CRYSTALLINE INDUCIBLE KYNURENINASE OF *NEUROSPORA CRASSA*

Katsuyuki TANIZAWA, Tatsuo YAMAMOTO and Kenji SODA*

Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan

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1. Introduction

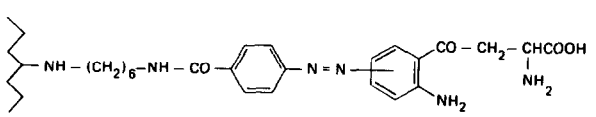
Kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) catalyzes the hydrolysis of L-kynurenine to L-alanine and anthranilate, and plate an important role as a key enzyme of the aromatic and NAD pathways in tryptophan metabolism. The properties of partially purified kynureninase from a pseudomonad [1], *Neurospora crassa* [2] and rat liver [3] have been reported. Evidence for the occurrence of two types of kynureninase in *N. crassa* and their physiological functions were reported [4,5]. Recently the enzyme has been purified to homogeneity from the extract of *Pseudomonas marginalis*, and crystallized [6,7]. It was also demonstrated that kynureninase activity is under the control of α-transamination of the bound pyridoxal 5′-phosphate (pyridoxal-P) catalyzed by the enzyme itself [8,9].

In this communication, we describe the purification and crystallization of inducible kynureninase of *N. crassa* and some of its properties to compare with those of the bacterial enzyme.

2. Materials and methods

L-Kynurenine and D-kynurenine were synthesized from L- and D-tryptophan, respectively, by the method of Warnell and Berg [10]. *N*′-Formyl-L-kynurenine was prepared from L-kynurenine with formic acetic anhydride according to the method of Dalgliesh [11]. L-3-Hydroxykynurenine was obtained from Calbiochem, San Diego, Calif.

L-Kynurenine-bound Sepharose 4B was prepared by the similar procedure to the preparation of the pyridoxal-P-bound Sepharose [12]. L-Kynurenine (50 mg) was coupled with diazotized *p*-aminobenzamidohexyl-Sepharose 4B (30 ml) in 50 ml of 0.4 M Na-acetate buffer (pH 5.6) at 4°C for about 16 h to form L-kynurenine-bound Sepharose whose structure is as follows:



N. crassa IFO 6068 was grown in Vogel's minimal medium [13] supplemented with 2% sucrose and 0.1% L-tryptophan. The cultures were grown in 2-liter flasks containing 750 ml of the medium at 30°C for two days under aeration. The harvested cells were washed twice with 0.85% NaCl solution, and with 0.01 M potassium phosphate buffer (pH 7.8) containing 10^{-5} M pyridoxal-P and 0.01% 2-mercaptoethanol.

The standard assay mixture consisted of 60 μmol of Tris–HCl buffer (pH 8.5), 0.05 μmol of pyridoxal-P, 0.195 μmol of L-kynurenine, and enzyme in a final volume of 1.0 ml. The decrease in absorbance at 360 nm due to hydrolysis of kynurenine was measured at 25°C with a Shimadzu MPS-50L recording spectrophotometer. One unit of enzyme is defined as the amount of the enzyme that catalyzes the hydrolysis of 1 μmol of kynurenine per min. Specific activity is expressed as units per mg of protein. Protein was determined by the method of Lowry et al. [14] using egg albumin as a standard; with most column fractions, protein elution pattern was estimated by the 280 nm absorption.

* To whom correspondence should be addressed

3. Results and discussion

3.1. *Purification and crystallization*

All operations were performed at 0–5°C. Every buffer employed here contained 2×10^{-5} M pyridoxal-P and 0.01% 2-mercaptoethanol.

Step 1. The washed cells (about 140 g, wet weight) were divided into 3 portions and ground thoroughly in a mortar with a half times its weight of sea sand (20–50 mesh). The crude enzyme was extracted from the slurry with 0.01 M potassium phosphate buffer (pH 7.8) followed by centrifugation.

Step 2. To the cell-free extract was added 1.0 ml of 1% protamine sulfate solution (pH 7.2) per 100 mg of protein with stirring. After 20 min, the mixture was centrifuged.

Step 3. The supernatant solution was brought to 40% saturation with ammonium sulfate and the precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant to 75% saturation. The precipitate was dissolved in 0.01 M potassium phosphate buffer (pH 7.8) and dialyzed overnight against 100 vol of the same buffer. The insoluble materials formed during the dialysis were removed by centrifugation.

