**Note**

**Substrate Specificity of Alkaline Proteases from *Cephalosporium* sp. KM388**

Katsumi TSUCHIYA,* Kazuyuki SEKI, Tsutomu ARAI, and Toshinari MASUI

Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350–02, Japan

Received May 7, 1993

Serine alkaline proteases from *Cephalosporium* sp. KM388 were specific against esters of aromatic and hydrophobic amino acids. Against oxidized insulin B-chain, the enzymes initially cleaved the site of Leu-Tyr(15–16). The cleavage specificity of KM388 protease D was broader than those of other alkaline proteases, and the site of Arg-Gly(22–23) was cleaved, which is a specific site for trypsin-like protease.

Two kinds of serine alkaline proteases (designated KM388 proteases C and D) from *Cephalosporium* sp. KM388 were purified and characterized for our previous paper. The substrate specificity of alkaline proteases from various microbial origins has been investigated with various synthetic substrates and oxidized insulin A- and B-chains. But the substrate specificity of serine alkaline proteases from *Cephalosporium* has not been investigated with any natural peptides. Many reports have appeared on the reverse reactions of proteases, i.e., peptide bond synthesis. A protease with a broad substrate specificity is thought to be useful for enzyme-catalyzed peptide synthesis. Therefore, the substrate specificity of serine alkaline proteases of *Cephalosporium* sp. KM388 was investigated using various synthetic substrates and oxidized insulin B-chain.

Activity on amino acid esters was measured by titration with 5 to 10 mM NaOH using a pH-stat apparatus (model AT-117, Kyoto Electronics, Kyoto) at pH 8.0 and 30°C. Substrate was dissolved in 0.1 M KCl containing 10 mM CaCl$_2$ and 10% CH$_3$CN. The reaction was started by adding the enzyme solution of 0.2 ml to the substrate solution of 7.8 ml, which was incubated for 5 min at 30°C. The amino acid esters used in this work, except for benzoyl arginine ethyl ester (Bz-Arg-OEt), tosyl arginine methyl ester (Tos-Arg-OMe) and Bz-Tyr-OMe, were synthesized by the method of Whitaker. Activities for amino acid amides and peptides were measured by the ninhydrin method after reaction with 0.1 mM of substrate concentration in 10 mM borate buffer (pH 10.0) containing 10 mM CaCl$_2$ and 10% CH$_3$CN for 20 min at 30°C. KM388 proteases C and D had similar specificity against synthetic amino acid esters as shown in Table. Both KM388 proteases showed the highest activity against Bz-Tyr-OEt, an ester of an aromatic amino acid with a polar side chain. But the enzymes had less activity against Bz-Phe-OEt, an ester of an aromatic amino acid with a nonpolar side chain, than those against Bz-Leu-OMe and Bz-Met-OMe, esters of hydrophobic amino acids with large aliphatic side chains. However, the enzymes hardly hydrolyzed amino acid esters with a substituent on the β-carbon such as Bz-Ile-OMe, Bz-Thr-OMe, and Bz-Val-OMe or a small side chain such as Bz-Ala-OMe, and basic amino acid esters such as Bz-Arg-OEt and Tos-Arg-OMe. These specificity of KM388 proteases for synthetic amino acid esters is similar to that of subtilisin-like proteases. However, the amidase activity of the enzymes was not so high. KM388 protease D hydrolyzed Bz-Leu-NH$_2$ and carboxybenzoyl phenylalanine-tyrosyl-leucine (Z-Phe-Tyr-Leu), although the enzyme did not hydrolyze Bz-Tyr-NH$_2$ or Z-Phe-Tyr (data not shown). This enzyme also cleaved Z-Gly-Phe, but not Gly-Phe, without modification of the amino group of glycine (data not shown).

For identification of the cleavage site, oxidized insulin B-chain (1 mM) in 10 mM borate buffer (pH 10.0) containing 10 mM CaCl$_2$ was hydrolyzed by KM388 protease C or D (each 1 μg) for 10 min or 1 h at 30°C. The reaction was stopped by freezing the solution with liquid nitrogen. The hydrolyzed peptide fragments were purified by high pressure liquid chromatography and lyophilized. A part of the peptide preparations were hydrolyzed with 6 N HCl.

