Esterolytic Properties of Leucine-Proteinase, the Leucine-Specific Serine Proteinase from Spinach (Spinacia oleracea L.) Leaves

A STEADY-STATE AND PRE-STEADY-STATE STUDY

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ABSTRACT

Steady-state and pre-steady-state kinetics for the hydrolysis of p-nitrophenyl esters of N-α-carbobenzoxy(-L-)-amino acids catalyzed by leucine-proteinase were determined between pH 5 and 10 (1 = 0.1 molar) at 23 ± 0.5°C. For the substrates considered: (a) the acylation step is rate-limiting in catalysis; (b) the pH profiles of kcat and kcat/Km reflect the ionization of two groups with pk values ranging between 6.5 and 6.9, and 8.1 and 8.3 (probably, the histidine residue involved in the catalytic triad and the N-terminus, respectively); and (c) values of Km are pH independent. Among the substrates examined, N-α-carbobenzoxy-L-leucine-p-nitrophenyl ester shows the most favorable catalytic parameters and allows to determine an enzyme concentration as low as 5 × 10^-10 molar at the optimum pH value (approximately 7.5).

In the course of solubilization and purification of fusococcin binding sites present in the microsomal fraction of spinach leaves (1), a new serine proteinase with a remarkable specificity for the leucine residue (hereafter Leu-proteinase) was identified and partially characterized (2, 3). The peculiar primary specificity of this serine proteinase, together with the role in spinach protein metabolism suggested for this enzyme by its presence not only in leaves, but also in seeds and roots (P Aducci, unpublished data), prompted our interest in a detailed kinetic investigation on the Leu-proteinase catalyzed hydrolysis of p-nitrophenyl esters of N-α-carbobenzyoxyp(-L-)-amino acids. Steady-state and pre-steady-state kinetics of Leu-proteinase have been analyzed in parallel with those of plant and animal serine endopeptidases.

MATERIALS AND METHODS

Leu-proteinase has been purified from the soluble fraction of spinach leaves by acetone precipitation and a combination of gel filtration, ion exchange and adsorption chromatography (3). The purity of Leu-proteinase was checked by PAGE and isoelectrofocusing; nonenzyme protein contaminants were less than 5% (3). ZGlyONp, ZAlaONp, ZValONp, ZLeuONp, ZArgONp, and ZLysONp, and acetonitrile were obtained from Sigma Chemical Co. ZArgONp was synthesized according to Glass and Pelzig (9). The characterization of substrates has been reported elsewhere (5, 6). The Leu-proteinase catalyzed hydrolysis of p-nitrophenyl esters was monitored spectrophotometrically at 360 nm and 23 ± 0.5°C with a VARIAN double-beam spectrophotometer (Cary 219) and a DURRUM-Gibson rapid-mixing stopped-flow apparatus (4).

Values of steady-state and pre-steady-state parameters were obtained from the experimental data according to the standard treatment of the catalytic mechanism of serine proteinases (4):

\[
\begin{align*}
E + S \rightleftharpoons E \cdot S \rightarrow E \cdot P \rightarrow E + P_2, \\
+ P_1
\end{align*}
\]

where \(E\) is the Leu-proteinase, \(S\) is the substrate, \(K_1\) is the dissociation preequilibrium constant for the formation of the reversible Leu-proteinase - substrate complex \((E \cdot S)\), \(k_{+2}\) is the acylation rate constant, \(E \cdot P\) is the acyl - intermediate, \(k_{-2}\) is the decylation rate constant, and \(P_1\) and \(P_2\) are the hydrolysis products. An average error value of ±8% was evaluated for kinetic parameters (4).

For other experimental details, see Aducci et al. (3) and Antonini and Ascenzi (4).

RESULTS AND DISCUSSION

Over the whole pH range explored (pH 5–10), the Leu-proteinase catalyzed hydrolysis of ZGlyONp, ZAlaONp, ZValONp, ZLeuONp, ZArgONp, and ZLysONp conforms to simple Michaelis-Menten kinetics, and steady-state and pre-steady-state data may be consistently fitted to the minimum three-step mechanism (Scheme 1), as indicated from the excellent correlation of the \(k_{cat}/K_m\) values (at \([S]_0 \approx 5 \times [E_0]\)) with those of \(K_{cat}/K_m\) (at \([E_0]_0 \approx 5 \times [S_0]\)) (Fig. 1 and Table I). Data shown

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Table 1. Values of Steady-State and Pre-Steady-State Parameters for the Leu-Proteinase Catalyzed Hydrolysis of p-Nitrophenyl Esters of N-o-Carboxybenzoyl-β-Amino Acids

