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Purification and Characterization of an L-Amino Amidase from *Mycobacterium neoaurum* ATCC 25795

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An L-amino amidase from *Mycobacterium neoaurum* ATCC 25795 responsible for the enantioselective resolution of DL-α-methyl valine amide was purified and characterized. The purification procedure included ammonium sulfate fractionation, gel filtration, and anion-exchange chromatography, which resulted in a homogeneous preparation of the enzyme with a native molecular mass of 136 kDa and a subunit molecular mass of 40 kDa. The purified enzyme displayed the highest activity at 50°C and at pH 8.0 and 9.5. The enzyme was strongly inhibited by the metal-chelating agent 1,10-phenanthroline, the disulfide-reducing agent dithiothreitol, and the cysteine proteinase inhibitor iodoacetamide. The purified amino amidase showed a unique L-enantioselective activity towards a broad range of both α-H- and α-alkyl-substituted amino acid amides, with the highest activity towards the cyclic amino acid amide DL-proline amide. No activity was measured with DL-mandelic acid amide nor with the dipeptide L-phenylalanine-L-leucine. The highest catalytic efficiency (k_{cat}/K_m ratio) was measured with DL-α-allyl alanine amide, DL-α-methyl phenylalanine amide, and DL-α-methyl leucine amide.

Enantiomerically pure, α-alkyl substituted amino acids constitute an interesting group of chiral synthons for pharmaceutical and agrochemical specialties, which find applications as inhibitors of essential enzyme activities. Illustrative examples are L-α-methyl-aminobutyric acid (L-isovaline), a natural constituent of several antibiotics (7), and L-α-methyl-(3,4-dihydroxy)phenylalanine, which is used as an antihypertensive agent (15). More recently, peptide analogs containing α-alkyl-substituted amino acids have become of interest for a variety of medical applications. The relation between the structure of these peptide analogs and their resistance to enzymatic degradation is receiving much attention (6, 20).

Several routes for the production of enantiomerically pure α-alkyl-substituted amino acids have been investigated (1, 5, 12, 13, 16, 17), and an enzymatic kinetic resolution process based on *Mycobacterium neoaurum* ATCC 25795 as a biocatalyst has been developed (10). As reported previously, whole cells of the organism are capable of enantioselective hydrolysis of a broad range of α-alkyl-substituted amino acid amides (9–11, 18). The purification and detailed characterization of the L-amino amidase enzyme system of *M. neoaurum* ATCC 25795, responsible for the enantioselective resolution of DL-α-methyl-valine-amide (DL-α-CH_3-Val-NH_2) is reported in this article.

MATERIALS AND METHODS

Microorganism and cultivation conditions. *M. neoaurum* ATCC 25795 was maintained on GYC agar slants (glucose, 10 g · liter^{-1}; yeast extract, 10 g · liter^{-1}; $CaCO_3$, 10 g · liter^{-1}; and agar, 12 g · liter^{-1}). Cultivation of the strain was performed in 20 liters of standard medium containing yeast carbon base (Difco; 10 g · liter^{-1}), urea (4 g · liter^{-1}), and nitrilotriacetic acid (4.8 g · liter^{-1}) at 37°C and pH 7.2 in a 30-liter fermentor. Cells were harvested by centrifugation at 18,000 × *g* for 20 min, washed once with 50 mM phosphate buffer (pH 7.5), and centrifuged again. The pellet fraction (approximately 800 g [wet weight]) was stored at −80°C.

The racemic and enantiomerically pure amino acids and amides were prepared by the enzymatic resolution processes of DSM Research by using L-aminopeptidase from *Pseudomonas putida* ATCC 12633 and L-amino amidase from *M. neoaurum* ATCC 25795 as described earlier (10). For α-H amino acids (amides), the Strecker synthesis starts from an aldehyde, and for the α-alkyl-substituted amino acids and derivatives, it starts from the corresponding ketone.

Purification of the L-amino amidase. All purification steps were performed at 4°C.

