CRYSTALLINE KYNURENINASE FROM *PSEUDOMONAS MARGINALIS*

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Summary The preparation of crystalline kynureninase from *Pseudomonas marginalis* is described. The enzyme is homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis. The molecular weight is 100,000 and 1 mole of pyridoxal 5'-phosphate is bound per mole of enzyme as a coenzyme. The enzyme exhibits absorption maxima at 280, 337 and 430 mμ. The coenzyme is resolved from the enzyme by the treatment with hydroxylamine or L-alanine.

Kynureninase (L-Kynurenine hydrolase EC 3.7.1.3) is a key enzyme of the kynurenine-niacin pathway in tryptophan metabolism and catalyzes the hydrolysis of L-kynurenine to L-alanine and anthranilate. The properties of partially purified kynureninase from pseudomonad (1), *Neurospora crassa* (2) and rat liver (3) have been reported. It has been also shown that pyridoxal 5'-phosphate is required as a coenzyme for the reaction (4,5). The induced kynureninase from *N. crassa* was recently found to occur in two forms (6).

The present communication describes the purification, crystallization and some of the properties of kynureninase from *Pseudomonas marginalis*.

Purification and crystallization

Ps. marginalis IFO 3925 was grown in the medium containing 0.1% L-tryptophan, 0.2% peptone, 0.1% glycerin, 0.05% yeast extract, 0.1% KH_2PO_4, 0.2% K_2HPO_4 and 0.01% $MgSO_4 7H_2O$. The pH was adjusted to about 7.0 with sodium hydroxide. The cultures were grown at 28° for 18 hr under aeration. The harvested cells were washed twice with 0.85% sodium chloride solution and subsequently with 0.01 M potassium phosphate buffer (pH 7.2). The yield of cells was approximately 4 g (wet weight) per liter of the medium. All operations were carried out at 0-5°. Every buffers employed here contained 20 μM pyridoxal 5'-phosphate

and 0.01% 2-mercaptoethanol.

Step 1. The washed cells (1300 g wet weight) were divided into 80-g portions and each portion was mixed with 2 to 3 times its weight of levigated alumina and grounded thoroughly in a mortar. After 2 hr, 0.01 M potassium phosphate buffer, pH 7.2, was added to the mortar and well mixed with the paste. The resultant slurry was centrifuged in order to separate the cell-free extract from the alumina and cellular debris.

Step 2. To the cell-free extract (7560 ml) was added 1.0 ml of 1% protamine sulfate solution (pH 7.2) per 100 mg of protein with stirring. The mixture was centrifuged and the bulky inactive precipitate was discarded. The supernatant solution was dialyzed against two changes of 50 volumes of 0.01 M potassium phosphate buffer (pH 7.2). The insoluble materials formed during dialysis were removed by centrifugation.

Step 3. The enzyme solution (8260 ml) was brought to 20% saturation with ammonium sulfate and the precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant to 55% saturation. The precipitate collected by centrifugation was dissolved in 0.01 M potassium phosphate buffer, pH 7.2. The enzyme solution was dialyzed overnight against 100 volumes of the same buffer.

Step 4. The dialyzed enzyme solution (704 ml) was placed on two DEAE-cellulose columns (5.5 x 55 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 7.2. The columns were washed thoroughly with the same buffer in order to remove the unadsorbed protein. After the columns were washed again with the buffer containing 0.1 M sodium chloride, the enzyme was eluted with the buffer supplemented with 0.2 M sodium chloride at the flow rate of 100 ml per hr. The active fractions were pooled and concentrated by ammonium sulfate precipitation (70% saturation)

Step 5. The precipitate obtained was dissolved in 0.01 M potassium phosphate buffer, pH 7.2. The enzyme solution (71 ml) was applied to three Sephadex G-150 columns (3.4 x 100 cm) equilibrated with the above-mentioned

buffer, and eluted with the same buffer. The active fractions were pooled and concentrated by addition of ammonium sulfate (70% saturation). The precipitate was dissolved in 0.01 M potassium phosphate buffer, pH7.2, and dialyzed against the same buffer.

