CRYSTALLINE HOLOTRYPTOPHANASE FROM *PROTEUS RETTGERI*

Hajime YOSHIDA, Hidehiko KUMAGAI and Hideaki YAMADA
The Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan

Received 27 June 1974

1. Introduction

Tryptophanase is an enzyme which catalyzes degradation of L-tryptophan into indole, pyruvate and ammonia (reaction I), and requires pyridoxal 5'-phosphate (PLP) as a cofactor.

$$\text{L-Tryptophan} + H_2O \rightarrow \text{indole} + \text{pyruvate} + NH_3 \quad (1)$$

Recently, it has been shown that in addition to the reaction I, the enzyme catalyzes a series of α,β-elimination and β-replacement reactions [1–3]. The reversal of α,β-elimination reaction was also shown to be catalyzed by the enzyme [3–6].

Tryptophanase was first crystallized as apoenzyme by Newton and Snell [1] from a mutant of *Escherichia coli* B, which produces this enzyme constitutively. Subsequently, we crystallized an inducible apotryptophanase from *Proteus rettgeri* grown in a medium supplemented with L-tryptophan [7,8]. Since the preparations of apotryptophanase are rather unstable [9], many attempts have been made to crystallize the enzyme in the holoenzyme form. However, no satisfactory procedures have been so far reported.

During the course of investigations on tryptophanase from *Proteus rettgeri,* we have developed a procedure to prepare the crystalline holoenzyme. This crystalline enzyme is much more stable and shows about 4 times the specific activity of the formerly crystallized apoenzyme [7]. In this report, the procedure to prepare the crystalline holotryptophanase from *Proteus rettgeri* and some of properties of this enzyme are described.

2. Materials and methods

All chemicals used in this work were commercial products. Tryptophanase was assayed by measuring the amount of pyruvate formed from L-tryptophan. A reaction mixture contained 10 μmoles of L-tryptophan, 0.4 μmole of PLP, 200 μmoles of potassium phosphate buffer, pH 8.0, and the enzyme in a total volume of 4 ml. The reaction was carried out at 30°C for 20 min and stopped by the addition of 1 ml of 30% trichloroacetic acid. The amount of pyruvate formed was determined with the deproteinized filtrate according to the method of Friedemann and Haugen [10]. One unit of the enzyme activity was defined as the amount of enzyme which catalyzed the formation of 1 μmole of pyruvate per min under the assay conditions described. Specific activity is expressed as units per mg of protein. Protein determination was performed by measuring the absorbance at 280 nm. An E value of 11.0 for 10 mg/ml and 1-cm light path which was determined by absorbance and dry weight determinations, was used throughout.

Disc electrophoresis was carried out in Tris–glycine buffer, pH 8.3, according to the method of Davis [11]. Sedimentation coefficients were measured with a Spinco model E ultracentrifuge operating at 56 100 rev/min and 20°C. Diffusion constants were measured with the same apparatus operating at 12 590 rev/min and 20°C, with the boundary condition at the meniscus in the sector-shaped centrifuge cell [12].

3. Results and discussion

3.1. *Purification of the enzyme*

Proteus rettgeri (AJ 2770) was grown in a medium

 North-Holland Publishing Company – Amsterdam

containing 0.6% L-tryptophan, 1.0% soybean hydrolyzate, 6.0% corn steep liquor, 4.0% Sorpol W-200, 0.3% yeast extract, 0.06% L-cysteine, 0.03% D,L-methionine, 0.03% L-proline, 0.06% L-arginine hydrochloride, 0.3% succinic acid, 0.3% KH_2PO_4 and 0.3% $MgSO_4 \cdot 7H_2O$ in tap water. The pH of the medium was adjusted to 7.0 with 5 N KOH. Cultivation was carried out at 30°C for 16 hr in 2 liter shaking flask containing 500 ml of the medium, with reciprocal shaking. Cells were harvested, washed and suspended in 0.01 M potassium phosphate buffer, pH 7.0. All subsequent procedures were carried out at 0–5°C. Potassium phosphate buffers used in the purification procedures contained 1 mM mercaptoethanol and 0.01 mM PLP unless otherwise specified.

Step 1 The cells (400 g, wet weight) were disrupted by ultrasonic oscillation (20 kc, 90 min) and centrifuged.