(i) Step 1. Preparation of cell extract. Frozen cell paste (400 g [wet weight]) was quickly thawed and resuspended in 50 mM Tris-H_2SO_4 buffer (pH 7.8; standard buffer, 500 ml). By using aliquots of 20 ml, cells were disrupted by one passage through a French pressure cell at 1,500 MPa (2.1 × 10^5 kN · m^{-2}). Nondisintegrated microbial cells and cell debris were removed by centrifugation at 70,000 × *g* for 45 min to yield a cell-free supernatant fraction.

(ii) Step 2. Ammonium sulfate fractionation. The ammonium sulfate fractionation was performed by adding solid ammonium sulfate to the supernatant fraction. The protein fraction precipitating between 40 and 65% ammonium sulfate saturation was collected by centrifugation (30,000 × *g* for 45 min), dissolved in the standard buffer (60 ml), and desalted by gel filtration of 2.5-ml aliquots on Sephadex PD-10 columns (Pharmacia).

(iii) Step 3. Gel filtration. The desalted 40 to 65% ammonium sulfate fraction (85 ml) was applied to a HiLoad 26/60 Superdex 200 preparative separation column (Pharmacia fast protein liquid chromatography [FPLC] system), equilibrated, and eluted with standard buffer containing 150 mM ammonium sulfate. The protein fractions (3 ml) containing

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the highest activity were collected, pooled, and desalted as described in step 2.

(iv) Step 4. Anion-exchange chromatography. The protein solution was loaded on a Mono Q HR 5/5 anion-exchange column (Pharmacia FPLC system) previously equilibrated with the standard buffer. Active protein fractions were eluted from the column at a flow rate of 1.0 ml · min^{-1} by applying a 50-ml linear gradient of ammonium sulfate between 80 and 300 mM. Fractions of 1 ml were collected. After desalting, the purified enzyme solutions were used directly for determination of the enzyme characteristics.

Enzyme assay for L-amino amidase activity. L-Amino amidase enzyme activity assays were performed in glass vials closed with a screw cap in a reaction mixture (total volume, 2.5 ml) with 400 mM DL-α-CH_3-Val-NH_2 as substrate in 50 mM Tris-H_2SO_4 buffer (pH 8.0). After temperature equilibration at 37°C, the reaction was initiated by the addition of the (purified) enzyme fractions. After 45 min, the reaction was terminated by the addition of 1.25 ml of 1 M phosphoric acid. In all of the assays, the observed rate of hydrolysis was linear for at least 45 min and proportional to the amount of enzyme added.

An ion-selective ammonia electrode system (Orion model 95-12) was used for the quantitative determination of the enzyme activity. For this purpose, the ammonia production, equivalent to the amino acid production, was measured in the reaction mixtures mentioned above. The detection limit for NH_3 measurement via this method is approximately 10^{-6} M.

Progress in enzyme purification was also monitored by a rapid, multiple-sample, qualitative assay for enzyme activity, by using silica gel 60 F_{254} thin-layer chromatography plates (Merck). After application of 4 μl of the reaction mixture and subsequent drying, the plates were placed in the solvent system chloroform-methanol-ammonia (60:45:20 [vol/vol/vol]). After elution and drying for 5 min at 135°C, the separated α-CH_3-Val-NH_2 and α-CH_3-Val were developed by using a 2.5% hypochlorite solution and a 1% KI-starch spray.

Chiral thin-layer chromatography analysis (CHIRALPLATE silica gel RP modification coated with Cu^{2+} and chiral reagent; Macherey-Nagel, Düren, Germany) was used to detect hydrolysis (and possible racemization of the amino acids produced) of the dipeptide substrate L-Phe–L-Leu. Samples were first diluted with the same volume of methanol-ammonia (3:1 [vol/vol]) and centrifuged (6,000 × *g* for 10 min). Subsequently, 2 μl of the supernatants was applied to the plates. The solvent system consisted of methanol-water-acetonitrile (50:50:200 [vol/vol/vol]). After elution and drying, spots were visualized with a 0.3% ninhydrin solution.

