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ALKYLAMIDASE OF SHEEP LIVER*

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SUMMARY

1. An amidase from sheep liver microsomes which catalyzes the hydrolysis of N-monosubstituted and N,N-disubstituted amides was purified 59-fold by hydroxylapatite and benzyl-DEAE-cellulose column chromatography.

2. Acrylamide gel electrophoresis of the final enzyme preparation indicated that it was a single protein.

3. The enzyme had an optimum at approximately pH 9, was not stimulated by divalent cations and had a molecular weight, as determined by gel filtration, of 230 000 to 250 000.

4. The enzyme was unable to hydrolyze the lower members of a series of *N*-methyl-substituted amides and only had a trace of activity to *N*-methyl butyramide. The activity reached a maximum with *N*-methyl caproamide and decreased with an increase in chain length. The enzyme hydrolyzed *N*-methyl, *N*-ethyl and *N*-propyl caproamide at approximately equal rates. Of all the compounds investigated as substrates, *N*-phenyl caproamide had the shortest enzymatic half-life. Of the *N,N*-dialkyl-substituted caproamides evaluated, only *N,N*-dimethyl caproamide was hydrolyzed.

INTRODUCTION

Only a limited number of reports have appeared concerning amidases and their deamidation of naturally occurring amides and their analogs[1–4]. BRAY and co-workers reported[5–9] that many primary amides of aliphatic and aromatic carboxylic acids underwent hydrolysis in the presence of crude liver extracts. Amidases which hydrolyze 2 to 3 carbon amides have also been reported in microorganisms[10–12]. An enzyme from rabbit liver microsomes with activity toward monoethyl glycinexylidide has been purified 45-fold[13]. In almost every study the substrates were primary amides. Therefore, it appeared to be of interest to investigate the hydrolysis of substituted amides since several *N*-alkyl- and *N,N*-dialkyl-substituted amides which are xenobiotics undergo hydrolysis in biological systems[14,15].

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This study is concerned with a microsomal amidase which hydrolyzes N-monosubstituted and N,N-disubstituted aliphatic amides. The purification, substrate specificity, and some of the properties of this enzyme are reported.

EXPERIMENTAL PROCEDURE

Materials

Various N-substituted and N,N-disubstituted amides were synthesized according to the procedure of D'ALELIO AND REID[16]. NAD^+, $NADP^+$, NADH, NADPH, bovine liver catalase (2 times crystallized), rabbit muscle lactate dehydrogenase Type II, and bovine serum albumin were purchased from the Sigma Chemical Co. Carbowax 20 M was purchased from Applied Science Laboratory, Inc. Fresh sheep liver was obtained from a local abattoir and was stored at $-20°$ until used.

Methods

Amidase assay. The amidase activity to various substituted and unsubstituted amides was determined using the method of CHEN AND DAUTERMAN[17]. *N*-Methyl caproamide was used as the substrate during the purification and one unit of amidase activity was defined as that amount of enzyme which catalyzed the hydrolysis of 1 μmole of substrate per h at 37° and pH 7.8. Specific activity was defined as enzyme units/mg of protein.

Protein determination. Protein determination was carried out using the method of LOWRY *et al.*[18]. The protein concentration of individual fractions from column chromatography was estimated by measuring the absorbance at 280 nm in a 1.0-cm cuvette. Both bovine serum albumin monomer and dimer were estimated by the extinction at 230 and 220 nm[19].

Column chromatography. Rapid-flow hydroxylapatite was prepared according to the method of ANACKER AND STOY[20]. Benzyl-DEAE-cellulose was prepared and washed as described by KREMZNER AND WILSON[21]. Column chromatography was performed at 4°.

Molecular weight estimation. Molecular weight estimations were carried out using the methods of ANDREWS[22] utilizing a Sephadex G-200 column which was equilibrated with 0.01 M phosphate buffer (pH 7.8) at 4°. The pure proteins were applied separately and in different combinations to eliminate any possible interactions. One ml (0.4 mg protein) of the enzyme solution, after concentration over Carbowax 20 M, was applied to the column for molecular weight determination.