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_{cat} \times 10^{-1}$</th>
<th>$k_m \times 10^{-1}$</th>
<th>$k_{cat}/K_m$</th>
<th>$k_{cat}/k_m$</th>
<th>$K_i \times 10^{-1}$</th>
<th>$k_{cat}/K_i$</th>
<th>$k_{cat}/k_m$</th>
<th>$k_{cat}/k_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZGlyONp</td>
<td>2.1</td>
<td>52.0</td>
<td>0.040</td>
<td>2.4</td>
<td>60.0</td>
<td>0.040</td>
<td>16.2</td>
<td></td>
</tr>
<tr>
<td>ZAlaONp</td>
<td>9.4</td>
<td>12.0</td>
<td>0.78</td>
<td>11.0</td>
<td>14.0</td>
<td>0.79</td>
<td>65.3</td>
<td></td>
</tr>
<tr>
<td>ZValONp</td>
<td>14.0</td>
<td>6.6</td>
<td>2.1</td>
<td>16.0</td>
<td>7.6</td>
<td>2.1</td>
<td>108.8</td>
<td></td>
</tr>
<tr>
<td>ZIleONp</td>
<td>14.0</td>
<td>6.5</td>
<td>2.2</td>
<td>16.0</td>
<td>7.4</td>
<td>2.2</td>
<td>113.8</td>
<td></td>
</tr>
<tr>
<td>ZLeuONp</td>
<td>43.0</td>
<td>1.8</td>
<td>23.9</td>
<td>50.0</td>
<td>2.1</td>
<td>23.8</td>
<td>303.5</td>
<td></td>
</tr>
<tr>
<td>ZArgONp</td>
<td>4.3</td>
<td>28.0</td>
<td>0.15</td>
<td>5.0</td>
<td>32.0</td>
<td>0.16</td>
<td>32.9</td>
<td></td>
</tr>
<tr>
<td>ZLysONp</td>
<td>4.5</td>
<td>26.0</td>
<td>0.17</td>
<td>5.1</td>
<td>30.0</td>
<td>0.17</td>
<td>35.7</td>
<td></td>
</tr>
</tbody>
</table>

in Table 1 also indicate that $k_2$ (the acylation step) is rate limiting (i.e. $k_{cat} \approx k_2 < k_3$ and $K_m = K_i$) in the Leu-proteinase catalyzed hydrolysis of $p$-nitrophenyl esters considered, as previously reported for serine proteinases acting on cationic and noncationic substrates (4, 7), over the same pH range.

Between pH 5 and 10, values of $k_{cat}$ and $k_{cat}/K_m$ for the Leu-proteinase catalyzed hydrolysis of ZGlyONp, ZAlaONp, ZValONp, ZIleONp, ZArgONp, and ZLysONp are both affected by two ionizing groups with $p_K$ values ranging between 6.5 and 6.9, and 8.1 and 8.3, respectively, while values of $K_m$ are always pH independent (Fig. 1). These results agree with those reported on the pH dependence of steady-state parameters for the Leu-proteinase catalyzed hydrolysis of N-o-benzoyl-L-leucine p-nitroanilide and azocasein (3).

Since no structural data are available for Leu-proteinase, the $p_K$ assignments are only tentative and are based on the similarity between the pH effects observed in Leu-proteinase catalysis with those for serine proteinases acting on cationic and noncationic substrates, taken as molecular models (see Refs. 7, 8, and 10–12 for reviews). Thus, the observed pH effects might be related to the acid-base equilibrium of the histidine residue involved in the catalytic triad (3), around neutrality, and of the N-terminus, at alkaline pH values.

In agreement with the unique primary specificity of Leu-proteinase for leucyl derivatives (3), data shown in Figure 1 and Table I indicate that, among the substrates examined, ZLeuONp shows the most favorable catalytic parameters in terms of the highest values of $k_{cat}$, $k_{cat}/K_m$, $k_2$, $k_3$, and $k_{cat}/K_i$, and the lowest values of $K_m$ and $K_i$, over the whole pH range explored (pH 5–10); thus, simple calculations, based on $k_{cat}$ values, indicate that the assay with ZLeuONp performed at the optimum pH (approximately 7.5, see Fig. 1) allows the determination of a Leu-proteinase concentration as low as $5 \times 10^{-10}$ M.

According to the values of kinetic parameters found in the present paper, the enzyme:substrate specificity can be arranged as follows: Leu > Ile = Val > Ala > Arg = Lys > Gly. The unique Leu-proteinase specificity profile is intermediate among those of pancreatic elastase and bovine chymotrypsin, acting on aliphatic and aromatic amino acid residues, respectively (see Refs. 7, 8, and 10–12 for reviews). However, at variance with these two pancreatic serine enzymes, Leu-proteinase not only is unable to catalyze the hydrolysis of aromatic $p$-nitroanilides (3), but also of $ZTyrONp$ even after prolonged incubation (2–6 h).

As a whole, results here reported indicate that the catalytic behavior of Leu-proteinase towards $p$-nitrophenyl esters of $N$-α-
carbobenzoxy(-L)-amino acids parallels those of serine proteinases (see Refs. 7, 8, and 10-12 for reviews).

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LITERATURE CITED