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THE PURIFICATION AND PROPERTIES OF FORMAMIDASE FROM RAT LIVER

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SUMMARY

Formamidase (aryl-formylamine amidohydrolase, EC 3.5.1.9, formerly known as kynurenine formamidase) was purified by heat treatment, $(NH_4)_2SO_4$ fractionation, gel filtration by Sephadex G-200 and DEAE-cellulose column chromatography. The purified enzyme preparation appears as a single peak in ultracentrifuge and in acrylamide electrophoresis.

The properties of the highly purified formamidase are as follows:

1. Formamidase has a broad pH optimum in the range between pH 5.5 and 9.0.

2. The K_m values for formylkynurenine and formylanthranilic acid were 50 μM and 211 μM, respectively.

3. The activity of formamidase was inhibited by Ag^+, Hg^{2+}, Cu^{2+}, $NaAsO_2$, $Na_2S_2O_4$, $HaHSO_3$ and NaF but not affected by Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, NH_2OH, $NaHAsO_4$, sodium formate and $Na_2S_2O_5$.

4. Anthranilic acid and *o*-aminobenzamide showed a mixed-type inhibition. Furthermore, *o*-aminophenol and *o*-aminotoluidine could activate formamidase in the presence of formylanthranilic acid as its substrate.

INTRODUCTION

HEIDELBERGER *et al.*[1] suggested that *N'*-formyl-L-kynurenine is an intermediate during the degradation of tryptophan into kynurenine. KNOX AND MEHLER[2] found formamidase (aryl-formylamine amidohydrolase, EC 3.5.1.9, formerly known as kynurenine formamidase) in rat liver which hydrolyzes formylkynurenine to kynurenine. This enzyme also has been found in the liver of horse, rabbit and pig, further in Drosophilla, *Neurospora crassa* and *Anagasta kuhniella*.

According to the report of JAKOBY[3], formamidase was purified from *Neurospora crassa* between 15- and 20-fold with a recovery of approx. 40% of the enzyme by Norit and protamine treatment, fractionation with $(NH_4)_2SO_4$ and absorption on calicium phosphate gel. This enzyme has also been purified with a 35–50-fold increase

Abbreviation: PCMB, *p*-chloromercuribenzoate.

in activity per mg of preparations from rat liver by MEHLER AND KNOX[4]. Formamidase which has been purified from various sources had different properties. For example formamidase from *Neurospora crassa* was inhibited by KCN but not by *p*-chloromercuribenzoate (PCMB), and formamidase from rabbit liver was inhibited by PCMB but not by KCN.

In this report, the purification and properties of formamidase from rat liver will be studied.

MATERIALS AND METHODS

Livers of Wistar-strain rats were used as the enzyme source. The animals weighed from 150 to 200 g.

Formylanthranilic acid was used as the substrate for the standard assay of formamidase activity. The standard assay system contained 0.5 ml of 0.2 M phosphate buffer (pH 7.5), 1.0 ml of 0.02 M formylanthranilic acid (prepared by dissolving with cautious neutralization), purified enzyme and water in a total volume of 3.0 ml. The reaction was started by the addition of the substrate. Incubation was carried out at 37° and the readings were taken at 1-min intervals for 20 min. The reaction was stopped by 0.5 ml of 0.1 M $NaHSO_3$, and the enzyme activity was measured spectrophotometrically as the increase in absorbance at 330 nm due to the formation of anthranilic acid.

A change of 0.15 in the absorbance at 330 nm is equal to the formation of 100 nmoles of product per ml. A unit of formamidase activity was defined as that quantity of enzyme forming 1 μmole of anthranilic acid from formylanthranilic acid in 60 min under the conditions mentioned above.

Formylanthranilic acid was prepared by the method of VON MEYER AND BELLMANN[5]. Formanilide, formyl-*o*-aminophenol and formylkynurenine were prepared by the methods of TOBIAS[6], LADENBURG[7] and DALGLIESH[8], respectively. Formyl-*p*-aminoacetophenone was prepared by the synthetic method of formyl-*o*-aminophenol according to BISCHLER[9].

RESULTS

Purification of formamidase from rat liver

Livers (450 g), excised from normal rats immediately after death, were homogenized in a Waring blender in 4 vol. of cold 0.14 M KCl containing 2.5 mM NaOH.

