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PARTIAL PURIFICATION AND PROPERTIES OF *N*-ACETYLHISTAMINE DEACETYLASE

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Summary

The enzyme catalyzing the deacetylaction of *N*-acetylhistamine was partially purified about 160-fold from rat liver extract and its properties were investigated. The purification procedure included DEAE-cellulose chromatography, precipitation with ammonium sulfate and DEAE-cellulose rechromatography. The enzyme contains a labile -SH group that is essential for its activity. Mn^{2+} and Co^{2+} enhanced the deacetylation reaction at low concentration. The molecular weight of the deacetylase was estimated to be about 70 000 from gel-filtration. Among various acetyl derivatives tested so far, *N*-acetylhistamine and to a lesser extent *N*-acetyltyramine served as the substrates. The K_m value was 0.3 mM at the optimum pH 8.0 for *N*-acetylhistamine. Diphenhysramine, an antihistaminergic agent, inhibited the deacetylation remarkably.

Introduction

Some acylases catalyzing the deacetylation of *N*-acetylamino acids are widely distributed among animal tissues [1—3], however, no enzymes deacetylating acetyl-biogenic amines are yet known. On the other hand, it has been known that mammalian tissues contain an enzyme catalyzing the *N*-acetylation of a number of biogenic amines [4—6]. In the previous study, the authors observed the deacetylating activity for *N*-acetylhistamine in the tissues of rats, mouse and guinea-pigs. Some enzymatic properties tested were similar among these tissues [7]. The present study was undertaken to purify and characterize the enzyme of rat liver which contains the highest activity found in rat tissues.

Materials and Methods

Materials

N-acetylhistamine was synthesized by Merwe's method [8]. P-Cellulose

(capacity: 1.18 mequiv/g) and DEAE-cellulose (capacity: 0.92 mequiv/g) were obtained from Brown Co. (U.S.A.). Sephadex G-200 and SP-Sephadex (C-25) (capacity: 2.3 mequiv/g) were obtained from Pharmacia Fine Chemicals (Sweden). *N*-acetylserotonin, melatonin, *N*-acetyl-L-tryptophan, *N*-acetyl-L-histidine, *N*-acetyl-L-asparagine, L-His-Gly, L-Pro-Gly and L-Leu-Gly were obtained from Sigma Chemical Co (U.S.A.). *o*-Phthalaldehyde, *N*-acetyl-L-alanine, acetanilide, *p*-acetaminobenzaldehyde, *p*-acetaminophenol, *p*-acetaminobenzoic acid and phenacetin were obtained from Nakarai Chemicals, Ltd. (Japan). *N*-acetyl-DL-methionine, *N*-acetyl-L-leucine, *N*-acetylglycine, *N*-acetyl-D-glucosamine, *o*-nitroacetanilide and *N*-acetyl-α-naphthylamine were obtained from Tokyo Chemical Industry Co. Ltd. (Japan). Diphenhydramine was obtained from Kowa Co. Ltd. (Japan). *N*-acetyltyramine was a generous gift from Dr. Fujimoto, Hamamatsu University School of Medicine (Japan). All other reagents were obtained from Wako Chemical Industry, Ltd. (Japan).

Enzymatic reaction and assay procedure

Deacetylation of N-acetylhistamine. Enzymatic activity was assayed by estimation of histamine produced. The standard reaction mixture contained 0.4 ml of 0.2 M borate buffer (pH 8.0) prepared from 0.2 M H_3BO_3 and 0.05 M borax, 0.5 μmol of $MnCl_2$, 1 μmol of GSH, 4 μmol of *N*-acetylhistamine and 0.1 ml of enzyme solution in a final volume of 1.0 ml. The reaction was carried out at 37°C for 30 min and terminated by lowering the pH to 2—3 with 0.2 ml of 0.1 M H_3PO_4. Histamine in the reaction mixture was determined by the author's method described previously [9] with some modifications as follows. The reaction mixture was applied to a P-cellulose column (0.6 × 3 cm) equilibrated with 0.06 M phosphate buffer (pH 6.2) and unadsorbed materials including *N*-acetylhistamine and GSH were washed out from the column with 5 ml of 0.06 M phosphate buffer (pH 6.2) and 1.5 ml of 0.1 M phosphate buffer (pH 7.5). Histamine was eluted in the next 3 ml of 0.1 M phosphate buffer (pH 7.5). Histamine in the eluate was determined fluorimetrically after reaction with *o*-phthaldehyde.

