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N-ACYL-L-AROMATIC AMINO ACID DEACYLASE IN ANIMAL TISSUES

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Summary

An enzyme deacylating preferentially *N*-acyl-L-aromatic amino acids was partially purified from rat kidney. The purification procedure included DEAE-cellulose column chromatography, $(NH_4)_2SO_4$ fractionation, gel-filtration on a Sephadex G-200 column and further DEAE-cellulose chromatography. The enzyme was thus separated from aminoacylase (*N*-acylamino-acid amidohydrolase, EC 3.5.1.14) (acylase I). Although the enzyme preparation contained other acylases, the experimental data (effect of *p*-chloromercuric benzoate, heat stability and inhibition between substrates) suggest that the enzyme acts preferentially on *N*-acyl derivatives of L-tryptophan, L-tyrosine, L-phenylalanine and L-histidine. This enzyme appears to be present in many animal tissues.

Introduction

Mammalian tissues contain acylases which act on *N*-acylamino acids. The following acylases are known: aminoacylase (*N*-acylamino-acid amidohydrolase, EC 3.5.1.14) (acylase I) [1—3], aspartoacylase (EC 3.5.1.15, acylase II) [1,4,5], acyl-lysine deacylase (EC 3.5.1.17) [6], *N*-acetyl-β-alanine deacetylase (EC 3.5.1.21) [7]. Acylase I acts on various substrates and has been proposed to be identical with dehydropeptidase II, histozyme, hippuricase and benzamidase [2,8]. However, the hydrolysis of *N*-acyl-tryptophan, -tyrosine and -phenylalanine by acylase I are very slow compared with other *N*-acylamino acids. From the activity profile of renal acylases toward various *N*-acylamino acids, it was suggested that an acylase was present which acted on *N*-acyl-L-aromatic amino acids and designated the enzyme tentatively as acylase III [1]. In the present study, this enzyme was partially purified from rat kidney extract and its properties are examined.

Materials and Methods

Materials

N-acyl-derivatives were obtained from Sigma Chemical Co. (U.S.A.) or Wako Chemical Industry, Ltd. (Japan). *N*-acetyl-histamine was synthesized according to Merwe's method [9]. *N*-acetyl-tyramine was a generous gift from Dr. Fujimoto, Hamamatsu University School of Medicine (Japan). DEAE-cellulose (capacity: 0.92 mequiv/g) was 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). Other reagents were obtained from Wako Chemical Industry, Ltd. (Japan). *N*-acyl-derivatives were dissolved in water and the pH of the solutions was adjusted to 7—8 with 0.4 M NaOH. The following animals were used: male rats (Wistar, 200—350 g), male guinea-pigs (Hartley, 200—250 g) and male mice (ddI, 20—25 g).

Enzyme assay procedure

The reaction mixture contained 0.3 ml 0.2 M borate buffer (pH 8.0, prepared from 0.2 M H_3BO_3 and 0.05 M borax), a substrate (4 μmol) and enzyme solution (0.1—0.3 ml) in a final volume of 1.0 ml. The reaction was carried out at 37°C for an appropriate period and terminated by the addition of 20% (w/v) trichloroacetic acid (0.2 ml). After the mixture was centrifuged (3000 rev./min, 5 min), an aliquot (0.1—1.0 ml) of the supernatant was taken, neutralized with 0.4 M NaOH and made to 3 ml with 0.2 M borate buffer (pH 8.0). The amino acid formed was determined as follows: A solution of 2,4,6-trinitrobenzene sulfonate (0.067% in 0.2 M borate buffer, pH 8.0, 0.5 ml) was added to the sample and the mixture incubated at 50°C for 30 min. The reaction mixture was cooled in a water bath and the absorbance at 420 nm measured. In the determination of aspartic acid, the reaction was carried out at pH 9.0.

During the purification procedure, acylase III activity was followed using *N*-acetyl-L-tryptophan as a substrate. The enzymatic reaction was carried out as described above but was terminated by the addition of 0.1 M H_3PO_4 (0.2—0.3 ml). The tryptophan formed was separated on a small SP-Sephadex column (0.6 × 3 cm) and determined fluorometrically as described previously [10]. The deacetylating activity for *o*-nitroacetanilide was assayed by the method of Hoagland and Graf [11].

