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Physical, Biochemical, and Immunological Characterization of a Thermostable Amidase from *Klebsiella pneumoniae* NCTR 1

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An amidase capable of degrading acrylamide and aliphatic amides was purified to apparent homogeneity from *Klebsiella pneumoniae* NCTR 1. The enzyme is a monomer with an apparent molecular weight of 62,000. The pH and temperature optima of the enzyme were 7.0 and 65°C, respectively. The purified amidase contained 11 5,5-dithiobis(2-nitrobenzoate) (DTNB)-titratable sulfhydryl (SH) groups. In the native enzyme 1.0 SH group readily reacted with DTNB with no detectable loss of activity. Titration of the next 3.0 SH groups with DTNB resulted in a loss of activity of more than 70%. The remaining seven inaccessible SH groups could be titrated only in the presence of 8 M guanidine hydrochloride. Titration of SH groups was strongly inhibited by carboxymethylation and $KMnO_4$, suggesting the presence of SH groups at the active site(s). Inductively coupled plasma-atomic emission spectrometry analysis indicated that the native amidase contains 0.33 mol of cobalt and 0.33 mol of iron per mol of the native enzyme. Polyclonal antiserum against *K. pneumoniae* amidase was raised in rabbits, and immunochemical comparisons were made with amidases from *Rhodococcus* sp., *Mycobacterium smegmatis*, *Pseudomonas chlororaphis* B23, and *Methylophilus methylotrophus*. The antiserum immunoprecipitated and immunoreacted with the amidases of *K. pneumoniae* and *P. chlororaphis* B23. The antiserum failed to immunoreact or immunoprecipitate with other amidases.

Acrylamide, a carcinogen, teratogen, and neurotoxicant (10, 20), is used in numerous industrial processes. Extensive usage and indiscriminate discharge have led to the contamination of terrestrial and aquatic ecosystems (9, 31). Microbial amidases (e.g., acylamide amidohydrolase [EC 3.5.1.4]) deaminate aliphatic amides to their carboxylic acids and ammonia, and this reaction is highly substrate specific. Although several microorganisms capable of degrading aliphatic amides have been reported (1, 7, 15–17, 25, 26) and the amidases of some microorganisms have been purified to apparent homogeneity (3, 8, 18), these organisms poorly degrade acrylamide.

We have isolated numerous microorganisms capable of degrading acrylamide (27, 28, 30), and we detailed the physical characteristics of an acrylamide-degrading amidase from *Rhodococcus* sp. (30). We report herein the purification and characterization of an amidase from an acrylamide-degrading strain of *Klebsiella pneumoniae*. We have studied the regulation and involvement of functional groups in the catalysis of this amidase or the maintenance of its tertiary structure. Analysis of functional groups could lead to the design of specific inhibitors for the enzyme. It would be useful to have inhibitors because amidolytic microorganisms are economically important and are implicated in the development of some human disorders (5, 33–35). Elevated soil amidase activity levels can undermine the efficiency of nitrogenous fertilizers and the persistence of certain herbicides such as triazine (14, 23), thus contributing to poor seed germination and seedling growth. In addition, some amidases have been used for detoxification of acrylamide and as biocatalysts for the industrial production of ammonium acrylate and acrylic acid, compounds of intense industrial use (24).

We report here the characterization of a novel thermostable amidase from an acrylamide-degrading strain of *K. pneumoniae*.

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MATERIALS AND METHODS

Materials. DEAE-Sepharose Fast Flo, Mono Q (HR 10/10), an HR 10/30 (Superose 12) prepacked column, and molecular mass standard kits were purchased from Pharmacia LKB Biotechnology (Piscataway, N.J.). Antibodies for *K. pneumoniae* amidase were obtained from Alpha Diagnostic International (San Antonio, Tex.).

