SHORT COMMUNICATION

Construction of novel subtilisin E with high specificity, activity and productivity through multiple amino acid substitutions

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Through three cumulative amino acid substitutions, we constructed novel mutant subtilisins E of Bacillus subtilis, all with high specificity, activity and productivity. The substitution of conserved Gly127, constituting P1 substrate-binding pocket, with Ala and Val showed a marked preference for the small P1 substrate. Leu was then substituted for Ile31 next to the catalytic Asp32 to enhance the catalytic activity. Both double mutants (I31L/G127A and I31L/G127V) showed a 3–5-fold increase in catalytic efficiency due to a large kcat without any change in the specificity of the mutants at position 127. Molecular modeling suggests that large P1 residues were unable to access the pocket because of steric hindrance. A third mutation was introduced by replacing Tyr(–1) with Ala in the propeptide essential for autoprocessing to active mature subtilisin in vivo. A prominent 7–20-fold increase in active enzyme production occurred in the triple mutants (Y-1A/I31L/G127V). The replacement of Tyr(–1) in the propeptide for the small P1 side chain, due to the steric hindrance in the pocket, was found to be an essential factor for the autoprocessing of mature subtilisin, due to the little activity toward Tyr(–1). In the present study, we attempted to enhance the catalytic activity and the yield of mutant enzymes with high specificities by site-directed mutagenesis.

Materials and methods

Construction of plasmids containing mutant subtilisin E gene

Using pH212 harboring the wild-type subtilisin E (Takagi et al., 1988), the plasmids with the substitutions (I31L/G127A and I31L/G127V) were constructed by site-directed mutagenesis and called pHT212L, pTMG127A and pTMG127V, respectively. In this work, the above mutant plasmids were digested with BamHI and HindIII, and then 0.8 kb fragment of pTMG127A and pTMG127V and 7.6 kb fragment of pTI31L were mixed, ligated and introduced into E.coli JAI21 to construct two mutant plasmids, pTO-LA and pTO-LV, having the double mutations of I31L/G127A and I31L/G127V, respectively. The replacement of Tyr(–1) in the propeptide of subtilisin E by Ala was performed by polymerase chain reaction (PCR) with 5′-phosphorylated primers, 5′-GCTTCCGGCTC GTA TAA-3′ (LAC), 5′-AACTGCGTG- CCTGGATAT-3′ (P60), 5′-GCACA T GAAC*G*TGCGCAA-3′ (+YA) and 5′-TTGGCAG*CTTATGTGC-3′ (–YA), using a DNA Thermal Cycler 480 (Perkin Elmer). The asterisks show the locations of mismatches. Primers LAC and P60 were synthesized to complement regions 75 bp upstream of the XbaI restriction site and 23 bp downstream of a HindIII site in pHI212 including Tyr(–1) of subtilisin E, respectively.

Primers +YA and –YA were used as primers for mutagenesis. The first PCR was carried out using primers LAC and –YA or +YA and P60 in an independent microtube. The unique amplified bands of 429 and 202 bp by use of the primers LAC and –YA, and +YA and P60, respectively, were purified by agarase gel electrophoresis, and then used as a template for the following PCR using primers LAC and P60, as in the corresponding reactions of first PCR. After the second PCR, the expected band (601 bp) of PCR products was digested with XbaI and HindIII to recover the fragment of 478 bp, and ligated to the large fragments of plasmids pTO-LA and pTO-LV and pH7212L digested with XbaI and HindIII. The resultant plasmids (pTO-YLA and pTO-YLV) contain the triple mutations Y-1A/I31L/G127A and Y-1A/I31L/G127V, respec-
tively, in subtilisin E gene. Plasmid pTO-YL, which contains Y-1A/I31L substitutions, was also constructed as a control. The mutations were confirmed on a Model 373A DNA sequencer from Applied Biosystems using dideoxy chain termination sequencing.

Expression and purification of wild-type and mutant subtilisins E

Various subtilisins E were expressed and purified as described previously (Takagi et al., 1996). Enzyme concentration was determined using the Bio-Rad Protein Assay kit. Bovine serum albumin was used as the standard protein.

Assay of enzymatic activity

For synthetic peptide substrates, assays were performed as described previously (Takagi et al., 1988).