One unit (U) of activity was defined as that amount forming 1 μmol of product per min under the conditions described above. The protein concentration was estimated by the method of Lowry et al. [12] with bovine serum albumin as a standard.

Inhibition of the deacetylation of N-acetyl-L-histidine by other substrates

The reaction mixture contained 0.3 ml 0.2 M borate buffer (pH 8.0), *N*-acetyl-L-histidine (4 μmol), another *N*-acetyl-L-amino acid (2 μmol) and the diluted Step 5 enzyme solution (0.1 ml) in a final volume of 1.0 ml. The reaction was carried out at 37°C for 1 h and terminated by the addition of 0.1 M H_3PO_4 (0.2 ml). The histidine formed in the reaction mixture was separated on a SP-Sephadex column and determined fluorometrically as described previously [10].

Results

Distribution of the activities deacetylating N-acetyl-L-tryptophan and N-acetyl-DL-methionine among various animal tissues

Extracts from various animal tissues were assayed for these activities (Table I). The ratio between these activities varies depending on the tissue. The enzyme which deacetylates *N*-acetyl-L-tryptophan was designated tentatively as acylase III. Like other acylases [1,4,6,7], acylase III activity is also highest in kidney. The acylase III activity of the cortex region of rat kidney was 2—3 times higher than that of the medulla region. More than 85% of acylase III activity was observed in the 20 000 × *g* supernatant fraction of the homogenate of rat kidney.

Separation of acylase I and III by DEAE-cellulose column chromatography

Acylase III activity in the kidneys of rat and mouse could be separated from acylase I by DEAE-cellulose column chromatography (Fig. 1A). On the other hand, these activities in the kidneys of guinea-pig and hog were not separated by the same chromatography (Fig. 1B).

Purification of acylase III

Rats were decapitated and the kidneys were taken out and stored in a deep-freezer at between −15 and −20°C until use. The kidneys (12.5 g) were homogenized in ice-cold 0.04 M borate buffer (pH 8.0, 130 ml). The homogenate was centrifuged (20 000 × *g*, 20 min) and the supernatant was

TABLE I

DISTRIBUTION OF THE ACTIVITIES DEACETYLATING *N*-ACETYL-L-TRYPTOPHAN AND -DL-METHIONINE AMONG VARIOUS ANIMAL TISSUES

Tissues were homogenized with 10 vols. of 0.04 M borate buffer (pH 8.0) and the homogenate was centrifuged (20 000 × *g*, 20 min). An appropriate amount of the supernatant was assayed for the activities. The activity values are expressed as nmol/h per 10 mg tissue.

Tissue	*N*-Acetyl-L-tryptophan	*N*-Acetyl-DL-methionine
Rat		
Kidney	40	8 900
Liver	4.4	926
Brain	0.7	210
Spleen	0.6	123
Testis	0.3	69
Pancreas	1.5	730
Thymus	0.5	219
Mouse		
Kidney	214	4 350
Liver	14.8	1 280
Brain	0.5	58
Spleen	0.3	68
Guinea-pig		
Kidney	171	17 700
Hog		
Kidney	11	96 800

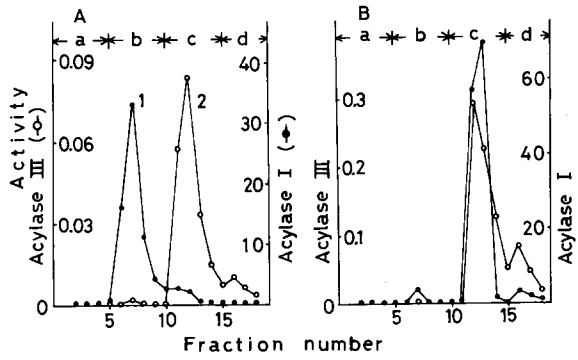

Fig. 1. Separation of acylase I and III by DEAE-cellulose column chromatography. Tissues were homogenized in 10 vols. of 0.04 M borate buffer (pH 8.0). The homogenate was centrifuged (20 000 × *g*, 20 min) and 2 ml of the supernatant was applied to a DEAE-cellulose column (1 × 13 cm) equilibrated with 0.04 M borate buffer (pH 8.0). The elution was carried out step by step with the borate buffers containing NaCl of different concentrations, a: without NaCl, b: 0.1 M, c:0.2 M, d: 0.4 M. Fractions (5 ml) were collected at a flow rate of about 5 ml/7 min. Activities are expressed as mU/0.1 ml of the enzyme solution. A: rat kidney, B: guinea-pig kidney.

