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NEW FORMAMIDASE HAVING SUBSTRATE SPECIFICITY FOR *o*-FORMYLAMINOACETOPHENONE IN PIG LIVER

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Summary

The supernatant (13 000 × *g*, 20 min) of pig liver homogenate was filtered with Sephadex G-200 and formamidase (aryl-formylamine amidohydrolase, EC 3.5.1.9) activity in each fraction was measured. When formylkynurenine was used as substrate, two peaks of formamidase activity were observed but, with formylaminoacetophenone as substrate, only one peak was observed. Formamidase in the lower molecular weight fraction is known as kynurenine formamidase (FA I), formamidase found here in the higher molecular weight fraction has not been previously reported. This form, designated FA II has been purified about 160-fold from pig liver.

The formamidase obtained has substrate specificity for *o*-formylaminoacetophenone only and could not hydrolyze formylkynurenine. The optimal pH was 8.5 and the K_m for *o*-formylaminoacetophenone was $1.66 \cdot 10^{-3}$ M. This formamidase was considered to be a new enzyme and was different from FA I in molecular weight and substrate specificity. This new formamidase was present in pig, rabbit and guinea pig liver and not present in rat or mouse liver.

Introduction

Knox et al. [1,2] isolated formamidase, formerly known as kynurenine formamidase (aryl-formylamine amidohydrolase, EC 3.5.1.9) which hydrolyzes formylkynurenine into kynurenine and reported some properties of the partially purified enzyme. Later, the formamidase in *Neurospora* was purified by Jakoby [3]. Santti et al. [4,5] reported the substrate specificity and other enzymic characteristics of formamidase, which had been isolated from liver of the guinea pig. In his report the purification method and some properties of new formamidase (FA II) are described.

Materials and Methods

Fresh pig liver was used as the enzyme source. The standard enzyme assay contained 0.5 ml of 0.2 M phosphate buffer, pH 8.5, 1.0 ml of 6.0 mM *o*-formylaminoacetophenone, enzyme solution and distilled water in a total volume of 3.0 ml. The reaction was started by the addition of enzyme after the substrate had been preincubated at 37°C for 5 min. After incubation (usually 10 min), the reaction was stopped by 0.5 ml of 0.2 M $NaHSO_3$, and the activity was measured spectrophotometrically as the increase in the absorbance at 370 nm due to the formation of *o*-aminoacetophenone. A change of 0.316 in the absorbance at 370 nm is equal to the formation of 0.1 μmol of product per ml. A unit of FA II was defined as the enzyme quantity forming 0.1 μmol of *o*-aminoacetophenone in 60 min under the conditions mentioned above.

o-Formylaminoacetophenone was prepared by adding 1.0 g of *o*-aminoacetophenone to 2.0 g of 99% formic acid and heating in a boiling water bath for 10 min. When the reaction mixture was poured into ice water, the crude *o*-formylaminoacetophenone was crystallized. Recrystallization was obtained by ethanol. Formylkynurenine and formylanthranilic acid were prepared by the methods of Auerbach and Knox [6] and Von Meyer and Bellmann [7], respectively. Protein was determined by the biuret method and ultraviolet absorption.

Calcium phosphate gel was prepared as follows. One part of cold 0.067 M Na_2HPO_4 containing 0.067 M ammonia water was poured into one part of cold 0.1 M $CaCl_2$ under continuous agitation. The gel formed was washed repeatedly with water until the supernatant of the suspension became negative to Nessler reagent. The washed gel was suspended in 0.01 M phosphate buffer, pH 7.5, and left for one night. The preparation of hydroxyapatite was followed by the method of Tiselius et al. [9].

Results

Presence of kynurenine formamidase. A total of 3.0 g of pig liver was homogenized in a waring blender in 20 volumes of cold 0.14 M KCl containing 2.5 mM NaOH. The homogenate was centrifuged at 13 000 × *g* for 20 min. The supernatant was fractionated with solid $(NH_4)_2SO_4$ between 0.25 and 0.45 saturation. The precipitate collected with centrifugation was dissolved in 20 ml of 10 mM phosphate buffer (pH 7.5). 4.0ml of this solution were filtered on a Sephadex G-200 column (2.5 × 76 cm), which had been equilibrated with 10 mM phosphate buffer, pH 7.5. Formamidase activity in each 10 ml fraction collected was assayed by using formylkynurenine, *o*-formylaminoacetophenone and formylanthranilic acid as substrates. When formylkynurenine and formylanthranilic acid were used as substrates, enzyme activities were found in tube Nos. 28—38, but two peak activities, which could hydrolyze *o*-formylaminoacetophenone were found in tube Nos. 18—28 and 28—38. The substrate specificity in fraction Nos. 28—38 could be regarded as kynurenine formamidase (FA I) which had been previously reported [8]. The enzyme in fraction Nos. 18—28 was different from FA I with respect to molecular weight and substrate specificity. Formamidase, which has such substrate specificity has not

been reported until now. Therefore, the authors purified this FA II and examined some properties of the enzyme.

