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N-ACETYL-β-ALANINE DEACETYLASE IN HOG KIDNEY

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SUMMARY

(1) An enzyme which catalyzed the hydrolysis of the acetylamino group of *N*-acetyl-β-alanine was found in mammalian kidney.

(2) The enzyme was purified to about 100 fold from hog kidney.

(3) The optimal pH of the enzyme was 7.6, K_m value for acetyl-β-alanine was $2.5 \cdot 10^{-3}$ M and the enzyme was inhibited by *p*-chloromercuribenzoate and monoiodoacetate.

(4) Among various acylamino acids tested so far, *N*-acetyl-β-alanine and to less extent *N*-acetyl-taurine served as the substrate.

(5) A name, *N*-acetyl-β-alanine amidohydrolase (trivial name: *N*-acetyl-β-alanine deacetylase) was suggested.

INTRODUCTION

Aminoacylase (*N*-acylamino-acid amidohydrolase, EC 3.5.1.14) is widely distributed among animal tissues, fungi and bacteria and has been studied extensively (for review see ref. 1). This enzyme catalyzes the hydrolysis of various *N*-acyl-α-amino acids but does not act on *N*-acetyl-β-amino acids[1]. No enzymes which hydrolyze acyl-β-amino acids are yet known.

The present paper describes the occurrence of an enzyme which hydrolyzes *N*-acetyl-β-alanine in mammalian kidney. The enzyme, *N*-acetyl-β-alanine deacetylase, was partially purified from hog kidney and the properties were studied. A preliminary report of the work has appeared[2].

METHODS

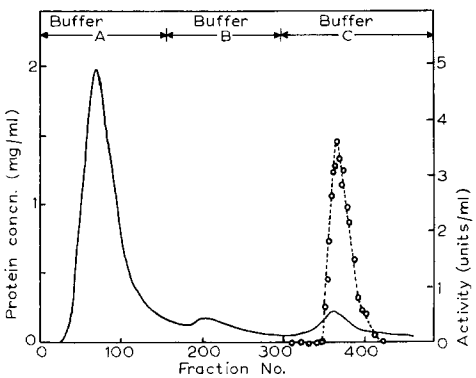
Preparation of N-acyl amino acids

N-Acetyl amino acids and peptides were prepared by acetylation of amino acids and peptides with acetic anhydride[1]. Amino acids or peptides (400 mg) were dissolved in water (1–2 ml) and placed in an ice bath. Acetic anhydride (0.8 g) was added drop-wise to the solution, which was kept at about pH 5 by the addition of 4 M NaOH.

After the reaction mixture showed a negative ninhydrin reaction on filter paper, it was diluted to 50 ml and passed through an Amberlite CG-120 column (H^+ form, 1.8 cm × 30 cm), and the effluent and the washings were evaporated under reduced pressure. The concentration of the acyl derivatives was estimated by hydrolyzing an aliquot of the solution with 6 M HCl at 110° for 16 h and determining the liberated amino group[3]. Formylation of amino acid was carried out according to the method of SHEEHAN AND YANG[4].

Enzymatic reaction and assay procedure

N-Acyl-amino acid (6.6 μmoles) was incubated with the enzyme preparation and borate–phosphate buffer, pH 7.6, (0.3 ml, prepared as described below) at 37° for 30 min. The total volume of the incubation mixture was 0.8 ml. After incubation, the reaction was terminated by adding 0.2 ml of 20% trichloroacetic acid. The mixture