recovered (Step 1). Ammonium sulfate was added to Step 1 enzyme solution kept in an ice bath to 40% saturation. After 30 min, the precipitate was collected by centrifugation (8000 × *g*, 10 min), dissolved in 0.04 M borate buffer (pH 8.0, 30 ml) and dialyzed against 2 changes of 0.04 M borate buffer (pH 8.0, 1000 ml) (Step 2). Step 2 enzyme solution was applied to a DEAE-cellulose column (3 × 33 cm) equilibrated with 0.04 M borate buffer (pH 8.0). The elution conditions were the same as those described in the legend to Fig. 1 and acylase III fraction was recovered (Step 3). $(NH_4)_2SO_4$ was added to Step 3 enzyme solution to 40% saturation. The precipitate recovered as described above was dissolved in 0.04 M borate buffer (pH 8.0, 4 ml) and applied to a Sephadex G-200 column (2.6 × 74 cm) equilibrated with 0.04 M borate buffer (pH 8.0). The elution was carried out with the same buffer at room temperature. Single peak of the activity was observed and the enzyme fraction was recovered (Step 4). Step-4 enzyme solution was rechromatographed on a DEAE-cellulose column (1.2 × 12 cm) under the same elution conditions as described above and the enzyme fraction was recovered (Step 5). By this procedure, about 100-fold purification was attained (Table II). Step-5 enzyme solution was used in the following experiments. The enzyme preparation was stable for at least 3 months when stored frozen at −15 to −20°C.

Properties of the enzyme

Substrate specificity. The deacylation rates of various substrates are shown in Table III, column A. No deacylation was observed with the following substrates: D-isomers of *N*-acetyltryptophan and *N*-acetylphenylalanine, hippuric acid, *N*-acetyl-serotonin, -histamine, -tyramine, melatonin and *o*-nitroacetanilide. The substitution of acetyl group to formyl or chloroacetyl group increased the rate of the hydrolysis of *N*-acyl-L-tryptophan.

The K_m values at pH 8.0 were 0.096 mM for *N*-acetyl-L-tryptophan, 0.22 mM for *N*-acetyl-L-tyrosine, 0.26 mM for *N*-acetyl-L-phenylalanine, 1.43 mM for *N*-acetyl-L-histidine and 0.67 mM for *N*-acetyl-L-methionine.

TABLE II

PURIFICATION OF ENZYME

Details of the procedure are described in the text.

Purification step	Volume (ml)	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Recovery (%)
1	115	679	1890	0.36	100
2	54	584	935	0.63	86
3	193	541	85.3	6.34	80
4	42	357	13.2	27.0	53
5	18	216	5.7	38.0	32

Effect of p-chloromercuribenzoate. Since the preparation of acylase III showed activity for many substrates, the activities for these substrates were compared between in the presence and absence of *p*-chloromercuribenzoate (Table III). In the presence of the reagent, the inhibition on the deacetylations on non-aromatic *N*-acetyl-L-amino acids was stronger than that of *N*-acyl-L-aromatic amino acids. Fig. 2 illustrates clearly the effect of the reagent.

Effect of pH. The effect of pH on the deacetylations of different substrates is illustrated in Fig. 3. The pH optima are near pH 8 for *N*-acetyl-L-aromatic amino acids and pH 7.5 for *N*-acetyl-L-methionine.

Heat stability. The enzyme solution (0.5 ml) was heated at 50, 55 and 60°C for 5 min, rapidly cooled in an ice bath and assayed for the activities toward *N*-acetyl-L-tryptophan, -histidine and -methionine. The activities for the former two substrates decreased in parallel and were more sensitive to heat than that for *N*-acetyl-L-methionine, i.e., 30% reduction at 55°C and 80% at 60°C for the

TABLE III

DEACRYLATION RATE IN THE PRESENCE AND ABSENCE OF *p*-CHLOROMERCURIC BENZOATE (PCMB)

Substrate concentration is 4 mM for L-isomers and 8 mM for DL-derivatives. The concentration of PCMB is 0.5 mM. Deacylation rate values are expressed as nmol/min in the presence of 0.1 ml of Step 5 enzyme solution.

