J. Biochem. 128, 585–589 (2000)

Enhanced Thermostability of the Single-Cys Mutant Subtilisin E under Oxidizing Conditions[1]

Hiroshi Takagi,[2] Kazunori Hirai, Masaru Wada, and Shigeru Nakamori

Department of Bioscience, Fukui Prefectural University, 4-1-1 Kenjojima, Matsuoka-cho, Fukui 910-1195

Received May 15, 2000; accepted July 21, 2000

We obtained enhanced thermostability by replacing Ser161 with Cys in subtilisin E from *Bacillus subtilis*, a cysteine-free alkaline serine protease. The Ser161Cys mutant subtilisin E was purified from the culture supernatant of the recombinant *B. subtilis* in an oxidizing environment. SDS–polyacrylamide gel electrophoresis and mass spectrometry under oxidizing conditions indicated that the mutant enzyme in part formed an oligomeric protein, which may contain an intermolecular disulfide bond between two surface Cys residues at position 161. Further, no free sulfhydryl groups were detected in the mutant enzyme, suggesting the sulfhydryl modification in a monomeric form under oxidizing conditions. The Ser161Cys mutant enzyme showed a catalytic efficiency equivalent to that of the wild-type enzyme. The half-life of thermal inactivation of the mutant was found to be 2–4 times longer than that of the wild-type enzyme. The optimum temperature of the mutant was 55°C, which was 5°C higher than that of the wild-type enzyme. Under reducing conditions, however, the characteristics of the mutant enzyme reverted to those of the wild-type enzyme. Similar results were obtained for another Cys mutant as to position 194 (wild-type, Ser), which is the same surface residue as Ser161. Possible reasons for the enhanced thermostability of the single-Cys mutant subtilisins E under oxidizing conditions are discussed in terms of two different mechanisms.

Key words: oxidizing conditions, site-directed mutagenesis, subtilisin E, sulfhydryl modification, thermostability.

Subtilisin, a cysteine-free alkaline serine protease produced by various *Bacillus* species, has been extensively investigated as a promising target for protein engineering (*1–3*). For instance, many attempts have been made to enhance its thermostability through the introduction of unnatural intramolecular disulfide bond(s) (*4–7*). However, none of the mutants produced in these experiments was dramatically stabilized on the introduction of a *de novo* designed disulfide bridge, indicating that the finding of positions in folded globular proteins which seem capable of accommodating an unstrained disulfide bond is not a clear-cut task.

In previous work (*8*, *9*), we introduced disulfide bonds engineered based on structural similarity to aqualysin I of *Thermus aquaticus* YT-1 (a thermophilic subtilisin-type serine protease containing two disulfide bonds) (*10*) into subtilisin E of *B. subtilis* I168. We found that the NH_2-terminal disulfide bond enhanced the thermostability without any change in the catalytic efficiency (*8*), and that the COOH-terminal disulfide improved the stability in polar organic solvents, whereas the thermal stability was less than that of the wild-type (*9*).

In the process of these studies, it is noteworthy that a single-Cys mutant subtilisin E as to position 161 (wild-type Ser; S161C) was heat-stable relative to the wild-type enzyme (Takagi, H., unpublished observations). Recently, Zhao and Arnold (*11*) also identified amino acid substitution S161C as one of the thermostable mutations in subtilisin E created through directed evolution, but they did not provide a possible explanation for this increased stability. In this study, we examined the mechanism underlying the enhanced stability of the single-Cys mutant subtilisin E.

MATERIALS AND METHODS

Materials—A *B. subtilis* three protease-deficient strain DB403 (*trpC2 aprE⁻ eprE⁻ nprE⁻*), a gift from Dr. R.H. Doi, and *Escherichia coli* strain JA221 (*hsdM⁺ trpE5 leuB6 lacY recA1/F′ lacI^q lac⁺ pro⁺*) (*12*) were used as host cells for the production of wild-type and mutant subtilisins E. Plasmid pHY300PLK (Takara Shuzo, Kyoto) and the isopropyl-β-D-thiogalactopyranoside (IPTG)–inducible pIN-III-ompA vector (*13*) were used for the expression and secretion of subtilisins E in *B. subtilis* and *E. coli*, respectively. *E. coli* C600 (*recA⁺ F⁻ thi-1 thr-1 leuB6 lacY1 tonA21 supE44λ⁻*) was used for the construction of expression plasmids for *B. subtilis*. All the enzymes for DNA manipulations were obtained from Takara Shuzo. The subtilisin substrate *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (AAPF) was purchased from Sigma (St. Louis, USA).