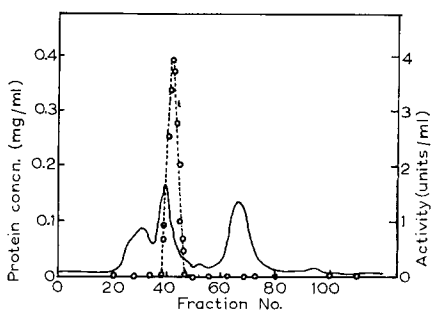


Fig. 1. Elution of acetyl-β-alanine deacetylase from DEAE-cellulose. Heat-treated extract (210 ml) was placed on a 3 cm × 84 cm DEAE-cellulose column and eluted successively with borate–phosphate buffer (pH 8.0) prepared from 0.05 M $Na_2B_4O_7$ and 0.1 M KH_2PO_4 (Buffer A), that from 0.067 M $Na_2B_4O_7$ and 0.13 M KH_2PO_4 (Buffer B) and that from 0.1 M $Na_2B_4O_7$ and 0.2 M KH_2PO_4 (Buffer C). Fractions (about 10 ml each) were assayed for protein (280 mμ absorbance) and for the deacetylase activity. ———, protein concentration; ○ · · · · ○, enzyme activity.

Fig. 2. Elution of acetyl-β-alanine deacetylase from Sephadex G-200. DEAE-cellulose eluate was lyophilized, taken up in water (12 ml) and placed on a Sephadex G-200 column (3 cm × 91 cm) and eluted with borate–phosphate buffer (pH 8.0) prepared from 0.05 M $Na_2B_4O_7$ and 0.1 M KH_2PO_4. Fractions (about 7 ml) were assayed for protein concentration (280 mμ absorbance) and for the enzyme activity. ———, protein concentration; ○ · · · · ○, enzyme activity.

was centrifuged (600 × *g* for 10 min). The amount of amino groups liberated by the reaction was determined by the trinitrobenzene sulfonate method[3] on an aliquot of the supernatant. One unit of the enzyme was defined as the amount of enzyme required to liberate 1 μmole of amino group under the conditions described above. The protein concentration of the enzyme preparation was estimated by the absorption at 280 mμ or by the method of LOWRY *et al.*[5] with bovine serum albumin as a standard. Borate–phosphate buffer was prepared by adding 0.1 M KH_2PO_4 to 0.05 M $Na_2B_4O_7$ until the pH of the mixture reached the desired pH.

RESULTS

Preparation and purification of acetyl-β-alanine deacetylase from hog kidney

Fresh frozen hog kidney (25 g) was thawed, minced and homogenized with a Waring Blendor for 1 min in borate–phosphate buffer, pH 8.0 (225 ml). The homogenate was centrifuged at 11 000 × *g* for 20 min and the supernatant solution was used as the crude enzyme preparation. The crude homogenate was heated at 60° for 2 min in a water bath, cooled immediately in an ice bath and centrifuged at 6200 × *g* for 10 min. The supernatant solution was applied on a DEAE-cellulose column (3 cm × 84 cm, equilibrated with borate–phosphate buffer (pH 8.0) prepared from 0.05 M $Na_2B_4O_7$ and 0.1 M KH_2PO_4). Step-wise elution was carried out with borate–phosphate buffer (pH 8.0) of various concentrations. As shown in Fig. 1, acetyl-β-alanine deacetylase activity was found in the eluate with the buffer prepared from 0.1 M $Na_2B_4O_7$ and 0.2 M KH_2PO_4. The fractions which contained high enzyme activity were collected and lyophilized. The residue was taken up in 12 ml of water and applied on a Sephadex G-200 column (3 cm × 91 cm) and eluted with borate–phosphate buffer, pH 8.0 (Fig. 2). By these procedures, about 100-fold purification was attained as shown in Table I.

TABLE I

PURIFICATION OF ENZYME

Details of the procedure were described in the text.

Fraction	*Volume (ml)*	*Activity (units)*	*Protein (mg)*	*Specific activity (units/mg)*
Crude extract	219	1508	4770	0.32
Heat treatment	211	1325	2765	0.48
DEAE-cellulose eluate	218	403	47.5	8.5
Sephadex G-200 eluate	20.5	58.5	1.74	33.5

Time course and extent of the reaction

Fig. 3 shows the time course of the reaction when a limited amount of acetyl-β-alanine was incubated with a large amount of enzyme. Almost all the acetyl-β-alanine added was hydrolyzed in 40 min.

Substrate specificity

The crude enzyme preparation from hog kidney contains strong deacylating activities for acetyl-α-alanine (aminoacylase I activity) and acetyl-aspartate (aminoacylase II activity) as well as acetyl-β-alanine. The purified preparation, however, was inactive on acetyl-α-alanine or acetyl-aspartate (Table II). These results confirm that acetyl-β-alanine deacetylase is an enzyme different from aminoacylases I and II.

