Enhancement of the Thermostability of Subtilisin E by Introduction of a Disulfide Bond Engineered on the Basis of Structural Comparison with a Thermophilic Serine Protease*

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Sites for Cys substitutions to form a disulfide bond were chosen in subtilisin E from Bacillus subtilis, a cysteine-free bacterial serine protease, based on the structure of aqualysin I of Thermus aquaticus YT-1 (a thermophilic subtilisin-type protease containing two disulfide bonds). Cys residues were introduced at positions 61 (wild-type, Gly) and 98 (Ser) in subtilisin E by site-directed mutagenesis. The Cys-61/Cys-98 mutant subtilisin appeared to form a disulfide bond spontaneously in the expression system used and showed a catalytic efficiency equivalent to that of the wild-type enzyme for hydrolysis of a synthetic peptide substrate. The thermodynamic characteristics of these enzymes were examined in terms of enzyme autolysis (tₐ) and thermal stability (Tm). The half-life of the Cys-61/Cys-98 mutant was found to be 2–3 times longer than that of the wild-type enzyme. Similar results were obtained by differential scanning calorimetry. The disulfide mutant showed a Tm of 63.0 °C, which was 4.5 °C higher than that observed for the wild-type enzyme. Under reducing conditions, however, the characteristics of the mutant enzyme were found to revert to those of the wild-type enzyme. These results strongly suggest that the introduction of a disulfide bond by site-directed mutagenesis enhanced the thermostability of subtilisin E without changing the catalytic efficiency of the enzyme.

One of the most important aims of protein engineering is to enhance the thermostability of enzymes. Several strategies have been undertaken for this purpose: e.g. introduction of hydrophobic interaction or disulfide bond(s) into protein molecules by means of site-directed mutagenesis (1–3).

There have been conflicting reports concerning the thermostability of subtilisin BPN’, a cysteine-free bacterial serine protease (4, 5) in which a designed disulfide bond has been introduced between positions 22 and 87 with the aid of computer modeling. Recently, based upon the computer program, five other disulfide variants of subtilisin BPN’ have been synthesized by site-directed mutagenesis (6). The disulfides were chosen on the basis of structural homology with proteinase K, a fungal protease that contains two disulfide bonds (7), and the stabilizing effect of a structural calcium atom (8). In some cases, the disulfide bond-containing protein was stabilized relative to its reduced form. However, none of the disulfide mutants was substantially more stable than wild-type subtilisin BPN’.

We have previously cloned and expressed the gene for Bacillus subtilis subtilisin E in Escherichia coli (9). In addition, using this system, we were able to isolate mutant subtilisin E with higher activity (10) and we examined the role of an α-helix containing the catalytic Ser-221 of subtilisin E (11) using site-directed mutagenesis. In the present study, sites for Cys substitutions for formation of a disulfide bond were chosen in subtilisin E of B. subtilis based on the structure of aqualysin I of Thermus aquaticus YT-1. Aqualysin I, an alkaline serine protease belonging to the subtilisin family, is secreted into the culture medium by T. aquaticus YT-1, an extreme thermophile (12). The optimum temperature for its caseinolytic activity is 80 °C. Aqualysin I contains 4 Cys residues, which form two disulfide bonds, Cys-67/Cys-99 and Cys-163/Cys-194 (13). These disulfide bonds seem to be responsible for the thermostability of aqualysin I. We found that the introduction of the disulfide bond enhanced the thermostability of subtilisin E without any change in its catalytic efficiency.

EXPERIMENTAL PROCEDURES

Materials—An E. coli strain, JA221 (hmdM trpE5 leuB6 lacY recA11F lacP lac+ pro+) (14), was used as the host cell. The isopropyl-β-D-thiogalactopyranoside-inducible pN-III-ompA vector (15) was used for the expression and secretion of mutant and wild-type subtilisin E. All enzymes used for DNA manipulation were from Takara Shuzo and the reaction conditions employed were those recommended by the supplier. Oligonucleotides were synthesized on a model 380A DNA synthesizer from Applied Biosystems using phosphoramidite chemistry (16) and purified by high performance liquid chromatography. The subtilisin substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide was from Sigma.

Site-directed Mutagenesis—To introduce amino acid substitutions, site-directed mutagenesis was carried out according to the method of Inouye and Inouye (17) directly on the plasmids and the mutations were confirmed by dideoxy sequencing (19) on the pUC plasmid. The placement of Gly-61, Ser-98, Ser-161, and Glu-195 by Cys was performed with 5'-phosphorylated oligonucleotide primers having the sequences 5'-CCAGGACTGCCAGTTCT-3', 5'-TGCTTGATGTTG-3', and 5'-ATCGCGGAGCAGGAAG-3', respectively (asterisks show the locations of mismatches). A plasmid harboring the wild-type sequence (pH212) (10) was used as a template DNA for site-directed mutagenesis.