Construction of Expression Plasmids—The replacement of Ser161 and Ser194 by Cys was performed by polymerase chain reaction (PCR) with oligonucleotide primers, 5′-ATC-

[1] This work was supported by a grant from the Fukui Prefectural Scientific Research Foundation (H.T.).

[2] To whom correspondence should be addressed. Tel: +81-776-61-6000, Fax: +81-776-61-6015, E-mail: hiro@fpu.ac.jp

Abbreviations: AAPF, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide; β-ME, β-mercaptoethanol; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SH, sulfhydryl.

© 2000 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

CGGAT*GCACAAGC-3′ (primer 161+), 5′-GCTTGTGCA-*TCCGGAT-3′ (primer 161–), 5′-GCAGGTTG*TGAGCTT-GA-3′ (primer 194+), and 5′-TCAAGCTCAC*AACCTGC-3′ (primer 194–), respectively, using a Gene Amp PCR system 2400 (Perkin-Elmer Applied Biosystems, Foster City, USA). The asterisks indicate the locations of mismatches. pIOE (*9*), containing the wild-type subtilisin E gene inserted into pHY300PLK, was used as a template DNA for site-directed mutagenesis. In addition, primers 5′-CGGAATTCTAAAT-AGAGAT-3′ (the underlined sequence is the position of a *Eco*RI site) and 5′-GCCCTAGGGCTTGTGAAGATTTT-3′ (the underlined sequence is the position of a *Bam*HI site) were used to complete regions of the *Eco*RI restriction site and the *Bam*HI restriction site in pIOE, respectively. The unique amplified band material of 1,437 bp was digested with *Eco*RI and *Bam*HI, and then ligated to the *Eco*RI and *Bam*HI site of pHY300PLK. The mutations were confirmed with a Model 377 DNA sequencer (Perkin-Elmer Applied Biosystems) using dideoxy chain termination sequencing. The resultant plasmids carrying the mutant subtilisin E genes (S161C and S194C) were constructed in *E. coli* C600, and designated as pIOC161 and pIOC194, respectively. These plasmids were then introduced into *B. subtilis* DB403 by electroporation (*14*).

For gene expression in *E. coli*, the above mutant plasmids and a plasmid, pHI212 (*15*), harboring the wild-type subtilisin E gene inserted into pIN-III-ompA, were digested with *Hin*dIII and *Bam*HI. The 0.8 kb fragment of pIOC161 or pIOC194 containing mutation S161C or S194C, respectively, was then ligated to the large fragment of plasmid pHI212.

Expression and Purification of Wild-Type and Mutant Subtilisins E—Various subtilisins E were expressed and purified from the culture supernatant of *B. subtilis* DB403 or from the periplasmic fraction of *E. coli* JA221, as described previously (*8*, *9*). To purify the mutant subtilisins E, a recombinant *B. subtilis* strain was grown at 37°C for 24 h in Luria-Bertani medium containing tetracycline (20 μg/ml) (*9*).

Assaying of Subtilisin E Activity—For the synthetic peptide substrate AAPF, assays were performed in 50 mM Tris-HCl (pH 8.5) and 1 mM $CaCl_2$ at 37°C as described previously (*15*). The amount of *p*-nitroaniline released was measured as the absorbance at 410 nm, and the activity was calculated as units/mg protein. One unit is defined as the activity releasing 1 mmol of *p*-nitroaniline/min.

