Restriction of substrate specificity of subtilisin E by introduction of a side chain into a conserved glycine residue

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Received 10 July 1996; revised version received 26 August 1996

Abstract Substitution of the conserved Gly127 for residues having a side chain markedly changed the substrate specificity of subtilisin E from Bacillus subtilis. The crystallographic findings suggested that Gly127 is responsible for accepting even the large P1 substrates, and the marked change of specificity was attributed to the introduction of a side chain in this position. To test this hypothesis, Gly127 was replaced with 3 non-charged amino acids, Ala, Ser and Val. When assayed with synthetic peptide substrates, all mutants purified from the periplasmic space in Escherichia coli showed a marked preference for small P1 substrate up to 150-fold relative to the wild-type. The kinetic data and molecular modeling analysis suggest that large hydrophobic P1 residues were unable to access the binding pocket due to steric hindrance.

Key words: Subtilisin; Substrate specificity; Site-directed mutagenesis

1. Introduction

Although many proteases with various substrate specificities exist in nature, one of the most important aims of protein engineering is to design and create new proteases with high preferences for specific substrates, which can be applied for site-specific proteolysis. Extensive studies on well-characterized enzymes have shown that substrate specificities can be modified by mutating the amino acid residues to which the substrate directly binds [1-3]. For instance, in view of its industrial applications in detergents and food processing, subtilisin produced by various Bacillus species has been extensively studied using site-directed mutagenesis [4,5]. From crystallographic analysis [6], the substrate binding pocket in subtilisins, which are characterized by broad specificities, is made up both of the main chain of Ser125-Leu126-Gly127 and the main and side chains of Ala152-Ala153-Gly154, and Gly166 is located at the bottom of the pocket for the P1 substrate side chain and plays a critical role in determining their specificities. In subtilisins, the pocket is large and hydrophobic, which explains the broad specificity of this enzyme with a preference for aromatic or large non-polar P1 and P4 substrate residues [7]. Previous studies on subtilisin [7-10] have shown that the P1 specificity can be changed by substitution of amino acid residues to which a substrate binds directly. In fact, all 19 possible amino acid substitutions for Gly166 in subtilisin BPN' have been tried by cassette mutagenesis, and the effects of hydrophobic interaction on substrate specificity have been systematically investigated [8]. On the other hand, Gly127 is absolutely conserved in the bacterial subtilisin family [11], and it contacts the P1, P3, and P4 substrate residues according to the X-ray coordinates for bound products in subtilisins BPN' and Carlsberg [7]. Therefore, the residue at position 127 is thought to be an important site for determining the specificity of subtilisin, however, the possible contribution of side chains at this position to substrate binding is unknown.

Using an Escherichia coli expression system [12,13], we also analyzed the substrate specificity of Bacillus subtilis subtilisin E based on the structure of a new alkaline elastase produced by the alkalophilic Bacillus strain, which has very high elastolytic activity and indicated that deletion of the four amino acids, Ser161-Thr164, in the vicinity of the P1 binding pocket appeared to influence not only its substrate specificity but also its catalytic efficiency [14]. In the present study, we focused upon the conserved residue constituting P1 substrate binding pocket, and found that the substitution of Gly127 with Ala, Ser and Val showed high specificity towards the small P1 side chain, whereas the catalytic activity toward large P1 side chains and casein as the substrates for wild-type subtilisin was severely impaired.

2. Materials and methods

2.1. Materials

An E. coli strain JA221(hsdM + trpE5 leuB6 lacY recA11F lacF lauf lac' pro') [15], was used as a host. The isopropyl-~D-thiogalactopyranoside (IPTG)-inducible plN-III-ompA vector [16] was used for the expression and secretion of mutant and wild-type subtilisin E. All enzymes for DNA manipulations were obtained from Takara Shuzo and used under conditions recommended by the supplier. Oligonucleotides were synthesized on a model 380A DNA synthesizer from Applied Biosystems using phosphoramidite chemistry [17] and purified by high-performance liquid chromatography. Synthetic peptide substrates were purchased from Bachem Feinchemikalien AG and Sigma. Casein (Hammarsten) was obtained from Merck.