Microorganism and culture conditions. *K. pneumoniae* NCTR 1, previously isolated from a wastewater facility, was used (27, 29). Cells were routinely grown in 1 liter of mineral salts medium (pH 7.2) containing 10 g of glucose, 17.5 g of K_2HPO_4, 13.4 g of KH_2PO_4, 0.5 g of $MgSO_4$, 10 ml of a micronutrient solution (27), and 4 g of acrylamide per liter at 30°C. The cells (100 g) from 40 liters of media were harvested after 48 h of incubation by centrifugation at 10,000 × *g* for 20 min at 5°C. The cells were washed with potassium phosphate buffer (K_2HPO_4-KH_2PO_4; 100 mM; pH 7.0) and pelleted by centrifugation.

Purification of amidase. The protocol for purification of amidase was adapted from one described previously (30). The amidase was purified by acetone (45 to 65%) precipitation followed by chromatographic separation on DEAE-Sepharose, ammonium sulfate (45 to 60%) fractionation, and gel filtration (30). The column dimensions, flow rates, and salt gradients used were the same as those reported earlier (30).

Enzyme assays and ammonia and protein content determinations. Amidase activity was assayed at 37°C by measuring the ammonia liberated from acrylamide (30). The amount of ammonia released was determined by the indophenol method (19). Protein content was determined by the Bradford method (4). One unit of enzyme activity is defined as the amount of enzyme required for the liberation of 1 μmol of ammonia from acrylamide per min.

Determination of molecular weights. The M_r of the purified amidase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions as described by Laemmli (21). The M_r of native amidase was determined by using a Superose column equilibrated with phosphate buffer (30). The M_r was also determined by nondenaturing PAGE with a commercial kit (Sigma Chemical Co., St. Louis, Mo.).

Determination of K_m, pH, and temperature optima. The pH optimum was determined by using 100 mM phosphate-citrate buffer, 100 mM KH_2PO_4-KOH, and 100 mM carbonate buffer for pH ranges from 4.0 to 7.6, 7.5 to 8.5, and 8.5 to 11.0, respectively. The temperature optimum of amidase was determined by incubations at various temperatures between 10 and 75°C. The thermostability of the enzyme was determined by incubating 1 ml of the enzyme solution (containing 1 mg of protein per ml) at 4, 30, 40, and 65°C. Amidase activity was determined at various times. Apparent K_m values for acrylamide were determined by incubating the enzyme at 65°C with various concentrations of acryl-

TABLE 1. Purification of amidase from *K. pneumoniae*

Step	Total activity (U)[a]	Sp act[b]	Yield (%)	Purification (fold)
Cell extract	21,000	16.0	100	0
Acetone (45 to 65%)	16,000	64.0	76	4.0
DEAE	7,000	1,540	33	96.0
Ammonium sulfate	4,500	1,820	21	115.0
Gel filtration	2,200	2,690	10	168.0

[a] Total activity = specific activity × total protein content.
[b] Specific activity is represented in micromoles of NH_3 per minute per milligram of protein.

amide (0.2 to 10.0 mM) in phosphate buffer (100 mM; pH 7.0). Lineweaver-Burk plots were used to determine the K_m.

Determination of enzyme metal content. Enzyme metal content analysis was performed by inductively coupled plasma-atomic emission spectrometry coupled with a Thermo Jarrell Ash (Franklin, Mass.) ICAP 61E spectrometer operating at 27.12 MHz as detailed earlier (30).

Determination of numbers of SH groups. Numbers of free sulfhydryls (SHs) were determined spectrophotometrically with 30 mM 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) at 25°C in 0.1 M KH_2PO_4-K_2HPO_4 buffer (pH 7.0) with 5 mM EDTA (13). The reaction mixture containing the buffer and DTNB (plus denaturant when required) served as the blank.

Correlation between loss of amidase activity and reaction of SH groups with DTNB. An enzyme solution containing 50 to 200 μg of protein was added to 800 μl of phosphate buffer (100 mM; pH 7.0). The reaction was started by the addition of 30 mM DTNB. At various incubation times after titration of SH groups at A_{412}, an aliquot (50 μl) was removed and subjected to chromatocentrifugation (11) to separate the protein from the reagent and determine the amidase activity.

Effects of iodoacetate and $KMnO_4$ on the SH groups of amidase. Enzyme solution (1 ml) containing 200 μg of protein was incubated with 5 mM iodoacetate or $KMnO_4$, vortexed, and incubated for 30 min at 5°C. After incubation, excess reagents were separated from the protein by chromatocentrifugation. Guanidine hydrochloride (8 M) was added when necessary, and the numbers of SH groups were determined.