1 munit (mU) of activity is defined as the activity of the enzyme forming 1 nmol of product per min in the reaction mixture. Protein concentration was estimated by measuring the absorbance at 280 nm (A^{1cm}_{280}). The specific activity was defined as the number of mU per A^{1cm}_{280} of the enzyme solution.

The deacetylating reactions of other possible substrates were carried out under the same conditions as in the case of N-acetylhistamine and the activity was assayed as follows.

Deacetylation of N-acetylserotonin and melatonin. An aliquot of the reaction was applied to a P-cellulose column (0.6 × 3 cm) equilibrated with 0.01 M phosphate buffer (pH 6.2) and unadsorbed materials including the substrate were washed out with 10 ml of 0.01 M phosphate buffer (pH 6.2). The deacetylated product (serotonin or 5-methoxytryptamine) was eluted with 3 ml of 0.1 M phosphate buffer (pH 7.5) and determined by measuring the fluorescence at 303/335 nm.

Deacetylation of N-acetyltyramine and N-acetyltryptophan. An aliquot of the reaction mixture was applied to an SP-Sephadex column (0.6 × 3 cm) equilibrated with 0.02 M acetic acid and unadsorbed materials including the sub-

strate were washed out with 10 ml 0f 0.02 M acetic acid. The deacetylated product (tyramine or tryptophan) was eluted with 3 ml of 0.1 M phosphate buffer (pH 7.5). Tyramine in the eluate was determined by measuring its fluorescence at 280/305 nm. Tryptophan in the eluate was determined by measuring the fluorescence at 287/348 nm after the addition of 0.4 ml of 0.4 M NaOH.

Deacetylation of N-acetylhistidine. An aliquot of the reaction mixture was applied to an SP-Sephadex column (0.6 × 3 cm) equilibrated with 0.02 M acetate buffer (pH 4.7) and unadsorbed materials including GSH that interfere with the fluorimetric determination of histidine were washed out with 12 ml of 0.02 M acetate buffer (pH 4.7). Histidine was eluted with 3 ml of 0.1 M phosphate buffer (pH 7.5) and determined fluorimetrically following the method of Håkanson et al. [10].

Deacetylation of other N-acetylamino acids. The enzymatic reaction was terminated by adding 0.2 ml of 20% trichloroacetic acid and the mixture was centrifuged (3000 rev./min for 5 min). The amount of amino group liberated by the reaction was determined by the trinitrobenzene sulphonate method [11] on an aliquot of the supernatant.

Deacetylation of o-nitroacetanilide. Aryl acylamidase activity was assayed by the method proposed by Hoagland and Graf [12] using *o*-nitroacetanilide as a chromogenic substrate.

Estimation of molecular weight

The molecular weight of the enzyme was estimated from its elution volume on Sephadex G-200 gel-filtration following the method of Andrews [13]. The enzyme precipitated by the addition of ammonium sulfate in Step 3 (described in the following section) was dissolved in 5 ml of 0.2 M borate buffer (pH 8.0) containing 3 mM GSH. 1 ml of the enzyme solution was applied to a Sephadex G-200 column (2.6 × 74 cm) equilibrated with 0.2 M borate buffer (pH 8.0) containing 3 mM GSH. The elution was carried out with the same buffer at 15—20°C. The proteins used as standards were bovine serum albumin dimer (M_r = 134 000), bovine serum albumin (M_r = 67 000), ovalbumin (M_r = 45 000) and chymotrypsinogen (M_r = 25 000).