Substrate	Deacylation rate		Inhibition (%)
	A(—PCMB)	B(+PCMB)	
N-Acetyl-L-tryptophan	1.44	1.08	25
N-Formyl-DL-tryptophan	4.31	3.23	25
N-Chloroacetyl-L-tryptophan	12.76	9.83	23
N-Acetyl-L-histidine	3.22	2.33	27
N-Acetyl-L-tyrosine	13.00	9.77	25
N-Acetyl-L-phenylalanine	11.62	8.48	27
N-Acetyl-L-methionine	5.61	1.06	81
N-Acetyl-DL-methionine	5.63	0.83	85
N-Acetyl-glycine	0.26	0.04	84
N-Acetyl-L-alanine	1.27	0.22	82
N-Acetyl-L-leucine	2.94	0.40	86
N-Acetyl-L-aspartic acid	0.31	0.07	68

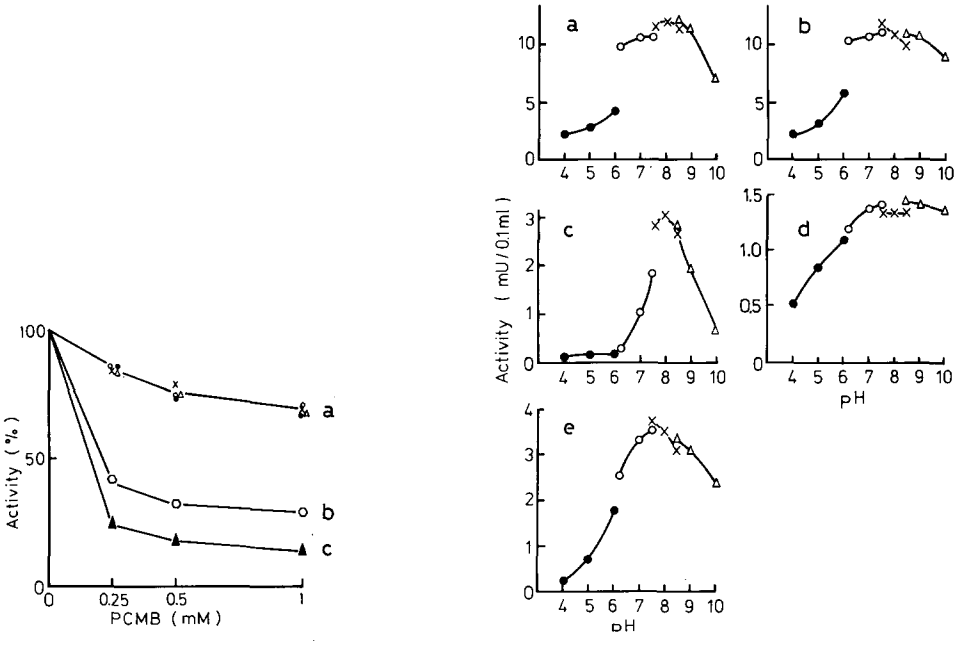

Fig. 2. Effect of PCMB on the deacetylating activities for different substrates. a: *N*-acetyl-L-aromatic aminoc acids (tryptophan, histidine, tyrosine and phenylalanine). b: *N*-acetyl-L-aspartic acid. c: *N*-acetyl-L-methionine.

Fig. 3. Effect of pH on the deacetylating activities for different substrates. The following buffers (0.3 ml) were used in the experiment: pH 4—6: 0.2 M CH_3COOH/0.2 M CH_3COONa; pH 6.2—7.5: 0.2 M NaH_2PO_4/0.2 M Na_2HPO_4; pH 7.6—8.5: 0.2 M H_3BO_3/0.05 M borax; pH 8.5—10.0: 0.2 M H_3BO_3/0.2 M Na_2CO_3. a: *N*-acetyl-L-tyrosine; b: *N*-acetyl-L-phenylalanine; c: *N*-acetyl-L-histidine; d: *N*-acetyl-L-tryptophan; d: *N*-acetyl-L-methionine. The activity values are expressed as mU/0.1 ml of Step 5 enzyme solution.

former two substrates and 10% at 55°C and 35% at 60°C for the latter substrate.