Step 2 Solid $(NH_4)_2SO_4$ was added to the supernatant to 0.40 saturation and the solution was warmed to 60°C in 70°C water bath. After standing at 60°C for 10 min, the solution was rapidly cooled in an ice bath. After standing overnight, the precipitate was removed by centrifugation. Solid $(NH_4)_2SO_4$ was added to the supernatant to 0.80 saturation. The precipitate was collected and dialyzed for 24 hr against 0.05 M phosphate buffer, pH 7.0.

Step 3 The dialyzed enzyme was applied to a DEAE-Sephadex column (11 × 36 cm) previously equilibrated with 0.05 M phosphate buffer, pH 7.0. After the column was washed with 0.05 M phosphate buffer, pH 7.0, the enzyme was eluted with a linear gradient of KCl concentration from 0 to 0.3 M in 0.05 M phosphate buffer, pH 7.0. Active fractions were combined and concentrated by the addition of $(NH_4)_2SO_4$ to 0.80 saturation. The precipitate was collected and dialyzed for 24 hr against 0.05 M phosphate buffer, pH 7.0.

Step 4 The dialyzed enzyme was fractionated with $(NH_4)_2SO_4$ (0.60–0.70 saturation), followed by dialysis for 24 hr against 0.05 M phosphate buffer, pH 7.0.

Step 5 The dialyzed enzyme was subjected to a hydroxylapatite column (5 × 10 cm) previously equilibrated with 0.05 M phosphate buffer, pH 7.0. After the column was washed with 0.05 M phosphate buffer, pH 7.0, the enzyme was eluted with a linear gradient of $(NH_4)_2SO_4$ concentration from 0 to 0.3 M in 0.05 M phosphate buffer, pH 7.0. Active fractions were combined and concentrated by the addition of $(NH_4)_2SO_4$ to 0.80 saturation. The precipitate was collected and dialyzed for 24 hr against 0.05 M phosphate buffer, pH 7.0.

Step 6 The dialyzed enzyme was again fractionated with $(NH_4)_2SO_4$ (0.60–0.70 saturation). The precipitate was dissolved in a minimum amount of 0.1 M potassium phosphate buffer, pH 8.0, containing 10 mM mercaptoethanol, 0.1 mM PLP and 20% (v/v) glycerol, and dialyzed for 24 hr against the same buffer.

Step 7 Finely powdered $(NH_4)_2SO_4$ was cautiously added to the dialyzed enzyme solution until it became slightly turbid. Crystallization began after about 2 hr and virtually completed within a month. Fig. 1 shows a photomicrograph of the crystalline tryptophanase which appears as thin square plates with a bright yellow color. Recrystallization was carried out by repeating the last step. Specific activity of the enzyme did not increase by further recrystallization. Approximately 231-fold purification was achieved with an over-all yield of 7.2%. A summary of typical purification procedure is shown in table 1.

3.2. *Properties of the enzyme*

Solutions of purified enzyme were relatively unstable, particularly in preparations with a specific activity greater than 3.0. In the presence of mercaptoethanol and PLP, high concentrations of ammonium

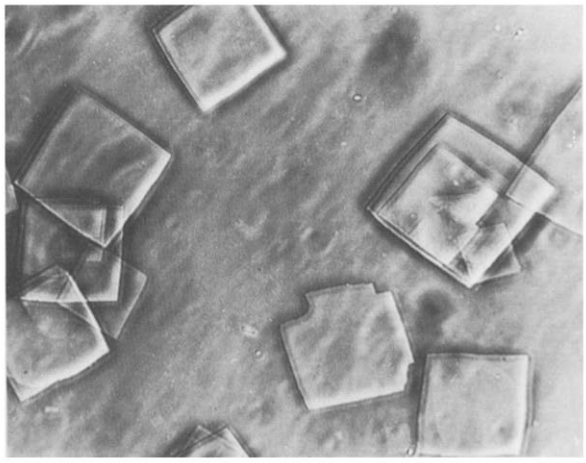

Fig. 1. Photomicrograph of crystalline holotryptophanase.