Results

Purification of N-acetylhistamine deacetylase from rat liver

Step 1. Preparation of crude enzyme. Sprague-Dawley rats weighing 300—400 g were decapitated and the livers (35 g) were homogenized in 7 vol. of ice-cold 0.04 M borate buffer (pH 8.0, prepared from 0.04 M H_3BO_3 and 0.01 M borax) containing 1 mM GSH. The homogenate was centrifuged at 13 000 × *g* for 20 min and the supernatant was used as the crude enzyme.

Step 2. DEAE-cellulose column chromatography. The crude extract was applied to a DEAE-cellulose column (3 × 33 cm) equilibrated with 0.04 M borate buffer (pH 8.0). The elution was carried out step by step with the borate buffer containing 1 mM GSH and an increasing amount of NaCl at 2—5°C. As shown in Fig. 1, the activity of *N*-acetylhistamine deacetylase was found in the eluate containing 0.4 M NaCl. In this step, the enzyme deace-

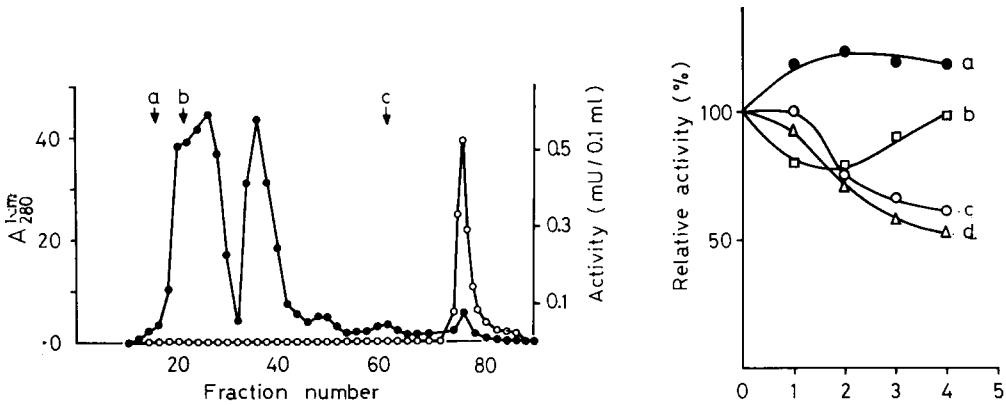

Fig. 1. DEAE-cellulose column chromatography. Crude extract (250 ml) was applied to a DEAE-cellulose column as described in the text, and eluted step by step with 0.04 M borate buffer (pH 8.0) containing different amounts of NaCl (a: no NaCl, b: 0.2 N NaCl, c: 0.4 M NaCl). All these buffers contained 1 mM GSH. An aliquot (0.1 ml) of each fraction was subjected to assay for activity. •: A^{1cm}_{280}, ○: activity; 1 fraction = 15 ml; flow rate = 15 ml/5 min.

Fig. 2. Effects of thiols on the activity. The enzyme used was eluted from a DEAE-cellulose column with thiol-free buffer. The reaction was carried out in the presence of each reagent under the conditions described in the text. a: GSH; b: cysteine; c: 2-mercaptoethanol; d: dithiothreitol.

tylating *N*-acetylmethionine (aminoacylase) [1] and *o*-nitroacetanilide (aryl acylamidase) [12] were eluted almost completely before *N*-acetylhistamine deacetylase. The enzyme fractions were collected and subjected to the following ammonium sulfate fractionation.

Step 3. Ammonium sulfate fractionation. Ammonium sulfate was added to the enzyme solution of Step 2 (kept in an ice bath) to 30% saturation. After 30 min the precipitate was collected by centrifugation (8000 × *g*, 10 min) and dissolved in 20 ml of 0.04 M borate buffer (pH 8.0) containing 1 mM GSH.

