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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-aminoc 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 CαH 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-aminoc acid amide racemates (1, 8).

The racemate mixtures of amino acid amides used were obtained as follows. Starting out from the corresponding aldehydes, aminonitriles were derived via the Strecker reaction (HCN, NH₃) 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 chemoenzymatic processes involving resolution of racemic mixtures have found commercial applications.

1. *P. putida* ATCC 12633 was grown in 10 liters of mineral medium (1.55 g of KH₂PO₄, 0.79 g of NaH₂PO₄·H₂O, 0.077 g of MgCl₂·6H₂O, 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-NH₂; 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-NH₂ (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 × 10⁵ 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 MgSO₄ (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

<table>
<thead>
<tr>
<th>TABLE 1. Purification of L-aminopeptidase from <em>P. putida</em> ATCC 12633</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Purification step</strong></td>
</tr>
<tr>
<td>----------------------</td>
</tr>
<tr>
<td>Whole cells</td>
</tr>
<tr>
<td>Cell envelopes</td>
</tr>
<tr>
<td>Cell envelope extract</td>
</tr>
<tr>
<td>Mono S</td>
</tr>
</tbody>
</table>

* Corresponding author.

**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 Hifoad 26/60 Superdex 200 column) and subunits of 53 kDa (by sodium dodecyl sulfate [SDS]-

---

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.)

4330
p-chloromercuribenzoate and iodoacetamide
the involvement of divalent (6).
produced from the 
lated proteinases (6).
chelators phosphate. The
proteinase activity (at 
stimulated with 
Mg2+, comparable to,
ular weight 
ions (Fig. 1).
Divalent cations 
Ca2+ and the thiol 
alkaline serine 
products observed with 
the serine 
substrate 
function of 
substrate 
substrates (50 to 100 
(Table 2) and dipeptides (2 mM). The ammonium ions 
substrate amides tested
high
function of 
substrate amides tested
activity of the nonpreincubated enzyme, with 100% activity corresponding to 363 
mlol · 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: Mg2+ and Ca2+ (2- to 3-fold), and especially Mn2+ ions (12-fold) stimulated activity (at 0.2 to 2 mM), whereas treatment with Cu2+ and Cd2+ 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 protease 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 
substrate amides were determined with 
an ammonia electrode system (Orion model 95-12). Amino 
substrate amides were determined with acids produced from dipeptides were qualitatively analyzed 
on silica gel. The enzyme was tested with compounds to be tested in 12.5 mM Tris · H2SO4, 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.