Immunoblotting and Amino Acid Sequence Analysis—The purified subtilisins E were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and then either stained with Coomassie Brilliant Blue or electrotransferred to a poly(vinylidene difluoride) membrane. Recombinant subtilisins E were detected by Western blot analysis using a Vectastain ABC kit (Vector Laboratories, Burlingame, USA) and an anti-mature subtilisin E polyclonal antibody. Protein bands were also excised for NH_2-terminal amino acid sequence analysis by automated Edman degradation with a Model 476A pulsed liquid protein sequencer (Perkin-Elmer Applied Biosystems).

Mass Spectrometry—Mass spectrometric analyses were carried out with a Voyager Elite mass spectrometer (PerSeptive Biosystems, Framingham, USA) using a 25 kV accelerating voltage. Mass spectra were acquired by adding the individual spectra from 32 laser shots. The samples were run in the linear mode. The protein solutions were diluted 1:1 (v/v) with the matrix solution, 10 mg/ml α-cyano-4-hydroxycinnamic acid (Aldrich, Gillingham, England) in 50% CH_3CN in 0.1% TFA, and then allowed to air-dry on the sample target before analysis. Cytochrome *c* (12,384 Da; Wako, Osaka) and bovine serum albumin (664,299 Da; Wako) were used as external standards.

Thermal Stability of Subtilisin E—To determine the autolytic stability, the purified enzymes (25 μg/ml) in 10 mM sodium phosphate buffer (pH 6.2) containing 1 mM $CaCl_2$ were either treated or not treated with 5 mM dithiothreitol (DTT) at room temperature for 30 min. The activity remaining after heating for various times at 55–65°C was determined at 37°C using AAPF as the substrate.

Molecular Modeling—Molecular modeling was performed with the computer programs Insight II and Homology (Molecular Simulations, San Diego, USA). Modeling of subtilisin E was based on the X-ray crystal structure of subtilisin E with propeptide (PDB codes 1SCJ) (*16*).

RESULTS

Structure of the S161C Mutant Subtilisin E under Oxidizing Conditions—The wild-type and mutant subtilisin E genes were expressed in *B. subtilis* DB403, followed by purification from the culture fluid to give a single band on SDS-PAGE. Interestingly, a new band corresponding to a higher molecular mass (~100 kDa) than that of the mature protein was observed for the S161C mutant under nonreducing conditions without the use of β-mercaptoethanol (β-ME) as a reducing agent (Fig. 1A). Further, Western blot analysis showed that the new band arose from subtilisin E (Fig. 1B). The percentage of the ~100-kDa protein was judged to be approximately 16% on densitometric estimation of the amounts obtained on SDS-PAGE. Similar results were obtained for the culture supernatant of the recombinant *B. subtilis* without further purification and for the enzymes purified from the *E. coli* periplasmic fraction. The NH_2-terminal amino acid sequence of the higher molecular mass protein was AQSVPYGISQIKAP-, which coincides with that of the mature subtilisin E. These results suggest that the S161C mutant enzyme was in part spontaneously oligomerized under oxidizing conditions.

Matrix-assisted laser desorption and ionization mass spectra of the wild-type and mutant enzymes were obtained to accurately determine the molecular masses of the proteins (Fig. 2). In the case of the S161C mutant subtilisin E, a prominent peak (molecular mass, 55,838 Da) was observed in addition to that of a monomer with an apparent molecular mass of 27,879 Da, and was assigned as a dimer in solution. These results suggest that an oligomeric protein from the S161C mutant enzyme may contain the disulfide-linked dimeric structure.

Further, free sulfhydryl (SH) groups were determined according to the method of Ellman (*17*) using 5,5′-dithiobis(2-nitrobenzoic acid) under denaturing conditions (6 M urea). No free SH groups were detected in the S161C mutant subtilisin E, suggesting the spontaneous modification of SH groups with small SH compounds and the formation of an intermolecular disulfide bond in the mutant enzyme (data not shown). The monomeric form of the S161C mutant enzyme had an apparent molecular mass of 27,879 Da, which was larger than the 352 Da observed for

