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 dipetides and α-H amino acid amides, e.g., L-phenylglycine amide.

<table>
<thead>
<tr>
<th>TABLE 1. Purification of L-aminopeptidase from <em>P. putida</em> ATCC 12633</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purification step</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>

* 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 Hiloa 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.)
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\(^{2+}\), Ca\(^{2+}\) (2- to 3-fold), and especially Mn\(^{2+}\) (12-fold) stimulated activity (at 0.2 to 2 mM), whereas treatment with Cu\(^{2+}\) and Cd\(^{2+}\) 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 \(\mu\)g) 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 \(\alpha\)-amino acid amides with a H atom at the \(C_a\) position. Highest activities were observed with L-Leu-NH\(_2\) and L-PG-NH\(_2\). The enzyme was inactive with L-\(\alpha\)-CH\(_3\)-Val-NH\(_2\), the single \(\alpha\)-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 \(V_{\text{max}}\) values) of the purified enzyme (1.8 \(\mu\)g) 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-\(\alpha\)-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 (\(C_\beta\)) adjacent to the \(C_a\) atoms in L-Val-NH\(_2\) and L-Ile-NH\(_2\) appears to result in relatively high \(K_m\) and low \(V_{\text{max}}\) values and consequently a low catalytic efficiency and specificity (\(k_{\text{cat}}/K_m\) values) for these substrates. The highest \(k_{\text{cat}}/K_m\) value was observed for L-Leu-NH\(_2\), where this methyl group at the \(C_\beta\) 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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Formula</th>
<th>R'</th>
<th>R²</th>
<th>R³</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetamide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CH₃-C(ONH₂)</td>
<td>-H</td>
<td>-H</td>
<td>-H</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Propionamide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CH₃-C₂H₂-C(ONH₂)</td>
<td>-H</td>
<td>-CH₃</td>
<td>-H</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Butyramide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CH₃-C₂H₄-C(ONH₂)</td>
<td>-H</td>
<td>-CH₂CH₃</td>
<td>-H</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Isobutyramide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(CH₃)₂C(ONH₂)</td>
<td>-H</td>
<td>-CH₃</td>
<td>-CH₃</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Acrylamide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CH₂-C(ONH₂)</td>
<td>-H</td>
<td>-CH₂</td>
<td>.</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Fluoroacetamide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CH₂F-C(ONH₂)</td>
<td>-H</td>
<td>-H</td>
<td>-F</td>
<td>&lt;5</td>
</tr>
<tr>
<td>D.L-Mandelic acid amide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>C₅H₆-C(OH)-C(ONH₂)</td>
<td>-H</td>
<td>-C₂H₅</td>
<td>-OH</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Pivalamide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(CH₃)₂C(ONH₂)</td>
<td>-CH₃</td>
<td>-CH₃</td>
<td>-CH₃</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Methacrylamide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CH₂-C(CH₃)-C(ONH₂)</td>
<td>-CH₃</td>
<td>-CH₂</td>
<td>.</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Nicotinamide&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;5</td>
</tr>
<tr>
<td>Glycine amide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>H-C(CH₃₂-NH₂)-C(ONH₂)</td>
<td>-H</td>
<td>-H</td>
<td>-NH₂</td>
<td>&lt;5</td>
</tr>
<tr>
<td>L-Alanine amide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CH₃-C(CH₃₂-NH₂)-C(ONH₂)</td>
<td>-H</td>
<td>-CH₃</td>
<td>-NH₂</td>
<td>&lt;5</td>
</tr>
<tr>
<td>L-α-Aminobutyramide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CH₃-C₂H₂-C(CH₃₂-NH₂)-C(ONH₂)</td>
<td>-H</td>
<td>-CH₂CH₃</td>
<td>-NH₂</td>
<td>10</td>
</tr>
<tr>
<td>L-Valine amide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(CH₃)₂CH-C(CH₃₂-NH₂)-C(ONH₂)</td>
<td>-H</td>
<td>-CH(CH₃₂)</td>
<td>-NH₂</td>
<td>5</td>
</tr>
<tr>
<td>L-Leucine amide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(CH₃)₂C₂H₂-C(CH₃₂-NH₂)-C(ONH₂)</td>
<td>-H</td>
<td>-CH₂CH(CH₃₂)</td>
<td>-NH₂</td>
<td>215</td>
</tr>
<tr>
<td>L-Isoleucine amide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CH₃-C₂H₂-C(CH₃₂-NH₂)-C(ONH₂)</td>
<td>-H</td>
<td>-CH(CH₃₂)-CH₂CH₃</td>
<td>-NH₂</td>
<td>15</td>
</tr>
<tr>
<td>L-Phenyglycine amide&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>C₆H₅-C(CH₃₂-NH₂)-C(ONH₂)</td>
<td>-H</td>
<td>-C₆H₅</td>
<td>-NH₂</td>
<td>100</td>
</tr>
<tr>
<td>L-Phenylalanine amide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>C₆H₅-C₆H₂-C(CH₃₂-NH₂)-C(ONH₂)</td>
<td>-H</td>
<td>-CH₂C₆H₅</td>
<td>-NH₂</td>
<td>15</td>
</tr>
<tr>
<td>L-Methionine amide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CH₅S-C₂H₂-C(CH₃₂-NH₂)-C(ONH₂)</td>
<td>-H</td>
<td>-CH₂CH₅-S-CH₃</td>
<td>-NH₂</td>
<td>60</td>
</tr>
<tr>
<td>D.L-Proline amide&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>-H</td>
<td>-CH₂CH₃-CH₂</td>
<td>-NH₂</td>
<td>&lt;5</td>
</tr>
<tr>
<td>L-Tryptophan amide&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>-H</td>
<td>-OH</td>
<td>-NH₂</td>
<td>15</td>
</tr>
<tr>
<td>L-Serine amide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>HO-C₂H₂-C(CH₃₂-NH₂)-C(ONH₂)</td>
<td>-H</td>
<td>-CH₂OH</td>
<td>-NH₂</td>
<td>&lt;5</td>
</tr>
<tr>
<td>L-Glutamic acid amide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>HOOC-C₂H₂-C(CH₃₂-NH₂)-C(ONH₂)</td>
<td>-H</td>
<td>-CH₂CH₂-COOH</td>
<td>-NH₂</td>
<td>&lt;5</td>
</tr>
<tr>
<td>D.L-α-Methyl valine amide&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(CH₃)₂CH-C(CH₃₂-NH₂)-C(ONH₂)</td>
<td>-CH₃</td>
<td>-CH(CH₃)</td>
<td>-NH₂</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reactions were performed in Tris-H₂SO₄ (pH 8.0); activity towards L-PG-NH₂ (100%) was 180 μmol·min⁻¹·mg of protein⁻¹.

