The intact cells were removed by centrifugation at 5,000 *g* for 15 min. Step 2. The turbid supernatant containing the extract and cell debris (about 180 g of protein) was brought to 30% saturation with ammonium sulfate. The precipitate was collected by centrifugation at 15,000 *g* for 15 min, suspended in 5 mM Tris-HCl buffer, pH 8.0, and dialyzed against two changes of the same buffer. Step 3. To a 1-liter portion of the suspension, was added slowly 250 ml of chilled n-butanol (-5°) under vigorous stirring. After standing for 30 min under stirring, the mixture was brought to 35% saturation with ammonium sulfate. The aqueous layer obtained by centrifugation was dialyzed against four changes of 5 mM Tris-HCl buffer, pH 8.0. The dialyzed enzyme was concentrated by addition of ammonium sulfate (75% saturation). The precipitate was dissolved in 0.01 M potassium phosphate buffer, pH 7.3, containing 2×10^{-5} M pyridoxal 5'-phosphate. The enzyme solution was dialyzed against the same buffer. Step 4. The enzyme was applied to a DEAE-cellulose column (5.5 x 45 cm) equilibrated with the dialysis buffer. After the column was washed thoroughly with the buffer containing 0.07 M sodium chloride, the enzyme was eluted with the buffer supplemented with 0.14 M sodium chloride. The active fractions were pooled, concentrated by addition of ammonium sulfate (75% saturation) and dissolved in 0.01 M potassium phosphate buffer, pH 7.3, containing 2×10^{-5} M pyridoxal 5'-phosphate. Step 5. The enzyme was applied to a Sephadex G-150 column (3 x 80 cm) washed with 0.01 M potassium phosphate buffer, pH 7.3, containing 2×10^{-5} M pyridoxal 5'-phosphate and eluted with the same buffer. The active fractions were pooled, concentrated by addition of ammonium sulfate (75% saturation) and dissolved in the same buffer, followed by dialysis. Step 6. The enzyme was applied to a DEAE-Sephadex (A-50) column equilibrated with the same buffer in Step 5. After the column was washed with the buffer

containing 0.1 M sodium chloride, the enzyme was eluted with the buffer containing 0.18 M sodium chloride, pooled and concentrated by addition of ammonium sulfate (75% saturation). The enzyme was dissolved in 0.01 M potassium phosphate buffer, pH 7.3, containing 2×10^{-5} M pyridoxal 5'-phosphate. Step 7. The enzyme solution was placed on a Sephadex G-150 column (1 x 90 cm) equilibrated as described in Step 5. The enzyme was eluted with 0.01 M potassium phosphate buffer, pH 7.3, containing 2×10^{-5} M pyridoxal 5'-phosphate. The active fractions were pooled and concentrated by addition of ammonium sulfate (75% saturation). The enzyme was dissolved in a small volume of 0.01 M potassium phosphate buffer, pH 7.3, containing 2×10^{-5} M pyridoxal 5'-phosphate. The insoluble materials were removed by centrifugation. Step 8. Ammonium sulfate was added slowly to the enzyme solution, maintaining the pH at 7.2 to 7.4 with 3 N ammonium hydroxide,