Preparation of antisera, immunotitration, and immunoblotting. Purified amidase (100 μg) was emulsified in an equal volume of complete Freund's adjuvant and injected into 2-month-old New Zealand White rabbits. The second immunization was given 2 weeks later in the same way except that incomplete adjuvant was used. High-titer antiserum was obtained at 7 weeks after the first injection. Immunoprecipitation was performed with 1 mg of purified amidase from *K. pneumoniae*, *Rhodococcus* sp. (30), *Methylophilus methylotrophus* (39), *Mycobacterium smegmatis* (22), or *Pseudomonas chlororaphis* B23 (8) per ml. Protein samples (10 μg) were incubated with different amounts of *K. pneumoniae* antiserum (1 to 10 μl). The suspensions were incubated for 1 h at 4°C and then centrifuged (10 min at 10,000 × *g*). The residual amidase activity in the supernatant was measured. Preimmune serum was used as a control in all immunochemical experiments. Immunoblotting was performed by the method of Burnette (6). Briefly, purified amidases (2 μg) from *K. pneumoniae*, *P. chlororaphis*, *M. methylotrophus*, *Rhodococcus* sp., or *M. smegmatis* were electroblotted onto a nitrocellulose membrane (0.45-μm pore size; Bio-Rad, Hercules, Calif.) and immunoblotted with antiamidase antibody, and the band of amidase was detected with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G and developed with bromochloroindolylphosphate-nitroblue tetrazolium substrate as recommended by the manufacturer.

RESULTS

Characteristics of amidase from *K. pneumoniae* NCTR 1. The bacterium utilized several aliphatic amides (acetamide, acrylamide, butyramide, propionamide, methacrylamide, and succinamide) as the sole nitrogen source (29). Crude cell extracts contained an amidase responsible for the deamination of these aliphatic amides. A summary of the purification of aliphatic amidase from cell extracts of *K. pneumoniae* is presented in Table 1. Acetone precipitation, DEAE-Sepharose, ammonium sulfate precipitation, and gel filtration yielded a purified amidase fraction having an overall purification factor of over 168-fold and a yield of 10%. The final product had a specific activity of 2,690 U/mg of protein.

Molecular, physical, and kinetic properties of purified amidase. The molecular weight of the native enzyme was estimated by gel filtration to be 62,000 (data not shown). Upon native PAGE and SDS-PAGE, the purified enzyme preparation migrated as a single band, indicating a homogeneous monomeric protein with a molecular weight of 62,000 (Fig. 1). The amidase was active in the pH range from 5.0 to 8.5, with maximum activity at pH 7.0, and the optimum temperature was 65°C. The half-lives of the enzyme at 30, 40, and 55°C were determined to be 72 h, 60 h, and 15 min, respectively. The K_m for acrylamide was estimated to be 1.7 mM. The K_{cat} is 517 mol of NH_3 per min per mol of enzyme.

Metal analysis. Analysis of the amidase indicated the presence of cobalt and iron in each protein preparation. The levels of iron and cobalt in the different enzyme preparations were directly proportional to the amount of protein, indicating the close association of the metal with the enzyme. The cobalt and iron contents in the enzyme preparations were similar and were estimated to be 0.33 mol/mol of protein. Treatment and incubation of the enzyme with EDTA (10 mM) resulted in the chelation of both metals accompanied by a loss of amidase activity of less than 5% (data not shown).

Effects of various amino acid-modifying reagents on amidase activity. No significant losses of enzyme activity were observed with histidine-, lysine-, serine-, or arginine-modifying reagents (Table 2). However, cysteine-SH-modifying reagents had significant effects on the amidase activity. Complete inhibition of enzyme activity was observed with iodoacetate and *para*-chloromercuribenzoate. DTNB and iodoacetamide also significantly inhibited the enzyme (Table 2).