Other enzyme assays. The activity of bovine liver catalase was measured by the method of CHANCE AND MAEHLY[23]. Lactate dehydrogenase was assayed by the method of KORNBERG[24].

Acrylamide gel electrophoresis. Disc electrophoresis using the method of DAVIS[25] was utilized to check the purity of the various enzyme preparations. The gel was loaded with 25 μg of protein and run for 1 h at 2 mA/tube at 5°. The gel concentration was 7%.

Determination of K_m and v_{max} values. The K_m and v_{max} values were determined using the assay method of CHEN AND DAUTERMAN[17]. The values were obtained from Lineweaver–Burk plots utilizing 5 to 7 different concentrations of the various substrates.

RESULTS

Amidase purification

Step 1. A 15-g sample of sheep liver was homogenized in 150 ml of 0.01 M phosphate buffer (pH 7.8). Microsomes were obtained by centrifuging the post-mitochondrial supernatant at 100 000 × *g* for 60 min after which the microsomal pellet was resuspended in 40 ml of the buffer.

Step 2. The suspension was frozen in a dry ice–acetone mixture for 5 min then thawed in a water bath at 30°. This process was repeated 10 times. The suspension was recentrifuged at 100 000 × *g* for 60 min.

Step 3. The supernatant of Step 2 was added to a 2 × 25-cm hydroxylapatite column which had been previously equilibrated with distilled water in the cold room. After absorption of the protein, the column was washed with 20 ml of distilled water. The enzyme was then eluted with a linear gradient consisting of 100 ml of distilled water and 100 ml of 0.2 M phosphate buffer pH 7.8. The flow rate was approximately 15 ml/h and 5-ml fractions were collected.

Step 4. The enzyme fraction recovered from the hydroxylapatite column was placed on a benzyl-DEAE-cellulose column previously equilibrated with 0.01 M phosphate buffer (pH 7.8). The column was eluted utilizing a gradient consisting of 150 ml each of 0.01 M phosphate buffer and 0.4 M NaCl at pH 7.8. The elution proceeded at a rate of 25 ml/h and 5-ml fractions were collected. The active fractions were pooled and dialyzed overnight against 0.01 M phosphate buffer at pH 7.8.

Table I summarizes the results of a typical purification procedure. It should be

TABLE I

THE PURIFICATION OF SHEEP LIVER AMIDASE

Step	*Vol. (ml)*	*Units*	*Specific activity*	*Recovery (%)*	*Purification (-fold)*
1. Microsomes	40	44.7	0.126	100	1
2. Solubilization	32	42	0.965	94	7.6
3. Hydroxylapatite	38	35.6	1.53	79	12
4. Benzyl-DEAE-cellulose	32	30	7.43	65	59

emphasized that the reproducibility is excellent having replicated the purification procedure at least ten times. Fig. 1A shows the elution profiles from a hydroxylapatite column and Fig. 1B from a benzyl-DEAE-cellulose column. Acrylamide gel electrophoresis of the various enzyme fractions after each purification step was compared. The presence of a single protein band when stained with Amido black possibly indicates homogeneity of the final preparation (Fig. 2).

