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Purification and Characterization of an Amidase from an Acrylamide-Degrading *Rhodococcus* sp.

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A constitutively expressed aliphatic amidase from a *Rhodococcus* sp. catalyzing acrylamide deamination was purified to electrophoretic homogeneity. The molecular weight of the native enzyme was estimated to be 360,000. Upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the purified preparation yielded a homogeneous protein band having an apparent molecular weight of about 44,500. The amidase had pH and temperature optima of 8.5 and 40°C, respectively, and its isoelectric point was pH 4.0. The amidase had apparent K_m values of 1.2, 2.6, 3.0, 2.7, and 5.0 mM for acrylamide, acetamide, butyramide, propionamide, and isobutyramide, respectively. Inductively coupled plasma-atomic emission spectrometry analysis indicated that the enzyme contains 8 mol of iron per mol of the native enzyme. No labile sulfide was detected. The amidase activity was enhanced by, but not dependent on Fe^{2+}, Ba^{2+}, and Cr^{2+}. However, the enzyme activity was partially inhibited by Mg^{2+} and totally inhibited in the presence of Ni^{2+}, Hg^{2+}, Cu^{2+}, Co^{2+}, specific iron chelators, and thiol blocking reagents. The NH_2-terminal sequence of the first 18 amino acids displayed 88% homology to the aliphatic amidase of *Brevibacterium* sp. strain R312.

Acrylamide, an aliphatic amide, is extensively used in numerous industrial processes. Global production of acrylamide has been estimated to be over 200,000 tons (ca. 180,000 metric tons) (20). Widespread use and indiscriminate discharge of acrylamide has resulted in the contamination of terrestrial and aquatic ecosystems (6, 7, 25). Removal of acrylamide is of paramount importance because of its deleterious health effects (8, 17). Some aliphatic amides are either active ingredients or metabolites of herbicide degradation (26, 32).

Microbial amidases (amidohydrolase, EC 3.5.1.4) catalyze the hydrolysis of aliphatic amides (Fig. 1) to their corresponding carboxylic acids and ammonia (3, 9). Although numerous microorganisms catabolize aliphatic amides (1, 3, 5, 10, 11, 13, 14, 19, 21, 22, 30), acrylamide, because of its inhibitory effect on sulfhydryl proteins (4), inhibits their growth. Therefore, few microorganisms capable of degrading acrylamide have been isolated.

Previously we isolated strains of a *Pseudomonas* sp. and *Xanthomonas maltophilia* (24) which can utilize acrylamide as the sole source of carbon and nitrogen, and an amidase in cell extracts of these strains was reported to degrade acrylamide. Little information is available on the purification and characterization of amidases from acrylamide-utilizing bacteria. Such an enzyme may detoxify acrylamide and may also be useful as a biocatalyst for the large-scale production of acrylic acid, a compound of intense industrial application (20). We report here the isolation of a soil bacterium, a *Rhodococcus* sp., which utilized acrylamide as the sole growth substrate and the purification and properties of the amidase involved in the degradation of acrylamide.

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MATERIALS AND METHODS

Materials. DEAE-Sepharose Fast Flo, Mono Q (HR 10/10), HR 10/30 (Superose 12) prepacked column, low- and high-molecular-mass standard kits, and isoelectric focusing kits were purchased from Pharmacia LKB Biotechnology (Piscataway, N.J.). All values presented in this paper are means from at least three replications.

Microrganism and culture conditions. A bacterium was isolated by an enrichment procedure (24) from soil which had been exposed to alachlor. The bacterium was morphologically and biochemically characterized (12) and was tentatively identified as a *Rhodococcus* sp. Axenic cultures were routinely grown in 1 liter of mineral salts medium amended with micronutrients (23). Acrylamide (62.8 mM) was supplied as the sole growth substrate. The cells used for enzyme purification were grown in batch cultures (40 × 1 liter) at 30°C on a shaker. The cells were harvested at 10,000 × *g* for 25 min at 4°C. The cells were washed with potassium phosphate buffer (100 mM, pH 7.2) and pelleted by centrifugation.