Inhibition of the deacetylation of N-acetyl-L-histidine by other substrates. The deacetylation of *N*-acetyl-L-histidine was inhibited strongly by *N*-acetyl-L-tryptophan, -tyrosine and -phenylalanine at 2 mM, i.e., 97, 89 and 88%, respectively. The K_m values of these substrates are significantly lower than that of *N*-acetyl-L-histidine as described above. On the other hand, *N*-acetyl-L-methionine, of which K_m value is also lower than that of *N*-acetyl-L-histidine, did not inhibit the deacetylation of *N*-acetyl-L-histidine.

Effect of metal salts, EDTA and thiols. The effect of these reagents on the deacetylation of *N*-acetyl-L-tryptophan was examined. $CaCl_2$, $MgCl_2$, $MnCl_2$, $CoCl_2$, $ZnSO_4$ and $FeSO_4$ showed no appreciable effect on the activity at the concentrations of 0.1, 0.5 and 1.0 mM. EDTA, cysteine, glutathione (reduced), dithiothreitol and 2-mercaptoethanol did not affect the activity at 1.0 and 5.0 mM. $HgCl_2$, $CuSO_4$ and $CdCl_2$ were inhibitory, i.e., 84, 82 and 32% inhibition were observed at 1.0 mM, respectively.

Molecular weight. The molecular weight of acylase III was estimated by gel-filtration using a Sephadex G-200 column (2.6 × 74 cm). The proteins used

as standards were bovine serum albumin dimer and monomer, and ovalbumin. A molecular weight of about 55 000 was estimated.

Discussion

Although the purified enzyme preparation showed activity for many substrates, the results including the effect of *p*-chloromercuric benzoate, heat stability and inhibition between substrates suggest that the activities for *N*-acyl-L-aromatic amino acids are due to a single enzyme (acylase III) that is different from the enzyme acting on non-aromatic *N*-acetyl-L-amino acids. The activities for non-aromatic *N*-acetyl-L-amino acids are not due to the contamination of acylase I corresponding to peak 1 shown in Fig. 1A, because the activity corresponding to the peak was not observed in the repeated DEAE-cellulose column chromatography with Step 5 enzyme preparation.

The separation profiles of the activities of rat kidney for *N*-acetyl-L-tyrosine and -phenylalanine were essentially the same as that for *N*-acetyl-L-tryptophan as shown in Fig. 1A. However, the separation profile for *N*-acetyl-L-histidine showed that acylase I fraction contained 3—4 times higher activity for this substrate than acylase III fraction. The deacetylating activity for *N*-acetyl-L-histidine in acylase I fraction was inhibited by *N*-acetyl-L-methionine. From these results, acylase III seems to be a main component to deacetylate *N*-acetyl-L-tryptophan, -tyrosine and -phenylalanine, but a main component to deacetylate *N*-acetyl-L-histidine seems to be acylase I.

Acylase III activity was observed in all tissues tested. The separation patterns of acylase I and III of rat liver, brain and pancreas in DEAE-cellulose column chromatography were also the same as those of the kidneys of rat and mouse shown in Fig. 1A. Although these enzymes of guinea-pig kidney could not be separated, essentially the same effect of *p*-chloromercuric benzoate was observed also with the enzyme fraction obtained as described in Fig. 1B. There-

TABLE IV

ACTIVITIES OF SOME ACYLASES IN RAT KIDNEY

Number in square brackets are the corresponding references. TP = This paper.

Enzyme and substrate	Activity			
	μmol/h per g tissue	ratio	μmol/h per ng N	ratio
Acylase I				
N-Acetyl-methionine	890 (TP)	1000	44 [1]	1000
Acylase II				
N-Acetyl-aspartic acid	28.5 [4]	32	1.2 [1]	27
Acylase III				
N-Acetyl-tryptophan	4 (TP)	4.5	—	—
N-Acetyl-tyrosine	37 (TP)	42	—	—
N-Acetyl-phenylalanine	33 (TP)	37	—	—
Acetyl-β-alanine deacetylase				
N-Acetyl-β-alanine	46 [7]	52	—	—
Acyl-lysine deacylase				
ε-*N*-Acetyl-lysine	—	—	0.14 [6]	32

fore, acylase III appears to be present widely in animal tissues.

Some acylases acting on different *N*-acylamino acids are present in animal tissues. On the basis of the data reported by other investigators, the activities of some acylases in rat kidney are compared (Table IV). Recently the author found an enzyme deacetylating *N*-acetyl-histamine in animal tissues [10,13]. Although the physiological role of these acylases has not been established, it is of interest that these characteristic acylases are present widely in animal tissues.

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