The activities of the purified enzyme on various acylated compounds were examined (Table II). It was found that the enzyme was rather specific to *N*-acetyl-β-alanine with respect to both acyl and amino acid moieties. *N*-Acetyl-α-amino acids, *N*-acetyl-γ-amino acids and *N*-acetyl-peptides were not hydrolyzed. Among acylated

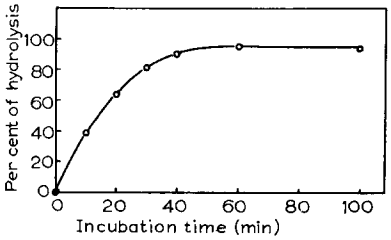


Fig. 3. Time course of the reaction. 5.5 μmoles of acetyl-β-alanine was incubated with 14 units of crude enzyme in a total volume of 4 ml. An aliquot (0.4 ml) of the reaction mixture was taken, assayed for liberated amino group and the per cent of acetyl-β-alanine hydrolyzed was calculated.

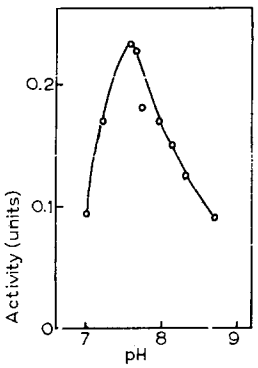


Fig. 4. pH–activity curve of acetyl-β-alanine deacetylase. Acetyl-β-alanine (6.6 μmoles) and purified enzyme (DEAE-cellulose eluate) were incubated in borate–phosphate buffer of various pH values. Assay condition was described in the text.

β-amino acids, acetyl-β-amino-*n*-butyrate, acetyl-β-amino-iso-butyrate, pantothenate (pantoyl-β-alanine) and formyl-β-alanine were not hydrolyzed. Acetyl-taurine, however, was found to be hydrolyzed by the purified enzyme preparation, although the reaction rate was about $^1/_3$ of that of acetyl-β-alanine. Since the enzyme was not purified as a single homogeneous protein, it is not yet clear whether the same enzyme protein catalyzes the hydrolysis of acetyl-β-alanine and acetyl-taurine.

Heat stability

As shown in Table III, more than 80% of the enzyme activity remains after

TABLE II

SUBSTRATE ACTIVITY OF CRUDE AND PURIFIED ENZYME

Enzyme activity was measured in 0.8 ml volume, buffered at pH 7.6 (borate–phosphate buffer) and incubated for 30 min at 37°. One unit of enzyme liberates 1 μmole of amino group under these conditions.

Substrate	*Concentration (mM)*	*Units/mg of protein*	
		Crude extact	*DEAE-cellulose eluate*
N-Acetyl-β-alanine	8.25	0.43	11.9
N-Acetyl-DL-alanine	16.5	0.74	0.0
N-Acetyl-DL-aspartate	14.2	0.05	0.0
N-Acetyl-taurine	8.25		4.4
N-Acetyl-γ-aminobutyrate	8.25		0.0
N-Acetyl-DL-β-amino-*n*-butyrate	8.25		0.0
N-Acetyl-DL-β-amino-iso-butyrate	8.25		0.0
D-Pantothate	8.25		0.0
N-Formyl-β-alanine	8.25		0.0
N-Acetyl-glycyl-glycine	8.25		0.0
N-Acetyl-glycyl-β-alanine	8.25		0.0

TABLE III

HEAT STABILITY OF THE ENZYME

Crude homogenate in borate–phosphate buffer (pH 8.0) was heated at various temperatures for 2 min, immediately cooled in an ice bath and assayed for the enzyme activity.

Treatment	*Recovery of the activity (%)*
None	100
50°, 2 min	95
60°, 2 min	83
70°, 2 min	30

TABLE IV

EFFECT OF INHIBITORS

Purified enzyme (DEAE-cellulose eluate, 0.4 unit) was used for tests.