Step 1. Heat treatment. The homogenate was heated at 60° for 5 min in the presence of 1 mM anthranilic acid and cooled rapidly in ice water, and the precipitate was removed by centrifugation at 13 000 $\times g$ for 30 min.

Step 2. $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to the supernatant obtained from the previous step to 0.25 saturation. After standing, the precipitate was removed by centrifugation. The $(NH_4)_2SO_4$ concentration of the supernatant was increased to 0.5 saturation by the addition of solid $(NH_4)_2SO_4$. The resulting precipitate was collected by centrifugation at 13 000 $\times g$ for 10 min and dissolved in a small amount of 1 mM phosphate buffer, pH 7.5.

Step 3. Sephadex G-25 filtration. This solution was applied to a Sephadex G-25 column which had been equilibrated with 1 mM phosphate buffer, pH 7.5. The enzyme

solution was placed on the column and the column was developed with 1 mM phosphate buffer, pH 7.5. The enzyme was eluted with 1 mM phosphate buffer (pH 7.5) at a flow rate of 1 ml/min, in 10-ml fractions. The fractions containing enzyme activity were combined to give 180 ml.

Step 4. Streptomycin treatment. 20 ml of a solution containing 0.4 g streptomycin sulfate were added to 180 ml of the eluted solution with stirring. After 30 min, the precipitate was discarded by centrifugation. At this stage the supernatant solution does not contain tryptophan oxygenase. After removing the precipitate, the supernatant solution was then concentrated by the addition of $(NH_4)_2SO_4$ to 0.5 saturation. The precipitate was collected by centrifugation at 10 000 $\times$ *g* for 10 min and dissolved in a minimum amount of 10 mM phosphate buffer, pH 7.5.

Step 5. Sephadex G-200 filtration. The enzyme solution obtained in Step 4 was applied to a Sephadex G-200 column (3 cm $\times$ 130 cm) which had been equilibrated with 10 mM phosphate buffer, pH 7.5. The enzyme solution was placed on the column and the enzyme was then eluted with 10 mM phosphate buffer at a flow rate of 20 ml/h, in 10-ml fractions. The elution of protein was followed by measuring its absorbance at 280 nm as well as by determining the enzyme activity. The elution curve from Sephadex G-200 is shown in Fig. 1. The fractions containing formamidase activity were collected and concentrated by ultrafiltration.

Step 6. DEAE-cellulose column chromatography. The concentrated solution in Step 5 was adjusted to the 2 mM buffer concentration, and it was applied to a DEAE-cellulose column which had been equilibrated with 2 mM phosphate buffer, pH 7.5. The enzyme solution on the column was eluted stepwise with 2, 5, 10, 20 and 50 mM phosphate buffer, each pH 7.5. A flow rate of about 10 ml/h was usually obtained, and 10-ml fractions were collected.

The formamidase activity and protein content of the eluted fractions are illustrated in Fig. 2. A summary of the purification procedures of the enzyme is shown in Table I.

The homogeneity of the purified formamidase was tested by means of disc electrophoresis on acrylamide gel and by sedimentation velocity with a Spinco model

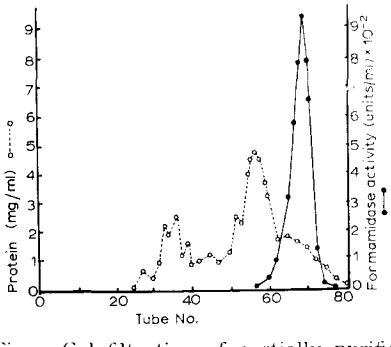


Fig. 1. Gel filtration of partially purified formamidase on Sephadex G-200. The column was eluted with 10 mM phosphate buffer (pH 7.5), and 10-ml fractions were collected. The ordinate on the left and open circles refer to protein concentrations. The ordinate on the right and filled circles represent formamidase activities.

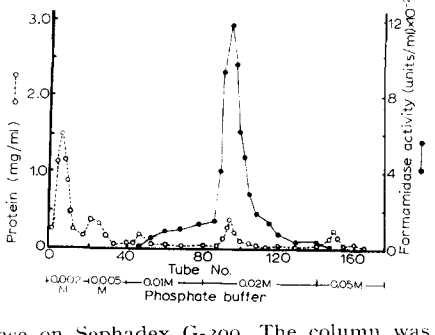


Fig. 2. Elution of partially purified formamidase from a DEAE-cellulose chromatographic column. The ordinate on the left and open circles refer to protein concentrations. The ordinate on the right and filled circles represent formamidase activities.