Protein determination. Protein concentrations between 0.1 and 1 mg · ml^{-1} were measured by the method of Bradford (2) with crystalline bovine serum albumin (BSA) as the standard. Lower concentrations were determined by using the bicinchoninic acid method (19), again with the BSA standard.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in PhastGel gradient gels (8 to 25%) by using the Pharmacia PhastSystem. Protein bands were visualized after gel electrophoresis by the silver staining method as described in the instruction manual of the PhastGel silver kit (Pharmacia). Proteins from a Pharmacia low-molecular-weight calibration kit were used as molecular weight markers. Isoelectrofocusing with Pharmacia PhastGel IEF 3-9 gels was used to determine the isoelectric point of the purified enzyme with Pharmacia broad-pI calibration kit proteins as marker proteins.

Molecular weight estimation of the native enzyme. To determine the molecular weight, the purified L-amino amidase was applied to a HiLoad 26/60 Superdex 200 column, calibrated with cytochrome *c* (12.5 kDa), chymotrypsinogen A (25 kDa), hen egg albumin (45 kDa), BSA (68 kDa), rabbit muscle aldolase (158 kDa), beef liver catalase (240 kDa), and ferritin (450 kDa). The conditions for the gel filtration step were identical to those used in the purification procedure. All gel chromatography calibration proteins were obtained from Boehringer Mannheim.

Effect of pH on enzyme activity. A quantitative analysis of the enzyme activity as a function of pH values between 4.0 and 11.0 was carried out by using a 400 mM DL-α-CH_3-Val-NH_2 solution in a universal buffer system described by Davies (3) containing 25 mM citric acid, 25 mM KH_2PO_4, 25 mM Tris, 12.5 mM $Na_2B_4O_7$, and 25 mM KCl (total volume, 2.5 ml). The pH value of the buffer was adjusted by the addition of KOH or H_2SO_4. The reactions were performed with 3 μg of purified enzyme for 45 min at 37°C.

Effect of temperature on enzyme activity and stability. A quantitative determination of the activity of the purified enzyme at temperatures ranging from 20 to 70°C was performed by using a 400 mM DL-α-CH_3-Val-NH_2 solution in Davies buffer (optimum pH, 9.0; see Results). After equilibration of the reaction mixture (total volume, 2.5 ml), 3 μg of enzyme was added to start the reaction.

For analysis of the thermal stability, 3 μg of the purified enzyme was preincubated for 30 min at temperatures ranging from 30 to 75°C with 0.6 ml of Davies buffer (pH 9.0). The remaining activities were measured after the addition of 2.0 ml of an 800 mM DL-α-CH_3-Val-NH_2 solution in Davies buffer (pH 9.0) and reaction for 45 min at 50°C (optimum temperature; see Results).

Effect of proteinase inhibitors. The effect of proteinase inhibitors (0.01 to 10 mM) was determined after preincubation with the L-amino amidase (2.4 μg) at 50°C for 12 min in 100 mM Tris-H_2SO_4 (pH 8.0; total volume, 1.2 ml). Residual activities were determined after the addition of 1.2 ml of 800 mM DL-α-CH_3-Val-NH_2 in 100 mM Tris-H_2SO_4 (pH 8.0) and then incubation for 45 min at 50°C.

Determination of substrate specificity. The standard enzyme assay was used for the measurement of enzyme activity towards different types of amide substrates. The assay was performed with 100 mM of the substrate to be tested in 50 mM Tris-H_2SO_4 buffer (pH 8.0), except for the dipeptide L-Phe–L-Leu (2 mM). The reaction (in a total volume of 2.5 ml) was started by the addition of 4.5 μg of purified enzyme and stopped after 0, 90, and 180 min. Incubation was performed at 40°C to avoid autohydrolysis of some of the substrates, especially the lower aliphatic amides.

Determination of kinetic properties. K_m and V_{max} values were calculated from double-reciprocal and direct-linear plots of the initial rate of enzyme activity as a function of the substrate concentration. At least four substrate concentrations of several amino acid amides were used to assay the corresponding enzyme activities. Incubations (2.5 ml) were performed in 50 mM Tris-H_2SO_4 buffer (pH 8.0) and at 50°C. The reaction was started by the addition of 2.5 μg of purified enzyme. The time periods used varied, depending on the different substrates and concentrations chosen (1 to 400 mM). Under these assay conditions, autohydrolysis of the substrates tested was limited. The k_{cat} values were calculated on the basis of a subunit molecular mass of 40 kDa.

