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Purification and Characterization of an L-Aminopeptidase from *Pseudomonas putida* ATCC 12633

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An L-aminopeptidase of *Pseudomonas putida*, used in an industrial process for the hydrolysis of D,L-amino acid amide racemates, was purified to homogeneity. The highly L-enantioselective enzyme resembled thiol reagent-sensitive alkaline serine proteinases and was strongly activated by divalent cations. It possessed a high substrate specificity for dipeptides and α-H amino acid amides, e.g., L-phenylglycine amide.

Various chemoenzymatic processes involving resolution of racemic mixtures for the production of chiral compounds have found commercial applications (16). Examples are processes for enantiomerically pure amino acids, versatile chiral building blocks for the synthesis of pharmaceuticals, agrochemicals, and food or feed additives (7–9, 12, 13). Knowledge on the substrate specificity and enantioselectivity of the proteinases, amidases, and aminopeptidases involved in these processes is limited (6, 7, 10, 11, 14). Here we report the purification and characterization of an L-aminopeptidase from *Pseudomonas putida* ATCC 12633, used as a whole-cell biocatalyst for the enantioselective hydrolysis of a broad range of D,L-amino acid amide racemates (1, 8).

The racemic mixtures of amino acid amides used were obtained as follows. Starting out from the corresponding aldehydes, aminonitriles were derived via the Strecker reaction (HCN, NH_3) and converted into the amides under alkaline conditions in the presence of a catalytic amount of a ketone. Enantiomerically pure amide and acid derivatives of phenylglycine (PG) and valine were prepared according to the enzymatic resolution process of DSM (1, 8). Other enantiomerically pure amides were from Bachem (Bubendorf, Switzerland).

P. putida ATCC 12633 was grown in 10 liters of mineral medium (1.55 g of K_2HPO_4, 0.79 g of $NaH_2PO_4 \cdot H_2O$, 0.077 g of $MgCl_2 \cdot 6H_2O$, 0.2 ml of trace element solution [15] in 1 liter of distilled water, all heat sterilized) with 15 mM sodium citrate (heat sterilized) and 10 mM L-valine amide (L-Val-NH_2; filter sterilized) at 30°C and pH 7 to 7.5 in a 15-liter fermentor. Cells (about 40 g [wet weight]) were harvested from the late stationary phase by centrifugation (15,000 × *g* at 4°C for 15 min).

The L-aminopeptidase, routinely assayed at 40°C in 200 mM Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) · KOH, pH 9.0, with L-PG-NH_2 (133 mM) as a substrate, was purified (at 4°C) to homogeneity by a combination of French pressure cell disintegration (at 1,000 MPa; 1.4×10^5 kN · m^{-2}), isolation of cell envelopes by centrifugation (at 70,000 × *g* at 4°C for 45 min), extraction of the enzyme from the cell envelopes with $MgSO_4$ (100 mM), and Mono S HR 5/5 cation-exchange chromatography (Table 1; Fig. 1). Detergents such as Triton X-100, successfully used to extract an arginine aminopeptidase from membranes of *Streptococcus sanguis* 903 (4), were ineffective in this case. Further characterization of the protein revealed a native molecular mass of approximately 400 kDa (by gel filtration chromatography on a Hiload 26/60 Superdex 200 column) and subunits of 53 kDa (by sodium dodecyl sulfate [SDS]-

TABLE 1. Purification of L-aminopeptidase from *P. putida* ATCC 12633

Purification step	Total activity (μmol · min^{-1})	Total protein (mg)	Sp act (μmol · min^{-1} · mg^{-1})	Recovery (%)	Purification (fold)
Whole cells	4,507	657	7	100	1
Cell envelopes	2,342	194	12	52	1.7
Cell envelope extract	1,453	24	62	32	9
Mono S	946	3	338	21	49