Step 4. Rechromatography. After centrifugation (8000 × *g*, 10 min) of the enzyme solution from Step 3, the supernatant was applied to a DEAE-cellulose column (1.8 × 22 cm) equilibrated with 0.04 M borate buffer (pH 8.0) containing 1 mM GSH. The elution was carried out under similar conditions to those of Step 2.

The purification in each step is summarized in Table I. The enzyme was partially purified about 160-fold through these procedures. Aminoacylase and aryl acylamidase, whose activities are more than 1000 times higher than *N*-acetylhistamine deacetylase activity in the crude enzyme solution, were removed from the fraction of *N*-acetylhistamine deacetylase. Further purification (about 300-fold) was attained by column chromatography using Separose 6B on concentrated enzyme from Step 4, however, the total recovery of the enzyme decreased to less than 10%. Moreover the column chromatography on Sepharose 6B showed that the preparation of Step 4 was not homogeneous.

Properties of N-acetylhistamine deacetylase

Heat stability. Crude enzyme solution (0.5 ml in each test tube) was exposed to hot water (50, 55 and 60°C) for 2 min, immediately cooled in an ice bath and assayed for enzyme activity. The activity was significantly reduced by this

TABLE I

PURIFICATION OF THE ENZYME

Details of the procedures are described in the text.

Purification step	Volume (ml)	Activity (mU/0.1 ml)	Protein (A_{280}^{1cm})	Specific activity	Total activity	Recovery (%)
1	250	0.106	69	0.0015	265	100
2	200	0.10	1.35	0.074	200	76
3	20	0.65	4.83	0.135	130	49
4	40	0.24	1.00	0.24	96	36

treatment, i.e., 23% reduction at 50°C, 38% at 55°C and 93% at 60°C.

Effects of thiols on the stability of the enzyme. The previous study [7] suggested that the deacetylase contained an SH group essential for its activity, so the effect of thiols was examined. Table II shows the stability of the deacetylase at 0°C in the presence of thiols. GSH, dithiothreitol and 2-mercaptoethanol are effective for the protection of the enzyme from inactivation during storage. In the absence of these thiols, the activity was lost rapidly. These results suggest that the deacetylase is unstable when the -SH group of the enzyme is not protected from oxidation. Cysteine showed no effect on the protection of the enzyme. The effects of thiols on the reaction of deacetylation are shown in Fig. 2. GSH enhanced the activity only slightly and other thiols were rather inhibitory. On the basis of these results, GSH was added to the reaction mixture.

Effects of pH and metal salts. The activity of the deacetylase was measured in the range pH 7—9 with H_3BO_3/ borax buffer. A pH optimum of 8.0 was observed.

The effects of metal salts are shown in Table III. $CaCl_2$ and $MgCl_2$ were not effective. $CuSO_4$, $ZnSO_4$, $HgCl_2$ and $CdCl_2$ were inhibitory. $CoCl_2$ enhanced the activity at low concentration and inhibited at high concentration. The deacetylating activity was markedly activated by $MnCl_2$. These effects of $MnCl_2$ and $CoCl_2$ are illustrated in Fig. 3. The response of the activity to $CoCl_2$ was more sensitive when the concentration of GSH in the reaction mixture was low, i.e., the peak of the activity curve was observed at 0.06 mM of $CoCl_2$ in the presence of 0.3 mM GSH. The concentration of $MnCl_2$ optimum for the activ-

TABLE II

EFFECTS OF THIOLS ON THE STABILITY OF THE ENZYME

The enzyme used was eluted from a DEAE-cellulose column with buffer, not containing thiol agent. In the assay of the activity, no further thiol agent was added to the reaction mixture.

Time (h)	None *	GSH **	Cysteine **	Dithiothreitol **	2-Mercaptoethanol **
1	100	105	102	113	108
25	65	100	30	106	97
48	28	97	8	106	92

* Enzyme solution without any treatment was kept in an ice bath.