Western blot analysis

To compare the efficiencies of autoprocessing among the various subtilisins, cell-free crude extracts were prepared by sonic oscillation (200W) for 5 min under cooling, and analyzed by SDS–PAGE. Recombinant subtilisins E were detected by Western blot analysis using the Vectastain ABC kit (Vector Laboratories) and an anti-mature subtilisin E polyclonal antibody.

Amino acid sequence analysis

Partial purified subtilisins, after passage through a CM-Sepharose Fast Flow column, were subjected to SDS–PAGE and then electrotransferred on to a poly(vinylidene difluoride) membrane (Broome and Gilbert, 1978). Protein bands were stained with Coomassie Brilliant Blue and excised for N-terminal amino acid sequence analysis by Edman degradation (Edman and Begg, 1967).

Molecular modeling

Molecular modeling was performed with the computer program Insight II and Homology (MSI Softwares). Modeling of subtilisin E was based on the X-ray crystal structure of subtilisin BPN’ and the primary sequence of subtilisin E.

Results

Experimental design for enhanced activity of mutant subtilisin with high specificity

In a previous study (Takagi et al., 1996), we found that the substitution of Gly127 with Ala and Val showed high specificity toward the small P1 side chain, because the crystallographic findings suggested that Gly127 is responsible for accepting even the large P1 substrates, and the marked change in specificity was attributed to the introduction of a side chain in this position. However, when assayed with synthetic peptides, the mutants were found to have decreased activity compared with those of wild-type subtilisin E. On the other hand, our previous work showed that replacing Ile31, which is adjacent to catalytic site Asp32, were generated for potential improvements in catalytic efficiency, and that the Leu31 mutant showed a remarkable increase in specific activity due to a larger $k_{cat}$ for substrates (Takagi et al., 1988). Therefore, we assumed that the specific activity of G127A and G127V mutants would be recovered by the Leu replacement at position 31. To examine this possibility, we constructed the double mutants I31L/G127A and I31L/G127V as described under Materials and methods.

Catalytic properties of double mutant subtilisins

The wild-type and the various mutant enzymes were purified from the periplasmic fraction to give a single band upon SDS–PAGE. To examine the effect of Leu substitution, kinetic constants $k_{cat}$ and $K_m$ were determined by using synthetic peptide substrates, N-succinyl-l-Ala-l-Ala-l-Pro-l-X-p-nitroanilide [AAPX; X = Ala (A), Phe (F), Lys (K), Leu (L) and Met (M)]. AAPF has been used as an authentic substrate for subtilisin and AAPA has been regarded as a typical P1 residue having a small side chain. As shown in Table I, the I31L mutant showed a general increase in $k_{cat}/K_m$ ranging from two to sixfold for all the substrates caused by an alteration in $k_{cat}$ and not in $K_m$, in agreement with previous results (Takagi et al., 1988). In the case of single Gly127 mutants, the catalytic efficiency toward all the substrates tested was significantly decreased, as we reported recently (Takagi et al., 1996). It was impossible to obtain the individual kinetic parameters for most of the substrates, owing to the marked increase in $K_m$, which indicates little affinity of the enzymes for them. However, G127A and G127V mutants for AAPA had 20–70% of the specific activity of the wild-type enzyme. Consequently, the relative activity of AAPA/AAPF of G127A mutant was 34-fold higher than that of the wild-type. When residue 31 was replaced with Leu in the Gly127 mutants, a significant increase in catalytic efficiency occurred, ranging from three- to fivefold for AAPA relative to those of Gly127 mutants and, notably, more than twofold compared with the wild-type enzyme. Also, it was found that the changes in the $k_{cat}/K_m$ ratio were predominantly caused by an increase in $k_{cat}$ and not in $K_m$ for small P1 substrates. It is worth noting that the AAPA/AAPF ratio of the double mutants followed essentially the same trend as observed for G127A mutant (from 33- to 280-fold higher than that of the wild-type).

Computer modeling was applied to predict the structure around the substrate-binding pocket and the catalytic triad in the enzymes, and the modeling of the double mutants suggested that the steric hindrance due to the introduction of a side chain of Ala and Val at position 127 might occur at the substrate-binding cleft, which is formed by the segments Ser125–Leu126–Gly127 and Ala152–Ala153–Gly154 (Takagi et al., 1996). Steric hindrance occurring in the double mutants is apparent judging from the predicted distances between the carbon backbones at positions 152–153–154 and at position 127 (Table II).

