The Acylamide Amidohydrolase of *Candida utilis*: Purification and Properties

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SUMMARY

The amidohydrolase formed in the later stages of growth on glucose peptone medium by *Candida utilis* was purified 60-fold from cell-free extracts. The purified enzyme hydrolysed mono- and di-carboxylic acid amides, benzamide, nicotinamide, pyrazinamide and certain β -hydroxyl substituted fatty acid amides, was inactive on L-asparagine and L-glutamine and was competitively inhibited by urea and *N*-methylurea. Acetamide and nicotinamide together showed mutual competition.

INTRODUCTION

Hydrolysis of aliphatic amides by micro-organisms has been reported as well as by liver and kidney tissue of rabbits (Bray, James, Thorpe & Wasdell, 1950) and other animals. Bönicke (1962) used the capacity to hydrolyse a series of such amides to classify species of the genus *Mycobacterium*; Muftic (1963) and van Uden, Vidal-Leiria & Buckley (1964) classified yeasts in a similar manner.

Gorr & Wagner (1933) found that fresh and dried *Candida utilis* ('*Torula utilis*') hydrolysed acetamide, propionamide and lactamide, whereas brewing and baking yeasts did not. Steiner (1959) extended to 10 the list of amides hydrolysed by dried *C. utilis* in buffer. An amidase from *Pseudomonas fluorescens* was purified 20-fold by Jakoby & Fredericks (1964), another from *P. aeruginosa* 35-fold by Kelly & Kornberg (1964), and one from *Mycobacterium smegmatis* by Draper (1967) 2.2-fold.

Enzymes which hydrolyse nicotinamide have been reported more frequently: Hughes & Williamson (1953) in cell-free extracts of *Lactobacillus arabinosus*; Pichappa & Shanmugasundaram (1962) in a 40-fold purified preparation from *Saccharomyces cerevisiae*; Petrak, Greengard, Craston & Sheppy (1965) in 38-fold purified material from rabbit and rat liver; Kirchner, Watson & Chaykin (1966) in a 60-fold purified preparation from rabbit liver. Often the hydrolysis of other amides was not investigated. Others have had negative results with such hydrolyses: Oka (1954) in crude cell-free extracts of 'beer yeast' and Joshi & Handler (1962) on an 850-fold purified nicotinamidase from *Candida pseudotropicalis* ('*Torula cremoris*').

Since *Candida utilis* gave promise of a capacity to hydrolyse a wide range of amides, it seemed worthwhile to purify the active material and to ascertain whether it consisted of one or more enzymes.

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METHODS

Organism. *Candida utilis*, IGC 3093, maintained in the culture collection of this laboratory.

Amide hydrolysis was tested as described by Jakoby & Fredericks (1964). For routine tests during the course of purification, incubation was for 1 hr at 37° with 0.01 M-acetamide in 0.05 M-tris (pH 9.0); total vol. 0.2 ml; for estimation of the kinetic constants with various substrates, incubation was for 20 min. and substrate concentrations appropriate to the affinity of the enzyme for the particular substrate were used. Ammonia was estimated by Nessler's reagent (Vanselow, 1940) in the range 0.04 to 0.38 $\mu\text{mole NH}_4^+/\text{ml}$. One 'unit' is the amount of enzyme which catalyses the formation of 1 $\mu\text{mole NH}_3/\text{min.}/\text{ml}$.

Compounds with a substituent on the amide nitrogen, since they do not form NH_3 on hydrolysis, were examined as possible inhibitors of the hydrolysis of acetamide or of nicotinamide. The Michaelis-Menten constants and relative maximum velocities were obtained from double reciprocal plots (Lineweaver & Burke, 1934).

The low solubilities of some compounds limited their use in kinetic studies because enzyme affinities were generally low. Substances labile at pH 9.0 were also assessed at low concentrations.

N-acetyl- β -naphthylamine was tested as a substrate by the colorimetric method involving the formation of an azo dye (Goldbarg & Rutenburg, 1958).

Glutamine hydrolysis was investigated at pH 7.0 and assayed by measuring any increase in glutamic acid over a control without added enzyme. After 1 hr at 25°, 1 ml. of enzyme + substrate mixture was passed through a column of Amberlite IRA-401 (acetate form) and six 1 ml. fractions were eluted with water followed by six 1 ml. fractions eluted with 0.2 M-acetate buffer (pH 4.8). Glutamine and glutamate concentrations in the relevant eluate fractions were estimated with ninhydrin (Moore & Stein, 1948).