Enzyme assays and ammonia, protein, labile sulfide, acrylamide, and acrylic acid determinations. Amidase activity was assayed at 37°C by measuring the ammonia liberated from acrylamide (24). Unless otherwise stated, the assay mixture consisted of 990 μl of 100 mM potassium phosphate buffer (pH 7.2), 28.4 mM acrylamide, and 10 μl of enzyme solution and mixtures were incubated for 30 min. The ammonia liberated was measured colorimetrically (16). Boiled enzyme served as the control. One unit of the enzyme activity is defined as the amount of enzyme required for the formation of 1 μmol of ammonia from the substrate per min. Protein content was determined by the Bradford method (2). Labile sulfide was measured by the methylene blue method (27). Detection and quantification of acrylamide and acrylic acid were done on a Varian 3400 gas chromatograph (24).

Purification of amidase. Enzyme purification was performed

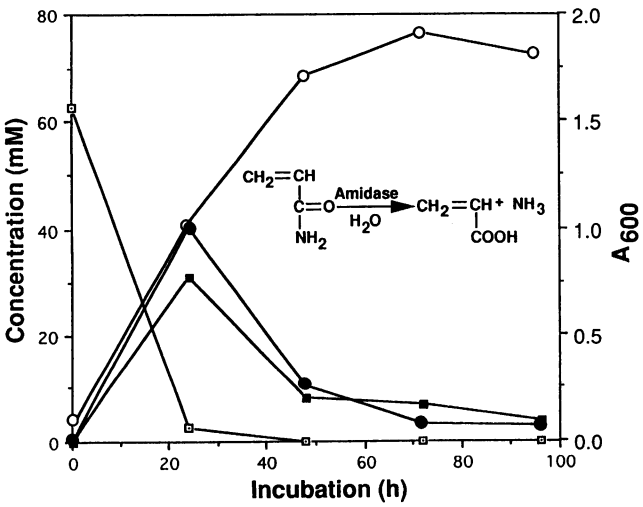

FIG. 1. Growth of a *Rhodococcus* sp. on acrylamide and the formation of metabolites. Cultivation was carried out at 28°C in phosphate-buffered medium containing 62.8 mM acrylamide. Symbols: ⊡, acrylamide; ●, acrylic acid; ■, ammonia; ○, growth. (Insert) Deamination of acrylamide to acrylic acid and ammonia by amidase.

at 4°C. The phosphate buffer used in the study contained K_2HPO_4:KH_2PO_4 (100 mM, pH 7.2) and 1 mM β-mercaptoethanol.

(i) Preparation of cell extracts. A cell pellet (ca. 150 g) was suspended in 1 liter of phosphate buffer, and the cells were disrupted by three successive passages of the cell suspension through a cold French press (SLM Instruments, Urbana, Ill.) operated at 15,000 lb/in^2. The resultant suspension was centrifuged at 10,000 × *g* at 4°C for 60 min to remove cell debris. The supernatant fluid was designated the crude cell extract and stored at 4°C.

(ii) Acetone precipitation. Cold acetone (−20°C) was slowly added with constant stirring to the crude cell extract (ca. 1,000 ml) to a 45% (vol/vol) solution. The mixture was stirred for 45 min, and the precipitate was removed by centrifugation at 10,000 × *g* and discarded. The supernatant fluid was further treated with acetone to a 70% solution, stirred, and centrifuged as before. The pellet was suspended in 150 ml of phosphate buffer and dialyzed overnight against 4 liters of the same buffer.

(iii) Ammonium sulfate fractionation. The protein solution was brought to 45% saturation with finely ground ammonium sulfate. The suspension was stirred for 30 min, and the resulting precipitate was removed by centrifugation. The supernatant was brought to 75% saturation and stirred for 30 min, and the resulting precipitate was pelleted by centrifugation. The protein pellet was resuspended in 40 ml of phosphate buffer and dialyzed overnight against 100 volumes of phosphate buffer.

(iv) DEAE-Sepharose chromatography. All column chromatography procedures described below were performed on a fast protein liquid chromatograph (Pharmacia) at 5°C. The dialyzed protein solution was applied to a DEAE-Sepharose column (3.5 by 60 cm) previously equilibrated with 500 ml of potassium phosphate buffer. The bound proteins were eluted with a linear NaCl gradient (0 to 0.35 M) in 1 liter of phosphate buffer at a flow rate of 4 ml/min. Amidase-positive fractions were pooled and concentrated (ca. 25 ml) in an ultrafiltration cell (Amicon Corp., Danvers, Mass.) with a PM-10 membrane.