Experimental design for improved autoprocessing of mutant subtilisin with high specificity and activity

From DNA sequence analysis of subtilisin, the primary gene product consists of a long peptide (pre-sequence) having a unique 77-residue sequence between the signal peptide (pre-sequence) and a 275-residue mature sequence. The prosequence has been shown to be essential for the formation of enzymatically active subtilisin and is then autocatalytically cleaved between the C-terminus of propeptide [Tyr(−1)] and the N-terminus of mature sequence (Ala1) to give the mature enzyme in vivo (Shinde and Inouye, 1993). In this study, the productivity of mature subtilisin in the double mutants was significantly low, probably because the double mutant subtilisins, having a narrow specificity, might not cleave large hydrophobic side chains, such as Tyr, causing prosubtilisin to accumulate. To improve the autoprocessing rate from prosubtilisin, the replacement of Tyr(−1) with Ala in the double
Table I. Substrate specificities of the wild-type and the various mutant subtilisins E using substrates with different residues at P1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Residue at P1</th>
<th>Ala&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Phe&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Lys&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Leu&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Met&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td></td>
<td>3.1</td>
<td>9.3</td>
<td>5.2</td>
<td>6.2</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2.5, 0.81)</td>
<td>(18, 2.0)</td>
<td>(30, 5.7)</td>
<td>(30, 4.9)</td>
<td>(15, 0.97)</td>
</tr>
<tr>
<td>I31L</td>
<td></td>
<td>6.4</td>
<td>58</td>
<td>21</td>
<td>2.2</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5.4, 0.83)</td>
<td>(120, 2.0)</td>
<td>(120, 5.7)</td>
<td>(110, 4.9)</td>
<td>(63, 1.0)</td>
</tr>
<tr>
<td>G127A</td>
<td></td>
<td>2.1</td>
<td>N.D.&lt;sup&gt;f&lt;/sup&gt;</td>
<td>N.D.</td>
<td>N.D.</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.9, 1.9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G127V</td>
<td></td>
<td>2.4</td>
<td>0.21</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4.2, 1.7)</td>
<td>(0.55, 2.6)</td>
<td></td>
<td></td>
<td>(0.36, 3.5)</td>
</tr>
<tr>
<td>I31L/G127A</td>
<td></td>
<td>6.2</td>
<td>0.067</td>
<td>1.1</td>
<td>N.D.</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(10, 1.7)</td>
<td>(0.3, 4.6)</td>
<td>(8.8, 8.0)</td>
<td></td>
<td>(31, 3.4)</td>
</tr>
<tr>
<td>I31L/G127V</td>
<td></td>
<td>13</td>
<td>1.2</td>
<td>0.68</td>
<td>0.68</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(21, 1.7)</td>
<td>(3.1, 2.6)</td>
<td>(6.7, 9.8)</td>
<td>(4.2, 6.3)</td>
<td>(2.9, 0.92)</td>
</tr>
</tbody>
</table>

Assays were performed in 50 mM Tris–HCl (pH 8.5) and 1 mM CaCl<sub>2</sub> at 37°C. V values represent k<sub>cat</sub>/K<sub>m</sub> (s<sup>–1</sup>/mM); values in parentheses represent k<sub>cat</sub> (s<sup>–1</sup>) and K<sub>m</sub> (mM<sup>–1</sup>), respectively. Variations in the values were below 5%.

<sup>a</sup>-Succinyl–L-Ala–L-Ala–L-Pro–L-Met

<sup>b</sup>-Succinyl–L-Ala–L-Ala–L-Pro–L-Lys

<sup>c</sup>-Succinyl–L-Ala–L-Ala–L-Pro–L-Ala

<sup>d</sup>-Succinyl–L-Ala–L-Ala–L-Pro–L-Leu

<sup>e</sup>-Succinyl–L-Ala–L-Ala–L-Pro–L-Phe

<sup>f</sup>, not determined since individual k<sub>cat</sub> and K<sub>m</sub> values could not be obtained owing to limited substrate solubility.