2.2. Site-directed mutagenesis

Amino acid substitution was carried out according to the method of Inouye and Inouye [18] directly on the plasmids, and the mutations were confirmed by dideoxy-chain termination sequencing [19]. The amber (UAG) termination codon at position 127 was introduced with 5'-CATGACCTTATTGGCCGACCTAC-3' (asterisks show the locations of mismatches). To derive active mutants with various amino acid substitutions at position 127, a plasmid harboring the complete subtilisin gene introducing termination codon at position 127 was used as a template DNA for mutagenesis, and the following mixture of oligonucleotides was used as a primer:

5'-CATGACCTTGGGACCTAC-3'

CCCAAA

TT

The replacement of Gly127 by Ala, Ser and Val was performed with 5'-TGAGCCTTGCCGAGCCCTAC-3', 5'-TGAGCCCTTGCCGAGCCCTAC-3', and 5'-TGAGCCTTGGCCGACCTAC-3', respectively. A plasmid harboring the wild-type sequence (pH212) [20] was used as a template DNA for site-directed mutagenesis.
Isolation of suppressor mutant subtilisins by site-directed mutagenesis

The UGA termination codon at position 127 in subtilisin E was first introduced by site-directed mutagenesis, and the cells carrying the mutated gene did not form any halo on a casein agar plate due to the structural destruction. We then attempted to isolate suppressors from the incomplete subtilisin by introducing various amino acid residues in place of the termination codon as described in Section 2. From approx. 10^6 colonies, several halo-forming colonies were isolated on a casein agar plate. Although all subtilisin genes from these positive colonies were sequenced, they were all wild-type subtilisin genes having Gly at position 127. In this study, none of the mutant subtilisins showing caseinolytic activity were obtained. This suggests that Gly at position 127 plays an important role in the expression of caseinolytic activity.

Construction and expression of mutant enzymes

To introduce the side chains with different volumes in this residue, three mutant plasmids, pTMG127A, pTMG127S, and pTMG127V, were constructed to replace Gly with Ala, Ser, and Val, respectively. Wild-type and mutant subtilisin E genes were expressed in E. coli. We first examined their abilities to form halos on a casein agar plate. However, none of the cells carrying the mutations formed clearly discernible halos even after 7 days incubation at 25°C. This suggested that the substitution of Gly with Ala, Ser, and Val resulted in decreased protease activity compared to the wild-type enzyme.

Catalytic properties of mutant enzymes

After passage through two ion-exchange columns, all mutant enzymes were purified from the periplasmic fraction to give a single band upon SDS-polyacrylamide gel electrophoresis, and their enzymatic properties were investigated. Wild-type subtilisin E purified from E. coli harboring plasmid pH1212 was examined as a control. Purified enzymes were assayed with N-succinyl-L-Ala-L-Ala-L-Pro-L-β-nitroanilide (AAPX; X=Phe (F), Ala (A), Met (M), Leu (L), and Lys (K)). AAPF has been used as an authentic substrate for subtilisin. AAPA has been regarded as a typical P1 residue having site-directed mutagenesis, tilisin. AAPA has been regarded as a typical P1 residue having

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To examine this possibility, we chose the residue at position 127 for site-directed mutagenesis.
Fig. 2. Hydrolysis of peptide substrates by the wild-type and the various mutant subtilisins E. Assays were performed in 50 mM Tris-HCl (pH 8.5) and 1 mM CaC\textsubscript{2} at 37°C using AAPX (succinyl-Ala-Ala-Pro-X-p-nitroanilide; X is Phe (F), Met (M), Leu (L), Lys (K), and Ala (A)). The wild-type and mutants Gly\textsubscript{127}→Ala, Ser, and Val enzymes are shown as WT, G127A, G127S, and G127V, respectively. Variations in the values were below 5%.

...a small side chain. Fig. 2 shows the specific activity toward five peptide substrates, and the relative activity of AAPA/ AAPF of the enzymes. The specific activity toward AAPF, AAPK, and AAPL as substrates for subtilisin was severely impaired when the side chain was introduced at position 127, in particular, all the mutants had less than 1% of the wild-type enzymatic activity for AAPF. Consequently, in the case of casein as a natural substrate, the hydrolyzing activities of the mutants were greatly diminished relative to that of the wild-type enzyme (less than 100 units/mg for the mutants vs. 896 units/mg for the wild-type). However, the activity of the mutants for AAPA remained approx. 15–35% of that of the wild-type enzyme. It is worth noting that all mutants showed a marked increase in the AAPA/AAPF ratio ranging from 40- to 150-fold.