TABLE I

THE PURIFICATION OF FORMAMIDASE FROM RAT LIVER

Step	*Fraction*	*Volume (ml)*	*Protein concn. (mg/ml)*	*Total protein (mg)*	*Activity (units/ml)*	*Total activity (units)*	*Specific activity (units)*	*Purification*
	20% Homogenate	2073	46.4	96 187.2	35.1	72 762.3	0.76	1
1	Heat treatment	1711	8.8	15 056.8	46.1	78 877.1	5.25	6.9
2	0.25–0.50 satd. $(NH_4)_2SO_4$ fraction	92	88.9	8 178.8	848.7	78 080.4	9.55	12.6
3	Sephadex G-25 column eluate	296	14.5	4 292.0	213.2	63 107.2	14.64	19.3
5	Sephadex G-200 column eluate	355	0.4	142.0	99.3	35 251.5	248.25	326.6
6	DEAE-cellulose column eluate	2	5.3	10.6	72 500.0	14 500.0	1367.92	1799.9

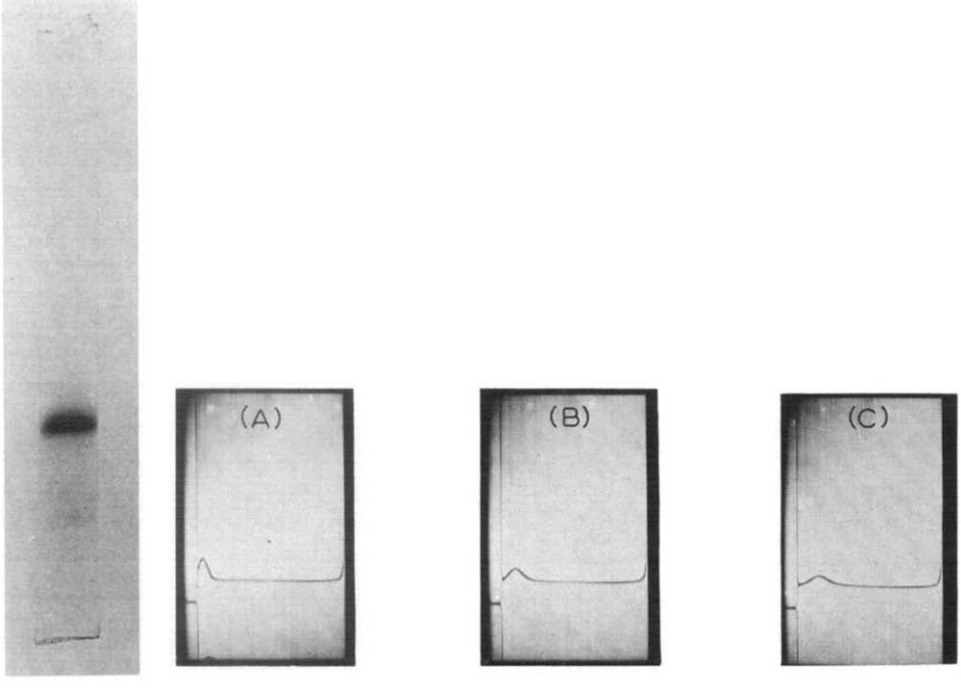


Fig. 3. Acrylamide-gel disc electrophoresis of purified formamidase. Electrophoresis was carried out according to DAVIS[11]. About 500 μg of purified formamidase were applied to the stocking gel and subjected to the electrophoresis at 1.0 mA for 45 min. After the electrophoresis, the gel was stained with 1% amido black, destained electrophoretically, and stored in 7% acetic acid.

Fig. 4. Schlieren patterns of sedimentation velocity of purified formamidase in an ultracentrifugal field. The enzyme, 5 mg/ml in 20 mM phosphate buffer (pH 7.5) was centrifuged at 60 000 rev./min in a Spinco model E ultracentrifuge at 20°. Photographs were taken at 9(A), 27(B) and 45(C) min. The centrifugal direction is toward the right.

E ultracentrifuge with schlieren optics. The purified enzyme preparation gave a single band on acrylamide gel electrophoresis carried out at pH 9.5 (Fig. 3). The enzyme preparation sedimented as a single symmetric peak in the ultracentrifuge in 10 mM phosphate buffer, pH 7.5 (Fig. 4). The sedimentation coefficient of formamidase was calculated from the plot of log x (the distance of boundary to axis of rotation) with respect to t (the time in minutes) according to the equation by SCHACHMANN[10], and found to be 2.92 S.