Purification method of FA II from pig liver. 500 g of pig liver were homogenized in a Waring blender in four vols. of cold 0.14 M KCl containing 2.5 mM NaOH. The homogenate was centrifuged at 13 000 × *g* for 20 min, and the supernatant was collected.

Step I. Solid $(NH_4)_2SO_4$ was added to the supernatant to 0.35 saturation at pH 5.0. After standing for 30 min at 5°C, 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 × *g* for 10 min, and dissolved in a small amount of 0.01 M phosphate buffer (pH 7.5). This solution was dialyzed against 0.01 M phosphate buffer (pH 7.5) for 2 days.

Step II. 500 ml of an aged suspension of calcium phosphate gel in 0.01 M phosphate buffer (pH 7.5), which was prepared from 1500 ml of 0.1 M $CaCl_2$ and 1500 ml of 0.067 M Na_2HPO_4 containing 0.067 M ammonia water, were added to the dialyzed solution, and stirred for 30 min. The adsorbed gel was collected by centrifugation at 5000 × *g* for 5 min and resuspended in 500 ml of 0.03 M phosphate buffer (pH 7.5) and stirred for 30 min. After centrifugation, the gel was suspended in 300 ml of 0.075 M phosphate buffer (pH 7.5) and stirred for 60 min. The suspension was centrifuged. FA II activity was present in the supernatant. The gel was washed once with 200 ml of the same buffer. The washing was mixed with the supernatant.

Step III. Solid $(NH_4)_2SO_4$ was added to the eluate from calcium phosphate gel to 0.50 saturation. The precipitate was collected by centrifugation at 13 000 × *g* for 10 min and dissolved in 40 ml of 0.01 M phosphate buffer (pH 7.5). This solution was filtered on Sephadex G-200 column (2.5 × 80 cm) which had been previously equilibrated with 0.01 M phosphate buffer (pH 7.5). The fractions containing FA II activity were pooled. Solid $(NH_4)_2SO_4$ was added to the fractions to 0.50 saturation. The precipitate which was formed was collected by centrifugation and dissolved in 0.01 M phosphate buffer (pH 7.5). The protein solution were desalted with Sephadex G-25 equilibrated with 0.01 M phosphate buffer (pH 7.5). The fractions containing protein were collected.

Step IV. DEAE-Sephadex was suspended in 1.0 M NaCl and was allowed to stand for 4—5 h. The DEAE-Sephadex was then filtered with glass filter and washed with 0.01 M phosphate buffer (pH 7.5). The gel was suspended in 0.01 M phosphate buffer (pH 7.5) for one night and was packed in the column tube for chromatography. Before chromatography, 0.01 M phosphate buffer (pH 7.5) was passed through the column. Fractions containing proteins (Step IV) were applied to the column. Unadsorbed protein was eluted with 0.01 M phosphate buffer (pH 7.5), and followed by elution using a continuous linear gradient of NaCl (0—0.5 M) in 0.01 M phosphate buffer (pH 7.5). Proteins of each 4.0 ml sample were collected. Activity was appeared in tube Nos. 24—44. These fractions which were pooled and concentrated to 2.0 ml were passed through Sephadex G-25 (2.0 × 30 cm). Also the protein fractions were pooled.

Step V. Cellulose powder was mixed with hydroxyapatite to prevent slowing of the flow rate. This mixed suspension was packed in a column (2.5 × 15 cm).

Eluted protein fraction from Sephadex G-25 was applied into the column and unadsorbed fractions were eluted with 0.01 M phosphate buffer (pH 7.5). Elution of FA II was carried out using a continuous gradient of 150 ml of 0.01 M phosphate buffer (pH 7.5) and 150 ml of 0.2 M phosphate buffer (pH 7.5). Fractions of 4.6 ml were collected. FA II was eluted in tube Nos. 16—24. The fraction containing FA II activity was concentrated to 1.5 ml with a collodion bag under depressure. The results of purification are shown in Table I.