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Expression and Purification of Wild-type and Mutant Subtilisin E—Wild type and mutant subtilisin E genes were expressed in E. coli strain JA221. Cultivation was performed at 37 °C in M9 medium (19) supplemented with ampicillin (50 μg/ml), CaCl2 (1 mM final concentration), and casamino acids (2%). When A660 reached 0.3, the cultivation temperature was shifted to 23 °C and isopropyl-β-D-thiogalactopyranoside was added to the culture medium to a final concentration of 1 mM for induction of gene expression. After cultivation for 3 h at 23 °C, the cells were harvested by centrifugation. The periplasmic fraction was prepared by the method of Koshland and Botstein (20) and used for protease activity assay, since most of the activity was found in the periplasmic space, as described previously (9). To purify the wild-type and mutant subtilisin E, the periplasmic fraction of induced cells was applied to a CM-cellulose column and the enzyme was eluted with 80 mM NaCl according to the method of Estell et al. (21). Enzyme concentrations were determined spectrophotometrically (22). The eluted active fraction showed a single protein band of subtilisin E upon SDS-polyacrylamide gel electrophoresis.

Analysis of Subtilisin Activity—Subtilisin activity was assayed spectrophotometrically by the release of p-nitroaniline due to enzymatic hydrolysis of succinyl-Ala-Ala-Ala-L-Pro-L-Phe-p-nitroanilide, as described previously (9). The activity was calculated as units/mg protein. One unit was defined as the activity releasing 1 μmol of p-nitroaniline/min.

Differential Scanning Calorimetry—Calorimetric measurements were carried out using a heat flux-type SSC 560U instrument, obtained from Seiko Instruments & Electronics Ltd., Tokyo. The data were compiled using a PC-9801 personal computer and the subsequent data processing was conducted with a RS-232C interface. The thermal stability (T½: midpoint in the thermally induced transition from the folded to the unfolded state) of these enzymes was estimated in the presence of Ca²⁺ (Fig. 4) after treatment with phenylmethanesulfonyl fluoride, a serine protease inhibitor, to prevent autolysis accompanying the unfolding of the enzyme. The disulfide mutant showed a T½ of 63.0 °C, which was 4.5 °C higher than that observed for the wild-type enzyme. Under reducing conditions (100 mM DTT), however, the thermal stability of the mutant enzyme was found to be decreased to a level lower than that of the wild-type enzyme.

Similar results were obtained by differential scanning calorimetry. The thermal stability (T½; midpoint in the thermally induced transition from the folded to the unfolded state) of these enzymes was estimated in the presence of Ca²⁺ (Fig. 4) after treatment with phenylmethanesulfonyl fluoride, a serine protease inhibitor, to prevent autolysis accompanying the unfolding of the enzyme. The disulfide mutant showed a T½ of 63.0 °C, which was 4.5 °C higher than that observed for the wild-type enzyme. Under reducing conditions (100 mM DTT), however, the thermal stability of the mutant enzyme was found to be decreased to a level lower than that of the wild-type enzyme.

**RESULTS**

Design of Disulfide Mutants—The amino acid residues of subtilisin BPN', Asn-61, Ala-98, Ser-161, and Glu-195, corresponding to Cys residues at positions 67, 99, 163, and 194 of aqualysin I, respectively, upon alignment of their sequences (Fig. 1). Crystallographic data for subtilisin BPN' (23) have shown that the distance between the α-carbons of the residues is estimated to be 5.8 Å for Asn-61 and Ala-98, 12.7 Å for Ser-161 and Gln-195. As the sequence of subtilisin (26) is 86% identical with that of subtilisin BPN' (Fig. 1), positions 61 (Gly) and 98 (Ser) in subtilisin E were chosen as candidates for replacement with Cys residues.

Construction and Expression of Mutant Enzymes—Three mutant plasmids, pHCTC61, pHCTC98, and pHCTC61C98, were constructed by site-directed mutagenesis to replace Gly-61 (polycracylamide gel electrophoresis). The wild-type enzyme was also purified from the periplasmic fraction to a single band upon SDS-polyacrylamide gel electrophoresis. The wild-type enzyme was also purified to homogeneity from E. coli harboring plasmid pH212 (10) as the control. Disulfide bond formation can be detected by SDS-polyacrylamide gel electrophoresis under nonreducing conditions because a protein that contains a disulfide bond has a smaller radius of gyration and therefore migrates further down the gel. Fig. 3 shows that under nonreducing conditions Cys-61/Cys-98 mutant subtilisin indeed migrated faster than the wild-type enzyme, whereas under reducing conditions the two bands were virtually indistinguishable. This result suggested that the disulfide bond formed spontaneously between Cys-61 and Cys-98 in the present expression system. The evidence for disulfide formation in the mutant was supported by assay according to the method of Ellman (28) using 5,5'-dithiobis(2-nitrobenzoic acid). No free sulfhydryl groups were detected in the Cys-61/Cys-98 mutant and the wild-type enzyme, while the single-Cys mutants at positions 61 or 98 and the Cys-61/Cys-98 mutant under reducing conditions have free Cys residues (~2 μmol of Cys/μmol of protein in the Cys-61/Cys-98 mutant).