Transferase activity was determined by the method of Brammar & Clarke (1964) with incubation for 20 min. at 37°. The extinction of the FeCl_3 -hydroxamate complex was read at 500 nm. and compared with a standard curve prepared from acethydroxamate concentrations between 0.5 and 2.5 $\mu\text{mole}/\text{ml}$. One 'unit' is the amount of enzyme catalysing the formation of 1 $\mu\text{mole acethydroxamate}/\text{min.}/\text{ml}$.

Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Eggstein & Kreutz (1955), with bovine serum albumin as standard.

Preparation of cell-free extract. *Candida utilis* was grown for 5 days on a rotary shaker at 25° in glucose (2%, w/v), peptone (1%, w/v), yeast extract (0.5%, w/v) medium. The organisms were washed twice with 0.02 M-K phosphate buffer (pH 6.0), suspended in buffer (1 ml./g. wet wt) and shaken in 15 ml. batches with 50 g. glass beads 0.45 to 0.50 mm. diameter in a homogeniser (B. Braun, Melsungen, Model MSK) at 4000 cyc./min. for 2 min., temperature between 1° and 10°. Ice-cold butanol was added slowly to the brei, with stirring, over 1 hr, to a final concentration of 3.8% (v/v), and stirring continued for a further 30 min. After centrifugation at 12,500 g for 1 hr at 0°, the cell-free extract was dialysed overnight at 2° against 50 vol. 0.01 M-K phosphate buffer (pH 6.0).

Enzyme fractionation. Partially purified enzyme was prepared from cell-free ex-

tracts on four separate occasions, in the course of which the enzyme was purified between 28- and 80-fold. One such fractionation procedure is described below (and in Table 1). On other occasions a fractionation by adding solid $(\text{NH}_4)_2\text{SO}_4$ in the presence of 10% (v/v) ethanol was included, adsorption on calcium phosphate gel, and on one occasion a final fractionation on a column of DEAE-Sephadex.

Precipitation with protamine sulphate. Two successive precipitations with an aqueous solution of protamine sulphate, 20 mg./ml., adjusted to pH 5.0 with NaOH, were made at 2°. The protamine sulphate was added with stirring during 15 min. and stirring was continued for a further 30 min. followed by centrifugation at 7250 g for 20 min. at 4°. Each addition was made on the basis of μg . protamine sulphate/mg. protein in the cell-free extract before precipitation was started. The first to 85 μg /mg. protein, resulted in a heavy white precipitate which was discarded; secondly 35 μg . more protamine sulphate/mg. protein was added and produced a small translucent yellow precipitate which readily dissolved in 0.5 M- K_2HPO_4 , giving a solution with about 12 mg. protein/ml. During subsequent dialysis overnight against two changes of 160 vol. each of 0.03 M-tris (pH 8.0), at 4°, a heavy white precipitate appeared in the dialysis sac and was removed at 7000 g for 20 min. at 4°. The supernatant fluid is referred to as protamine fraction in Table 1.

Table 1. *Flow sheet of purification of acylamide amidohydrolase of Candida utilis*

Extract	Volume (ml.)	Units/ml.	Total Units	Protein mg/ml.	Total Protein (mg.)	Specific Activity	Yield (%)	Purification (fold)
Butanol-treated cell-free extract	196	6.25	1225	10.5	2058	0.59	100	1
Protamine fraction	21	32.19	676	3.1	65.1	10.4	55	17.6
Acetone precipitate 2 dissolved	6.4	43.4	278	2.1	13.4	20.7	22.7	34.8
Sephadex fractions 9, 10, 11 pooled	16	6.5	104	0.18	2.88	36	8.5	60.5

Acetone precipitation. The protamine fraction was diluted with tris to give 25 units of activity/ml. and two successive precipitations with cold acetone were done: the first to 37.5% (v/v) acetone, the second to 64% (v/v). Each precipitate was removed by centrifugation at 7000 g for 30 min.; the first was discarded and the second was taken up in 8 ml. 0.5 M- K_2HPO_4 and dialysed against 0.05-M-tris (pH 8.0).

Column chromatography on Sephadex G 200. A column of Sephadex G 200, 4.9 cm² × 31 cm., equilibrated with 0.05 M-tris (pH 8.0) containing 0.05 M-NaCl, was charged with the 6.4 ml. sample containing 13.4 mg. protein. The flow of tris + NaCl solution was maintained by gravity at a rate of 15 ml./hr; 5 ml. fractions were collected. The protein eluted from the column appeared as a single peak in a volume of 50 ml. after 30 ml. had passed through the column, and was coincident with the single peak of enzyme activity. The three most active fractions were combined and had a specific activity of 36, giving a 60-fold purification with a yield of 8.5%. This material retained its activity over 8 months at 4°.