Step 4. The enzyme was applied to a DEAE–cellulose column (3 X 35 cm) equilibrated with the dialysis buffer. After the column was washed with the same buffer, and then with 0.065 M potassium phosphate buffer (pH 7.8), the enzyme was eluted with 0.13 M potassium phosphate buffer (pH 7.8) at the flow rate of 25 ml/h. The other form of kynureninase (constitutive enzyme) reported by Turner et al. [4] was eluted with 0.2 M potassium phosphate buffer (pH 7.8), the activity of which was only slight compared with that of the inducible enzyme. The active fractions were pooled and concentrated by ultrafiltration through a Diaflo membrane (Amicon, Cambridge, Mass., USA).

Step 5. The enzyme was placed on a Sephadex G-200 column (3.4 X 100 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.8) containing 0.3 M KCl, and eluted with the same buffer. The active fractions were combined, concentrated by ultrafiltration, and dialyzed against 100 vol of 0.1 M potassium phosphate buffer (pH 7.8).

Step 6. The enzyme solution was applied to a column (1.2 X 15 cm) of L-kynurenine-bound

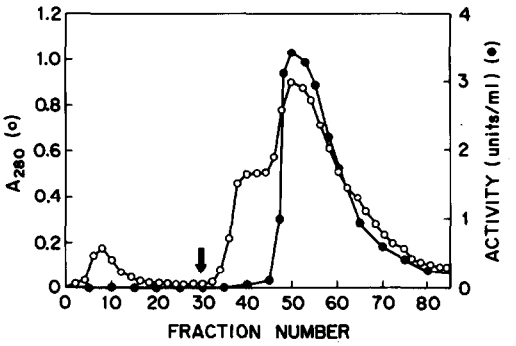


Fig.1. Affinity chromatography of kynureninase on L-kynurenine-bound Sepharose 4B. The flow rate was approximately 20 ml/h; 1.5 ml fractions were collected. At the point indicated by the arrow 0.1 M potassium phosphate buffer (pH 7.8) was replaced by the buffer containing 0.3 M KCl. ○, absorbance at 280 nm; ●, kynureninase activity.

Sepharose 4B previously equilibrated with the dialysis buffer, and the column was washed with the same buffer. The enzyme was eluted with 0.1 M potassium phosphate buffer (pH 7.8) containing 0.3 M KCl. Almost all the proteins applied onto the column were retarded, presumably through ion-exchange, and kynureninase was sufficiently separated from other proteins by increasing the ionic strenght of solution as shown in fig.1. The active fractions were concentrated by ultrafiltration.

Step 7. Ammonium sulfate was added gradually to

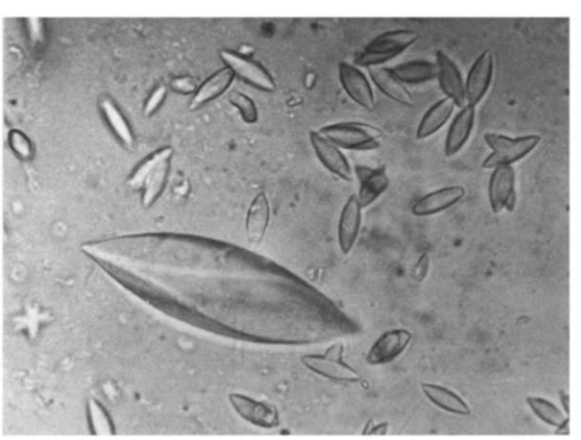


Fig.2. Crystals of inducible kynureninase of *N. crassa.* (× 600).

Table 1
Purification of inducible kynureninase of *N. crassa*

Step	Fraction	Total protein	Total units	Specific activity	Yield
1	Crude extract	932 (mg)	68.0	0.073	100(%)
2	Protamine treatment	688	66.0	0.096	97
3	Ammonium sulfate fractionation	510	63.2	0.124	93
4	DEAE–cellulose chromatography	42	60.7	1.45	89
5	Sephadex G-200 chromatography	20	43.6	2.21	64
6	Kyn-Sepharose 4B chromatography[a]	17	41.5	2.40	61
7	Crystallization	13	32.6	2.49	48

[a]L-Kynurenine-bound Sepharose 4B affinity chromatography

the enzyme solution until a faint turbidity appeared. On standing at 4°C for two days prismatic crystals formed (fig.2). A summary of the purification is presented in table 1.