(v) Mono Q HR 10/10. The concentrated protein solution (1 ml) was applied to a Mono Q column equilibrated with phosphate buffer. Bound proteins were eluted with a 500-ml linear NaCl gradient (0 to 1 M over 75 min). The active fractions were pooled, concentrated, and dialyzed overnight against 2 liters of phosphate buffer.

(vi) Gel filtration. This procedure was performed with a Superose 12 (HR 10/30) column (1 by 30 cm) previously equilibrated with 50 ml of phosphate buffer and 50 mM NaCl. The protein was eluted with the same buffer at a flow rate of 0.5 ml/min. Active fractions were pooled and concentrated.

Influence of metals and inhibitors on amidase activity. The effects of metals, inhibitors, and chelators on the enzyme activity were tested in two different ways: (i) the purified amidase was added to acrylamide (28.4 mM) in a buffer containing 5 mM divalent cation (as chloride salts) or chelator or inhibitor and (ii) the enzyme (25 μl) was incubated with a 25-μl aliquot of the test compound (10 mM) for 30 min at 4°C and the residual activity of 10 μl of enzyme solution was assayed.

MW and isoelectric point determinations. The molecular mass of the native enzyme was determined with a Superose 12 column (1 by 30 cm) equilibrated with potassium phosphate buffer (10 mM, pH 7.2). The column was run at 0.5 ml/min. K_{av} (the fraction of the stationary gel volume that is available for diffusion of a given solute species) was determined by the formula $(V_e - V_0)/(V_t - V_0)$, where V_e is elution volume, V_t is column volume, and V_0 is void volume. The molecular weight (MW) standards are aldolase (MW, 158,00), catalase (MW, 232,000), ferritin (MW, 444,000), and thyroglobulin (MW, 669,000). The subunit molecular mass was determined by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (18). Isoelectric focusing was performed on an ampholine PAGplate (pH 3.5 to 9.5) as described by Welch et al. (31).

Determination of kinetic constants, pH, temperature optima, and stability of the enzyme. The apparent K_m values were determined by using various aliphatic amide concentrations (0.34 to 11.4 mM). Lineweaver-Burk plots were used to calculate K_m and V_{max} values. The temperature optimum of amidase was determined at various temperatures between 10 and 65°C. The pH optimum was determined by using 100 mM phosphate-citrate buffer, 100 mM KH_2PO_4-NaOH, and 100 mM sodium carbonate buffer for pH ranges 3.0 to 7.6, 7.5 to 8.5, and 8.5 to 11.0, respectively.

Metal analysis. Enzyme metal content analysis was performed by inductively coupled plasma-atomic emission spectrometry utilizing a Thermo Jarrell Ash (Franklin, Mass.) ICAP 61E spectrometer operating at 27.12 MHz.

Amino acid analysis and protein sequencing. Purified amidase (10 μg) was hydrolyzed and derivatized with phenylisothiocyanate, and amino acid analysis was conducted with a 120A amino acid analyzer (Applied Biosystems Inc., Foster City, Calif.). The automated N-terminal sequencing was performed on an Applied Biosystems 470A PTH protein sequencer with an online 120A PTH amino acid analyzer.

RESULTS

Isolation and identification of an acrylamide-utilizing microorganism. A bacterium was isolated from enrichments with soils exposed to the herbicide alachlor. The microorganism was an aerobic, gram-positive rod, measuring 1.0 by 2.0 to 6.0 μm. It was nonmotile, non-endospore forming, catalase and phosphatase positive, and oxidase negative. It failed to hydrolyze either starch, sugars, or gelatin. However, it hydrolyzed urea

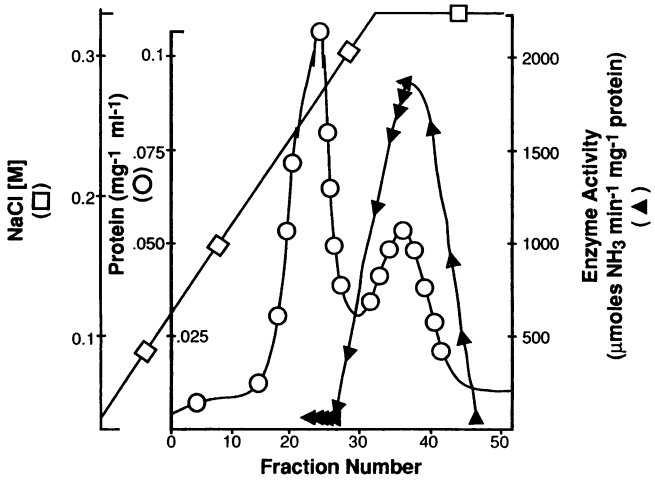

FIG. 2. Elution profile of amidase from a DEAE-Sepharose Fast Flo column.