TABLE 1. Purification (scheme) of the L-amino amidase from *M. neoaurum* ATCC 25795

Purification step	Total activity (μmol · min^{-1})	Total protein (mg)	Sp act (μmol · min^{-1} · mg^{-1})	Recovery (%)	Purification (fold)
Cell extract	900	4,500	0.2	100	1
Ammonium sulfate fractionation (40–65%)	139	140	1.0	15	5
HiLoad Superdex	94	10	9.4	10	47
Mono Q	112	2	56	12	280

Determination of enantioselectivity. The enantioselectivity of the purified L-amino amidase enzyme towards different racemic mixtures of α-H- and α-alkyl-substituted amino acid amides was determined with 100 mM DL-Val-NH_2, DL-phenylglycine amide (DL-PG-NH_2), DL-α-CH_3-Val-NH_2, DL-α-CH_3-Phe-NH_2, and 25 mM DL-Ala-NH_2 in 2.5 ml of 50 mM Tris-H_2SO_4 buffer (pH 8.0). The reaction was started by the addition of 3 μg of purified enzyme and stopped after overnight incubation at 50°C by the addition of 1.25 ml of 1 M H_3PO_4. The amino acid and amino acid amide enantiomers were determined by chiral high-performance liquid chromatography methods (4).

RESULTS

Enzyme purification. Several procedures were used to purify the L-amino amidase activity from *M. neoaurum* ATCC 25795. The most convenient procedure is summarized in Table 1. Disintegration of whole cells by French pressure cell treatment resulted in more than 60% release of the L-amino amidase into the extract. Alternative methods, like sonication and bead mill treatment, did not result in significant solubilization of the enzyme.

After the preparation of cell extract, the enzyme was purified to homogeneity by 40 to 65% ammonium sulfate fractionation, gel filtration, and anion-exchange chromatography steps. With DL-α-CH_3-Val-NH_2 as a substrate, only a single activity peak was consistently observed during these purification steps. When these procedures were used, the L-amino amidase was purified approximately 280-fold and with a 12% yield, resulting in a homogeneous preparation as judged by SDS-PAGE.

Molecular mass, subunit composition, and isoelectric point. The molecular mass of the native enzyme was estimated by FPLC gel filtration to be 136 kDa. The subunit molecular mass was determined by SDS-PAGE, yielding a single protein band corresponding to a molecular mass of 40 kDa. This indicates that the native enzyme most likely consists of three or four subunits of equal size. The isoelectric point of the purified enzyme was estimated to be 4.2 by isoelectric focusing.

Effect of pH on enzyme activity. The effect of pH in the range of 4.0 to 11.0 was investigated. L-Amino amidase activity towards DL-α-CH_3-Val-NH_2 occurred at pH values of 6.5 to 11.0, with a broad optimum at pH values of 8.0 to 9.5 (data not shown). No enzyme activity was detected at values below pH 6.0. Comparable results were obtained with permeabilized whole cells and DL-α-CH_3-Leu-NH_2 as a substrate.

Effect of temperature on activity and stability. With DL-α-CH_3-Val-NH_2 as a substrate, the purified L-amino amidase displayed an optimum temperature of approximately 50°C. At 35 and 60°C, only 50% of the optimal activity was found.

The stability of the L-amino amidase was determined by preincubation of the purified enzyme for 30 min at different temperatures. At temperatures up to 55°C, more than 90% of the optimum activity was retained. Above this temperature, the activity loss was considerable, e.g., about 95% at 70°C.

Effect of proteinase inhibitors. The purified enzyme system was further characterized by testing a number of inhibitors for their ability to affect the L-amino amidase activity towards DL-α-CH_3-Val-NH_2 (Fig. 1). Complete inhibition of enzymatic activity occurred in the presence of dithiothreitol. The enzyme was also inhibited by 1,10-phenanthroline and by higher concentrations of iodoacetamide (IAA), which is known to be an inhibitor of cysteine proteinases (43% inhibition at 10 mM). However, no inhibitory effect was observed with *p*-chloromercuribenzoate (another effector of cysteine proteinases), EDTA (an inhibitor of metalloproteinases), and diisopropyl fluorophosphate (DFP) and phenylmethylsulfonyl fluoride (serine proteinase inhibitors; data not shown). DFP even appeared to have a stimulating effect on the enzyme (Fig. 1).