General properties

The amidase was inhibited at $1 \cdot 10^{-6}$ M by paraoxon (*O,O*-diethyl *p*-nitrophenyl phosphate). However, this enzyme was insensitive to sulfhydryl inhibitors such as *p*-chloromercuribenzoate since only 20% of the amidase activity was inhibited at $1 \cdot 10^{-3}$ M and *N*-ethyl maleimide failed to inhibit the enzyme at the same concentration. Studies with various divalent cations such as Co^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, at $1 \cdot 10^{-6}$

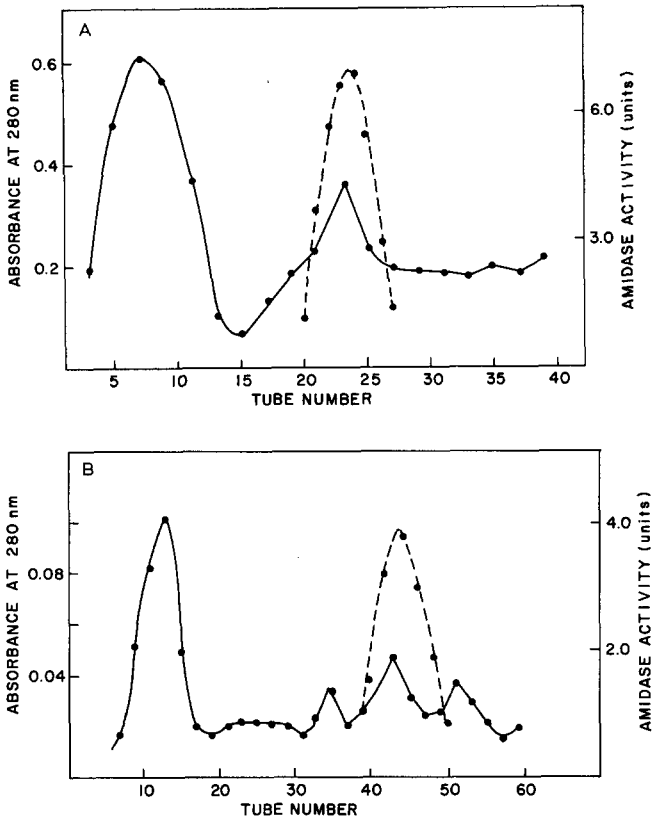


Fig. 1. Elution of sheep liver amidase from (A) hydroxylapatite column; (B) benzyl-DEAE-cellulose column; ●- - -●, amidase; ●—●, $A_{280\ nm}$.

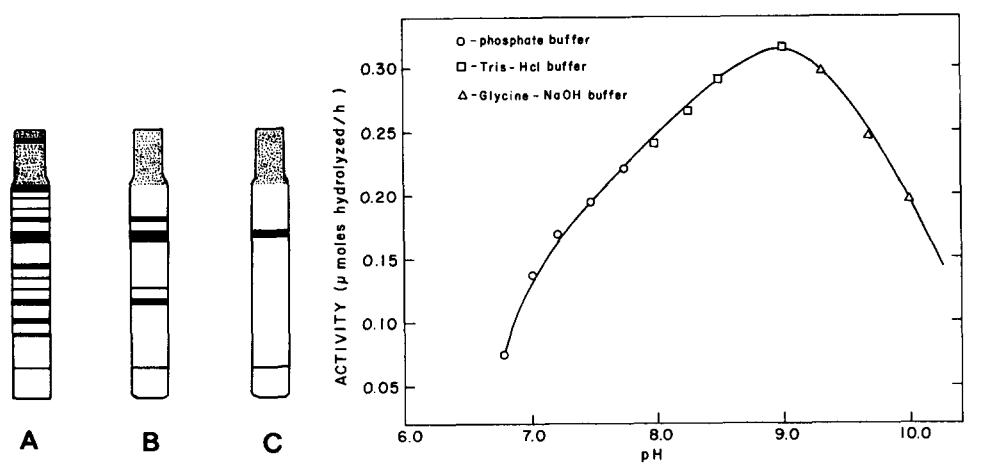
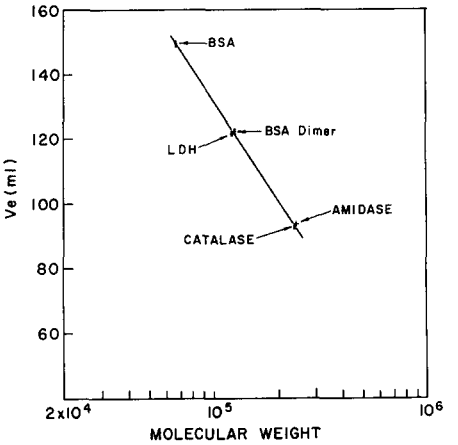


