Novel Aminopeptidase Specific for Glycine from Actinomucor elegans

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Glycyl aminopeptidase was purified 600-fold from a cell extract of Actinomucor elegans by ammonium sulfate fractionation and sequential chromatography on DEAE-Toyopearl, Toyopearl HW65C, and FPLC-Superdex 200 HR, with recovery of 3.3% of the activity. The enzyme highly specifically hydrolyzed Gly-X (amino acid, peptide, or arylamide) bonds. The enzyme hydrolyzed other amino acid residues but at a rate of less than one fifth that with Gly. The order was Gly >> Ala >> Met >> Arg >> Ser >> Leu. The K_m value for glycyl-2-naphthylamide was 0.24 mM. The enzyme was most active at pH 8.0 with glycyl-2-naphthylamide as the substrate and its optimal temperature was 40°C. The enzyme was inhibited by iodoacetic acid, and p-chloromercuribenzoate but not by diisopropylfluorophosphate, o-phenanthroline, or EDTA. Magnesium and calcium had no effect on enzymic activity, but the activity was suppressed by cadmium, zinc, and copper ions. The molecular mass was estimated to be 320 kDa by gel filtration on FPLC-Superdex 200 HR and 56.5 kDa by SDS-PAGE, so the enzyme probably was a hexamer.

Key words: glycine; aminopeptidase; glycyl aminopeptidase; Actinomucor; peptidase

Of the aminopeptidases that have been studied, most have been leucine aminopeptidases (EC 3.4.11.2) or membrane alanine aminopeptidases (EC 3.4.11.1).8-11) These enzymes have a broad substrate specificity. However, some enzymes with narrow specificity, methionine aminopeptidase (EC 3.4.11.19),12) prolyl aminopeptidase (EC 3.4.11.5),13) cystinyl aminopeptidase (EC 3.4.11.3),14) arginyl aminopeptidase (EC 3.4.11.6),15) glutamyl aminopeptidase (EC 3.4.11.7),16) and lysyl aminopeptidase (EC 3.4.11.15)17) also have been reported. Some of these enzymes have particular biological significance.

Methionine aminopeptidase acts in the processing of protein synthesis.12) Cystinyl aminopeptidase is also named oxytocinase, and is involved in the control of oxytocine during pregnancy.10)

Glycine is the smallest of the 20 most common amino acids that make up proteins and the only one that has no stereoisomer. A Gly-X bond is very slowly hydrolyzed even by leucine aminopeptidase6) or alanine aminopeptidases11) that have broad specificity. An aminopeptidase highly specific for glycine residues is needed for basic studies of substrate recognition and for complete hydrolysis of peptides to amino acids. By screening microorganisms, we found glycyl aminopeptidase activity in A. elegans. This is the first report of a glycyl aminopeptidase.

Materials and Methods

Materials. Arg-NH2(2-naphthylamide), Gly-NH2, Leu-NH2, Met-NH2, <Glu-NH2, Ser-NH2, and Trp-NH2 were obtained from Bachem (Bubendorf, Switzerland). The other substrates used were from our laboratory stock. Actinomucor elegans IFO 6408 was obtained from the Institute of Fermentation, Osaka, and was maintained on potato sucrose agar, at 24°C. The medium for screening and producing the enzyme contained cotton seed protein, 2.0%; soybean protein, 0.5%; glucose, 0.5%; NaCl, 0.3%; K_2HPO_4, 0.1%; CaCO_3, 0.2%; and MgSO_4·7H_2O, 0.5%. For glycinyl aminopeptidase production, the A. elegans was cultivated in a 5-liter culture in a jar fermentor at 24°C for 4 days. The rate of aeration was from 1 to 1.5 liters per minute and the speed of rotation was 100 rpm. After the cultivation, the mycelia of A. elegans were collected by filtration for enzyme purification. For screening of glycinyl aminopeptidase, Gly-NH2 as substrate and proteins in medium were used.