(Christensen's reaction). The strain reduced nitrate to nitrite. On the basis of these tests the bacterium was tentatively identified as a *Rhodococcus* sp. The bacterium utilized 62.8 mM acrylamide as the sole source of carbon and nitrogen, and analysis of culture filtrates indicated catabolism of acrylamide to acrylic acid and ammonia (Fig. 1). Maximum accumulations of acrylic acid (42.6 mM) and ammonia (31.2 mM) were detected after 24 h, and peak growth was observed between 72 and 96 h at 28°C (Fig. 1). The amidase was determined to be constitutive and intracellular.

Purification of the enzyme. Although DEAE-Sepharose chromatography (Fig. 2) removed most of the nonspecific proteins from the amidase, some contamination was still observed on SDS-PAGE gels; thus, additional purification was performed on Mono Q and Superose 12 to attain homogeneity. A typical purification scheme is summarized in Table 1. The amidase was purified approximately 138-fold from crude cell extracts with a yield of 20%.

Purity and physical characteristics of the enzyme. The purified amidase had a molecular mass of 360 kDa (Fig. 3A). SDS-PAGE of the purified amidase preparation showed a single band of protein with an MW of approximately 44,500 (Fig. 3B). On the basis of these results we suggest that the enzyme probably consists of eight subunits identical in MW. The isoelectric point of amidase was estimated to be pH 4.0 (data not shown), and the enzyme migrated as a single band. The enzyme showed high activity within the pH range of 5.5 to 9.0, and the optimum pH was 8.5. The optimum temperature at pH 8.5 was 40°C (data not shown).

TABLE 1. Purification of amidase from *Rhodococcus* sp. strain NCTR 4

Step	Total activity[a] (U)	Total protein (mg)	Sp act[b]	Yield (%)	Purification (fold)
Cell extract	27,800	1,071	26	100	1
Acetone (45–70%)	21,700	275	79	78	3.0
Ammonium sulfate	19,600	89	220	70	8.0
DEAE-Sepharose	17,700	7	2,520	63	96.0
Mono Q	8,300	2.6	3,200	30	123.0
Superose 12	5,800	1.6	3,600	20	138.0

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

Substrate specificity. The amidase was capable of catalyzing the deamination of several aliphatic amides. Michaelis-Menten kinetics (Fig. 4) indicated K_ms of 1.2, 2.6, 2.7, 3.0, and 5.0 mM for acrylamide, acetamide, propionamide, butyramide, and isobutyramide, with corresponding V_{max} values of 5,900, 20,000, 16,700, 7,700, and 5,000 μmol of $NH_3 \cdot min^{-1} \cdot mg$ of $protein^{-1}$, respectively. Aromatic amides inhibited the enzyme.

Effect of metals and other compounds. Various compounds were investigated for their effects on enzyme activity. Cu^{2+}, Hg^{2+}, Ni^{2+}, and Co^{2+} completely inhibited the enzyme (Table 2), whereas Mg^{2+} partially inhibited the enzyme activity. However, the amidase activity was significantly enhanced by Fe^{2+}, Cr^{2+}, and Ba^{2+}.