Fig. 2. Acrylamide gel electrophoresis of sheep liver amidase after various purification steps. (A) Zymogram after solubilization; (B) zymogram of the enzyme fraction after hydroxylapatite column; (C) zymogram of the enzyme fraction after benzyl-DEAE-cellulose column. Gels were stained with Amido black.

Fig. 3. Effect of pH on the hydrolysis of *N*-methyl caproamide by sheep liver amidase.



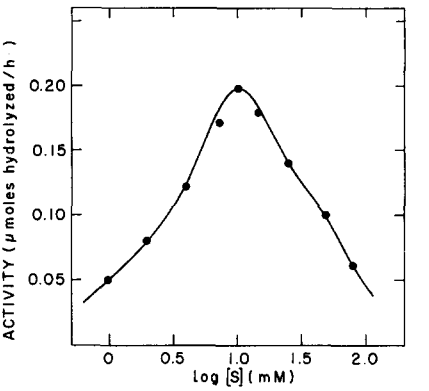


Fig. 4. Plot of elution volume, V_e, against log of the molecular weight for proteins on a Sephadex G-200 column at pH 7.8. BSA, bovine serum albumin; LDH, lactate dehydrogenase.

Fig. 5. The effect of *N*-methyl caproamide concentration (log[*S*]) on the activity of sheep liver amidase.

M did not stimulate the amidase activity indicating that this enzyme is not an arylamidase[26]. The enzyme had a pH optimum in the range of 9 (Fig. 3) which is much higher than that reported by BRAY *et al.*[9]. No distinct optimum ionic strength could be found in the range of 0.01 to 0.4. The relationship between elution volume (V_e) and the log of the molecular weight for several pure proteins is shown in Fig. 4. The mean elution volume of the amidase was 93 ± 2 ml which was almost identical to that of bovine liver catalase (93 ± 1 ml). The molecular weight of bovine liver catalase is considered to be approximately 230 000–250 000 (ref. 22). Therefore, the molecular weight of this enzyme was in the same range.

Substrate specificity

This enzyme was unable to hydrolyze the lower members of the *N*-methyl-

TABLE II

K_m, v_{max} AND THE ENZYMATIC HALF-LIFE OF SEVERAL N-SUBSTITUTED AMIDES

Substrate	K_m (*M*)	v_{max} (*μmoles/mg per h*)	*Enzymatic half-life* ($0.695\ K_m/v_{max} \times 10^{-3}$)
N-Methyl formamide	—	—	—
N-Methyl acetamide	—	—	—
N-Methyl butyramide	—	—	—
N-Methyl valeramide	$5 \cdot 10^{-3}$	2.82	1.232
N-Methyl caproamide	$6.6 \cdot 10^{-4}$	1.30	0.350
N-Methyl heptylamide	$1.25 \cdot 10^{-3}$	2.14	0.410
N-Methyl caprylamide	$3.7 \cdot 10^{-3}$	0.367	0.704
N-Ethyl caproamide	$8.3 \cdot 10^{-4}$	1.71	0.337
N-Propyl caproamide	$1.8 \cdot 10^{-3}$	3.42	0.366
N-Butyl caproamide	$2.2 \cdot 10^{-3}$	0.087	17.980
N-Phenyl caproamide	$2.5 \cdot 10^{-3}$	132.4	0.013
N,N-Dimethyl caproamide	$1.9 \cdot 10^{-4}$	0.564	0.234
N,N-Diethyl caproamide	—	—	—

substituted amides and had only a trace of activity to *N*-methyl butyramide. The kinetic constants for the various N-substituted amides are summarized in Table II. The enzyme was also inhibited at high concentrations of the various substrates. Fig. 5 shows the typical bell-shaped curve when activity was plotted against the log of the concentration of *N*-methyl caproamide indicating inhibition by excess substrate.