Optimal pH and ionic strength of buffer in the assay of formamidase

As shown in Fig. 5, formamidase has a broad pH optimum in the range between pH 5.5 and 9.0, but the activity of formamidase at alkaline pH was higher than at acid pH. The effect of ionic strength of phosphate buffer to formamidase activity was unchanged.

Effect of various substances to the activity of formamidase

As shown in Table II, the activity of formamidase was inhibited intensely by Hg^{2+} and Ag^{+}. Both percentages of the inhibitions were 100%. Moreover, $NaAsO_2$, $Na_2S_2O_4$, $NaHSO_3$ and $CuSO_4$ also inhibited the activity of formamidase.

The activity of formamidase was not affected by Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}

TABLE II

EFFECT OF VARIOUS INHIBITORS TO THE ACTIVITY OF FORMAMIDASE

Inhibitor	*Concentration (μM)*	*Inhibition (%)*
$AgNO_3$	1	15.0
	5	45.3
	10	71.6
	50	100.0
$HgCl_2$	4	12.0
	8	44.1
	20	86.6
	50	100.0
$CuSO_4$	1 000	31.5
	2 000	43.5
$NaAsO_2$	50	25.2
	500	63.6
	1 000	75.9
	2 000	86.9
$Na_2S_2O_4$	400	26.5
	1 000	46.9
	2 000	65.3
$NaHSO_3$	400	24.3
	1 000	46.8
	2 000	63.9
	6 000	100.0
NaF	400	8.9
	1 000	9.3
	2 000	15.8
	5 000	17.3
	10 000	27.1

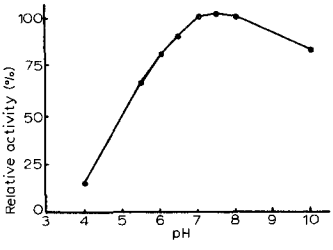


Fig. 5. Effect of pH on the formamidase activities. Acetate buffer between pH 4.0 and 5.5, phosphate buffer between pH 6.0 and 8.0, carbonate–bicarbonate buffer between pH 9.0 and 10 were used.

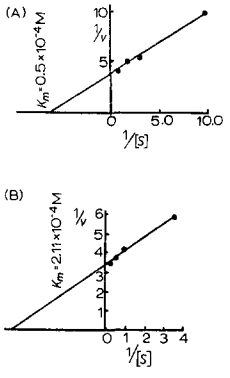


Fig. 6. K_m values of (A) formylkynurenine and (B) formylanthranilic acid as substrate.

as metal ions, ascorbic acid, cysteine, glutathione as reductants, PCMB, iodoacetic acid as SH-inhibitors, EDTA, KCN, K_3 Fe(CN)$_6$, NH_2OH, $Na_2S_2O_5$ and sodium formate.

Kinetics of formamidase

K_m values of formylkynurenine and formylanthranilic acid were 50 and 211 μM, respectively, as shown in Fig. 6. While the relative maximal velocity of formamidase was 100 when formylkynurenine was used as the substrate; it was 13.5 with formylanthranilic acid as the substrate. Relative rates of other substrates are shown in Table III.

TABLE III

THE RELATIVE RATE OF VARIOUS SUBSTRATE

Substrate	*Relative rate (%)*
Formylkynurenine	100.0
Formyl-*o*-aminoacetophenone	60.7
Formylanthranilic acid	13.5
Formanilide	1.3

Effect of anthranilic acid, o-aminobenzamide, o-aminophenol and o-amino-toluidine to formamidase activity

The inhibition of formamidase by anthranilic acid and *o*-aminobenzamide was observed, the percentages of which are shown in Table IV. The inhibition of formamidase by anthranilic acid showed a mixed type, which was the result of both competitive and noncompetitive inhibition (Fig. 7).