Step 6. The dialyzed enzyme solution (126 ml) was placed on a DEAE-cellulose column (3.8 x 44 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 7.2. After the column was washed with the same buffer containing 0.13 M sodium chloride, the enzyme was eluted with the buffer containing 0.2 M sodium chloride at the flow rate of 60 ml per hr. Active fractions were combined and concentrated by addition of ammonium sulfate (60% saturation). The precipitate was collected by centrifugation and dissolved in a minimum amount of 0.001 M potassium phosphate buffer, pH 7.2. The enzyme solution was dialyzed against three changes of 50 volumes of the same buffer for 24 hr.

Table 1. Purification of Kynureninase

Step	Fraction	Total Protein (mg)	Total Units	Specific Activity	Yield (%)
1.	Crude extract	99,883	2,227	0.0223	100
2.	Protamine treatment	67,245	2,192	0.0326	98
3.	Ammonium sulfate fractionation	44,472	2,019	0.0454	90
4.	First DEAE-cellulose chromatography	3,253	480	0.1476	21
5.	Sephadex G-150 chromatography	1,024	333	0.3255	15
6.	Second DEAE-cellulose chromatography	596	254	0.4261	11
7.	Hydroxylapatite chromatography	43	127	2.950	5.7
8.	Crystallization	25	77	3.071	3.4

The activity of kynureninase was determined by measuring the rate of decrease in absorbance at 360 mμ, derived from kynurenine, at 25°. The reaction mixture contained 200 μmoles of Tris-HCl buffer, pH 8.0, 1 μmole of L-kynurenine sulfate, 0.2 μmole of pyridoxal 5'-phosphate and enzyme in a final volume of 3.1 ml. One unit of enzyme is defined as the amount of the enzyme that catalyzes the hydrolysis of 1 μmole of kynurenine per min. Specific activity is expressed as units per mg of protein. The protein was determined by the method of Lowry et. al(8), or estimated from the absorbance at 280 mμ.

Step 7. The dialyzed enzyme solution (72.2 ml) was subjected to hydroxylapatite column chromatography. Hydroxylapatite was packed in a column (3.8 x 35 cm) and equilibrated with 0.001 M potassium phosphate buffer, pH 7.2. The enzyme was eluted with the same buffer. The active fractions containing the specific activities of greater than 2 were combined and concentrated by ammonium sulfate to 60% saturation. The precipitate was collected by centrifugation at 17,000 x g for 30 minutes and dissolved in 0.01 M potassium phosphate buffer, pH 7.2.

Step 8. Ammonium sulfate was added gradually to the enzyme solution until a faint turbidity was obtained. On standing at about 4° for 2-3 days, crystal formation occurred. The crystals took the form of thin rectangular leaflets. Approximately 130-fold purification was achieved with an over-all yield of 3%. A summary of the purification procedure is presented in Table 1.

Properties

The crystalline enzyme was shown to be homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis (Fig. 1). The sedimentation coefficient of the enzyme, calculated for water at 20° and zero protein concentration, is 5.87 S. The molecular weight was determined by the sedimentation equilibrium method (7). Assuming a partial specific volume of 0.74, a molecular weight of 100,000 was obtained.

The enzyme exhibits absorption maxima at 280, 337 and 430 mμ (Fig. 2). No appreciable spectral shifts occurred when pH (5.4-9.0) was varied. The enzyme was found to contain one mole of pyridoxal 5'-phosphate per 100,000 g of protein, when examined by the phenylhydrazine method (9) and the 3-methyl-2-benzothiazolone hydrazone method (10).

Incubation of the enzyme with 1 mM hydroxylamine solution (pH 7.2) or 10 mM L-alanine (pH 8.0), followed by dialysis against 0.01 M potassium phosphate buffer, pH 7.2, resulted in formation of the apoenzyme. The mechanism of resolution of the enzyme by incubation with L-alanine is now under investigation.