Formation of o-aminoacetophenone from o-formylaminoacetophenone by FA II. *o*-Formylaminoacetophenone was incubated with purified FA II at 37°C for 2 h. The product was investigated at pH 7.5 by spectrophotometer. The ultraviolet spectrum of the compound was the same as authentic *o*-aminoacetophenone and was positive to the Bratton-Marshall reaction, which is specific for detection of primary aromatic amines.

pH optimum. FA II was found to have a narrow pH optimum of pH 8.5.

Specificity for substrate. Rat liver FA I could hydrolyze aromatic formylamino compounds such as formylkynurenine, *o*-formylaminoacetophenone and formylanthranilic acid. The maximal relative rate of hydrolysis of *o*-formylaminoacetophenone and formylanthranilic acid by FA I was 60.7 and 13.5, respectively, as compared with that of formylkynurenine (100) [8]. In our experiments, purified FA II hydrolyzed only *o*-formylaminoacetophenone, the other compounds such as formylkynurenine, formylanthranilic acid, *N*-acetylanthranilic acid, formanilide and acetoanilide not be hydrolyzed.

The K_m value of *o*-formylaminoacetophenone for FA II was found to be $1.66 \cdot 10^{-3}$ M.

Effects of metal ions on FA II activity. The activity of rat liver FA I has been shown to be strongly inhibited by Ag^+, Hg^{2+} and Cu^{2+}, but was not affected by Mg^{2+}, Zn^{2+}, Fe^{2+} and Fe^{3+} [8]. The activity of FA II was inhibited by Ag^+ and Hg^{2+}, as shown in Table II, but Cu^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+} and Fe^{3+} did not inhibit the activity of FA II.

Effects of kynurenine and anthranilic acid. Rat liver FA I was inhibited completely by kynurenine (2.0 mM), anthranilic acid (2.0 mM) and *o*-aminoacetophenone (2.0 mM). The inhibition mechanism of kynurenine was uncompetitive, both anthranilic acid and *o*-aminoacetophenone showed mixed type inhibition to rat liver FA I, as reported in our previous paper [10]. On the other

TABLE I

PURIFICATION OF PIG LIVER FORMAMIDASE HAVING SUBSTRATE SPECIFICITY FOR *o*-FORMYLAMINOACETOPHENONE

Preparation stage	Total protein (mg)	Total activity (units)	Specific activity	Purity
Homogenate	78 138.5	92 196.0	1.2	1.0
Supernatant	47 144.5	64 580.3	1.4	1.2
0.35—0.5 saturation $(NH_4)_2SO_4$	12 312.0	20 430.3	1.7	1.4
$Ca_3(PO_4)_2$ gel	2168.0	10 423.2	4.8	4.0
Sephadex G-200	201.5	5355.0	26.6	22.2
DEAE-Sphadex	52.5	2445.7	46.6	38.8
Hydroxy apatite	10.0	1956.6	195.7	163.1

TABLE II

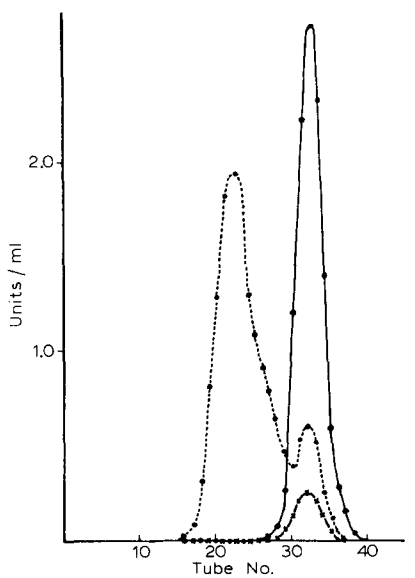
EFFECTS OF Ag^+ AND Hg^{2+} TO THE ACTIVITY OF NEW FORMAMIDASE

Inhibitor	Concentration (μM)	Inhibition (%)
$AgNO_3$	10	25.0
	20	32.4
	50	59.6
	100	75.9
$HgCl_2$	10	2.0
	20	19.6
	50	32.0
	100	49.6

hand, FA II of pig liver was inhibited intensely by kynurenine and its inhibition was a non-competitive type as shown in Fig. 2, but FA II was not inhibited by anthranilic acid.

Inhibition of diisopropylfluorophosphate. Diisopropylfluorophosphate (DFP) inhibited 100% of the enzyme activity at a concentration of 10^{-5} M suggesting that serine residue may be present in the active site of FA II (Table III).