Chemicals. All chemicals were of analytical grade wherever possible; most were commercial products. Nicotinamide was twice recrystallised from benzene. *N*-acetyl-naphthylamine was prepared by Professor Bernado Herold of the Instituto Superior

Técnico, Lisbon; oxytetracycline HCl and tetracycline HCl were gifts from Chas. Pfizer and Co. and the remaining tetracycline compounds from Lederle Laboratories.

Buffers. All buffers and solutions of substrates contained 0.5 mM-ethylenediamine-tetraacetic acid (EDTA).

RESULTS

Evidence that a single amidase is active on a wide range of substrates

Substrate specificity throughout the purification process. Whole cells of *Candida utilis* are able to hydrolyse a wide range of compounds containing amide groups. Cell-free extracts and the 60-fold purified material hydrolysed the amides listed in Table 2, with the exception of asparagine, which was hydrolysed by crude cell-free extracts but not by the purified enzyme, and isonicotinamide, which was weakly hydrolysed by the purified enzyme and not detectably by the cell-free extract.

Table 2. *Hydrolysis (as % of activity with acetamide) of various substrates by cell-free extract of Candida utilis and purified enzyme preparation*

Amide (80 mM)	Cell-free extract	60-fold purified preparation
Acetamide	100	100
N-Valeramide	90	59
Propionamide	82	59
N-Hexoamide	80	66
Pyrazinamide	78	27
Acrylamide	43	46
N-Butyramide	40	33
Nicotinamide	16	13
Malonamide	15	22
Asparagine	13	0
Isonicotinamide	0	4.5

During the course of several purifications the activity of the material was assayed by using as substrate 10 mM-acetamide or nicotinamide. The activity with nicotinamide was between 9 and 12 % of that with acetamide in the cell-free extract both before and after treatment with butanol, and in the supernatant fluid after the first precipitation with protamine sulphate. However, after the second precipitation with protamine sulphate, and throughout the subsequent purification, the activity on nicotinamide diminished to between 3 and 6 % of that on acetamide.

In one of the later purification experiments, when the Michaelis-Menten constant for nicotinamide of the purified enzyme had been found to lie between 5 and 10×10^{-2} M, the nicotinamidase activity of the second protamine sulphate supernatant fluid indicated that there was material present with a much higher affinity for nicotinamide, of K_m between 1 and 4×10^{-3} M. Elimination of this activity with the supernatant fluid accounts for the decrease in the ratio of nicotinamide:acetamide hydrolysis.

Simultaneous hydrolysis of acetamide and nicotinamide by the 60-fold purified material. Acetamide was taken as a typical representative of the range of simple aliphatic amides hydrolysed by the *Candida utilis* enzyme, and nicotinamide as a substrate more commonly hydrolysed by cells in general. Initial velocities were determined for three concentrations of acetamide and one concentration of nicotinamide, separately and mixed.

The velocities of hydrolysis in the mixed assays were lower than the sums of the velocities of the separate assays. The observed velocities of the mixed assays agreed well with calculated values based on competition of two substrates for one active site on the same enzyme (Table 3). The kinetic parameters used in the calculations were obtained from Lineweaver-Burk plots.

Table 3. *Hydrolysis of acetamide and nicotinamide, separately and together by the 60-fold purified enzyme preparation from Candida utilis*

Substrate concentration		Velocities with separate substrates	Sum of velocities with separate substrates	Velocities with mixed substrates v_{obs}	Calculated $v_1 + v_2$ in mixed substrates*
(a) Acetamide (M)	(b) Nicotinamide (M)				
$\mu\text{mole NH}_3/\text{ml.}/20 \text{ min.}$					
10^{-3}	—	0.675	2.4	2.1	2.5
—	2×10^{-1}	1.725			
2×10^{-3}	—	1.380	3.105	2.385	2.75
—	2×10^{-1}	1.725			
10^{-3}	—	5.355	7.08	4.605	4.88
—	2×10^{-1}	1.725			

$$* \text{From } v = v_1 + v_2 = \frac{V_a a K_b + V_b b K_a}{K_a K_b + a K_b + b K_a},$$

where V_a and V_b are maximum velocities and K_a and K_b Michaelis constants.