3.2. *Properties of enzyme*

The crystalline enzyme was shown to be homogeneous by the criteria of disc-gel electrophoresis and ultracentrifugation (fig.3). The sedimentation coefficient ($S^0_{20,w}$) of the enzyme is 6.55 S. A molecular weight of about 105 000 was obtained by the Sephadex G-200 gel filtration method of Andrews [15], with ovalbumin (Mol. wt 43 000), bacterial kynureninase (Mol. wt 91 000), bovine serum albumin (dimer, Mol. wt 136 000), bovine heart lactate dehydrogenase (Mol. wt 140 000), and bovine liver catalase (Mol. wt 240 000) as standard proteins. The enzyme exhibits absorption maxima at 280 (ϵ: 72 000) and 430 nm (ϵ: 10 500), and no spectral shifts occurred on varying the pH (5.5–9.0). The enzyme activity was strongly inhibited by hydroxylamine, phenylhydrazine, semicarbazide and sodium borohydride with concomitant disappearance of the

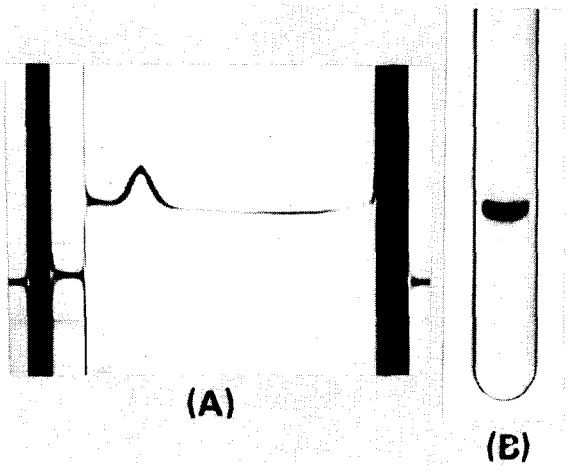


Fig.3. Sedimentation pattern (A) and disc-gel electrophoresis (B) of kynureninase. (A). Sedimentation pattern was obtained at 2.35 mg/ml of protein concentration in 0.01 M potassium phosphate buffer (pH 7.2) containing 0.1 M KCl and 2×10^{-5} M pyridoxal-P. The picture was taken at 38 min after achieving top speed (59 780 rev/min). (B). A sample of the crystalline enzyme preparation (65 μg) was electrophoresed under the conditions of Davis [16].

Table 2
Substrate specificity

Substrates	Relative activity	
	Ps. marginalis enzyme[a]	*N. crassa* enzyme
L-Kynurenine	100	100
L-3-Hydroxykynurenine	19	192
N'-Formyl-L-kynurenine	15	16

[a]Kynureninase of *Ps. marginalis* was prepared as described previously [7]. The specific activity was 9.52 when L-kynurenine was used as a substrate.

absorption peak at 430 nm. Pyridoxal-P fully reactivated the enzyme except when sodium borohydride was used. Incubation of the enzyme with 1 mM hydroxylamine solution (pH 7.2) followed by exhaustive dialysis against 0.01 M potassium phosphate buffer (pH 7.2) resulted in resolution of the enzyme. The Michaelis constant for the cofactor, pyridoxal-P, was determined to be 1.4×10^{-7} M. The enzyme showed also a high sensitivity to $HgCl_2$ and *p*-chloromercuribenzoate. Ethylene-diaminetetraacetic acid and divalent cations such as Mg^{2+}, Mn^{2+} and Ca^{2+} had no effect on the activity.

Although the inducible kynureninase of *N. crassa* is similar to the *Pseudomonas* enzyme, which also is formed inducibly, in the properties above mentioned, differs strikingly in the substrate specificity (table 2). The bacterial enzyme catalyzes preferably the hydrolysis of L-kynurenine, and L-3-hydroxykynurenine is hydrolyzed very slowly. However, L-3-hydroxykynurenine the best substrate for the fungal enzyme, and hydrolyzed about twice more effectively than L-kynurenine. The K_m values were calculated to be 1.8×10^5 M for L-kynurenine and 3.5×10^5 M for L-3-hydroxykynurenine. The enzyme exhibits the maximum reactivity at pH 8.5; this value is higher by 0.5 than that for the bacterial enzyme [7].

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