Among the chelators, direct addition of triethylenetetramine

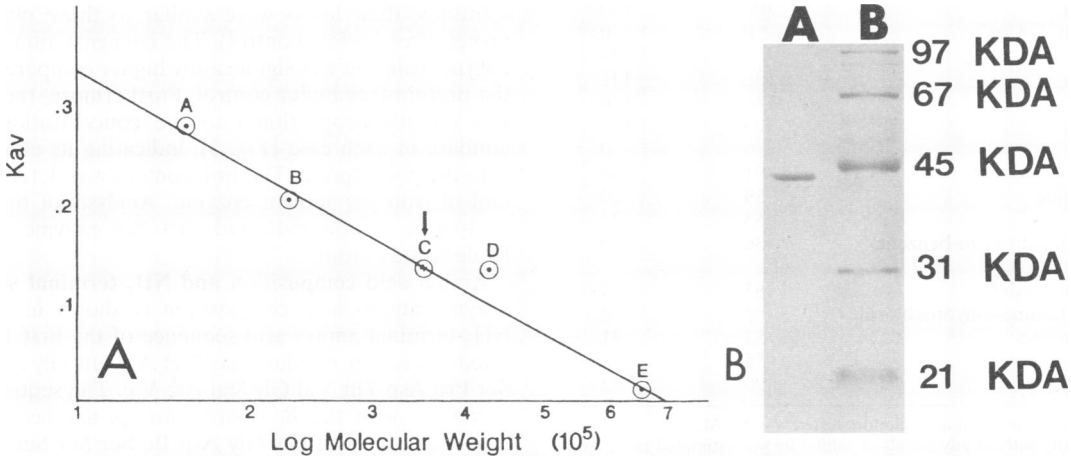

FIG. 3. M_r determination by gel filtration and SDS-PAGE. (A) Amidase and standard proteins were chromatographed on a phenyl-Superose as described in the text. MW standards (M_r; K_{av}): A, aldolase (158,000; 0.285); B, catalase (232,000; 0.208); C, amidase; D, ferritin (440,000; 0.145); E, thyroglobulin (669,000; 0.0125). (B) SDS-PAGE of *Rhodococcus* amidase. Lane A, purified amidase (5 μg); lane B, molecular mass standards (from top, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor).

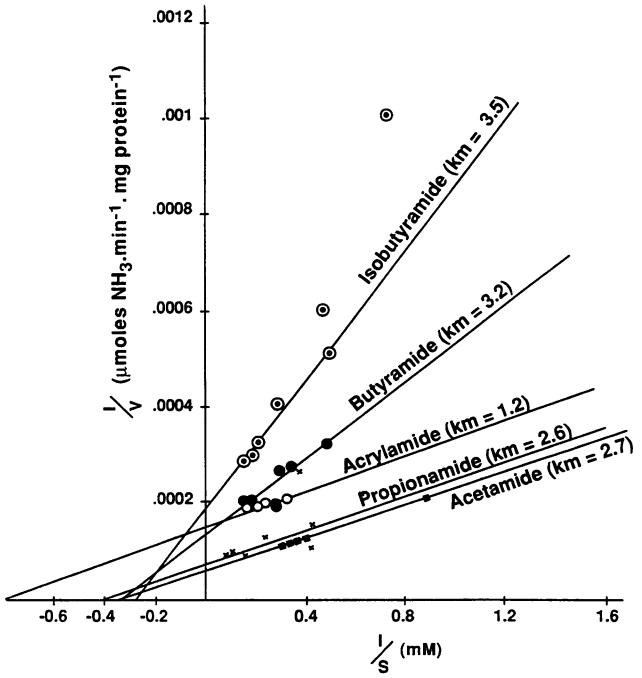

FIG. 4. Determination of K_m of *Rhodococcus* amidase for different aliphatic amide substrates. Substrate concentrations (S) varied from 0.34 to 11.4 mM. Velocity (V) was calculated on the basis of the specific activity. Lineweaver-Burk plots were used to calculate K_m.

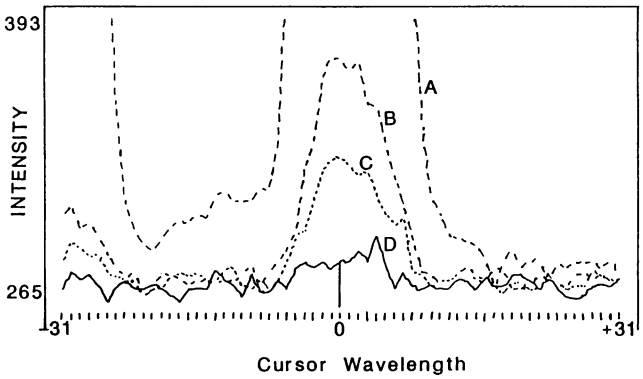

FIG. 5. Detection of iron in amidase by inductively coupled plasma-atomic emission spectrometry. The intensity is represented in absolute intensity units. An absolute intensity of about 265 is the level of the electronic zero for this line. The sample intensity is several times higher than the electronic zero level. The sample quantitation and instrument standardization were performed with the cursor wavelength of 259.94 nm. Curves: A, National Institute of Standards and Testing standard (iron; the iron content of the standard was 1.00 μg/ml); B, amidase (300 μg of protein); C, amidase (150 μg of protein); D, buffer (control) without the enzyme.