Substrate specificity. The substrate specificity of the purified enzyme was investigated by using a variety of the amide substrates available (Tables 2 to 4). This enzyme appears to have a high substrate specificity, restricted to α-amino acid amides, with an H atom at the C_α position (e.g., DL-Val-NH_2) or α-methyl-substituted molecules (e.g., DL-α-CH_3-Val-NH_2). Activity was also detected with DL-α-allyl-Ala-NH_2 (Table 3) and with α-ethyl- and propyl-substituted Phe-NH_2 but not with α-ethyl-, propyl-, allyl-, and benzyl-substituted PG-NH_2 or α-allyl- and isopropyl-substituted Phe-NH_2 (data not shown). Among the aliphatic amides tested, activity was observed with acetamide and propion-

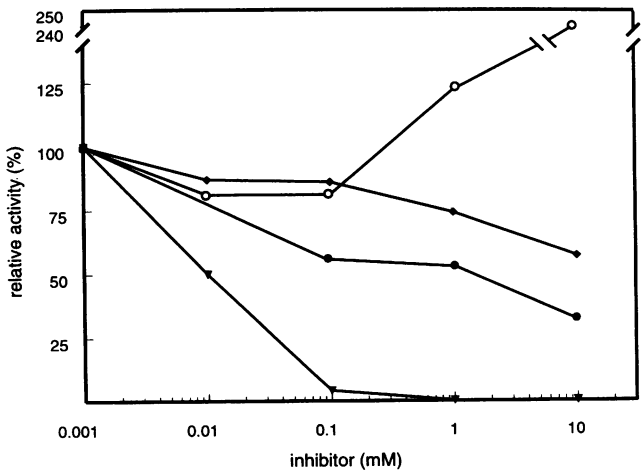
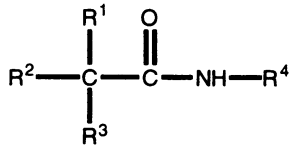


FIG. 1. Effect of several proteinase inhibitors on the L-amino amidase activity. The activity is expressed as a percentage of the activity of the nonpreincubated L-amidase enzyme. One hundred percent activity corresponds to 50 μmol · min^{-1} · mg of protein^{-1}. Symbols: ●, 1,10-phenanthroline; ▼, dithiothreitol; ◆, IAA; ○, DFP.

TABLE 2. Substrate specificity of the L-amino amidase enzyme from *M. neoaurum* ATCC 25795 with activity expressed relative to the activity towards DL-α-CH_3-Val-NH_2

Model



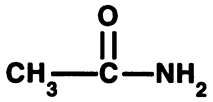
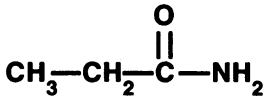
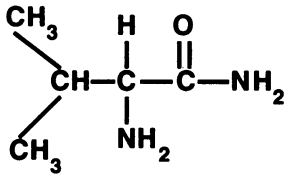
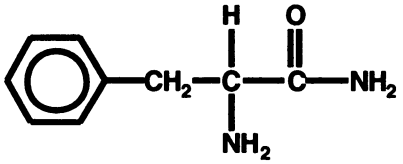
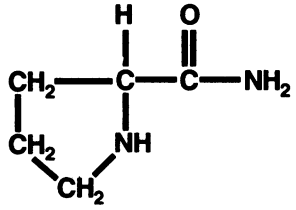
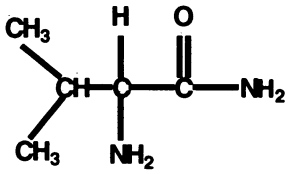
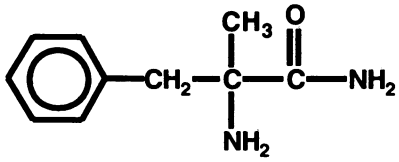
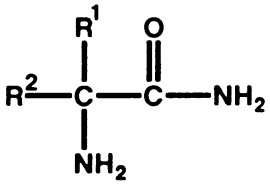
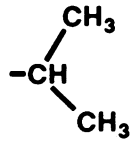
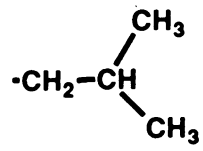
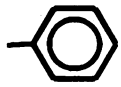
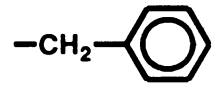
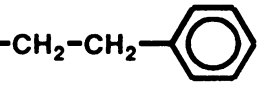
Substrate	Formula	Activity (%)
acetamide		10
propionamide		15
D,L-Val-NH_2		160
D,L-Phe-NH_2		60
D,L-Pro-NH_2		420
D,L-α-CH_3-Val-NH_2		100
D,L-α-CH_3-Phe-NH_2		70