<sup>b</sup> Reactions were performed in TAPS-KOH (pH 9.0); activity towards L-PG-NH₂ was 490 μmol·min⁻¹·mg of protein⁻¹.
matic amino acid amides tested, the lowest $K_m$ value was observed with L-Phe-NH$_2$ containing a C$_3$ 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$_3$ 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.

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. Kaptein for evaluations on the enantioselectivity and substrate specificity, and W. Harder for stimulating discussions.

**REFERENCES**

11. **Maestracci, M., K. Bui, A. Thiéry, A. Arnaud, and P. Galzy.** 1988. The amidases from a _Brevibacterium_ strain: study and

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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$V_{max}$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-PG-NH$_2$</td>
<td>1,565</td>
<td>20,580</td>
<td></td>
</tr>
<tr>
<td>L-Phe-NH$_2$</td>
<td>80</td>
<td>4,525</td>
<td></td>
</tr>
<tr>
<td>L-Phe-NH$_2$HCl</td>
<td>70</td>
<td>3,910</td>
<td></td>
</tr>
<tr>
<td>L-Val-NH$_2$HCl</td>
<td>110</td>
<td>715</td>
<td></td>
</tr>
<tr>
<td>L-Leu-NH$_2$HCl</td>
<td>1,915</td>
<td>99,215</td>
<td></td>
</tr>
<tr>
<td>L-Ile-NH$_2$HCl</td>
<td>270</td>
<td>1,604</td>
<td></td>
</tr>
</tbody>
</table>

$K_m$ is expressed in millimolar. 
$V_{max}$ is expressed in micromoles per minute per milligram of protein.
$k_{cat}$ is expressed per second.
$k_{cat}/K_m$ is expressed per second per millimeter.

**TABLE 4. Enantioselectivity of the L-aminopeptidase of _P. putida_ ATCC 12633**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>ee$_{L\text{-amino}}$</th>
<th>$E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D,L-PG-NH$_2$</td>
<td>0.96</td>
<td>79</td>
</tr>
<tr>
<td>D,L-Leu-NH$_2$</td>
<td>0.96</td>
<td>&gt;1,000</td>
</tr>
</tbody>
</table>

$ee_{L\text{-amino}} = (L\text{-acid} - d\text{-acid})/(L\text{-acid} + d\text{-acid})$. ee, enantiomeric excess.
$c = (L\text{-amine} - d\text{-amine})/(L\text{-amine} + d\text{-amine}) 	imes 100$, c, conversion.
$E = \ln(1 - c(1 + ee_{L\text{-amino}}))/\ln(1 - c(1 - ee_{L\text{-amino}}))$. 