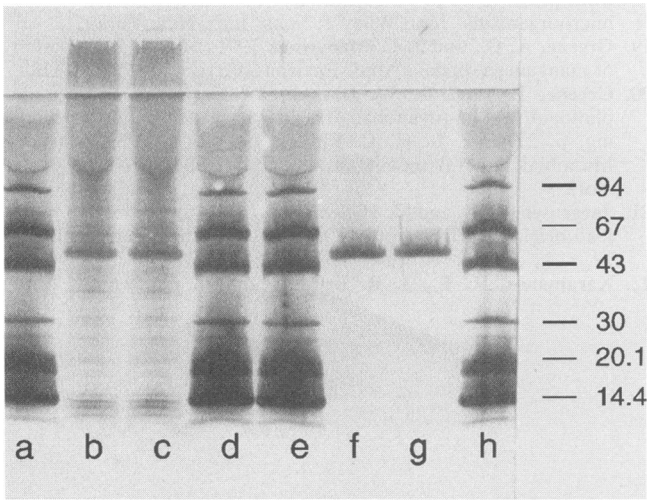

FIG. 1. SDS-PAGE of the enzyme fractions obtained during purification of the L-aminopeptidase. Electrophoresis was performed with molecular weight standard proteins (lanes a, d, e, and h), 100 ng of cell envelope extract (lanes b and c), and 50 ng of protein after Mono S HR 5/5 column chromatography (lanes f and g). Protein bands were visualized by silver staining. (Coreldraw 3.0 format.)

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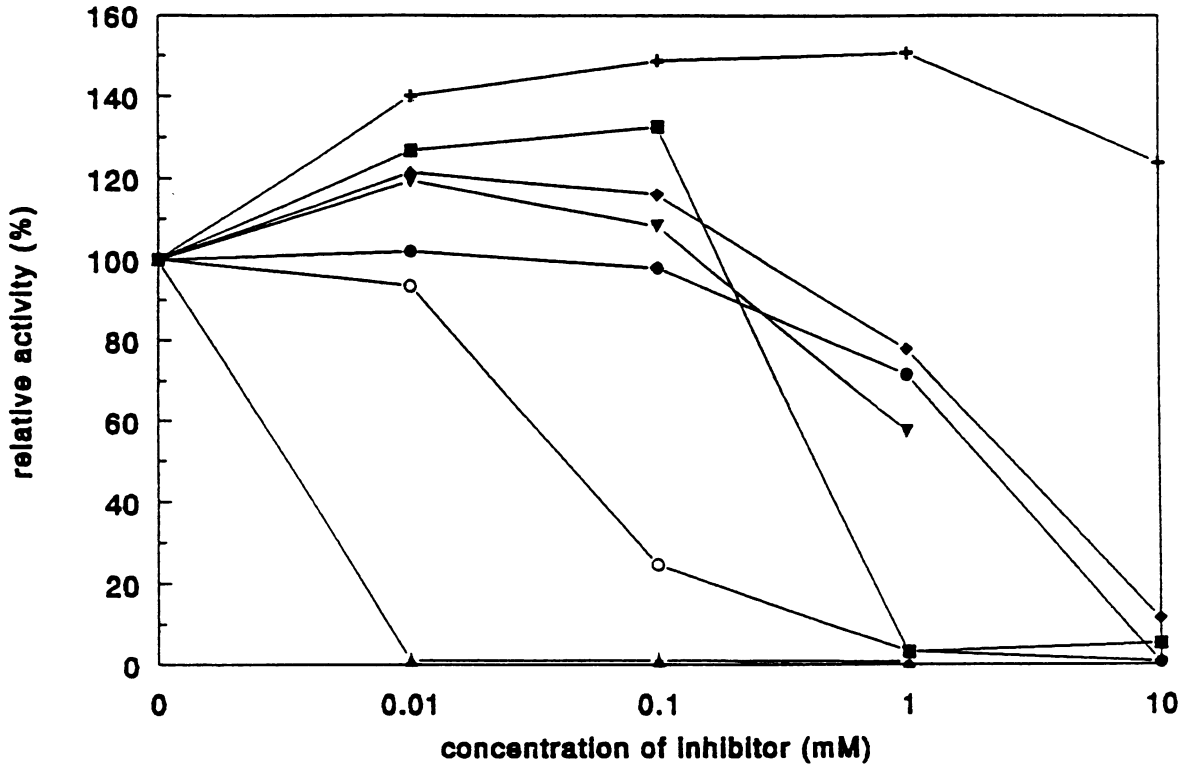