TABLE 3. K_m and V_{max} values for different amino acid amides hydrolyzed by the purified L-amino amidase enzyme from *M. neoaurum* ATCC 25795

Model 

Substrate	R^1	R^2	K_m[a]	V_{max}[b]	k_{cat}[c]	k_{cat}/K_m[d]
D,L-α-CH_3-Val-NH_2	$-CH_3$		100	75	50	500
D,L-α-CH_3-Leu-NH_2	$-CH_3$		10	125	85	8300
D,L-iso-Val-NH_2	$-CH_3$	$-CH_2-CH_3$	30	160	105	3600
D,L-α-allyl-Ala-NH_2	$-CH_3$	$-CH_2-CH=CH_2$	5	240	160	32000
D,L-α-CH_3-PG-NH_2	$-CH_3$		335	25	15	50
D,L-α-CH_3-Phe-NH_2	$-CH_3$		5	80	55	10700
D,L-α-CH_3-homoPhe-NH_2	$-CH_3$		5	20	15	2700
D,L-Pro-NH_2	-H	$-(CH_2)_3-NH$	40	175	115	2900

[a] K_m is expressed in millimolar.
[b] V_{max} is expressed in micromoles per minute per milligram of protein.
[c] k_{cat} is expressed per second.
[d] k_{cat}/K_m is expressed per second per molar.

amide but not with (iso)butyramide or (metha)acrylamide. The highest activity was observed with the cyclic amino acid amide DL-Pro-NH_2. The enzyme was not active with the hydroxy acid amide DL-mandelic acid amide nor with the single dipeptide tested, L-Phe–L-Leu (data not shown).

Kinetic properties. The initial rates of enzyme activity were studied as a function of the concentration of various DL-α-CH_3-amino acid amides and DL-Pro-NH_2. The enzyme displayed normal Michaelis-Menten type of kinetics, and the K_m and V_{max} values for eight amino acid amide substrates were derived from double-reciprocal and direct-linear plots (Table 3). From the calculated k_{cat}/K_m ratios, it can be

TABLE 4. Enantioselectivity of the L-amino amidase enzyme of *M. neoaurum* ATCC 25795[a]

Substrate	$ee_{L\text{-acid}}$[b]	c[c]	E values[d]
DL-Ala-NH_2	0.85	0.49	≥25
DL-Val-NH_2	>0.99	0.48	≥1,000
DL-PG-NH_2	>0.99	0.49	≥1,000
DL-α-CH_3-Val-NH_2	>0.99	0.27	≥500
DL-α-CH_3-Phe-NH_2	>0.98	0.34	≥400

[a] *ee*, enantiomeric excess; *c*, conversion.
[b] $ee_{L\text{-acid}} = (L_{acid} - D_{acid})/(L_{acid} + D_{acid})$.
[c] $c = [(L_{acid} + D_{acid})/(L_{amide\ (t\ =\ 0)} + D_{amide\ (t\ =\ 0)})] \times 100\%$.
[d] $E = \{\ln[1 - c(1 + ee_{L\text{-acid}})]\}/\{\ln[1 - c(1 - ee_{L\text{-acid}})]\}$.

concluded that an additional methylene group adjacent to the C_α atom in the substrate results in an increased affinity of the enzyme system, e.g., for DL-α-CH_3-Leu-NH_2, DL-α-CH_3-Phe-NH_2, DL-α-CH_3-homoPhe-NH_2, and DL-allyl-Ala-NH_2. In contrast, for substrates with a bulky group directly adjacent to the C_α position, such as DL-α-CH_3-Val-NH_2 or DL-α-CH_3-PG-NH_2, the affinity is much lower. Moderate affinities were determined for the substrates DL-isoVal-NH_2 and DL-Pro-NH_2.