Catalytic Properties of Mutant Enzymes—To investigate the kinetic constants, kcat and Km, enzyme assays were carried out at 37 °C using a 0.13 mM solution of the synthetic peptide N-ε-arginyl-Ala-Ala-Pro-L-Phe-p-nitroanilide, which is a well-known typical substrate for subtilisin. As shown in Table I, the single-Cys mutants at positions 61 or 98 have a slight decrease in catalytic efficiency relative to the wild-type enzyme. The changes in kcat/Km were predominantly caused by alterations in the Km value, not in the kcat value. On the other hand, the catalytic efficiency of the disulfide mutant containing Cys at both positions 61 and 98 was essentially equivalent to that of the wild-type enzyme. Besides, with a casein assay according to the method of Hagiwara et al. (29), the specific activity of the disulfide mutant was virtually unchanged from that of the wild-type enzyme (151 units/mg for the mutant versus 139 units/mg for the wild-type).

In addition, the relative activity toward the synthetic peptide versus assay temperature was investigated (data not shown). Although the optimum temperature of both enzymes was estimated to be 50–55 °C, the disulfide mutant was much more stable than the wild-type enzyme when assayed at 60 °C or above.

**DISCUSSION**

An unnatural intramolecular disulfide Cys-3/Cys-97 has been introduced by site-directed mutagenesis in T4 lysozyme (1), and has a stabilizing effect on the irreversible thermal inactivation. However, none of the disulfides introduced in subtilisin (4–6) provide greater stability against irreversible inactivation compared with the wild-type enzyme. In these cases, a computer-assisted method for finding suitable disulfide sites was employed. The disulfide bond mutants constructed are actually more stable than their reduced forms. However, the autolytic stability of the disulfide mutants is...
**Enhanced Thermostability of Subtilisin.**

FIG. 1. Comparison of amino acid sequence of aqualysin I (AQ) (23) with those of subtilisins E (E) (26) and BPN' (B) (25). The numbering above the sequences refers to aqualysin I and that below the sequences to subtilisins. Amino acids identical between the two enzymes are surrounded by rectangles. Asterisks indicate the active site residues, Asp, His, and Ser. Horizontal lines indicate the absence of a corresponding amino acid at that position.

The same or below that of the wild-type enzyme, indicating that finding positions in folded globular proteins which seem capable of accommodating an unstrained disulfide bond is not straightforward.

Recently, a disulfide bond in a natural subtilisin variant (proteinase K), which contains two disulfide bonds was introduced at an analogous position in subtilisin BPN' (6). The reduced form of proteinase K is much less stable than the oxidized form to irreversible inactivation. Yet the introduction of the disulfides Cys-29/Cys-119 or Cys-148/Cys-243 into subtilisin BPN' was not stabilizing. It is possible that there is some divergence of structural homology between these two proteins in the regions around the disulfides. In the present work, we investigated the thermostability of subtilisin E after introduction of a disulfide engineered from a structural comparison with a thermophilic serine protease, aqualysin I. The amino acid sequence of aqualysin I is about 40% identical with those of other subtilisin-type proteases, proteinase K (24) and subtilisin BPN' (25) is also about 40% (24). The main chain fold of proteinase K shows a high degree of tertiary homology with subtilisin BPN'
**Enhanced Thermostability of Subtilisin**

Fig. 3. SDS-polyacrylamide gel electrophoresis patterns for the purified wild-type and Cys-61/Cys-98 mutant subtilisin E. Purified enzymes were inactivated with phenylmethanesulfonyl fluoride before boiling in SDS solution to limit the autolysis that accompanies denaturation. For the samples in the two left lanes, the SDS solution also contained 1 mM β-mercaptoethanol (β-ME) as a reducing agent. The β-mercaptoethanol was omitted for the non-reduced samples in the other lanes. Five μg of protein was loaded in each lane. Molecular mass standards are shown at the left. SDS-polyacrylamide gel electrophoresis was done on a 17.5% polyacrylamide gel using the procedure described by Laemmli (33).