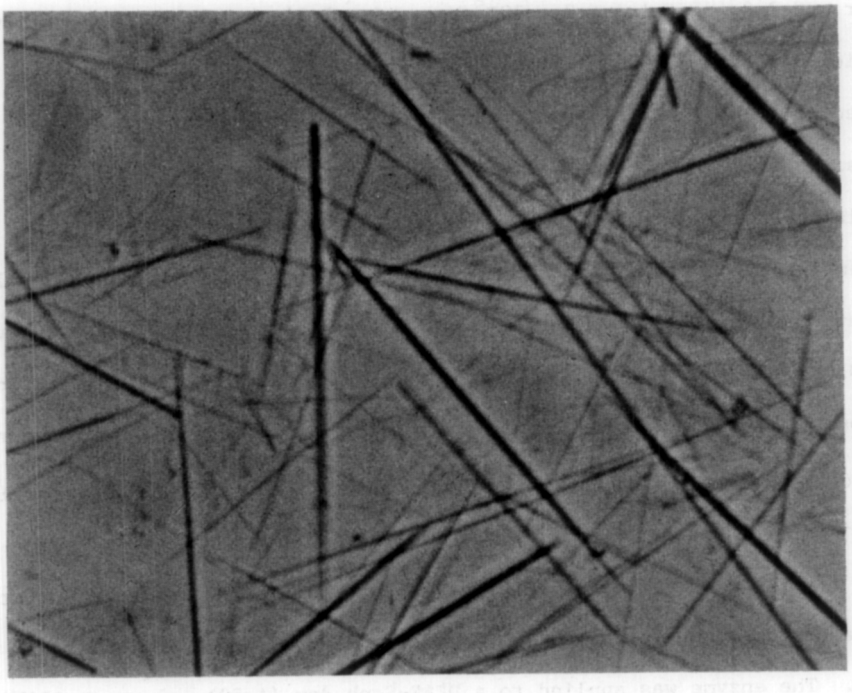

Fig. 1. Crystals of Arginine Racemase

until a faint turbidity was obtained. To the clear supernatant obtained by centrifugation, minimum amount of ammonium sulfate was added. On standing for 3 days at about 4°, the formation of crystals occurred. The crystals appear as needles with a yellow color (Fig. 1). A summary of the purification procedure is presented in Table 1.

Properties.

The crystalline enzyme was shown to be homogeneous using the criterion of ultracentrifugation ($S^{\circ}_{20,w}$ = 5.2 S). The molecular weight of the enzyme determined by the sedimentation equilibrium method (Yphantis, 1960) is 167,000, assuming a partial specific volume of 0.74. The spectrum of the enzyme exhibits two absorption maxima at 280 mμ and 420 mμ. The enzyme

TABLE I. SUMMARY OF PURIFICATION PROCEDURE

Step	Fraction	Total protein (mg)	Total units	Specific activity
1	Crude extract	180,000	97,200	0.54
2	Ammonium sulfate fractionation	98,000	92,100	0.94
3	n-Butanol treatment	10,400	78,000	7.50
4	DEAE-cellulose chromatography	1,440	75,600	52.5
5	Sephadex G-150 chromatography	690	71,700	104
6	DEAE-Sephadex chromatography	88	27,700	315
7	Sephadex G-150 chromatography	11.5	14,100	1,230
8	Crystals	10	12,500	1,250

Arginine racemase was assayed as follows. The reaction mixture contained 10 μmoles D-arginine·HCl, 40 μmoles glycine-KCl-KOH buffer, pH 10.2, and enzyme in a final volume of 1.0 ml. Incubation was carried out at 37° for 20 min. The determination of L-arginine formed is described in our previous paper (Soda *et al.*, 1967). Specific activity of the enzyme is defined as μmole of L-arginine formed per mg of protein per min. The protein was determined by the method of Lowry *et al.*(1951), or from the absorbance at 280 mμ.

contains firmly bound pyridoxal 5'-phosphate. The coenzyme was substantially completely resolved from the enzyme by ammonium sulfate precipitation from 0.01 M potassium phosphate buffer, pH 7.3, containing 10^{-3} M phenylhydrazine. The enzyme was found to contain one mole of pyridoxal 5'-phosphate per 42,000 g of protein, when examined by the method of Wada and Snell (1961). Arginine was the exclusive substrate among the various amino acids tested; alanine, threonine, methionine, phenylalanine, valine, α-aminobutyric acid, leucine and glutamic acid.

Acknowledgements.

The authors are indebted to Dr. H. Utiyama for ultracentrifugation studies. Thanks are also due to Dr. T. Yamamoto and Dr. H. Yamada for their helpful discussions.

Rererences.

Cardinale, G. J. and Abeles, R. H., Biochemistry 7, 3970 (1968).
Diven, W. F., Scholz, J. J., and Johnston, R. B., Biochim. Biophys. Acta 85, 322 (1964).
Free, C. A., Julius, M., and Barry, G. T., Fed. Proc. 27, 785 (1968).
Glaser, L., J. Biol. Chem. 235, 2095 (1960).
Ichihara, A., Furiya, S., and Suda, M., J. Biochem. (Japan) 48, 277 (1960).
Lowry, O. H., Rosebrough, J. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).
Rosso, G., Takashima, K., and Adams, E., Biochem. Biophys. Res. Commun. 34, 134 (1968).
Soda, K., Yorifuji, T., and Ogata, K., Biochim. Biophys. Acta 146, 606 (1967).
Tanaka, M., Kato, Y., and Kinosita, S., Biochem. Biophys. Res. Commun. 4, 114 (1961).
Wada, H., and Snell, E. E., J. Biol. Chem. 236, 2089 (1961).
Yphantis, D., Ann. N. Y. Acad. Sci. 88, 586 (1960).