Titration of native and denatured amidase with DTNB. Since SH-modifying reagents inhibit the amidase activity, we studied the nature and composition of the SH groups of the enzyme. Titration of SH groups by DTNB in the absence of a denaturant was slow. A maximum of 4.0 SH groups per mol of enzyme were titrated in 40 min with the native enzyme (Fig. 2). Titration of SH groups in the presence of guanidine hydrochloride (a denaturant) was quicker and more extensive than that in its absence. Addition of 8 M guanidine hydrochloride to the reaction mixture resulted in the titration of 11.0 SH groups per mol of the enzyme within 4 min (Fig. 2). Titration of SH groups in the presence of urea (a denaturant) was slow, and a maximum of 4.0 SH groups were titrated by 60 min (Fig. 2).

Correlation between loss of amidase activity and reaction of SH groups with DTNB. The data concerning the loss of amidase activity and the information on the number and reactivity of SH groups involved in inactivation were obtained at different times of incubation (Fig. 3). Although 1.0 SH group was titrated within 10 min of incubation, no loss of amidase activity was detected. Titration of the next 3.0 SH groups within 10 to

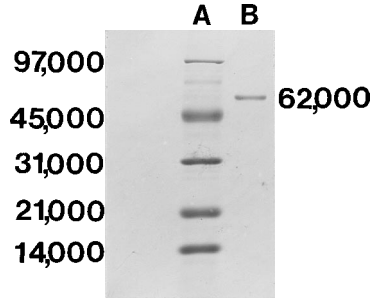

FIG. 1. SDS–12.5% PAGE of purified amidase from *K. pneumoniae* NCTR 1. Lane A, molecular mass standards (from top, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor); lane B, purified amidase (2.0 μg).

TABLE 2. Effects of amino acid-modifying reagents on the amidase activity of *K. pneumoniae*

Target amino acid	Reagent	Reagent concn (mM)	% Inhibition[a]
Histidine	Diethylpyrocarbonate	5	5
		10	5.9
Cysteine-SH	Iodoacetamide	1	29.0
		5	79.0
	Iodoacetate	1	70.0
		5	100.0
	p-Chloromercuribenzoate	2.5[b]	65.0
		5.0[b]	100.0
	DTNB	5	70.0
		10	70.0
Lysine	*N*-Ethylacetamide	5	3.0
		10	8.0
	Pyridoxal phosphate	5	8.9
		10	10.0
Arginine	Phenylglyoxal	5	5.2
		10	11.0
	2,3-Butadione	5	5.0
		10	7.5
Serine	Phenylmethylsulfonyl fluoride	5	8.3
		10	11.5

[a] Amidase activity of 100% corresponds to 2,690 U mg^{-1}.
[b] *p*-Chloromercuribenzoate concentrations are measured in micromoles.

80 min of incubation resulted in the loss of more than 70% of the amidase activity (Fig. 3). Additional incubation failed to completely inactivate the enzyme. No significant loss of amidase activity was detected in enzyme samples without DTNB (controls).

Effects of iodoacetate and $KMnO_4$ on the titration of SH groups. Treatment of the enzyme with 5 mM iodoacetate completely inhibited the titration of SH groups by DTNB. A significant increase in the titration of SH groups by DTNB relative to that obtained with native amidase was observed when the enzyme was treated with 8 M guanidine hydrochloride in the presence of iodoacetate. A maximum of 2.2 SH groups were titratable when denatured enzyme was carboxymethylated with iodoacetate (data not shown). Iodoacetate treatment completely inhibited the enzyme activity (data not shown).

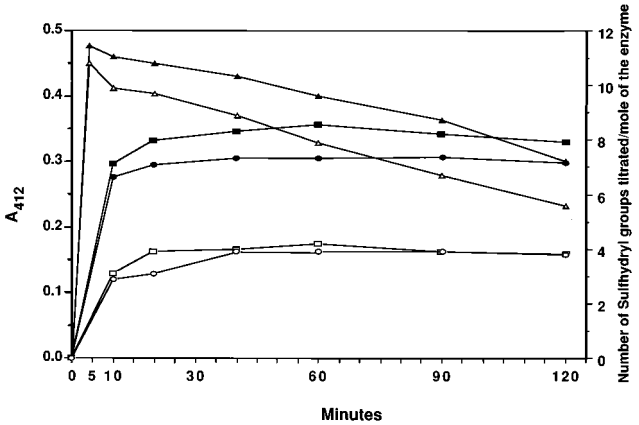

FIG. 2. Determination of numbers of SH groups in the native and denatured amidase. Guanidine hydrochloride (8 M) and urea (8 M) were used as denaturants. Symbols: ●, ▲, and ■, A_{412}s of native, guanidine hydrochloride-denatured, and urea-denatured amidase, respectively; ○, △, and □, numbers of SH groups in native, guanidine-HCl-denatured, and urea-denatured amidase titrated with DTNB, respectively.