...Kinetic constants $k_{\text{cat}}$ and $K_m$ were determined from initial rate measurements for hydrolysis of AAPF, AAPM, and AAPA. As shown in Table 1, it was impossible to obtain the individual kinetic parameters $k_{\text{cat}}$ and $K_m$ of the mutants for AAPF (except for the Val mutant) which reflects its catalytic efficiency, due to the marked increase in $K_m$ for this substrate and limited substrate solubility, which indicates little affinity of the enzyme for AAPF. On the other hand, the Gly\textsubscript{127} mutations resulted in only a 2-fold increase in $K_m$ values for AAPA with respect to wild-type enzyme. Therefore, the $k_{\text{cat}}/K_m$ ratio of the mutants remains approx. 7–25% of...
that of the wild-type enzyme, although the $k_{cat}$ value decreased significantly. These findings suggest that after introduction of the side chain into position 127, only the small P1 substrate could be accepted at the binding pocket, not the large ones, leading to the marked change in substrate preference. In addition, it might cause the structural change around the active site that showed considerably lower specific activity toward the substrates for subtilisin.

Computational modeling was conducted to predict the structure around the substrate-binding pocket in the mutant enzymes. A part of them (Ala substitution) is shown in Fig. 3. The modeling of the mutants suggested that the steric hindrance due to the introduction of Ala, Ser, or Val side chain at position 127 might occur at the entrance of the cleft, which is formed by the segments Ser125-Leu126-Gly127 and Ala152-Ala153-Gly154. The steric hindrance can be explained on the basis of the distances between the α-carbon backbones of the substrate binding pocket.

Table 1
Kinetic constants of the wild-type and the various mutant subtilisins E

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (s⁻¹ mM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAPF</td>
<td>Wild-type</td>
<td>17.6 ± 0.52</td>
<td>1.85 ± 0.05</td>
<td>9.48 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>G127A</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>G127S</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>G127V</td>
<td>0.55 ± 0.10</td>
<td>2.63 ± 0.11</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>AAPM</td>
<td>Wild-type</td>
<td>19.7 ± 0.48</td>
<td>1.03 ± 0.02</td>
<td>19.1 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>G127A</td>
<td>2.75 ± 0.08</td>
<td>3.23 ± 0.08</td>
<td>0.85 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>G127S</td>
<td>0.57 ± 0.03</td>
<td>3.23 ± 0.11</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>G127V</td>
<td>0.36 ± 0.02</td>
<td>3.33 ± 0.10</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>AAPA</td>
<td>Wild-type</td>
<td>2.89 ± 0.60</td>
<td>0.83 ± 0.03</td>
<td>3.44 ± 0.61</td>
</tr>
<tr>
<td></td>
<td>G127A</td>
<td>1.41 ± 0.07</td>
<td>1.79 ± 0.14</td>
<td>0.79 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>G127S</td>
<td>0.44 ± 0.01</td>
<td>1.79 ± 0.05</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>G127V</td>
<td>0.75 ± 0.01</td>
<td>1.72 ± 0.07</td>
<td>0.44 ± 0.01</td>
</tr>
</tbody>
</table>

Assays were performed in 50 mM Tris-HCl (pH 8.5) and 1 mM CaCl₂ at 37°C using AAPF (succinyl-Ala-Ala-Pro-Phe-p-nitroanilide), AAPM (succinyl-Ala-Ala-Pro-Phe-p-nitroanilide), and AAPA (succinyl-Ala-Ala-Pro-Ala-p-nitroanilide). N.D., not determined since individual $k_{cat}$ and $K_m$ values could not be obtained due to limited substrate solubility.

Fig. 3. Computer modeling of the P1 substrate binding pocket in the predicted structure of the α-carbon backbone structure of subtilisins E. Wild-type (Gly127) and Gly127Ala mutant subtilisins E are drawn as the predicted structure using program Insight II, and Homology (MSI softwares).
The distances are given between residue 127 and segment Ala 152- mutations are partly the result of large disruption in enzyme.