Abbreviations: tRNA, 2-naphthylamide; Boc, t-Butylxycabonyl; DFP, diisopropylfluorophosphate; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’'-tetraacetic acid; FPLC, fast protein liquid chromatography; NMMec, 7-(4-methyl)coumarylamide; PCMB, p-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; Z,N-benzylxycarbonyl

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Activity assay. The method used to measure enzyme activity was based on that of Yoshimoto et al. To 0.8 ml of 20 mM Tris-HCl buffer, pH 7.0, was added 0.1 ml of Gly-bNA solution (3 mM, dissolved in the same buffer), the mixture was first incubated at 37°C, 0.1 ml of enzyme solution was added, and incubation was continued at 37°C for 10 min. The reaction was stopped by the addition of 0.5 ml of Fast Garnet GBC solution (1 mg/ml) containing 10% Triton X-100 in 1 M acetate buffer (pH 4.0). The reaction mixture was left for a few minutes at 37°C. The absorbance at 550 nm was measured. One unit of enzyme activity was defined as the amount that released 1 nmol of β-naphthylamine per minute under the standard conditions.

Peptide hydrolyzing activity was assayed by the ninhydrin method and products were checked by TLC. After the enzyme was incubated with 3 mM substrate in 20 mM Tris-HCl buffer, pH 7.0, at 37°C, 10 ml of the reaction mixture was assayed by the ninhydrin method. Another 10-μl portion of the reaction mixture was removed for identification of a newly formed amino acids by TLC with n-BuOH:AcOH:H₂O (4:1:2) as the solvent. Comparison was done with authentic markers.

Purification of glycyl aminopeptidase. All procedures for enzyme purification were done at 4°C. After fermentation, mycerria were suspended in the same buffer containing 50 mM ammonium sulfate. The mycelial suspension was centrifuged at 12,000 × g for 10 min. The supernatant, which contained most of the glycyl aminopeptidase activity, was dialyzed against 20 mM Tris-HCl, pH 7.0, and put on a column of DEAE-Toyopearl equilibrated with the same buffer. The enzyme was eluted by dialysis against 20 mM Tris-HCl buffer, pH 7.0, and put on a column of Toyopearl HW 65C previously equilibrated with the same buffer containing 50 mM ammonium sulfate. Another 10-μl portion of the enzyme solution was added, and incubation was continued at 37°C for 10 min. The reaction mixture was left for a few minutes at 37°C. The absorbance at 550 nm was measured. The enzyme was defined as the amount that released 1 μmol of β-naphthylamine per minute under the standard conditions.

The enzyme was eluted with a linear gradient of NaCl from 0 to 1.0 M. The active fractions were collected, removed for identification of a newly formed amino acids by TLC with n-BuOH:AcOH:H₂O (4:1:2) as the solvent. Comparison was done with authentic markers.

Protein assay. Absorbance at 280 nm was measured to estimate the protein concentration throughout the purification. Absorbance at 280 nm of 1.0 was assumed to indicate a protein concentration of 1.0 mg/ml. The purified enzyme concentration was measured by the method of Bradford with bovine serum albumin as the standard.

Measurement of molecular weight. The molecular weight of glycyl aminopeptidase was measured by SDS-PAGE. A 12.5% gel was used. The standards used in the experiment were produced by Amersham Biosciences. The molecular mass of the native enzyme was measured by gel filtration on FPLC-Superdex 200 HR.

Results

Screening for and purification of glycyl aminopeptidase

After screening several microorganisms, we found glycyl aminopeptidase activity in A. elegans IFO 6408. The mycelia (590 grams, wet weight) were suspended in 590 ml of 20 mM Tris-HCl buffer, pH 7.0, and disrupted with a blender. The debris of the cell walls was separated by centrifugation at 12,000 × g for 10 min. More than 95% of the activity of glycyl aminopeptidase remained in the supernatant and the supernatant was purified further.