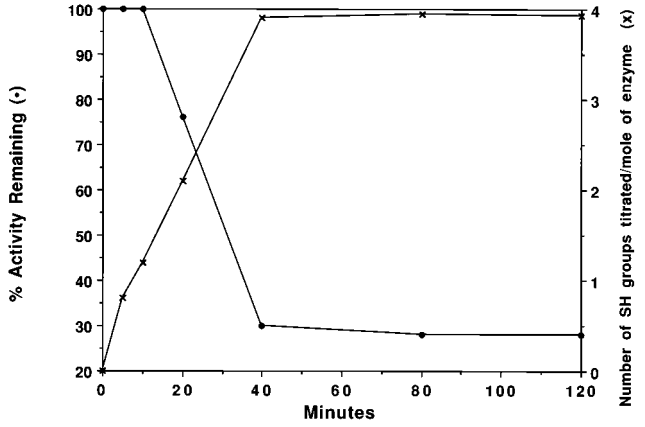

FIG. 3. Correlation between loss of amidase activity and reaction of SH groups with DTNB. Amidase was added to phosphate buffer, and the reaction was started by addition of DTNB. At various times after determination of the A_{412}, 50 μl of the aliquot was chromatocentrifuged and the amidase activity of the protein was determined.

Potassium permanganate (5 mM) totally inhibited the titration of SH groups in the native enzyme. Less than 1.0 SH group was titrated when denatured amidase was oxidized by $KMnO_4$, and oxidation totally inhibited the amidase activity (data not shown).

Serological relationships between amidases. To determine whether purified amidase from *K. pneumoniae* is immunologically related to the amidases from other microorganisms, purified amidases from *K. pneumoniae*, *P. chlororaphis* B23, *M. smegmatis*, *Rhodococcus* sp., and *M. methylotrophus* were subjected to immunoprecipitation and immunoblotting experiments with polyclonal antibodies raised against purified amidase from *K. pneumoniae*. We found that the *K. pneumoniae* amidase activity was significantly immunoprecipitated by the polyclonal antiserum raised against it. More than 80% of the enzyme activity was immunoprecipitated when equal amounts of the enzyme and antiserum were mixed and incubated (data not shown). The antiserum was also found to significantly immunoprecipitate the amidase activity of *P. chlororaphis*. A maximum of 60% immunoprecipitation was obtained when this enzyme was mixed and incubated with equal quantities of the antiserum. Regardless of the antiserum or protein concentration, the *K. pneumoniae* antibodies failed to immunoprecipitate the amidase activity of *M. smegmatis*, *Rhodococcus* sp., or *Methylophilus methylotrophus.*

SDS-PAGE immunoblotting experiments using a 10,000-fold serum dilution indicated a positive immunoreaction with the 62,000-M_r amidase from *K. pneumoniae* and the 54,000-M_r amidase from *P. chlororaphis* B23 (Fig. 4, lanes 4 and 5). No

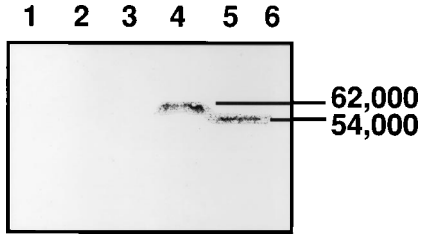

FIG. 4. Immunoblots after SDS-PAGE with a 10,000-fold serum dilution raised against amidase of *K. pneumoniae*. Lanes 1 to 3, purified amidases of *Rhodococcus* sp., *M. smegmatis*, and *M. methylotrophus* (10 μg of each protein), respectively; lanes 4 and 5, purified amidases from *K. pneumoniae* and *P. chlororaphis* B23 (1.0 μg each), respectively; lane 6, preimmune serum (control).

immunoreaction occurred between the antiserum and the amidase of *M. smegmatis*, *M. methylotrophus*, or *Rhodococcus* sp.