![Image of SDS-PAGE gel](https://example.com/image.png)

**TABLE I**

Kinetic constants of wild-type, Cys-61, Cys-98, and Cys-61/Cys-98 mutant subtilisins for the hydrolysis of succinyl-Ala-Ala-Pro-Phe-p-nitroanilide

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(K_m) (mM)</th>
<th>(k_{cat}) (s⁻¹)</th>
<th>(k_{cat}/K_m) (s⁻¹·mM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.9 ± 0.2</td>
<td>21 ± 4</td>
<td>11</td>
</tr>
<tr>
<td>Cys-61</td>
<td>1.5 ± 0.3</td>
<td>11 ± 3</td>
<td>7.3</td>
</tr>
<tr>
<td>Cys-98</td>
<td>1.4 ± 0.3</td>
<td>6.0 ± 2</td>
<td>4.3</td>
</tr>
<tr>
<td>Cys-61/Cys-98</td>
<td>2.2 ± 0.5</td>
<td>23 ± 4</td>
<td>10</td>
</tr>
</tbody>
</table>

**TABLE II**

Effect of mutation in subtilisin E on the half-life due to autolytic inactivation

Remaining activity after heating for various times at indicated temperatures in the presence of 1 mM CaCl₂ was determined at 37 °C using succinyl-Ala-Ala-Pro-Phe-p-nitroanilide as the substrate. Half-life due to autolytic inactivation was determined from semi-log plots of log₁₀[residual activity] versus time.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Half-life at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>45 °C</td>
</tr>
<tr>
<td>Wild-type</td>
<td>4.4</td>
</tr>
<tr>
<td>Cys-61</td>
<td>37</td>
</tr>
<tr>
<td>Cys-98</td>
<td>40</td>
</tr>
<tr>
<td>Cys-61/Cys-98</td>
<td>10.2</td>
</tr>
</tbody>
</table>

(7, 24), suggesting that the tertiary structure of aqualysin I is similar to those of proteinase K and subtilisin BPN'. The introduction of the disulfide bond Cys-61/Cys-98 into subtilisin E stabilized the protein against irreversible thermal inactivation. High sequence identity was observed in the regions around Cys positions in subtilisin E and aqualysin I. Both aqualysin I and proteinase K are disulfide-containing serine proteases in the subtilisin family. The positions of the Cys residues of the two proteases differ from each other, suggesting that the disulfide bonds in microbial serine proteases are required for enzyme stability, and are not traits which have been conserved in the process of evolution. X-ray crystallographic data for subtilisin BPN' (31) show that residues 61 and 98 are not α-helix and β-sheet or in a tight turn. Such positions in subtilisin E could produce a good geometric fit for a disulfide.

Pantoliano et al. (5) reported that a disulfide bond between positions 22 and 87 has a significant effect on the stability of subtilisin BPN', although the effect is less pronounced in 10 mM CaCl₂. On the other hand, our present results indicate that the engineered disulfide bond increases the thermostability of subtilisin E in the presence of calcium. Therefore, it is noteworthy that the mechanism whereby the tertiary structure is stabilized by calcium is not influenced by the introduction of the Cys-61/Cys-98 disulfide bond. Furthermore, the introduction of the disulfide enhances the thermostability of subtilisin E without changing its catalytic efficiency. To our knowledge, this is the first report of a dramatic improvement in thermal stability by introduction of a disulfide bond in a naturally occurring thermostable enzyme.

We also attempted replacements with Cys at positions 161...
and 195 in subtilisin E, corresponding to Cys-163/Cys-194 in aqualysin I, to test their effect on stability. However, in the mutant subtilisin with Cys-161 and Cys-195, disulfide bonds were not detected by SDS-polyacrylamide gel electrophoresis, probably because of the long distance between these 2 residues. Surprisingly, when Glu-195 alone was replaced with Cys, catalytic activity was severely impaired (less than 5% of the wild-type enzyme activity), whereas replacement at Ser-161 did not greatly affect the catalytic parameters of the enzyme (60% of the wild-type activity). From x-ray analysis of subtilisin BPN’ (31), it is known that there is a lysine at position 170 near Glu-195 and that the distance between the α-carbons of these residues is estimated to be 5.0 Å. It is possible that electrostatic interaction between Lys-170 and Glu-195 may cause the structural stability around the active site, hence the impairment of catalytic activity by the replacement. Considering that both of the residues are included in a β-turn region, not in the α-helix or the β-sheet region, of subtilisin, it is possible that introduction of 2 Cys residues into both of the above-mentioned positions for formation of a disulfide bond would create higher thermostability. We are now designing another disulfide bond variant around positions 161 and 195 in subtilisin E, predicted by computer graphics from the crystallographic data for subtilisin BPN’.

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