DISCUSSION

Attempts to solubilize this enzyme by extraction of the microsomal suspension with detergents, butanol and glycerol resulted in loss of enzyme activity. However, freezing and thawing solubilized almost all of the enzyme from the microsomes with the liberation of only about 20% of the microsomal protein. The solubilized enzyme was very labile to acetone, ethanol and $(NH_4)_2SO_4$. For example the presence of 1% acetone in the incubation mixture reduced the activity about 70%, and 1% ethanol reduced the amidase activity 30%. Therefore, fractionation of the enzyme with these reagents was avoided in the subsequent purification steps.

For column chromatography, hydroxylapatite prepared in the laboratory proved to be better than the commercial product because the flow rate was approximately twice as fast, and the recovery of the enzyme was greater. Attempts to use DEAE-cellulose and CM-cellulose columns were not successful since the enzyme did not absorb to these materials. Therefore, benzyl-DEAE-cellulose was utilized for chromatography.

Electrophoresis of the various enzyme fractions clearly showed that during the purification a number of protein-staining bands were removed with the final product containing only one electrophoretic band.

The enzyme had little or no activity toward short chained *N*-methyl amides. The affinity of the enzyme toward the *N*-methyl amides increased as the acyl group increased. It reached the maximum at *N*-methyl caproamide then decreased as the acyl group increased. This is in general agreement with an earlier report[8]. However, the size of the *N*-alkyl group also influenced the affinity. Changing the *N*-methyl to *N*-ethyl, the K_m increased slightly whereas the K_m increased almost 27-fold in the case of *n*-propyl caproamide.

Comparing the enzyme activity to the various substrates, it was difficult to determine the total overall effects of the various alkyl substituents. However, if one assumes that the reaction of the enzyme and substrate is first-order, one may derive a first-order constant referred to as an enzymatic half-life $(t_{0.5})_e = 0.695\ K_m/v_{max}$ which is the time necessary to reduce a given substrate concentration at a constant enzyme concentration to half of its initial value[27]. The $(t_{0.5})_e$ includes a measure of the affinity of the enzyme for the substrate as well as the rate of reaction (Table II). The findings with the *N*-methyl alkyl amides indicated that *N*-methyl caproamide and *N*-methyl heptylamide had similar $(t_{0.5})_e$ whereas the *N*-methyl valeramide and *N*-methyl caprylamide had much longer half-lives. Comparing the *N*-alkyl caproamides, it is interesting that the enzymatic half lives of the methyl, ethyl and *n*-propyl analogs were almost identical whereas the *N*-butyl caproamide was hydrolyzed very slowly. The largest v_{max} value was found with *N*-phenyl caproamide. Of all the compounds for which a K_m and v_{max} could be determined, the *N*-phenyl caproamide had the shortest enzymatic half-life followed by *N,N*-dimethyl caproamide. With the *N,N*-

diethyl caproamide, no hydrolysis occurred, possibly indicating that the addition of a second ethyl group prevented the binding of the compound at the site of hydrolysis due to the bulk size of the *N,N*-diethyl group.

The molecular activity (moles substrate per mole of enzyme per min) hydrolyzed was calculated for the enzyme and the substrates,*N*-methyl caproamide and *N*-phenyl caproamide. The enzyme had a low molecular activity, 5.4 min^{-1}, for *N*-methyl caproamide whereas the molecular activity for N-phenyl caproamide was 551 min^{-1}, indicating a preference for *N*-phenyl group.