Ala 152 7.06 6.30 6.41 6.56
Ala 153 7.76 6.91 7.02 7.23
Gly 154 6.40 5.68 5.93 6.39

while side chains of Ser 127 and Leu 126 extend outside of the pocket.

Table 2
Predicted distances between α-carbon backbones of the P1 substrate binding pocket of the wild-type and the various mutant subtilisins E

<table>
<thead>
<tr>
<th>Residue</th>
<th>Wild (Gly127)</th>
<th>Ala127</th>
<th>Ser127</th>
<th>Val127</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala152</td>
<td>7.06</td>
<td>6.30</td>
<td>6.41</td>
<td>6.56</td>
</tr>
<tr>
<td>Ala153</td>
<td>7.76</td>
<td>6.91</td>
<td>7.02</td>
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<td>Gly154</td>
<td>6.40</td>
<td>5.68</td>
<td>5.93</td>
<td>6.39</td>
</tr>
</tbody>
</table>

The most notable finding of the present study is the change of the P1 substrate specificity by introduction of side chains to position 127 in subtilisin. Our findings strongly suggest that the volume of side chain equivalent to one methyl group would be enough not to allow the large P1 substrate side chains to be accepted, since Ala substitution at position 127 caused a marked reduction in activity with AAPF, AAPL, and AAPK as the favored substrates for serine. The mutant enzyme with the substitution for Val showed hydrolyzing activity only for Ala as P1 substrate. The mutant enzymes might be useful in protein chemistry, and industry, even though their specific activity should be enhanced. The fact that the kcat and K_m values for AAPF in Gly127Ala and Gly127Ser mutants were unable to be determined support the possibility of the loss of binding toward large P1 substrate.

Indeed, when the α-carbon distances between residues forming the cleft were predicted by molecular modeling (Table 2), steric hindrance in the mutants would obviously occur. In particular, it was speculated that Ala substitution at position 127 led to the greatest hindrance effect caused by the shortest distance. Similarly, the crystal structures of mammalian serine proteases (trypsin and chymotrypsin) showed a correlation between the P1 specificity of the enzyme and the conformation of the polypeptide backbone at Gly127, which is the analog of Gly127 in these enzymes [26]. In mammalian elastases, Val126 plays a dual role both by providing a hydrophobic pocket for the P1 residue and by forming the main chain hydrogen bonds at position P3 [27]. Therefore, conversion of either trypsin or chymotrypsin to an elastase-like protease is predicted to require mutations sufficient to reorient the main chain of position 216 to an elastase-like conformation [26].

The question arises as to why the amino acid replacement at position 127 causes a significant decrease in catalytic efficiency, mainly caused by an alteration in kcat and not in K_m, whereas the substrate having the Ala residue in P1 was hydrolyzed effectively. The reason for the decrease in the catalytic efficiency of the mutants relative to the wild-type enzyme remains unknown.

A similar finding was obtained for the cysteine protease cathepsin B exhibiting broad P1 specificity [28]. These findings indicate that the specificity changes resulting from P1 pocket mutations are partly the result of large disruption in enzyme structure. An X-ray crystallographic study by Kraut et al. [29] revealed that there is a hydrogen bond between Asp92 and His54, but there is none between the catalytic side chains of Ser221 and His54 being constituents of the catalytic triad in subtilisin. Although the mechanism is not elucidated by the structural prediction around the substrate-binding pocket in the mutant enzymes, it seems that with the Ala, Ser, and Val substitutions at position 127, a slight change of distance between the catalytic triad may occur, diminishing proton transfer. On the other hand, it may appear surprising that the conserved Gly166 can be substituted to maintain activities almost equal to the wild-type enzyme with narrower specificity [8]. Further research on the active site of the mutants is currently in progress.

In conclusion, we found that it is possible to create a novel protease having highly limited substrate specificity from the wild-type enzyme by replacing the conserved residue related to the substrate binding pocket.

Acknowledgements: We thank Ryoka Systems Inc. (Chiba, Japan) for computational modeling, and Mr. Yukihiro Yabuta for the preparation of figures.

References