FURTHER PURIFICATION AND SOME PROPERTIES OF KYNURENINASE

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The enzyme kynureninase converts kynurenine and hydroxykynurenine to alanine and anthranilic acid and to alanine and hydroxyanthranilic acid, respectively[1~4]. Previously, Knox[5], Jakoby *et al.*[4], and Wiss *et al.*[6] have described the purification and some properties of kynureninase. According to their reports, this enzyme obtained from bacteria was activated with magnesium ions, while it from pig liver was not observed such effect. In the course of the studies on the relation between the increased excretion of xanthurenic acid and the enzyme activities in vitamin B_6 deficient rats[7], the author tried to further purification of this enzyme with DEAE-cellulose column chromatography. The present paper will be described about the purification procedures and some properties of kynureninase prepared from rat and pig livers.

EXPERIMENTAL

Materials—L-Kynurenine sulfate monohydrate and pyridoxal phosphate were purchased from California Corporation for Biochemical Research. All other chemicals were the commercially available reagent grade. DEAE-cellulose was obtained from the Brown Company. Hydroxylapatite was prepared by treating brushite with alkali[8].

Enzyme Assay—The reaction mixture contained the following compounds in 1.0 ml: 1.0 μ mole of L-kynurenine, 25 μg of pyridoxal phosphate, 100 μ moles of potassium phosphate buffer, pH 8.0. After 30 minute-incubation at 38°C, anthranilic acid formed was measured by the methods of Knox[5] and Price *et al.*[9].

Protein concentrations were determined by the biuret method and ultraviolet absorption.

Spectral Studies—All spectral observations were performed with a Hitachi EPS-II type spectrophotometer. The enzyme spectra were obtained at room temperature in 1-cm quartz cuvette with a 1-cm light path.

Electrophoresis—Starch zone electrophoresis was made at room temperature for 15 hours with an applied potential of 250 volts. The dimensions of the starch tray were 7 cm × 1 cm × 26 cm. Veronal buffer, pH 8.6, was employed.

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RESULTS AND DISCUSSION

1. Purification of Kynureninase

Step I and II. Homogenization, Heating and pH Treatment—After rats weighing about 200 g were sacrificed by stunning and decapitation, rat livers were removed immediately and chilled in ice. They were homogenized in 3 volumes of 0.14 M KCl in a Waring Blendor. The homogenate was treated with heating at 60°C for 5 minutes and then its pH was adjusted to 5.0 with 1.0 M acetic acid. The precipitate was removed by centrifugation at $20000 \times g$ for 10 minutes and the supernatant fluid was neutralized with 1.0 M KOH.

Step III. Ammonium Sulfate Fractionation—The neutralized supernatant from step II was made to 60% saturation with solid ammonium sulfate. The precipitate was collected by centrifugation at $20000 \times g$ for 15 minutes and dissolved in 0.005 M potassium phosphate buffer, pH 8.0. Then the enzyme solution was dialysed for about 10 hours against the same buffer.

Step IV. Second Heating and Ammonium Sulfate Fractionation—After pyridoxal phosphate being added into the enzyme solution up to 1.0×10^{-3} M, the solution was incubated for 15 minutes at 38°C. Then the temperature of the preparation was brought to 60°C for 5 minutes and cooled rapidly in an ice bath. The precipitate was removed by centrifugation. If the enzyme was heated without pyridoxal phosphate at this step, the activity of this enzyme was markedly reduced. The enzyme was precipitated between 30 and 40% saturation with ammonium sulfate. The precipitate was collected and dialysed against 0.005 M potassium phosphate buffer, pH 7.5.

Step V. Chromatography on DEAE-cellulose—The dialysed enzyme was placed on DEAE-cellulose column equilibrated with 0.005 M potassium phosphate

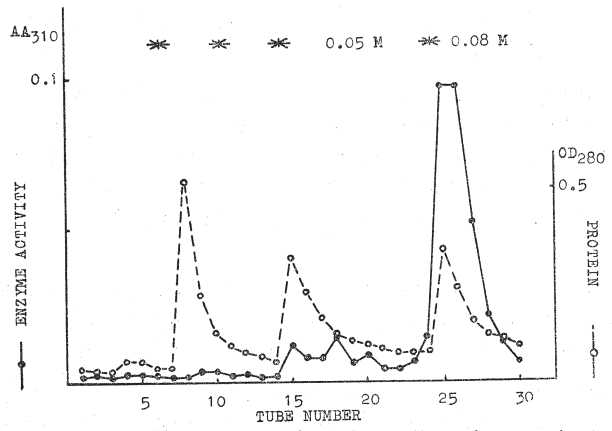

FIG. 1. Chromatography of rat liver kynureninase on DEAE-cellulose.

The enzyme was eluted with varying concentration of potassium phosphate buffer, pH 8.0.

buffer, pH 7.5. All the protein was adsorbed and the column was then washed with increasing concentration of phosphate buffer, pH 8.0. The elution pattern of this enzyme is shown in Fig. 1. The fraction which was eluted from the column with 0.08 M potassium phosphate buffer had the kynureninase activity. The results of the purification are summarized in Table I.