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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Formula</th>
<th>( R' )</th>
<th>( R^2 )</th>
<th>( R^3 )</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetamide (^a)</td>
<td>( \text{CH}_3-\text{CONH}_2 )</td>
<td>-H</td>
<td>-H</td>
<td>-H</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Propionamide (^a)</td>
<td>( \text{CH}_3-\text{CH}_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}_3</td>
<td>-H</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Butyramide (^a)</td>
<td>( \text{CH}_3-\text{CH}_2-\text{CH}_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}_3-\text{CH}_3</td>
<td>-H</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Isobutyramide (^a)</td>
<td>( (\text{CH}_3)_2-\text{CH}-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}_3</td>
<td>-\text{CH}_3</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Acrylamide (^a)</td>
<td>( \text{CH}_2-\text{CH}-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}=\text{CH}_2</td>
<td>-</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Fluoroacetamide (^a)</td>
<td>( \text{F-CH}_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{H}</td>
<td>-F</td>
<td>&lt;5</td>
</tr>
<tr>
<td>D.L-Mandelic acid amide (^a)</td>
<td>( \text{C}_6\text{H}_5-\text{CH(OH)}-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{C}_2=\text{H}_4</td>
<td>-\text{OH}</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Pivalamide (^a)</td>
<td>( (\text{CH}_3)_2-\text{CH}-\text{CONH}_2 )</td>
<td>-CH_3</td>
<td>-\text{CH}_3</td>
<td>-\text{CH}_3</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Methacrylamide (^a)</td>
<td>( \text{CH}_2=\text{C}(\text{CH}_3)-\text{CONH}_2 )</td>
<td>-\text{CH}_3</td>
<td>-\text{CH}=\text{CH}_2</td>
<td>-</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Nicotinamide (^a)</td>
<td>( \text{C}=\text{C}(\text{CH}_2)=\text{CONH}_2 )</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Glycine amide (^b)</td>
<td>( \text{H-CH(NH)}_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{H}</td>
<td>-\text{NH}_3</td>
<td>&lt;5</td>
</tr>
<tr>
<td>L-Alanine amide (^b)</td>
<td>( \text{CH}_3-\text{CH(NH)}_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}_3</td>
<td>-\text{NH}_2</td>
<td>&lt;5</td>
</tr>
<tr>
<td>L-(\alpha)-Aminobutyramide (^b)</td>
<td>( \text{CH}_3-\text{CH}_2-\text{CH(NH)}_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}_2-\text{CH}_3</td>
<td>-\text{NH}_2</td>
<td>10</td>
</tr>
<tr>
<td>L-Valine amide (^b)</td>
<td>( (\text{CH}_3)_2-\text{CH(NH)}_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}=\text{CH}(\text{CH}_3)_2</td>
<td>-\text{NH}_2</td>
<td>5</td>
</tr>
<tr>
<td>L-Leucine amide (^b)</td>
<td>( (\text{CH}_3)_2-\text{CH}-\text{CH(NH)}_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}=\text{CH}(\text{CH}_3)_2</td>
<td>-\text{NH}_2</td>
<td>215</td>
</tr>
<tr>
<td>L-Isoleucine amide (^b)</td>
<td>( \text{CH}_3-\text{CH}=\text{CH}(\text{CH}_3)-\text{CH(NH)}_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}(\text{CH}_3)-\text{CH}=\text{CH}_3</td>
<td>-\text{NH}_2</td>
<td>15</td>
</tr>
<tr>
<td>L-Phenylglycine amide (^ab)</td>
<td>( \text{C}_6\text{H}_5-\text{CH(NH)}_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{C}_6=\text{H}_4</td>
<td>-\text{NH}_2</td>
<td>100</td>
</tr>
<tr>
<td>L-Phenylalanine amide (^a)</td>
<td>( \text{C}_6\text{H}_5-\text{CH}_2-\text{CH(NH)}_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}_2-\text{C}_6=\text{H}_4</td>
<td>-\text{NH}_2</td>
<td>15</td>
</tr>
<tr>
<td>L-Methionine amide (^b)</td>
<td>( \text{CH}_3-\text{S-CH}_2-\text{CH}=\text{CH}(\text{CH}_3)-\text{CH(NH)}_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}_2-\text{S-CH}_2-\text{CH}_3</td>
<td>-\text{NH}_2</td>
<td>60</td>
</tr>
<tr>
<td>D.L-Proline amide (^a)</td>
<td>( \text{CH}_3-\text{CH(NH)}_2-\text{CH}=\text{CH}(\text{CH}_3)-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}=\text{CH}_3-\text{CH}_2-\text{CH}_3</td>
<td>-\text{NH}-</td>
<td>&lt;5</td>
</tr>
<tr>
<td>L-Tryptophan amide (^b)</td>
<td>( \text{C}=\text{C}(\text{CH}_2)=\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}=\text{CH}(\text{CH}_3)-\text{CONH}_2</td>
<td>-\text{NH}_2</td>
<td>15</td>
</tr>
<tr>
<td>L-Serine amide (^b)</td>
<td>( \text{HO-CH}=\text{CH}(\text{NH})_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}=\text{OH}</td>
<td>-\text{NH}_2</td>
<td>&lt;5</td>
</tr>
<tr>
<td>L-Glutamic acid amide (^b)</td>
<td>( \text{HOOC-CH}_2-\text{CH}=\text{CH}(\text{NH})_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}_2-\text{CH}(\text{COOH})</td>
<td>-\text{NH}_2</td>
<td>&lt;5</td>
</tr>
<tr>
<td>D.L-(\alpha)-Methyl valine amide (^a)</td>
<td>( (\text{CH}_3)_2-\text{CH}=(\text{CH}=\text{CH})_2-\text{CONH}_2 )</td>
<td>-H</td>
<td>-\text{CH}_3</td>
<td>-\text{CH}(\text{CH}_3)_2</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

\(^a\) Reactions were performed in Tris-H_2SO_4 (pH 8.0); activity towards L-PG-NH_2 (100%) was 180 \( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1} \).

\(^b\) Reactions were performed in TAPS-KOH (pH 9.0); activity towards L-PG-NH_2 was 490 \( \mu \text{mol} \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1} \).
matic amino acid amides tested, the lowest $K_m$ value was observed with L-Phe-NH$_2$ containing a C$_8$ 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$_\alpha$ atom has a strongly positive effect.

The enantioselectivity of the purified enzyme (2.1 $\mu$g) 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 $\alpha$-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) amidasises 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.

Thanks are due to W. Kortenoeven for preparation of the figures and tables, L. Kerkhoffs and B. Dassen for the preparation of the amino acids and derivatives, B. Kaptin for evaluations on the enantioselectivity and substrate specificity, and W. Harder for stimulating discussions.

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