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

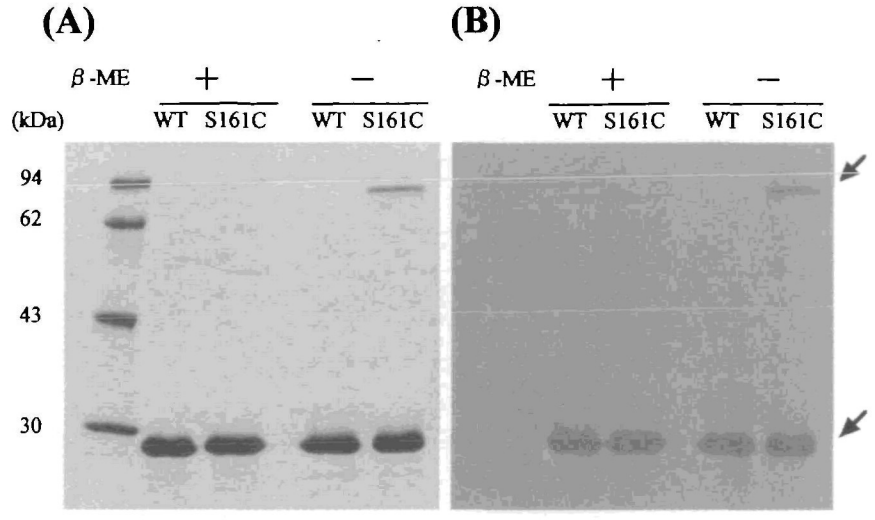

Fig. 1. **The wild-type and S161C mutant subtilisins E detected on (A) SDS-PAGE and (B) immunoblotting with anti-subtilisin E antiserum.** Both enzymes were purified from the culture fluid of *B. subtilis* DB403. Arrowheads indicate the positions of the dimer (upper) and monomer (lower) of subtilisin E. To limit the autolysis that accompanies denaturation, the enzymes were inactivated with phenylmethanesulfonyl fluoride before being boiled in the SDS solution. For the samples in the left two lanes, the SDS solution also contained 1 mM β-ME as a reducing agent. β-ME was omitted from the nonreducing samples in the other two lanes. Three micrograms of protein was loaded in each lane. SDS-PAGE was performed in a 15% polyacrylamide gel. The gel for SDS-PAGE was stained with Coomassie Brilliant Blue. Molecular mass standards are shown at the left.

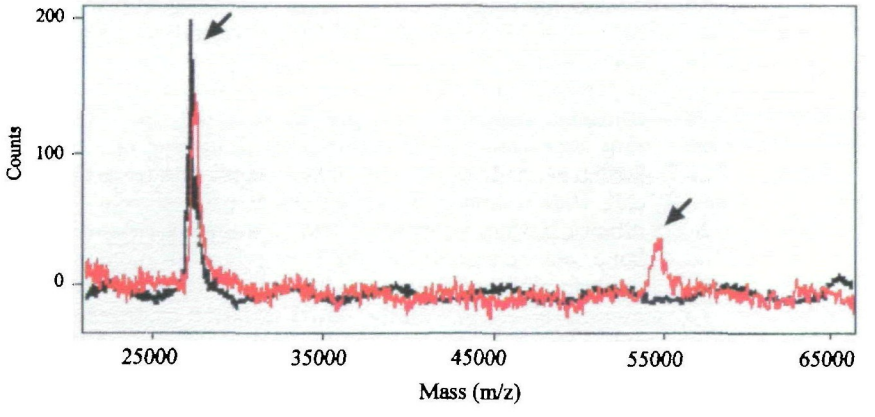

Fig. 2. **Matrix-assisted laser desorption and ionization mass spectrum of the wild-type (black) and S161C mutant (red) subtilisins E.** Arrowheads indicate the peaks of the monomer (left) and dimer (right) of subtilisin E.

the wild-type enzyme (27,527 Da; Fig. 2). Judging from the difference in molecular weight between the two enzymes, it can therefore be presumed that glutathione (5-L-glutamyl-L-cysteinylglycine) (307 Da) is a candidate small SH compound, which is linked through a covalent bond to the SH group in the S161C mutant enzyme.