FIG. 2. Effects of several proteinase inhibitors on the L-aminopeptidase activity. Purified enzyme (1.3 μg) was incubated with compounds to be tested in 12.5 mM Tris · H_2SO_4, pH 8.0, at 40°C for 30 min, and the residual activity was determined in the standard assay. Activity is expressed as a percentage of the activity of the nonpreincubated enzyme, with 100% activity corresponding to 363 μmol · min^{-1} · mg^{-1}. Symbols: ○, diisopropylfluorophosphate; ●, iodoacetamide; ▼, phenylmethylsulfonyl fluoride; ◆, *o*-phenanthroline; ▲, *p*-chloromercuribenzoate; ■, EDTA; +, dithiothreitol.

polyacrylamide gel electrophoresis [PAGE] [Fig. 1]), suggesting that it has an octameric structure. The native molecular weight of the *P. putida* enzyme is relatively high but comparable to, for instance, that of a *Sulfolobus* aminopeptidase (5). The isoelectric point of the protein was estimated at pH 10.5 (by using an IEF Phastgel with an expanded pH range), which is extremely high compared with those of other aminopeptidases (11). Highest enzyme activity was observed at pH 9.5 and 40°C.

Divalent cations had a marked effect on enzyme activity: Mg^{2+}, Co^{2+} (2- to 3-fold), and especially Mn^{2+} ions (12-fold) stimulated activity (at 0.2 to 2 mM), whereas treatment with Cu^{2+} and Ca^{2+} ions (at 2 mM) caused 70 and 40% inhibition, respectively. Similar observations have been made for various other aminopeptidases (11).

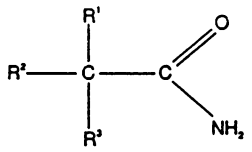
The *P. putida* L-aminopeptidase was sensitive to various proteinase inhibitors (Fig. 2), e.g., the serine protease inhibitors phenylmethylsulfonyl fluoride and diisopropylfluorophosphate. The enzyme thus resembles the alkaline serine proteinases (6). In addition, the enzyme was inhibited by the chelators EDTA and *o*-phenanthroline and the thiol reagents *p*-chloromercuribenzoate and iodoacetamide but was stimulated by dithiothreitol (Fig. 2). These phenomena may be due to the presence of a cysteine residue near the active site and the involvement of divalent metal ions in the catalytic mechanism.

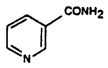
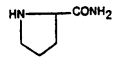
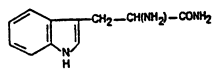
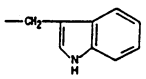
The substrate specificity of the purified enzyme (1.2 to 1.8 μg) was tested with a range of amide substrates (50 to 100 mM) (Table 2) and dipeptides (2 mM). The ammonium ions produced from the amide substrates were determined with an ammonia electrode system (Orion model 95-12). Amino acids produced from dipeptides were qualitatively analyzed on silica gel 60 F_{254} thin-layer chromatography plates (E. Merck AG, Darmstadt, Germany) by using a chloroform-methanol-ammonia (60:45:20) mixture as a mobile phase, followed by spraying with a 0.3% ninhydrin solution and incubation at 125°C for 5 min. The enzyme was only active with α-amino acid amides with a H atom at the C_α position. Highest activities were observed with L-Leu-NH_2 and L-PG-NH_2. The enzyme was inactive with L-α-CH_3-Val-NH_2, the single α-methyl-substituted amino acid amide tested (Table 2). High activities were also observed with the four aliphatic and aromatic dipeptides tested (L-Phe-L-Phe, L-Phe-L-Leu, L-Leu-L-Phe, and L-Leu-L-Leu), designating the purified enzyme as an L-aminopeptidase.