Discussion

One of the most important goals of protein engineering is to design and create new proteases with high preferences for specific substrates, which can be applied for site-specific proteolysis. Previous studies on Bacillus amyloliquefaciens subtilisin BPN’ have shown that the substrate specificity can be altered by the replacement of amino acid residues to which a substrate binds directly. A conserved Gly at position 166, one of the most important goals of protein engineering, is expected to cleave propeptide effectively, and then to produce a large amount of mature enzyme.

Efficiency of autoprocessing of triple mutant subtilisins in vivo

We examined the efficiency of autoprocessing in terms of SDS–PAGE in the total cellular protein and catalytic activity in the periplasmic fraction of the induced cells. When the total cellular protein was analyzed by SDS–PAGE (Figure 1A), the bands corresponding to mature subtilisin E were observed in the case of Y-1A/I31L/G127A (lane 6) and Y-1A/I31L/G127V (lane 7) mutants and also I31L mutant (lane 5). In contrast, only a small amount of mature subtilisin was detected in fractions containing Y-1A/I31L (lane 6), I31L/G127A (lane 7) and I31L/G127V (lane 9), whereas large amounts of prosubtilisin were observed. Similar results were obtained by Western blot analysis (Figure 1B).

Table III summarizes the relative activities of various subtilisins in the periplasmic fraction toward AAPA. It was found that the Ala(–1) replacement for I31L/G127A and I31L/G127V mutants caused a prominent 7–20-fold increase in activity, but that a significant decrease in enzymatic activity occurred when Ala was introduced at position –1 of I31L mutant. Judging from densitometric estimation of the amounts of accumulated subtilisin or prosubtilisin (Figure 1A), the level of gene expression seems to be almost the same for the samples. These results suggested that the rate of autoprocessing to mature subtilisin was markedly improved by optimizing the residue at the processing site. Subsequent purification of mutants substituting Tyr(–1) with Ala from the periplasmic fraction showed a catalytic efficiency equivalent to that of the parent enzymes, indicating that Ala(–1) substitution had little influence on the catalytic site (data not shown). Further, the N-terminal sequencing analysis supported the contention that the processing of the triple mutant subtilisins occurred at Ala(–1), indicating that the propeptide was autocatalytically cleaved. It is interesting that the double mutant subtilisins possessed three extra amino acid residues, His(–3)–Glu(–2)–Tyr(–1), upstream of the N-terminus of mature subtilisin (data not shown). These results indicate that efficient autoprocessing by optimizing the residue at the cleavage site for an individual enzyme leads to a higher yield of active subtilisin.
Fig. 1. The total cellular protein from cells harboring a plasmid containing
the various mutant subtilisin genes detected by (A) SDS–PAGE and (B) immunoblotting with anti-subtilisin antiserum. Cells were induced with 1 mM IPTG at 25°C for 4 h, and then 5 µg (A) and 0.1 µg (B) of protein were subjected to SDS–PAGE. Arrowheads show the positions of pro-
subtilisin E (upper) or mature subtilisin E (lower). The gel for SDS–PAGE
was stained with Coomassie Brilliant Blue. SDS–PAGE was performed on a
15% polyacrylamide gel using the procedure described by Laemmli (1970).
Lane 1, molecular mass standards (in kDa); lane 2, purified native subtilisin
E; lane 3, vector (pIN-III-ompA); lane 4, an active-center Asp32Asn
(D32N) mutant as control to identify the prosubtilisin (Ikemura
et al., 1987); lane 5, I31L; lane 6, Y -1A/I31L; lane 7, I31L/G127A; lane 8, Y -1A/
I31L: Tyr (–1) 1206 (1.0)
6
6
Ala (–1) 465 (20)
6
Ala (–1) 215 (0.18)
6
6
Ala (–1) 877 (7.2)
6
6
Ala (–1) 465 (20) ± 28
6
6
Ala (–1) 877 (7.2) ± 49
6
6
Table III. Specific activities of the various mutant subtilisins E toward
N-succinyl– L -Ala– L -Ala– L -Pro– L -Ala– n -nitroanilide