The amidase was also capable of hydrolyzing primary amides such as caproamide. This was demonstrated by thin-layer chromatography of a cyclohexane extract of the assay system. The product, 2,4-dinitroaniline was further confirmed by comparing the absorption spectrum with a maximum absorbance at 315 nm[28]. The assay method used for substituted amides did not permit an accurate quantitative measurement of ammonia since it was so volatile. Furthermore, the partition coefficient of 2,4-dinitroaniline was approximately 8 in favour of the alkaline aqueous phase. These deficiencies in the assay method limited the measurement of enzyme activity to substituted amides.

JAKOBY AND FREDERICKS[12] studied a microbial acetamidase and concluded the enzyme not only catalyzed the hydrolysis of acetamide but also was capable of transferring acyl groups. It is unlikely that this enzyme was able to transfer the large acyl groups.

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REFERENCES

1 A. MEISTER, *Physiol. Rev.*, 36 (1956) 103.
2 E. ROBERTS, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. IV, Academic Press, New York, 1960, p. 285.
3 P. R. KRISHNASWANY, V. PANILJANS AND A. MEISTER, *J. Biol. Chem.*, 237 (1962) 2932.
4 A. MEISTER, L. LEVINTOW, R. E. GREENFIELD AND P. A. ABENDSCHEIN, *J. Biol. Chem.*, 215 (1955) 441.
5 H. G. BRAY, S. P. JAMES, B. E. RYMAN AND W. V. THORPE, *Biochem. J.*, 42 (1948) 274.
6 H. G. BRAY, S. P. JAMES, I. M. RAFFAN, B. E. RYMAN AND W. V. THORPE, *Biochem. J.*, 44 (1949) 618.
7 H. G. BRAY, W. V. THORPE AND P. B. WOOD, *Biochem. J.*, 45 (1949) 45.
8 H. G. BRAY, S. P. JAMES, W. V. THORPE, M. R. WASDELL AND P. B. WOOD, *Biochem. J.*, 45 (1949) 467.
9 H. G. BRAY, S. P. JAMES, W. V. THORPE AND M. R. WASDELL, *Biochem. J.*, 47 (1950) 294.
10 M. KELLY AND H. L. KORNBERG, *Biochim. Biophys. Acta*, 64 (1962) 190.
11 T. KIMURO, *J. Biochem. Tokyo*, 46 (1958) 1271.
12 W. B. JAKOBY AND J. FREDERICKS, *J. Biol. Chem.*, 239 (1964) 1978.
13 G. HOLLUNGER, *Acta Pharmacol. Toxicol.*, 17 (1960) 374.
14 T. UCHIDA, W. C. DAUTERMAN AND R. D. O'BRIEN, *J. Agric. Food Chem.*, 12 (1964) 48.
15 I. M. FORD, I. J. MENN AND G. D. MEYDING, *J. Agric. Food Chem.*, 14 (1966) 83.
16 G. F. D'ALELIO AND E. E. REID, *J. Am. Chem. Soc.*, 59 (1937) 109.
17 P. R. CHEN AND W. C. DAUTERMAN, *Anal. Biochem.*, 38 (1970) 224.
18 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
19 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
20 W. F. ANACKER AND V. STOY, *Biochem. Z.*, 330 (1958) 141.

21 L. T. KREMZNER AND I. B. WILSON, *J. Biol. Chem.*, 238 (1963) 1714.
22 P. ANDREWS, *Biochem. J.*, 95 (1965) 595.
23 B. CHANCE AND A. C. MAEHLY, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 2, Academic Press, New York, 1955, p. 764.
24 A. KORNBERG, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 441.
25 B. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
26 F. J. BEHAL, G. H. LITTLE AND R. A. KLEIN, *Biochim. Biophys. Acta*, 178 (1970) 118.
27 W. C. DAUTERMAN AND A. R. MAIN, *Toxicol. Appl. Pharmacol.*, 9 (1966) 409.
28 M. J. KOLBEZEN, J. W. ECKERT AND B. F. BRETSCHNEIDER, *Anal. Chem.*, 34 (1953) 583.