dihydrochloride or disodium 4,5-dihydroxy-*m*-benzene disulfonate (Tiron) significantly inhibited the enzyme activity. However, complete inhibition of the enzyme was noted when the enzyme was preincubated with either reagent. Direct addition of other chelating agents (1,10-phenanthroline, 2,2'-dipyridyl, and 8-hydroxyquinoline) appreciably inhibited the enzyme activity. However, pronounced inhibition by these chelators was noted when the enzyme was preincubated with the reagent.

TABLE 2. Effects of various compounds on the amidase activity from the *Rhodococcus* sp.

Compound[a]	Relative activity (%)[b]	
	Direct addition	Preincubation
None (control)	100	100
Ni^{2+}, Hg^{2+}, Cu^{2+}, Co^{2+}	0	0
Mg^{2+}	55	39
Fe^{2+}	136	141
Cr^{2+}	146	136
Ba^{2+}	129	133
Iodoacetate	0	0
Iodoacetamide	91	88
N-Ethylmaleimide	59	20
2,2'-Dipyridyl	53	21
Disodium 4,5-dihydroxy-*m*-benzene disulfonate	36	0
EDTA (disodium salt)	83	54
Triethylene tetramine-dihydrochloride	40	0
1,10-Phenanthroline	52	31
8-Hydroxyquinoline	55	36
Sodium azide	90	92

[a] The final concentration of all inhibitors tested was 5 mM.

[b] Enzyme activity without any metals or inhibitors was estimated to be 3,600 U/mg of protein and was considered 100%. In the direct addition method, the test compound was added to the reaction mixture. The reaction was started with the addition of amidase. In the preincubation method, the enzyme was incubated for 30 min with the test compound and the residual activity of 10 μl (5 μg of protein) of enzyme solution was assayed.

The effects of thiol blocking reagents (iodoacetate, iodoacetamide, and *N*-ethylmaleimide) on amidase were ambiguous. Regardless of the treatment, iodoacetate completely inhibited the enzyme. However, pronounced *N*-ethylmaleimide inhibition of the enzyme was observed only when the enzyme was preincubated for 30 min with the reagent. Regardless of the treatment, iodoacetamide and sodium azide had very little inhibitory effect on the amidase activity.

Metal analysis. Metal analysis of the concentrated enzyme solution indicated the presence of iron, calcium, and zinc. Although calcium and zinc were detected in the enzyme solutions, their levels were similar to those observed in the protein-free buffer (control). The concentration of iron in the enzyme solution was significantly higher compared with that in the protein-free buffer control. Furthermore, the iron content was directly proportional to the concentration of purified amidase in each case (Fig. 5), indicating its close association with the polypeptide. The iron content was determined to be 8 mol of iron per mol of enzyme. Analysis of the enzyme for acid-labile sulfide indicated that the enzyme contained no labile sulfide atom.

Amino acid composition and NH_2-terminal sequence. The enzyme amino acid composition is shown in Table 3. The NH_2-terminal amino acid sequence of the first 17 residues of *Rhodococcus* sp. amidase was Met Arg His Gly Asp Ile Thr Ser Ser Pro Asp Thr Val Gly Val Ala Val. The sequence had 88% homology with the *Brevibacterium* sp. amidase that had the sequence Met Arg His Gly Asp Ile Ser Ser Ser Asn Asp Thr Val Gly Val Ala Val. The *Rhodococcus* amidase contained threonine and proline at positions 7 and 10, respectively, whereas *Brevibacterium* amidase contained serine at position 7 and asparagine at position 10.