Competitive inhibition by urea and N-methylurea. Urea was a competitive inhibitor of hydrolysis of acetamide and of nicotinamide by the 60-fold purified enzyme preparation. By using three different concentrations of inhibitor in each case, the inhibition constant, K_i , for urea was calculated. The average K_i urea obtained with acetamide (11×10^{-3} M) agreed well with the average with nicotinamide (8.4×10^{-3} M).

N-methylurea was a competitive inhibitor with even higher affinity for the enzyme. The average K_i of N-methylurea with acetamide was 7.5×10^{-4} M and with nicotinamide, 6.5×10^{-4} M. These results are additional evidence that only one enzyme was involved in the hydrolysis of both substrates.

Properties of the enzyme

Optimum pH value for amide hydrolysis. With 80 mM-acetamide in 0.05 M-phosphate buffer over a range pH 6.0 to 7.9, and in 0.05 M-tris + HCl buffer over a range pH 6.7 to 9.8, the optimum pH was 9.0. Within the region where both buffer systems operate, activity in phosphate buffer was approx. 15 % higher than activity in tris at the same pH value. With 80 mM-acetamide (pH 9.0) activity was linear over a period of 1 hr.

Substrate specificity and competitive inhibitors. The range of enzyme activity with respect to a number of compounds containing a free or substituted amide group is shown in Table 4. Straight-chain aliphatic monoamides with 2 to 6 carbon atoms had Michaelis-Menten constants of similar orders of magnitude, between 2 and 5×10^{-3} M and relative maximum velocities between 28 and 100 % of V_{max} of acetamide. Nicotinamide had an affinity for the enzyme approximately 10 times lower than had the series of aliphatic amides, while the relative maximum velocity of hydrolysis of nicotinamide lay within the same range as for the latter group.

Of the substrates tested as inhibitors, only urea and N-methylurea showed an effect, both being competitive inhibitors. Among the compounds tested several, such as NAD and carbamylphosphate, are physiologically active, while 4-amino-5-imidazole carboxamide is encountered in purine biosynthesis as the ribotide. Also tested were antibiotics of the tetracycline series containing an amide group, and certain nicotinamide analogues either found in nature or of pharmaceutical use. None showed activity either as substrate or inhibitor. However, several such compounds activated acetamide and nicotinamide hydrolysis. This group included products of the amidase reaction (acetate and nicotinate) and certain structural analogues of nicotinamide such as picolinate, quinolinate and N_1 methylnicotinamide.

Table 4. *Specificity of the 60-fold purified acylamide amideohydrolase from Candida utilis*

Compounds	Substrate	$V_{\max}$ *	$K_i \times 10^{-3}M$
Acetamide	+	100	5.0 (7.7)
Propionamide	+	63	2.1
<i>n</i> -Butyramide	+	28	2.0
Isobutyramide	+	—	—
<i>n</i> -Valeramide	+	88	3.2
<i>n</i> -Hexamide	+	88	4.2
Acrylamide	+	42	4.3
Malonamide	+	6	9.1
Succinamide	+ (weak)	—	—
Adipamide	+	—	—
Benzamide	+	—	—
Nicotinamide	+	32	100 (72)
Isonicotinamide (pyridine-4-carboxamide)	+ (weak)	—	—
Pyrazinamide	+	18	14.3
Glycolamide	+	36	125
DL-Lactamide	+	65	44.0

Inhibitors (competitive): Urea (K_i $11 \times 10^{-3}M$ with acetamide as substrate). N-methylurea (K_i $0.75 \times 10^{-3}M$ with acetamide as substrate).

Activators: N_1 -methylnicotinamide ($8 \times 10^{-3}M$ approx. doubles $V_{\max}$ acetamide); picolinate, nicotinate, quinolinate, acetate ($200 \times 10^{-3}M$ approx. doubles $V_{\max}$ acetamide).

Compounds not affected by the amidase: Formamide, oxamide, salicylamide, L-asparagine, L-glutamine, N-methylacetamide, N-phenylacetamide, *p*-acetamidophenol, N-acetylglucine, N-acetyl-DL-methionine, N-acetyl- β -D-glucosamine, N-acetyl- β -naphthylamine, allantoin, NAD, 4-amino-5-imidazole carboxamide HCl, carbamylaspartic acid, carbamylphosphate, 7-chloro-6-dimethyl-tetracycline HCl, chlorotetracycline HCl, 6-dimethyl-tetracycline AIX base, oxytetracycline HCl, tetracycline HCl.