In the case of pig liver kynureninase, hydroxylapatite column chromatography was added besides the procedures described above. The gel was prepared according to Tiselius in 0.001 M phosphate buffer, pH 6.8. The enzyme was adsorbed and then washed with potassium phosphate buffer, pH 8.0. The enzyme activity was selectively eluted by the first fractions collected with 0.03 M buffer. The pattern of elution of this enzyme is shown in Fig. 2.

TABLE I. Purification of Kynureninase from Rat Livers

	Volume	Protein (mg/ml)	Total activity	Specific activity	
Homogenate	650	70.8	262	0.0057	1.0
Heat, pH	650	14.2	197	0.0213	3.7
AS	53	41.2	150.5	0.069	12.1
Heat	74	21.3	213	0.135	23.7
AS	15.5	50.0	152.5	0.197	39.6
DEAE	10	0.7	8.4	1.2	210

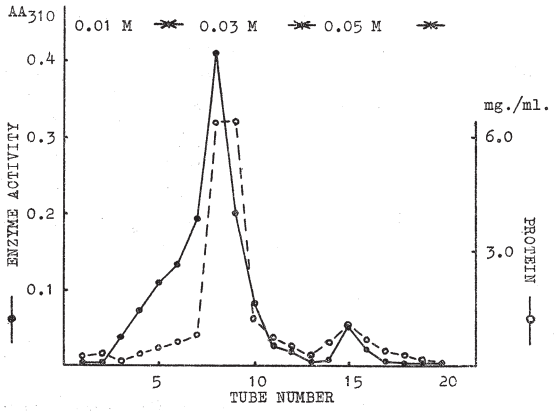

FIG. 2. Chromatography of pig liver kynureninase on hydroxylapatite.

The enzyme was eluted with varying concentration of potassium phosphate buffer, pH 8.0.

2. Stability to Heat

As shown in Fig. 3, the enzyme is comparatively stable to 5 minute-heating at 65°C, but at higher temperature, its activity was rapidly reduced. If pyridoxal phosphate was not added in the enzyme solution, the enzyme activity was rapidly lost. This result was similar to those of other pyridoxal phosphate-containing enzymes. This procedure was used in the step II and IV.

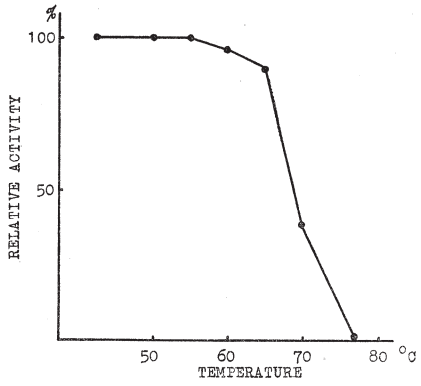

FIG. 3. Effect of heating on kynureninase.
The solution containing enzyme, pyridoxal phosphate and potassium phosphate buffer, pH 8.0, was incubated at 38°C for 15 minutes and then heated at various temperatures for 5 minutes. After heating, the enzyme activity was assayed with the method of Knox[5]. The figure shows the percentages of the heated enzyme activities to the control.

3. *Effect of Various Ions on Kynureninase Activity*

The enzyme was used in step V in this test. Jakoby *et al.* showed that kynureninase from *Neurospora crassa* was activated in the order of 15 to 45% with magnesium ions at a concentration of 2×10^{-3} M[4]. But such effect could not be observed with the enzyme from rat livers (Table II). This result was the same as the case from pig livers by Wiss *et al.*[6].

TABLE II. Effect of Ions on Kynureninase

Ions	Concentration	Relative activity
None		100%
Mg^{2+}	2×10^{-3} M	86
Mg^{2+}	2×10^{-4}	90
K^{+}	2×10^{-3}	85
K^{+}	2×10^{-4}	90
Mn^{2+}	1×10^{-3}	95
Ca^{2+}	〃	94
Co^{2+}	〃	80
Zn^{2+}	〃	89
Na^{+}	〃	97
NH_4^{+}	〃	98

TABLE III. Effect of Inhibitors on Kynureninase

Compounds	Concentration	Per cent inhibition
KF	1×10^{-3} M	0%
NH_2OH	1×10^{-3}	100
NH_2OH	1×10^{-4}	64
KCN	1×10^{-2}	85
KCN	1×10^{-3}	50
$NaHSO_3$	1×10^{-3}	0

4. *Effect of the Carbonyl Group Reagents on Kynureninase*

As shown in Table III, the carbonyl group reagents inhibit the kynureninase action as well as other pyridoxal phosphate-containing enzymes. Hydroxylamine showed 100 and 64% inhibition at concentration 1×10^{-3} and 1×10^{-4} M, respectively. Potassium cyanide inhibits kynureninase action in the order of 50% at 1×10^{-3} M.

5. *Effect of pH on Absorption Spectrum*

The absorption spectrum of the enzyme showed the maxima at 280 and 415 mμ at pH 7.0. The peak at 415 mμ did not shift from pH 4.5 to 10.0 range (Fig. 4).