** Each thiol agent was added to the enzyme solution (final concn. 1 mM) and the solution was kept in an ice bath.

TABLE III

EFFECTS OF METAL SALTS ON THE DEACETYLATION OF *N*-ACETYLHISTAMINE

Step 4 enzyme (diluted) was used in the experiment.

	Relative activity (%) for metal salts of concn. (mM):			
	0.05	0.1	1.0	0.5, in presence of 0.5 mM $MnCl_2$
None	13	13	13	—
$CaCl_2$	13	17	13	102
$MgCl_2$	13	14	20	98
$CoCl_2$	39	49	7	61
$CuSO_4$	12	3	0	4
$ZnSO_4$	0	0	0	5
$HgCl_2$	2	0	0	6
$CdCl_2$	0	0	0	0
$MnCl_2$	100	100	100	100 *

* In the presence of 0.5 mM $MnCl_2$ alone.

ity was in a range 0.05—0.6 mM. The activation by $MnCl_2$ was independent of the concentration of GSH.

Molecular weight of the deacetylase. The molecular weight of the deacetylase was estimated from its elution volume on Sephadex G-200 gel-filtration as described in the Materials and Methods section. The activity curve showed a single peak and the molecular weight was estimated to be about 70 000.

Substrate specificity. A variety of substances was examined as possible substrates or inhibitors of the deacetylase. Reaction mixtures containing purified enzyme, *N*-acetylhistamine (4 mM), and various acetyl or other derivatives were incubated and the formation of histamine was measured. The following compounds had no effect (less than 5%) on this reaction: *N*-acetylserotonin (4 mM), melatonin (4 mM), *N*-acetyltyramine (4 mM), *N*-acetyl-L-histidine (4 mM), *N*-acetyl-L-tryptophan (4 mM), *N*-acetyl-L-aspartic acid (4 mM), *N*-acetyl-DL-methionine (4 mM), *N*-acetylglycine (4 mM), *N*-acetyl-L-leucine (4 mM), *N*-acetyl-L-alanine (4 mM), L-carnosine (4 mM), L-His-Gly (4 mM), Gly-Gly (4 mM), L-Pro-Gly (4 mM), L-Leu-Gly (4 mM), *N*-acetyl-α-naphtylamine

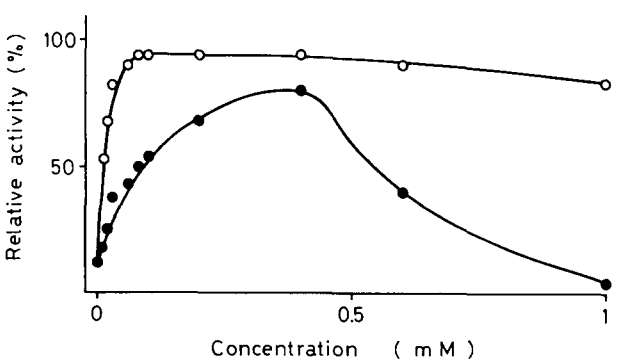

Fig. 3. Effects of $MnCl_2$ and $CoCl_2$ on the deacetylation of *N*-acetylhistamine. Step 4 enzyme (diluted) was used in the experiment. ○: $MnCl_2$; ●: $CoCl_2$.

TABLE IV

SUBSTRATE SPECIFICITY OF THE PURIFIED ENZYME

The complete reaction mixture contained 0.4 ml of Step 4 enzyme, 0.3 ml of 0.2 M borate buffer, 1 μmol of GSH, 0.5 μmol of $MnCl_2$ and each substrate (4 μmol) in a final volume of 1.0 ml. The reaction was carried out at 37°C for 2 h. The method of the assay for each deacetylating activity was described in the Materials and Methods section.