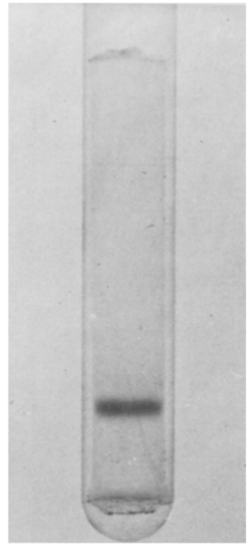

Fig. 1. Disc gel electrophoresis of the enzyme

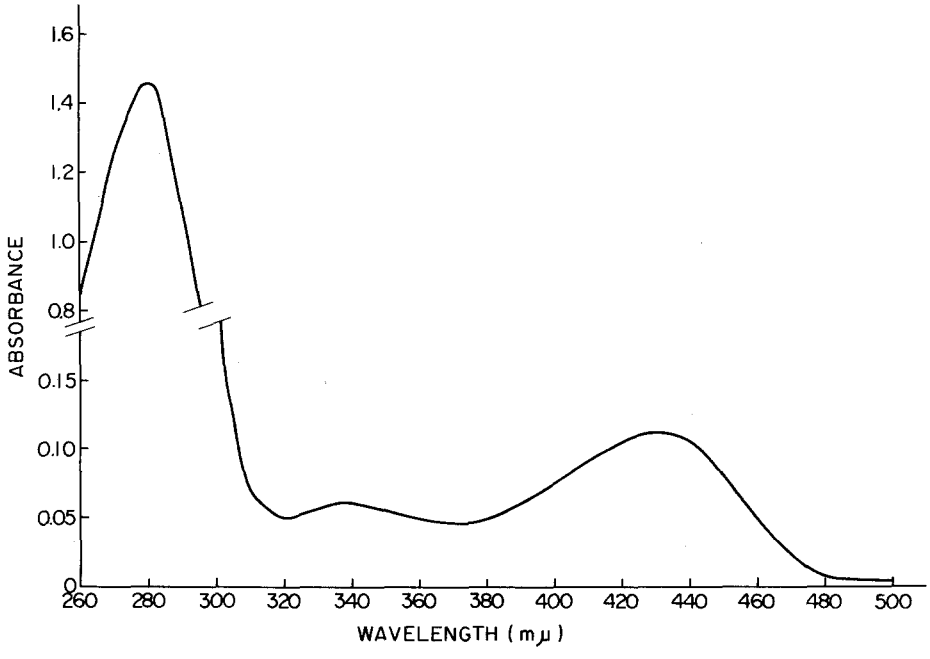

Fig. 2. Absorption spectrum of the enzyme in 0.01 M potassium phosphate buffer, pH 7.2.

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REFERENCES

1. Hayaishi, O., and Stanier, R. Y., J. Bacteriol. 62, 691 (1951).
2. Jacoby, W. B., and Bonner, D. M., J. Biol. Chem. 205,699, 709 (1953).
3. Knox, W. E., Biochem. J. 53, 379 (1953).
4. Braunstein, A. E., Goryachenkova, E. V., and Pashkina, T. S., Biokhimiya. 14, 163 (1949).
5. Wiss, O., Helv. Chim. Acta, 32, 1694 (1949).
6. Turner, J. R., and Drucker, H., Biochem. Biophys. Res. Commun., 42, 698 (1971).
7. Van Holde, K. E., and Baldwin, R. L., J. Phys. Chem., 62, 734 (1958).
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).
9. Wada, H., and Snell, E. E., J. Biol. Chem., 237, 127 (1962).
10. Soda, K., Yorifuji, T., Misono, H., and Moriguchi, M., 114,629 (1969).

ERRATUM

Volume 43, Number 1, April 2, 1971, in the Communication, "Effect of Indole-3-Acetic Acid on the Synthesis of Cyclic 3'-5'-Adenosine Phosphate by Bengal Gram Seeds" by S. Azhar and C. R. Krishna Murti, page 59, line 25:

the concentration of IAA should read 0.18 μM instead of 0.18 M.