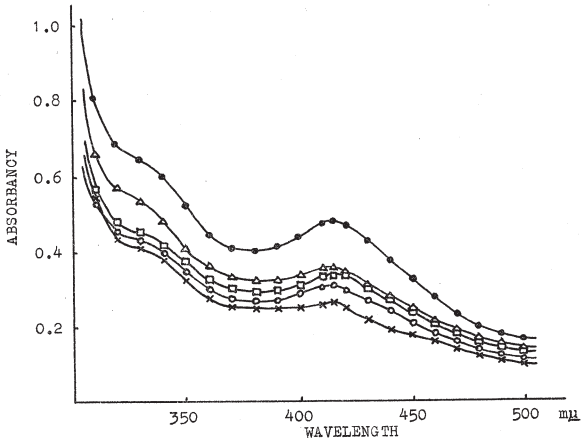

FIG. 4. Absorption spectra of kynureninase at various pH values.
—△—, pH 4.5; —□—, pH 5.8; —●—, pH 7.15; —○—, pH 8.1; —×—, pH 10.0.

Since Metzler showed that the Schiff bases between pyridoxal and several amino acids exhibit an absorption peak at 410 to 415 mμ[10], the same results were obtained from other amino acids[11)12)], some peptides[13)] and serum albumin[13~15)]. The peak at 415 mμ of kynureninase is probably thought as a Schiff base between pyridoxal phosphate and an amino group on the enzyme. Similar phenomena have been observed for the pyridoxal phosphate-containing enzymes: homoserine dehydratase[16)], serine transhydroxymethylase[17)18)], aspartate aminotranspherase[19)], alanine aminotranspherase[20)], phosphorylase[21)] and glutamate decarboxylase[22)].

From the effect of pH on absorption spectra, Jenkins differentiated those pyridoxal phosphate-containing enzymes to the following three groups[20)].

a) The peak at about 420 mμ (410–430 mμ) does not shift in the pH range of 5.0 to 10.0. This was first observed by Matsuo and Greenberg with homoserine dehydratase[16)].

b) The yellow color of the enzyme solution changes like a true pH indicator. This effect was first noted with the aspartate aminotranspherase[19)].

c) A rapid change in color is noted with pH 5.0 range. This was observed for phosphorylase[21)] and glutamate decarboxylase[22)].

Kynureninase resembles homoserine dehydratase and serine transhydroxymethylase in the effect of pH on the absorption spectrum. Other pyridoxal phosphate-containing enzymes exhibit a peak between 415 and 430 mμ in the pH range of 4.0 to 5.0, but at higher pH values the peak shifts to the 330 to 365 mμ region. The difference in response of these enzymes to pH changes undoubtedly reflects differences in their mode of binding pyridoxal phosphate.

6. Electrophoresis

Starch zone electrophoresis was carried out at room temperature for 15

hours with an applied potential of 250 volts. Veronal buffer, pH 8.6, was employed. The dimensions of starch tray were 7 cm × 1 cm × 26 cm. After 15 hours, starch was then cut serially into 1 cm sections and the enzyme was extracted with potassium phosphate buffer, pH 8.0. As shown in Fig. 5, the enzyme activity was located in the region 2 to 2.5 cm toward the cathode from the original slot.

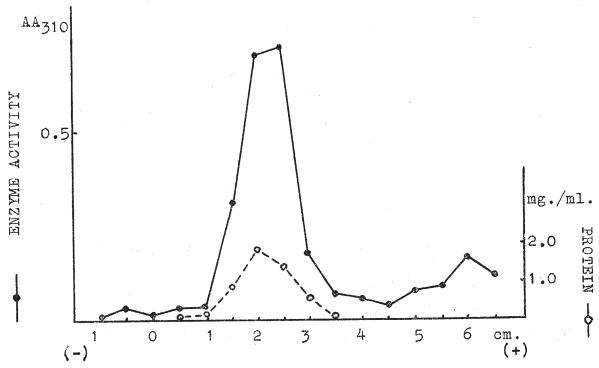

FIG. 5. Starch zone electrophoresis pattern of kynureninase.

This was carried out with 5 ml of the enzyme solution. The enzyme protein migrated toward the cathode.

SUMMARY

Further purification of kynureninase was carried out with the following procedures: heating, pH treatment, ammonium sulfate fractionation, DEAE-cellulose and hydroxylapatite column chromatography.

When pyridoxal phosphate was added in the enzyme solution, the enzyme was stable in spite of heat treatment at 60°C for 5 minutes.

The activation of kynureninase activity from rat liver was not observed with various ions. This result was different from that of the enzyme from *Neurospora*.

Carbonyl group reagents inhibited the enzyme activity as well as other pyridoxal phosphate-containing enzymes.

The absorption spectrum of this enzyme showed the maxima at 280 and 415 mμ. This peak at 415 mμ did not change from pH 4.5 to 10.0 range.

The kynureninase activity exhibited only one band on starch zone electrophoresis.

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