Substrate	Amount of product (nmol)		
	Complete	Without $MnCl_2$	Diphenhydramine *
N-Acetylhistamine	122	13	14
N-Acetylserotonin	0.4	—	—
Melatonin	0.5	—	—
N-Acetyltyramine	33	27	18
N-Acetyl-L-tryptophan	0.3	—	—
N-Acetyl-L-histidine	0.5	—	—

* Diphenhydramine (1 μmol) was added to the complete reaction mixture.

(3 mM), *o*-nitroacetanilide (4 mM), acetanilide (4 mM), *p*-acetaminophenol (4 mM), *p*-acetaminobenzaldehyde (4 mM), *p*-acetaminobenzoic acid (3 mM), phenacetin (3 mM), *N*-acetyl-L-glucosamine (4 mM), L-arginine (1 mM), L-histidine (1 mM), imidazoleacetic acid (1 mM), and neostigmine (1 mM). Imidazole (1 mM) showed a slight inhibition (13%). Diphenhydramine, an antihistaminergic agent, showed a large inhibition, i.e. 90% at 1 mM, 40% at 0.1 mM, and 24% at 0.05 mM. This inhibition was not reversed by excess substrate, indicating the noncompetitive nature of this reaction.

In addition to the above inhibition test, *N*-acetylserotonin, *N*-acetyltyramine, *N*-acetyl-L-tryptophan and *N*-acetyl-L-histidine were treated with a large amount of the enzyme and their deacetylation were measured by a sensitive fluorimetric method (Table III). Although *N*-acetyltyramine was deacetylated to some extent, the effects of $MnCl_2$ and diphenhydramine were not so remarkable as those in the deacetylation of *N*-acetylhistamine. The deacetylation of *N*-acetyltyramine showed also a pH optimum of 8. The deacetylations of *N*-acetylserotonin, melatonin, *N*-acetyltryptophan and *N*-acetylhistidine were negligible within a pH range of 7—9. The deacetylations of *N*-acetylmethionine (a sensitive substrate of amino acylase [1]) and *o*-nitroacetanilide (a substrate of aryl acylamidase [12]) were not detected by the trinitrobenzene sulphonate method.

Effect of substrate concentration on the rate of deacetylation. The reaction of deacetylation of various amounts of *N*-acetylhistamine with the purified enzyme followed Michaelis-Menten kinetics. The K_m value was 0.3 mM.

Comparison with other tissue enzyme. Rat brain and mouse liver were subjected to the same purification procedures as those for rat liver. The enzyme from those tissues showed similar behavior in DEAE-cellulose column chromatography and ammonium sulfate precipitation.

Discussion

There has been no documentation of the enzyme deacetylating *N*-acetyl biogenic amines. The present study demonstrated the existance of an enzyme

catalyzing the deacetylation of *N*-acetylhistamine. Among acetyl derivatives tested so far, *N*-acetylhistamine and, to a lesser extent, *N*-acetyltyramine were found to serve as substrates. It is still possible that the deacetylation of *N*-acetyltyramine is catalyzed by another enzyme, because *N*-acetyltyramine was not effective as an inhibitor, and because the effects of $MnCl_2$ and diphenhydramine were different in both deacetylations.

When the properties of the enzyme (stability, effects of -SH reagents and metal ions, distribution of the enzyme, etc.) are compared with known hydrolytic enzymes acting on the C-N bond, it is likely that the enzyme deacetylating N-acetylhistamine is different from these known enzymes.

The nature of the true physiological substrate of the enzyme remains to be elucidated further, although the enzyme could be called *N*-acetylhistamine aminohydrolase (EC 3.5.1.-, trival name, *N*-acetylhistamine deacetylase).

Previous studies suggested that *N*-acetylhistamine deacetylase was distributed widely among tissues of animals and possessed common properties with respect to effects of metal ions, -SH reagents and pH. The present study showed further similarities between enzymes from different tissues in their behavior during ammonium sulfate precipitation and column chromatography.

Little can be said at this time about the physiological role of this enzyme and the inhibition of deacetylation by diphenhydramine.

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