### Table

Kinetic Parameters for Various Synthetic Amino Acid Esters by Alkaline Proteases of *Cephalosporium* sp. KM388

<table>
<thead>
<tr>
<th>Amino acid esters</th>
<th>KM388 Protease C</th>
<th></th>
<th>KM388 Protease D</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$k_{cat}/K_m$ (mM$^{-1}$s$^{-1}$)</td>
<td>$K_m$ (mM)</td>
</tr>
<tr>
<td>Bz-Arg-OEt</td>
<td>10.7</td>
<td>23.1</td>
<td>2.2</td>
<td>14.1</td>
</tr>
<tr>
<td>Tos-Arg-OMe</td>
<td>116.0</td>
<td>78.8</td>
<td>0.7</td>
<td>65.8</td>
</tr>
<tr>
<td>Bz-Tyr-OEt</td>
<td>10.1</td>
<td>1015.4</td>
<td>100.5</td>
<td>5.7</td>
</tr>
<tr>
<td>Bz-Phe-OEt</td>
<td>16.6</td>
<td>359.6</td>
<td>21.7</td>
<td>10.5</td>
</tr>
<tr>
<td>Bz-Met-OMe</td>
<td>11.9</td>
<td>533.8</td>
<td>44.9</td>
<td>3.4</td>
</tr>
<tr>
<td>Bz-Ala-OMe</td>
<td>20.1</td>
<td>81.4</td>
<td>4.0</td>
<td>66.3</td>
</tr>
<tr>
<td>Bz-Leu-OMe</td>
<td>3.4</td>
<td>237.6</td>
<td>69.9</td>
<td>7.5</td>
</tr>
<tr>
<td>Bz-Thr-OMe</td>
<td>0.6**</td>
<td></td>
<td></td>
<td>0.7**</td>
</tr>
<tr>
<td>Bz-Val-OMe</td>
<td>0.4**</td>
<td></td>
<td></td>
<td>0.8**</td>
</tr>
</tbody>
</table>

Assays were done as described in the text.

* n.d.: Not detected.
** These values were calculated from the initial rate at the substrate concentration of 0.25 mM.

* To whom correspondence should be addressed.

Abbreviations: Bz, benzoyl; OMe, methyl ester; OEt, ethyl ester; Tos, tosyl; Z, carboxybenzoyl.
at 110°C for 24 h and analyzed by an amino acid analyzer (JLC-200A, JOEL, Tokyo). Another part of the peptide preparations was used for the analysis of N-terminal amino acids by the phenyl isothiocyanate method.9) Phenylthiohydantoin amino acids were identified by thin layer chromatographies on silica gel 60 plates.10,11) After hydrolysis for 10 min, KM388 protease C gave one major cleavage site at Leu-Tyr(15–16) and four minor cleavage sites at Ser-His(9–10), Glu-Ala(13–14), Phe-Phe(24–25), and Phe-Tyr(25–26). KM388 protease D also showed a similar cleavage pattern. The enzyme, however, gave a minor cleavage site at Gln-His(4–5), but not the site at Ser-His(9–10) (data not shown). These results show that both enzymes initially cleave the site of Leu-Tyr(15–16), which is a common preferred cleavage site among microbial serine alkaline proteases.8) When oxidized insulin B-chain was hydrolyzed for 1 h, both KM388 proteases showed many cleavage sites as shown in Fig. The cleavage specificity of KM388 protease C resembles those of Asp. flavid protease12) and the alkaline elastase from Bacillus sp. Ya-B.13) On the other hand, the cleavage specificity of KM388 protease D was broader than those of KM388 protease C and other alkaline proteases. It is noteworthy that KM388 protease D also cleaved the site of Arg-Gly(22–23) like thermite,17) of which the site is specific to trypsin-like protease.8)

The broad substrate specificity of alkaline protease D from Cephalosporium sp. KM388 resulted in high ability of peptide syntheses based on an aminolysis reaction. This ability of peptide syntheses will be reported elsewhere.

References