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I31L: Tyr (–1)</td>
<td>1206 (1.0) ± 48</td>
</tr>
<tr>
<td>Ala (–1)</td>
<td>215 (0.18) ± 9</td>
</tr>
<tr>
<td>I31L/G127A: Tyr (–1)</td>
<td>25 (1.0) ± 2</td>
</tr>
<tr>
<td>Ala (–1)</td>
<td>465 (20) ± 28</td>
</tr>
<tr>
<td>I31L/G127V: Tyr (–1)</td>
<td>121 (1.0) ± 17</td>
</tr>
<tr>
<td>Ala (–1)</td>
<td>877 (7.2) ± 49</td>
</tr>
</tbody>
</table>

The periplasmic fractions were used as enzyme sources. Enzyme assays
were performed in 50 mM Tris–HCl (pH 8.5) and 1 mM CaCl₂ at 37°C.
Values in parentheses are activities relative to that of Tyr(–1).

which is located at the bottom of the pocket for the P1 substrate
side chain, was replaced by several nonionic amino acids
(Estell et al., 1986). Owing to the hydrophobic and steric
environment, the catalytic efficiency toward small hydrophobic
substrates was increased by hydrophobic substitutions at posi-
tion 166. Wells et al. (1987) reported general changes in
specificity resulting from charged amino acid substitutions at
residues 156 and 166 in the P1 binding site, causing electrostatic
effects. Recently, several attempts focused on the P4 pocket
of subtilisin BPN’ and subtilisin 309 from Bacillus lentus to
alter the specificity (Bech et al., 1993; Rheinnecker et al.,
1993). However, most of these mutations in subtilisin and
other proteases, including trypsin and cathespin B (Craik et al.,
1985; Fox et al., 1995), caused a significant decrease in
catalytic efficiency.

Even though we succeeded in restricting the specificity of
subtilisin E by introducing a side chain into a conserved
Gly127, the hydrolyzing activity of the mutant enzyme was
considerably lower than that of the wild-type (Takagi et al.,
1996). To recover the catalytic efficiency, when Leu was
substituted for Ile31, a remarkable increase in specific activity
was observed due to a larger kₘ. The analysis of Asp102
mutant of trypsin suggested that its function is to stabilize
the His57 tautomer for accepting the serine hydroxyl proton (Craik
et al., 1987; Sprang et al., 1987). Although its function is
poorly understood, residue 31 supposedly serves to maintain
Asp32 accepting the His64 proton. Based on our kinetic data,
it is unlikely that an environmental change around the substrate-
binding pocket is occurring, since there is no alteration in the
Km values. We now speculate that a slight change in the
distance between Asp32 and His64 of the wild-type and Leu31
mutant may occur, facilitating proton transfer. This is despite
the fact that there was no significant difference when con-
ducting the modeling to predict the distances (data not shown).

As can be seen from Figure 1 and Table III, the rate
of autoprocessing of the Gly127 mutants was dramatically
improved by introducing the Y-1A mutation. This is supported
by the previous report that restricted specificity in the P4
pocket caused a reduction in subtilisin yields due to insufficient
autocatalytic processing in subtilisin BPN’, and that there was
a clear correlation between higher specificity and lower yields
(Bech et al., 1993). It is also known that the autoprocessing
of the propeptide is inhibited when one of catalytic residues
are damaged by amino acid substitution (Ikemura et al., 1987;
Li and Inouye, 1994; Shinde and Inouye, 1995). Crystallo-
graphic data for the propeptide–subtilisin BPN’ complex have
also shown that the C-terminus of propeptide binds in the
active site of subtilisin in a product-like manner, with Tyr(–1)
in the P1 binding pocket (Gallagher et al., 1995). It is
noteworthy that the autoprocessing of Gly127 mutants occurred
at His(–3) in the propeptide, probably since Ala(–4) could
play a role in a favorable P1 substrate for the mutants. In fact,
one can think that a peptide bond between Ala1 and Gln2
would be cleaved. However, it is possible that Tyr(–1) as a P2
residue is too large to access the substrate-binding pocket of
Gly127 mutants. The tertiary structure around the processing
site would be helpful for understanding the mechanism
involved. We are now designing another substitution at the
residue which would affect the specificity and form the
substrate-binding pocket of subtilisin for the detailed analysis
of its specificity.

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