The crude enzyme was first put on a DEAE-Toyopearl column (4 × 15 cm) and was purified 20-fold with a yield of 27%. Then, the enzyme was purified by hydrophobic interaction chromatography on the Toyopearl HW65C column (4 × 15 cm). The enzyme was eluted in fractions 45 to 60, with the peak at 54. Fractions 52–56 were collected and pooled. The enzyme was desalted by dialysis put on a Superdex 200 HR column, and washed out with the same buffer (Fig. 1). The active fractions were collected and pooled as the purified enzyme for further experiments.
Table 1. Steps and Results of Purification of Glycyl Aminopeptidase

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Activity (units)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>25000</td>
<td>600</td>
<td>0.024</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>329</td>
<td>160.4</td>
<td>0.49</td>
<td>27</td>
<td>20.4</td>
</tr>
<tr>
<td>Toyopearl HW65C</td>
<td>10</td>
<td>39.4</td>
<td>3.94</td>
<td>6.6</td>
<td>164</td>
</tr>
<tr>
<td>FPLC-Superdex 200 HR</td>
<td>1.4</td>
<td>19.9</td>
<td>14.2</td>
<td>3.3</td>
<td>592</td>
</tr>
</tbody>
</table>

Table 2. Substrate Specificity of the Glycyl Aminopeptidase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (μmol/min)</th>
<th>Relative activity value (%)</th>
<th>( K_m ) (mM)</th>
<th>( k_{cat} ) (sec(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-βNA</td>
<td>14.25</td>
<td>100</td>
<td>0.24</td>
<td>24.2</td>
</tr>
<tr>
<td>Ala-βNA</td>
<td>2.60</td>
<td>18.1</td>
<td>0.66</td>
<td>1.4</td>
</tr>
<tr>
<td>Met-βNA</td>
<td>0.91</td>
<td>6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg-βNA</td>
<td>0.90</td>
<td>6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ser-βNA</td>
<td>0.50</td>
<td>3.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu-βNA</td>
<td>0.37</td>
<td>2.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The following compounds were not hydrolyzed: Trp-βNA, Pro-βNA, Asp-βNA, Lys-βNA, Val-βNA, Cys-di-βNA, Glu-βNA, Thr-βNA, or <Glu-βNA.

Effects of pH and temperature on activity and stability of glycyl aminopeptidase

The optimum pH of the enzyme was 8.0 using Gly-βNA as the substrate (Fig. 3(A)). The enzyme retained more than 90% of its original activity between pH 6.0 and 10.0 after incubation at 30°C for 30 min (not shown). The optimum temperature was 40°C for a 10 min reaction and 50% of the initial activity was observed after incubation for 15 min at 30°C at pH 7.0 (Fig. 3(B)).

Substrate specificity of glycyl aminopeptidase

Gly-βNA (\( K_m \) of 0.24 mM and \( k_{cat} \) of 24.2 sec\(^{-1}\)) was the best substrate, and Ala-βNA (\( K_m \) of 0.66 mM and \( k_{cat} \) of 1.1 sec\(^{-1}\)), Met-βNA, Arg-βNA, Ser-βNA, and Leu-βNA also were hydrolyzed (Table 2). However, Trp-βNA, Pro-βNA, Asp-βNA, Lys-βNA, Val-βNA, Cys-di-βNA, Glu-βNA, Thr-βNA, and <Glu-βNA were not hydrolyzed at all. The enzyme also released the glycine from peptides such as Gly-Gly-Gly, Gly-Phe (0.71 μmol/min/mg), and Gly-Glu.
aminopeptidase N from for membrane-binding enzymes, for example the
However, it was not necessary to use detergent as was extracted from the mycelia with a homogenizer.
aminopeptidase is present inside cells, the enzyme was a hexamer. Aminopeptidases are of two varieties, monomers and oligomers, which usually exist in the membrane or periplasmic space. The aminopeptidases from Aspergillus oryzae and E. coli are monomers, 26.5 and 80 kDa, respectively. In contrast, the aminopeptidase from Aspergillus sojae is 220 kDa. A leucine aminopeptidase from humans is 360 kDa and cattle lens leucine aminopeptidase is 324 kDa. These are homohexamers. The crystal structure of the cattle lens enzyme is hexameric with a three-fold molecular axis. Glycyl aminopeptidase may have a hexameric structure similar to leucine aminopeptidase.