DISCUSSION

We observed the characteristics of the thermostable-acrylamide-degrading amidase from *K. pneumoniae* in this study to be distinctly different from those of other amidases. The amidases from *K. pneumoniae* and *M. smegmatis* (22) are monomers with molecular weights of 62,000 and 47,000, respectively, whereas the amidases from *M. methylotrophus* (39) and *P. chlororaphis* B23 (8) are dimers with apparent molecular weights of 123,000 and 105,000, respectively. The *Rhodococcus* (30) amidase is an octamer with an apparent molecular mass of 360,000 Da.

The pH and temperature optimum of *K. pneumoniae* amidase is also different from those of other amidases. The *K. pneumoniae* amidase has optimal activity at a temperature of 65°C at neutral pH (7.0). The amidases from *M. methylotrophus* (39), *Rhodococcus* sp. (30), and *M. smegmatis* (22) have maximal activity at pH 6.0, 8.0, and 8.5, respectively, at 37 to 40°C. The optimum pH and temperature for the *P. chlororaphis* enzyme (8) are pH 7.0 to 8.5 and 50°C. Considerable enzyme inactivation is reported to occur when amidases of *M. methylotrophus* (39), *Rhodococcus* sp. (30), *P. chlororaphis* (8), and *M. smegmatis* (22) are incubated above 50°C. Thus, the acrylamide-degrading *K. pneumoniae* amidase appears to be the first thermostable amidase reported.

Metal analysis indicated that the amidases of *K. pneumoniae* and *Rhodococcus* sp. (30) are the only metal-associated amidases. Iron is essential for the *Rhodococcus* amidase activity (30); treatment of the enzyme with chelators resulted in significant loss of activity. Contrarily, the *K. pneumoniae* amidase is independent of the metal requirement, because removal of the metals from the enzyme failed to significantly inhibit the amidase activity.

Amidases are considered to be SH proteins because they are inhibited by heavy metals such as mercury, copper, and lead (3). Such generalized enzyme classifications without chemical modification studies are misleading because most heavy metals, and mercury in particular, are known to modify histidine, cysteine, and carboxyl groups at the active site (12). The inhibition of the *K. pneumoniae* amidase by SH-modifying reagents suggests that SH groups are involved at the active site of the enzyme or important for the tertiary enzyme structure. Results of the chemical modification with DTNB in the present study suggest that some (three) but not all (one) of the SH groups are in or near an active site(s) of the native protein. The evidence presented in this investigation demonstrates that *K. pneumoniae* amidase contains at least three classes of SH groups. Although the first SH group was readily titratable, the modification of 1.0 SH group had no effect on the activity. It is unlikely that this residue has an important role at the active site or significantly affects tertiary or quaternary structures. The next 3.0 SH groups are catalytically required, and they could be in the active site because their titration parallels significant loss of activity. The remaining majority of the SH groups (7.0) exist in an environment into which the titrant cannot penetrate and are accessible only when the enzyme is denatured.

Additional information concerning the involvement of SH groups at the active site(s) was obtained by modifing the enzyme with iodoacetate, a known SH-alkylating reagent (2, 36–38), and potassium permanganate, a strong oxidant known to oxidize cysteine to cysteic acid (32). Both reagents completely inhibit the titration of SH groups in the native enzyme and significantly inhibit the titration in the denatured enzyme, and this inhibition is accompanied by enzyme inactivation. Our results unambiguously indicate the presence of SH groups at the active site(s) and show that their modification results in enzyme inactivation.

Immunoprecipitation and immunoreaction of amidases from *K. pneumoniae* and *P. chlororaphis* indicate immunological similarities between the two enzymes. The failure of the antiserum to immunoreact with the other acrylamide-degrading amidases suggests evolutionary diversity and spread of catabolic genes among different bacteria.

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