Table 1
Purification of holotryptophanase from *Proteus rettgeri*

Step	Fraction	Total protein (mg)	Total units	Specific activity
1	Crude extract	107 993	4258	0.039
2	Ammonium sulfate and heat	60 158	3321	0.055
3	DEAE-Sephadex	765	601	0.79
4	Ammonium sulfate (60–70%)	533	595	1.11
5	Hydroxylapatite	210	592	2.81
6	Ammonium sulfate (60–70%)	83	460	5.52
7	First crystals	40	350	8.75
8	Second crystals	34	310	9.01

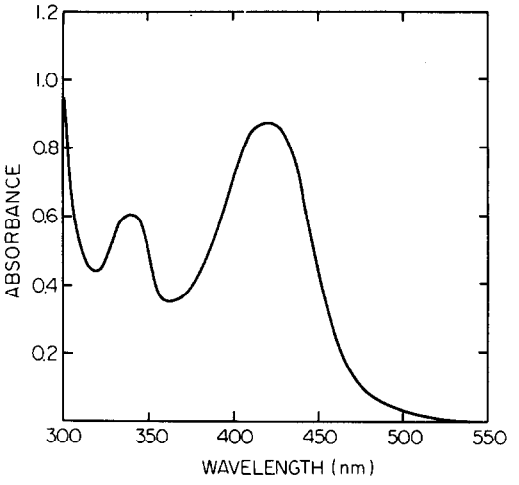

Fig. 2. Absorption spectrum of crystalline holotryptophanase (16.5 mg/ml) in 0.1 M potassium phosphate buffer, pH 8.0, containing 10 mM mercaptoethanol.

sulfate plus glycerol markedly stabilize the enzyme from denaturation.

The crystalline enzyme prepared by the above procedure is much more stable than the formerly crystallized apoenzyme [7]. This enzyme could be stored at 5°C as a suspension in 0.1 M potassium phosphate buffer, pH 8.0, containing 10 mM mercaptoethanol, 0.1 mM PLP, 20% (v/v) glycerol and 60% saturated $(NH_4)_2SO_4$, for periods of over five months without loss of activity. The binding of PLP may give to the enzyme molecule a stability greater than that of the apoenzyme [9].

The crystalline tryptophanase preparation gave a single band on disc electrophoresis, and sedimented as a single peak under ultracentrifugation. Extrapolation of the data from 5 ultracentrifuge runs to zero concentration gave a $s^0_{20,w}$ of 9.8 S. A diffusion constant, $D_{20,w}$, of 4.1×10^{-7} cm²/sec was determined for a 7.96 mg/ml solution of protein. A value of 222 000 was calculated [13] for the molecular weight of the enzyme using a partial specific volume of 0.739 which was obtained from the result of amino acid composition of the enzyme.

The crystalline enzyme showed its full activity even when diluted and assayed in the absence of added PLP. The crystalline enzyme is yellow in color and showed absorption maxima at 340 and 420 nm, at pH 8.0. These results indicate that the crystalline enzyme prepared by the above procedure is entirely in the holoenzyme form and that the formyl group of the enzyme bound PLP forms an azomethine linkage to an amino group of the protein, as reported in other PLP enzymes. The amount of PLP bound to the enzyme was determined [14] to be 4 moles per mole of the enzyme. The maximum velocity and K_m value for L-tryptophan (reaction 1, at 30°C) were 9.0 μmoles/min/mg of protein and 0.26 mM, respectively. Further investigations on the properties of holotryptophanase from *Proteus rettgeri* are in progress in our laboratory.

References

[1] Newton, W. A., Morino, Y. and Snell, E. E. (1965) J. Biol. Chem. 240, 1211–1218.
[2] Hoch, S. O. and DeMoss, R. D. (1973) J. Bacteriol. 114, 341–350.
[3] Yoshida, H., Kumagi, H. and Yamada, H. (1974) Agr. Biol. Chem. 38, 463–464.
[4] Nakazawa, H., Enei, H., Okumura, S., Yoshida, H. and Yamada, H. (1972) FEBS Lett. 25, 43–45.
[5] Nakazawa, H., Enei, H., Okumura, S. and Yamada, H. (1972) Agr. Biol. Chem. 36, 2523–2528.
[6] Watanabe, T. and Snell, E. E. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1086–1090.
[7] Yoshida, H., Utagawa, T., Kumagai, H. and Yamada, H. Agr. Biol. Chem. in press.
[8] Yoshida, H., Kumagai, H. and Yamada, H. Agr. Biol. Chem. in press.

[9] Raibaud, O. and Goldberg, M. E. (1973) J. Biol. Chem. 248, 3451–3455.
[10] Friedemann, T. E. and Haugen, G. E. (1943) J. Biol. Chem. 147, 415–442.
[11] Davis, B. J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427.
[12] Ehrenberg, A. (1957) Acta Chem. Scand. 11, 1257–1270.
[13] Svedberg, T. and Pederson, K. O. (1940) The Ultracentrifuge, Oxford University Press.
[14] Wada, H. and Snell, E. E. (1961) J. Biol. Chem. 236, 2089–2095.