The enzyme hydrolyzed Gly-X bonds. There are two endopeptidases specific for the glycine residue. One is lysostaphin (EC 3.4.24.75), which hydrolyzes a Gly-Gly bond in the pentaglycine subunit of the cell wall peptidoglycan. The enzyme is a zinc endopeptidase and has a molecular mass of 27 kDa. The enzyme hydrolyzes not only polyglycine but also glycine-rich proteins such as insoluble elastin. Another is glycyl endopeptidase (EC 3.4.22.25) from Carica papaya latex. This enzyme is a sulfhydryl enzyme. It is interesting that the enzyme is able to hydrolyze Ala-X bonds. Substitution of Ala for Gly in the Boc-Ala-Ala-Gly-NHMec substrate at position P1 decreases the $k_{cat}$ 60-fold. In its amino acid sequence, it was similar to other cysteine endopeptidases from papaya latex, being 81% identical to carcin, 70% to chymopapain, and 67% to papain. The crystal structure confirms the overall similarity to papain, but with a much-altered S1 subsite, as the side chains of Glu23 and Arg65 form a barrier across the binding pocket and sterically exclude residues with large side chains, making a small S1 pocket for the glycine residue.

Since glycyl aminopeptidase was inhibited by PCMB and iodoacetic acid, it seemed to be a sulfhydryl enzyme like cysteine endopeptidases from papaya. There are few examples of thiol aminopeptidases: aminopeptidase C (pepC) from Lactococcus lactis, which resembles bleomycin hydrolase, and pyroglutamyl peptidase, which releases pyroglutamic acid at the amino terminal. However, most of the aminopeptidases reported so far are metallo-enzymes.

Proline and glycine have unusual structures and characteristics for amino acids. Proline is unusual in that it is cyclic. Several kinds of proline-specific peptidases have been studied. It is interesting that glycine-specific peptidases are present in living things.

We found the enzyme in an A. elegans which is used for the production of sufu, a traditional soybean cheese in China. Many peptidases from Lactococcus species produce a special taste in cheese. There are reports that A. elegans produces an acid proteinase, and glycyl peptidase may affect the taste of sufu.

### Table 3. Effect of Chemicals on Glycyl Aminopeptidase

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>DFP</td>
<td>1.0</td>
<td>103.3</td>
</tr>
<tr>
<td>Iodoacetic</td>
<td>1.0</td>
<td>41.7</td>
</tr>
<tr>
<td>PCMB</td>
<td>0.1</td>
<td>12.5</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>1.0</td>
<td>105</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.0</td>
<td>78.8</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>0.1</td>
<td>18.5</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>0.1</td>
<td>1.3</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>0.1</td>
<td>61.9</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>0.1</td>
<td>80.1</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>0.1</td>
<td>58.0</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.1</td>
<td>100.1</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.1</td>
<td>101.1</td>
</tr>
</tbody>
</table>

### Discussion

A glycyl aminopeptidase was purified to homogeneity from A. elegans IFO 6408. Since glycyl aminopeptidase is present inside cells, the enzyme was extracted from the mycelia with a homogenizer. However, it was not necessary to use detergent as for membrane-binding enzymes, for example the aminopeptidase N from E. coli.

The molecular mass of the enzyme was estimated to be 320 kDa by gel filtration on FPLC-Superdex 200 HR and 56.5 kDa by SDS-PAGE, suggesting that the enzyme was a hexamer. Aminopeptidases are of two varieties, monomers and oligomers, which usually exist in the membrane or periplasmic space. The aminopeptidases from Aspergillus oryzae and E. coli are monomers, 26.5 and 80 kDa, respectively. In
References

29) Yoshimoto, T., and Ito, K., Prolyl oligopeptidase. In