* % of activity with acetamide as substrate.

Transferase activity. The 60-fold purified enzyme catalysed the transfer of the acyl group of acetamide to hydroxylamine. At pH 7.2 the number of units/ml. was 11.4 compared with 6.5 units of hydrolase activity at pH 9.0. Later, the optimum for transferase activity was found to be near pH 8, but the high blank values for the $FeCl_3$ mixture at alkaline pH values made precise evaluation difficult. No further study was made of the transferase activity of either the crude cell-free extracts or of the purified material.

DISCUSSION

The enzyme purified by Kelly & Kornberg (1964) from *Pseudomonas aeruginosa* catalysed the hydrolysis of aliphatic amides and the transfer of the acyl moiety of the amides or of the corresponding acids to hydroxylamine. McFarlane, Brammar & Clarke (1965) showed the same enzyme to be active on esters, though with a limited specificity and a low affinity for the substrates. According to Kelly & Kornberg, amidase activity is but one instance of acyl transfer from a suitable donor through an intermediate acyl protein to an acceptor of the type X-OH where X may be H or NH₂, and the enzymes should be more properly called acyl transferases rather than hydrolases. However, work with organisms of the genus *Mycobacterium* has shown (Kimura, 1959; Draper, 1967) that while in some species the amidase and transferase activities are intimately related and apparently properties of the same enzyme, in others they can be readily separated. Although the transferase activity of the *Candida utilis* enzyme was examined only with respect to acetamide as substrate, it was present to a marked extent in the 60-fold purified material and is probably characteristic of the enzyme.

The range of substrates hydrolysed by the *Candida utilis* amidase confirms and extends that cited by Steiner (1959) for dried organisms of the same species in buffer. In comparison with enzymes isolated from other organisms, the purified *C. utilis* enzyme had a wider substrate specificity than that from *Pseudomonas aeruginosa* (Kelly & Kornberg, 1964; Brown, Brown & Clarke, 1969) although both enzymes were similar in having no affinity for compounds with a substitution on the amide nitrogen and both retained the ability to transfer the acyl moiety of acetamide to hydroxamate. The wide substrate range of the *C. utilis* enzyme approaches that of the amidase from *Mycobacterium smegmatis* (Draper, 1967) which also was unable to hydrolyse N-substituted amides. The affinity of the *C. utilis* amidase for acetamide was within the same order of magnitude as that of the *P. fluorescens* enzyme (Jakoby & Fredericks, 1964), whereas for propionamide it was higher.

Inhibition by urea and N-methylurea of the *Candida utilis* enzyme, which was competitive, contrasts with non-competitive inhibition in the enzymes from *Pseudomonas* spp. (Kelly & Kornberg, 1964; Jakoby & Fredericks, 1964).

The low affinity for nicotinamide of the *Candida utilis* amidase contrasts with the usually higher affinity of enzymes which appear to function primarily in the conversion of nicotinamide to nicotinate in the metabolism of NAD and which appear not to be active on simple aliphatic amides. It seems possible that the nicotinamidase activity detected in the second supernatant fluid of the protamine sulphate precipitation of the *C. utilis* preparation (K_m 1 to 4×10^{-3} M) indicates an enzyme of the latter type.

The function of the acyl amidase of *Candida utilis* remains obscure. In glucose yeast-extract peptone medium amidase activity develops after the logarithmic phase of growth, when the glucose supply has been exhausted; in a similar medium without glucose it is formed without preliminary repression (Brady & van Uden, 1966). Brammar & Clarke (1964) showed the *Pseudomonas aeruginosa* amidase to be subject to catabolite repression. In exponentially growing cultures on succinate medium this pseudomonad enzyme was inducible by substrate and by certain non-substrate analogues of acetamide. It is not known whether the *Candida utilis* enzyme might be inducible under favourable conditions since all experiments to test substrates as in-

ducers were made in the presence of glucose. A search for possible natural substrates for the acyl amidase showed no activity with NAD, the 4-amino-5-imidazole carboxamide moiety of the corresponding ribotide, carbamylaspartic acid, carbamylphosphate or various tetracyclines. Despite the fact that the enzyme is active on dicarboxylic acid amides derived from aromatic and heterocyclic compounds, e.g. benzamide and nicotinamide, it seems appropriate to name it according to the substrates on which it is most active, namely as an acylamide amidohydrolase EC 3.5.1.4.

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