Inhibitor	*Concentration (mM)*	*Inhibition (%)*
$AgNO_3$	1	94
$HgCl_2$	1	100
$CuSO_4$	1	57
$CoCl_2$	1	0
$CaCl_2$	1	0
$MnCl_2$	1	0
$ZnCl_2$	1	0
$FeSO_4$	1	0
$MgCl_2$	1	0
EDTA	1	0
EDTA	10	27
α,α'-Dipyridyl	1	3
p-Chloromercuribenzoate	1	100
Monoiodoacetate	1	73
Mercaptoethanol	1	(+6)*
L-Cysteine	1	(+33)*
Diisopropylfluorophosphate	1	0

* Per cent stimulation.

TABLE V

DISTRIBUTION OF THE ENZYME

Organs were taken from freshly killed animals and crude homogenate was prepared by the same method as described with hog kidney in the text.

Animal	*Organ*	*Activity (units per g wet weight)*
Rat	Liver	3
Rat	Kidney	23
Rat	Heart	4
Rat	Muscle	0
Rat	Spleen	5
Rat	Brain	1
Hog	Kidney	69

heating the enzyme solution at 50° or 60° for 2 min. Therefore, heat treatment at 60° for 2 min was used in the purification procedures. Heat treatment at 70° for 2 min, however, destroyed about 70% of the activity.

pH dependence of the reaction

The pH–activity curve of the enzyme is shown in Fig. 4. The optimal pH was about 7.6.

Effects of inhibitors

Effects of various compounds on acetyl-β-alanine deacetylase activity were examined (Table IV). *p*-Chloromercuribenzoate, monoiodoacetate and $HgCl_2$ inhibited the reaction strongly and L-cysteine stimulated the reaction. These results suggest that a sulfhydryl group may be involved in the active site of the enzyme. Heavy metal ions such as Ag^+, Hg^{2+}, Cu^{2+} inhibited the reaction, but other metal ions (Co^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, and Mg^{2+}) neither inhibited nor stimulated the reaction. Metal chelating agents did not affect the reaction.

Effects of substrate concentration

Hydrolysis of varying amounts of acetyl-β-alanine with the enzyme followed the Michaelis–Menten kinetics (Fig. 5). The apparent K_m for the substrate was 2.5×10^{-3} M.

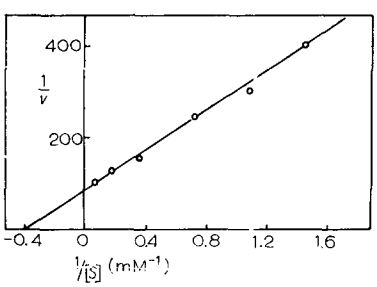


Fig. 5. Lineweaver–Burk plot of the reaction. Purified enzyme preparation (DEAE-cellulose eluate, 1.8 units) was incubated with various amounts of acetyl-β-alanine at 37°. Total volume was 3.0 ml. An aliquot (0.2 ml) was taken and assayed after 10, 20, 30, 40 and 60 min incubation and the initial velocity was estimated. Velocity (v) is expressed as μmoles of β-alanine liberated per min per ml of incubation mixture.

Distribution of the enzyme

Acetyl-β-alanine deacetylase activity was found in the various tissues of rat as well as in the hog kidney. Table V shows the activity levels of various organs. Although some activity was found in heart, spleen and liver, kidney appears to contain the strongest activity.

DISCUSSION

The enzyme activity which catalyzes the hydrolysis of the acetylamino group of *N*-acetyl-β-alanine was found in mammalian kidney and was purified to about 100 fold from hog kidney. Among the acylamino acids tested so far, *N*-acetyl-β-alanine,

and to less extent, *N*-acetyl-taurine, were found to serve as substrates. Therefore, the enzyme should be called as *N*-acetyl-β-alanine amidohydrolase (EC 3.5.1; trivial name, *N*-acetyl-β-alanine deacetylase). However, the occurrence of acetyl-β-alanine in animal tissues has not yet been noted and the physiological substrate of the enzyme remains to be elucidated.

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