TABLE IV

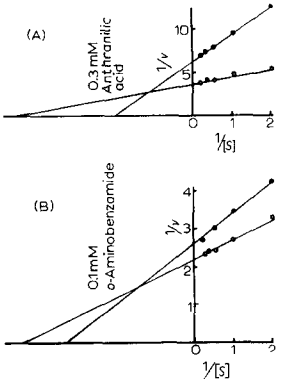
INHIBITIONS OF FORMAMIDASE BY ANTHRANILIC ACID AND *o*-AMINOBENZAMIDE

Inhibitor concentration (mM)	*Anthranilic acid inhibition (%)*	*o-Aminobenzamide inhibition (%)*
0.1	10.9	24.5
0.2	23.8	45.3
0.3	32.8	52.5
0.5	45.3	61.8

On the other hand, formamidase was activated by *o*-aminophenol and *o*-aminotoluidine in the concentrations as shown in Fig. 8. The reaction rate of the formamidase activity was decelerated by anthranilic acid of the reaction product when the absorbance at 330 nm was over 0.200 by using formylanthranilic acid as its substrate. It was very interesting that the inhibition of formamidase by anthranilic acid was not observed when *o*-aminophenol was added to the assay mixture which contained formylanthranilic acid as the substrate.

DISCUSSION

MEHLER AND KNOX[4] have purified formamidase 35-fold from rat liver, ICHIHARA *et al.*[12] partially from rabbit liver and JAKOBY[3] 20-fold from *Neurospora crassa*. The latter has discussed the properties of this purified enzyme. In their reports, ICHIHARA *et al.*[12] have mentioned that anthranilic acid formamidase from rabbit liver was inhibited by PCMB and determined it as a SH-enzyme. On the other hand, formamidase from *Neurospora crassa* was not inhibited by PCMB, but was inhibited by



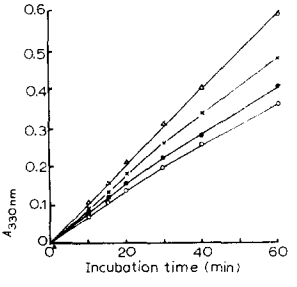


Fig. 7. The inhibition mechanism of (A) anthranilic acid and (B) *o*-aminobenzamide.

Fig. 8. The effect of *o*-aminophenol to formamidase activity. Enzyme and *o*-aminophenol were preincubated at 37° for 10 min and then the reaction was started by the addition of 1.0 ml of 20 mM formylanthranilic acid. Enzyme and formylanthranilic acid were incubated alone as a control (○—○), or with 0.2 mM *o*-aminophenol (○—○), or with 1.0 mM *o*-aminophenol (×—×), or with 2.0 mM *o*-aminophenol (△—△).

KCN, and formamidase from Drosophilla could not hydrolyze formylanthranilic acid. As mentioned above, various differences in properties of formamidase appeared in each species. As compared with highly purified enzyme from rat liver, the results were different from those of others; this purified enzyme could not be inhibited by PCMB, iodoacetic acid and KCN.

Of various metal ions, Hg^{2+} and Ag^{+} could intensively inhibit the activity of this purified enzyme (100% inhibition by 50 μM of each ions), and Cu^{2+} also.

From various data mentioned above, this enzyme must contain some metal, but it is not known so far. Formylkynurenine as a substrate of formamidase was much hydrolyzed and it was followed by formyl-*o*-aminoacetophenon, and then by formylanthranilic acid. K_m value for formylkynurenine was 50 μM. It showed that this enzyme was a very high affinity for formylkynurenine and was matched for the purpose in the relation to tryptophan oxygenase which has a very low activity. JAKOBY[3] had reported that formamidase was not inhibited by anthranilic acid. In this report, it has been confirmed that anthranilic acid exhibited a mixed-type inhibition to formamidase and *o*-aminobenzamide also did the same inhibition as shown in Table IV and Fig. 7. Judging from these results, anthranilic acid and *o*-aminobenzamide will connect with a part of the enzyme protein. The reaction rate of the formamidase activity was decelerated by anthranilic acid of the reaction product when the absorbance at 330 nm was over 0.200 by using formylanthranilic acid as its substrate.

This fact may be due to the accumulation of the enzymic product, *i.e.*, anthranilic acid from formylanthranilic acid. Formamidase was activated by addition of *o*-aminophenol to the incubation medium and, besides, the inhibition by the reaction product, *i.e.*, anthranilic acid, did not appear. The reaction proceeded linearly till the absorbance at 330 nm was 0.60. Furthermore, *o*-aminophenol could activate formamidase and excluded the inhibition of anthranilic acid through connecting of *o*-aminophenol with a part of the enzyme protein.

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