The kinetic properties (K_m and V_{max} values) of the purified enzyme (1.8 μg) were estimated from double-reciprocal and Eadie-Hofstee plots of initial rates of hydrolysis of amino acid amides as a function of substrate concentration (Table 3). The enzyme displayed normal Michaelis-Menten type of kinetics for the six L-α-amino acid amides tested (Table 3), the products of which were determined by high-pressure liquid chromatography (HPLC) analysis (2). The additional methyl groups at the C atoms (C_β) adjacent to the C_α atoms in L-Val-NH_2 and L-Ile-NH_2 appears to result in relatively high K_m and low V_{max} values and consequently a low catalytic efficiency and specificity (k_{cat}/K_m values) for these substrates. The highest k_{cat}/K_m value was observed for L-Leu-NH_2, where this methyl group at the C_β atom is missing (Tables 2 and 3). Similarly, amongst the two aro-

TABLE 2. Substrate specificity of the purified L-aminopeptidase of *P. putida* ATCC 12633

Model

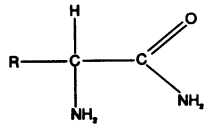


Substrate	Formula	R^1	R^2	R^3	Relative activity
Acetamide [a]	CH_3-$CONH_2$	-H	-H	-H	<5
Propionamide [a]	CH_3-CH_2-$CONH_2$	-H	-CH_3	-H	<5
Butyramide [a]	CH_3-CH_2-CH_2-$CONH_2$	-H	-CH_2-CH_3	-H	<5
Isobutyramide [a]	$(CH_3)_2$CH-$CONH_2$	-H	-CH_3	-CH_3	<5
Acrylamide [a]	CH_2=CH-$CONH_2$	-H	=CH_2	-	<5
Fluoroacetamide [a]	F-CH_2-$CONH_2$	-H	-H	-F	<5
D,L-Mandelic acid amide [a]	C_6H_5-CH(OH)-$CONH_2$	-H	-C_6H_5	-OH	<5
Pivalamide [a]	$(CH_3)_3$C-$CONH_2$	-CH_3	-CH_3	-CH_3	<5
Methacrylamide [a]	CH_2=C(CH_3)-$CONH_2$	-CH_3	=CH_2	-	<5
Nicotinamide [a]					<5
Glycine amide [b]	H-CH(NH_2)-$CONH_2$	-H	-H	-NH_2	<5
L-Alanine amide [b]	CH_3-CH(NH_2)-$CONH_2$	-H	-CH_3	-NH_2	<5
L-α-Aminobutyramide [b]	CH_3-CH_2-CH(NH_2)-$CONH_2$	-H	-CH_2-CH_3	-NH_2	10
L-Valine amide [b]	$(CH_3)_2$CH-CH(NH_2)-$CONH_2$	-H	-$CH(CH_3)_2$	-NH_2	5
L-Leucine amide [b]	$(CH_3)_2$CH-CH_2-CH(NH_2)-$CONH_2$	-H	-CH_2-$CH(CH_3)_2$	-NH_2	215
L-Isoleucine amide [b]	CH_3-CH_2-CH(CH_3)-CH(NH_2)CONH2	-H	-CH(CH_3)-CH_2-CH_3	-NH_2	15
L-Phenylglycine amide [a,b]	C_6H_5-CH(NH_2)-$CONH_2$	-H	-C_6H_5	-NH_2	100
L-Phenylalanine amide [b]	C_6H_5-CH_2-CH(NH_2)-$CONH_2$	-H	-CH_2-C_6H_5	-NH_2	15
L-Methionine amide [b]	CH_3-S-CH_2-CH_2-CH(NH_2)-$CONH_2$	-H	-CH_2-CH_2-S-CH_3	-NH_2	60
D,L-Proline amide [a]		-H	-CH_2-CH_2-CH_2-	-NH-	<5
L-Tryptophan amide [b]		-H		-NH_2	15
L-Serine amide [b]	HO-CH_2-CH(NH_2)-$CONH_2$	-H	-CH_2OH	-NH_2	<5
L-Glutamic acid amide [b]	HOOC-CH_2-CH_2-CH(NH_2)-$CONH_2$	-H	-CH_2-CH_2-COOH	-NH_2	<5
D,L-α-Methyl valine amide [a]	$(CH_3)_2$CH-C(CH_3)(NH_2)-$CONH_2$	-CH_3	-$CH(CH_3)_2$	-NH_2	<5