Enantioselectivity. The purified enzyme system displayed L-specific amino amidase activity towards both α-alkyl-substituted amino acid amides, e.g., α-CH_3-Val-NH_2 and α-CH_3-Phe-NH_2, and α-H amino acid amides, e.g., Ala-NH_2, Val-NH_2, and PG-NH_2. The calculated *E* values, as a measure for the level of enantioselectivity, varied from approximately 25 for Ala-NH_2 to more than 1,000 for Val-NH_2 and PG-NH_2 (Table 4).

DISCUSSION

This article describes the purification and characterization of the L-amino amidase enzyme system of *M. neoaurum* ATCC 25795. This system has found application as a whole-cell biocatalyst in a commercial chemoenzymatic resolution process, yielding enantiomerically pure D- and L-α-alkyl-substituted amino acids (10).

A combination of ammonium sulfate fractionation, gel filtration, and anion-exchange chromatography resulted in a homogeneous preparation of the L-amino amidase enzyme with a native molecular mass of 136 kDa and a subunit molecular mass of 40 kDa. These results suggest a tri- or tetrameric structure for the L-amino amidase enzyme. The enzyme system has a reasonably high temperature optimum (50°C) and is fairly stable.

Proteinase enzyme systems can be classified as serine, cysteine, aspartic, and metallo-proteinases as reviewed by Kalisz (8). The purified enzyme was strongly inhibited by 1,10-phenanthroline, an inhibitor of metalloproteinases, although EDTA had no significant effect (Fig. 1). Also, IAA and p-chloromercuribenzoate, typical inhibitors of cysteine proteinases, gave contradictory results. IAA caused a clear inhibition of enzyme activity, whereas p-chloromercuribenzoate had no significant effect. Serine proteinase inhibitors, like DFP or phenylmethylsulfonyl fluoride, displayed no inhibitory effect on the enzyme activity, and high concentrations of DFP (1 and 10 mM) even showed stimulating effects. The strong inhibitory effect of the disulfide-reducing agent dithiothreitol may indicate that secondary structures, such as disulfide bonds, are essential for maintaining enzyme activity. The inhibitory effects of the metal-chelating agent 1,10-phenanthroline and the sulfhydryl reagent IAA suggest that metal ions and sulfhydryl groups have a major role in the hydrolytic mechanism. The properties of this amidase enzyme are closely analogous to metallocysteine proteinases, but further studies are required for a full classification.

Classification of the enzyme on the basis of the spectrum of amide substrates appeared to be more convenient. In this respect, the enzyme clearly was an amino amidase with a broad specificity for α-H- and α-alkyl-substituted amino acid amides (Tables 2 and 3). The physiological function of the enzyme remains to be elucidated. The *M. neoaurum* enzyme combines the unique properties of a broad substrate specificity and high enantioselectivity for both α-H- and α-alkyl-substituted amino acid amides, resulting in sole hydrolysis of the L-enantiomer (Table 4). Only with an H atom or a methyl group at the R^1 position can the R^2 substitute be varied without losing activity (Table 2, model formula). If the R^1 substitute is larger than a methyl group, the R^2 substitute requires an additional methylene group to maintain enzyme activity, e.g., α-ethyl-PG-NH_2 versus α-ethyl-Phe-NH_2. In the case of a methyl group at the R^1 position, the presence of an additional methylene group between the C_α atom and the rest of the R^2 group, e.g. phenyl or isopropyl, causes a 10- to 60-fold increase in the affinity of the enzyme (Table 3).

Several (amino) amidase and aminopeptidase enzyme systems have been purified and characterized (reviewed in reference 14). The substrate specificity and enantioselectivity of the enzyme system from *M. neoaurum*, however, appear unique.

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