Presence of FA II in other animals. As shown above, FA II was present in pig liver, so an investigation was made to determine whether FA II is present in livers of other animals. The detection of FA II activity was followed, after gel



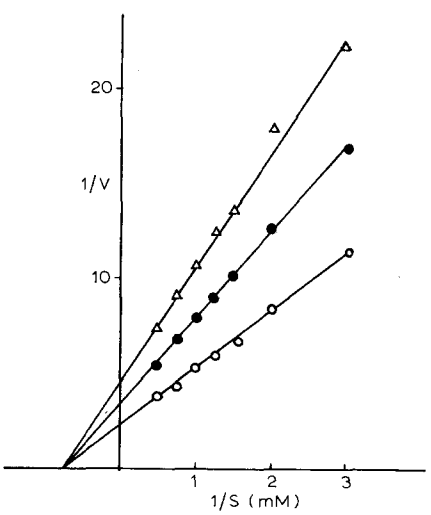


Fig. 1. Presence of FA I and FA II in pig liver. Sample was gel-filtered with a Sephadex G-200 column (2.5 × 76 cm). Each 10 ml fraction was collected. *o*-Formylaminoacetophenone (1.0 mM), formylkynurenine (0.5 mM) and formylanthranilic acid (6.7 mM) were used as substrates for the detection of formamidase activity. ●------●, *o*-formylaminoacetophenone; ●———●, formylkynurenine; X———X, formylanthranilic acid.

Fig. 2. The inhibition mechanism of kynurenine. ○———○, control; ●———●, kynurenine (0.1 mM); △———△, kynurenine (0.2 mM).

TABLE III

INHIBITION OF NEW FORMAMIDASE ACTIVITY BY DIISOPROPYLFLUOROPHOSPHATE

Diisopropylfluorophosphate (M)	Inhibition (%)
10^{-7}	70.5
10^{-6}	96.2
10^{-5}	100.0

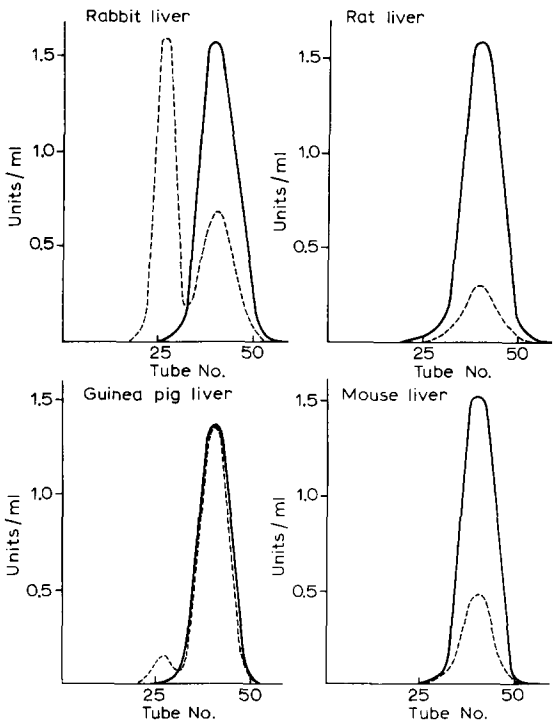


Fig. 3. Gel filtration of supernatant of various liver homogenates. ———, substrate was formylkynurenine; - - - - -, substrate was *o*-formylaminoacetophenone.

filtration of the supernatant of liver homogenate on Sephadex G-200 column. The results of this study are shown in Fig. 3. FA II was present in large amounts in rabbit liver, but in smaller amounts in guinea pig. In rat and mouse liver, FA II could not be detected.

Discussion

In tryptophan metabolism, it is known that FA I participates in the process by which formylkynurenine is hydrolyzed to kynurenine. The authors clarified the presence of new formamidase (FA II) and its some enzymic properties. It can be concluded from these studies that there is a difference in the specificities of FA I and FA II for substrate. Sedimentation coefficient of FA II was 7.43 S.

Santti and Hopsu-Havu [4] reported that FA I of guinea pig liver was inhib-

ited by Mintacol, and concluded the serine residue would be present in an active site of FA I. In our other study, it was found that FA I of rat liver and kidney was not inhibited by DFP, but FA I of rabbit, mouse and dog liver was completely inhibited by 10^{-5} M DFP [11]. Therefore, it was assumed that serine residue would be present in an active site of FA I in livers except of the rat. On the other hand, FA II of pig liver was readily inhibited by DFP, and indicated the presence of serine residue, too.

As shown in Fig. 3, FA II was not always present in the liver of all species, and it was concluded that tryptophan metabolism differs between those animals which possess or do not possess FA II, because the supply of *o*-formylaminoacetophenone or *o*-aminoacetophenone will be dependent on the presence of tryptophan [12,13]. It will be interest to study further the physiological significance of FA II.

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