TABLE 3. Amino acid composition of the aliphatic amidase from *Rhodococcus* sp. strain NCTR 4[a]

Amino acid	Composition (mol %)
Aspartic acid or asparagine	12
Glutamic acid or glutamine	10
Serine	5
Glycine	10
Histidine	3
Arginine	6
Threonine	6
Alanine	8
Proline	5
Tyrosine	5
Valine	7
Methionine	3
Isoleucine	6
Phenylalanine	4
Lysine	4
Leucine	5
Cysteine	Not determined
Tryptophan	Not determined

[a] The amino acids were determined from a 20-h hydrolysate.

DISCUSSION

This is the first report on the purification and characterization of an aliphatic amidase from an acrylamide-utilizing bacterium. Comparison of the characteristics of amidase from other non-acrylamide-utilizing microorganisms indicate that *Rhodococcus* amidase is constitutive whereas aliphatic amidases (1, 3, 15, 29) from other microbes were inducible. The *Rhodococcus* amidase by far has the highest affinity for acrylamide (K_m = 1.2 mM), whereas acrylamide inhibits amidases from a *Pseudomonas* sp. (1, 3, 15) and is poorly degraded (29) by a *Brevibacterium* sp. (K_m = 12 mM).

The amidase described herein also exhibited several properties that differentiate it from amidases of other microorganisms. Compared with the *Rhodococcus* amidase, which had an apparent M_r of 360,000 under nondenaturing conditions and an apparent subunit M_r of 44,500 when denatured, the amidases of *Pseudomonas aeruginosa* (3) and a *Pseudomonas* sp. (15) had apparent M_rs of 200,000 and 43,000, respectively, by gel filtration and subunit M_rs of 35,000 and 26,000, respectively, by SDS-PAGE. The amidase of a *Brevibacterium* sp. (29) had an M_r of 180,000 and was composed of four identical subunits (M_r, 43,000).

Because different assay systems have been used to assay purified amidases from two different strains of *Pseudomonas* sp. (3, 15) and a *Brevibacterium* sp. (29), it is difficult to compare specific activities among these preparations. Nevertheless, since they all present specific activities as micromoles of NH_3 per minute per milligram of protein, we attempted to compare the specific activities. Among the amidases reported, the *Rhodococcus* sp. amidase had the highest specific activity of ca. 3,500 μmol of $NH_3 \cdot min^{-1} \cdot mg$ of $protein^{-1}$. In contrast, the purified amidases of the two *Pseudomonas* sp. strains (3, 15) and the *Brevibacterium* sp. (29) exhibited specific activities of 7.14, 37.6, and 274.1 μmol of $NH_3 \cdot min^{-1} \cdot mg$ of $protein^{-1}$, respectively.

The presence of iron in the amidase described in this paper is a novel feature that differentiates it from other amidases. Although at present we are not certain of its role in the mechanism of amide hydrolysis, the inhibition of amidase activity by specific iron chelators suggests a crucial role of iron in the active site of the enzyme.

The effects of metals and inhibitors on amidase activity appear to be a function of the specific amidase analyzed. For example, our studies indicate that Fe^{2+} and Ba^{2+} significantly enhance enzyme activity whereas Ni^{2+}, Co^{2+}, Cu^{2+}, and Hg^{2+} inhibit amidase activity. In contrast, Kagayama and Ohe (15) indicated that Ni^{2+} and Co^{2+} enhanced amidase activity whereas Hg^{2+} and Cu^{2+} inhibited the activity of an aromatic amidase. Since *Rhodococcus* amidase is strongly inhibited by thiol blocking reagents, the amidase may be a sulfhydryl protein.

The NH_2-terminal amino acid sequence of the *Rhodococcus* sp. amidase presents a high degree of homology with the amidase of a *Brevibacterium* sp. (28). The variation of amino acid composition at position 7 is relatively insignificant because both amino acids (threonine and serine) are neutral and hydrophilic. The variation in amino acids at position 10 is a distinguishing characteristic, because proline in the *Rhodococcus* amidase sequence is structurally different from the asparagine in the *Brevibacterium* amidase sequence.

The application of biocatalysts for the synthesis of fine chemicals has attracted considerable interest (20). The *Rhodococcus* sp. amidase is the only biocatalyst that can efficiently deaminate acrylamide to acrylic acid under a wide range of pHs and temperatures. The described purification protocol is relatively simple and rapid, resulting in high yields of the purified amidase with high specific activity, compared with other methods.

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