[a] Reactions were performed in Tris-H_2SO_4 (pH 8.0); activity towards L-PG-NH_2 (100%) was 180 μmol · min^{-1} · mg of $protein^{-1}$.
[b] Reactions were performed in TAPS-KOH (pH 9.0); activity towards L-PG-NH_2 was 490 μmol · min^{-1} · mg of $protein^{-1}$.

TABLE 3. Kinetic parameters for the hydrolysis of different amino acid amides by purified L-aminopeptidase of *P. putida* ATCC 12633

Model



Substrate	-R	K_m[a]	V_{max}[b]	k_{cat}[c]	k_{cat}/K_m[d]
L-PG-NH_2	-C_6H_5	65	1,565	1,380	20,580
L-Phe-NH_2	-CH_2-C_6H_5	15	80	68	4,525
L-Phe-NH_2.HCl	-CH_2-C_6H_5	15	70	65	3,910
L-Val-NH_2.HCl	-$CH(CH_3)_2$	130	110	95	715
L-Leu-NH_2.HCl	-CH_2-$CH(CH_3)_2$	15	1,915	1,690	99,215
L-Ile-NH_2.HCl	-$CH(CH_3)$-CH_2-CH_3	150	270	240	1,604

[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.

matic amino acid amides tested, the lowest K_m value was observed with L-Phe-NH_2 containing a C_β atom without further substituents. However, in this case the highest catalytic efficiency was observed with L-PG-NH_2, indicating that the presence of an aromatic ring directly adjacent to the C_α atom has a strongly positive effect.

The enantioselectivity of the purified enzyme (2.1 μg) towards racemic mixtures of Leu-NH_2 and PG-NH_2 (133 mM) was studied by chiral HPLC (3) to determine the concentrations of both the amino acid and the amino acid amide enantiomers after overnight incubation. The enzyme system displayed high L-enantioselectivity towards both the aliphatic and the aromatic α-H amino acid amide. The enantiomeric excess of the L-amino acids formed varied from 96% for PG to more than 99% for Leu, corresponding with calculated E values of 79 and more than 1,000, respectively (Table 4).

Comparison of the characteristics of this *P. putida* aminopeptidase with those of other aminopeptidases and (amino) amidases described (e.g., reference 11) leads to the conclusion that this enzyme is unique not only with respect to its physicochemical characteristics but especially also concerning its high enantioselectivity and substrate specificity. Further studies are required to elucidate the physiological role of the enzyme and its reaction mechanism.

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TABLE 4. Enantioselectivity of the L-aminopeptidase of *P. putida* ATCC 12633

Substrate	$ee_{L\text{-acid}}$[a]	c[b]	E[c]
D,L-PG-NH_2	0.96	38	79
D,L-Leu-NH_2	>0.99	46	>1,000

[a] $ee_{L\text{-acid}}$ = (L-acid − D-acid)/(L-acid + D-acid). ee, enantiomeric excess.
[b] c = (L-acid + D-acid)/(L-$amide_{t=0}$ + D-$amide_{t=0}$) × 100. c, conversion.
[c] $E = \ln[1 - c(1 + ee_{L\text{-acid}})]/\ln[1 - c(1 - ee_{L\text{-acid}})]$.

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