Molecular Modeling of the Mutant Subtilisins E—Based on the three-dimensional structure of subtilisin E (*16*), the solvent-accessible surface area of each residue was calculated by means of computational modeling. The solvent-accessible surface area of Ser161 was estimated to be 67.7 Å^2, indicating that the Cys residue at position 161 is located on the protein surface and can be modified at a free SH group or form an intermolecular disulfide bond spontaneously. To further analyze the role of an engineered surface Cys residue in subtilisin E in the enzymatic properties, we focused on Ser194, which would be in the same environment as Ser161 (the solvent-accessible surface area of Ser194 was 75.2 Å^2), and constructed a single-Cys mutant, S194C, by means of site-directed mutagenesis. Judging from the findings on SDS-PAGE and the detection of free SH groups, the S194C mutant subtilisin E also in part forms a higher molecular mass protein (~100 kDa) under oxidizing conditions and has no free SH groups, which is in good agreement with the results regarding the S161C enzyme (data not shown).

TABLE I. **Specific activity and thermal stability of the wild-type and mutant subtilisins E.**

Enzyme	DTT	Specific activity (units/mg)	Half-life (min)		
			55°C	60°C	65°C
Wild-type	−	4,220	31	13	4.1
	+	4,250	31	12	4.2
S161C	−	4,040	137	38	8.1
	+	4,020	32	15	4.3
S194C	−	3,990	NT	NT	8.0
	+	4,000	NT	NT	4.1

The Purified enzymes were treated with or without 5 mM DTT at room temperature for 30 min. Specific activity and remaining activity after heating for various times at the indicated temperatures in the presence of $CaCl_2$ were determined at 37°C using succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. The half-life due to autolytic inactivation was determined from a semi-log plot of $\log_{10}$ [residual activity] *versus* time. The variations in the values were less than 5%. NT, not tested.

Characteristics of the S161C and S194C Mutant Subtilisins E—As shown in Table I, the specific activities toward AAPF as an authentic substrate for subtilisin of the mutant subtilisins E were essentially equivalent to those of the wild-type enzyme, regardless of whether or not they were pretreated with 5 mM dithiothreitol (DTT) for reduction.

It has been shown for some serine proteases including

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

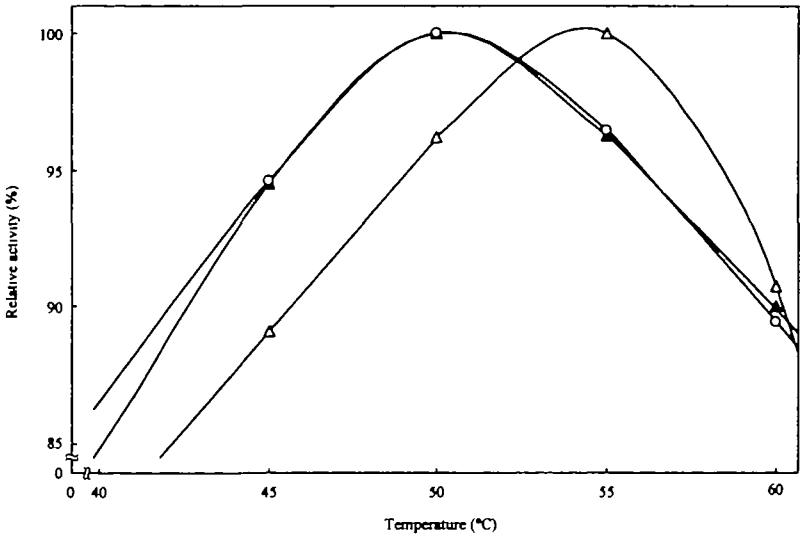

Fig. 3. **Temperature dependence of the relative activity for hydrolysis of AAPF by the wild-type (○) and S161C mutant (△) subtilisins E.** The S161C mutant was also pretreated with 5 mM DTT (▲). Relative activity was expressed as a percentage of the maximum activity. The variations in the values were less than 5%.

subtilisin that reduced autolysis leads to an apparent increase in instability (*8*, *9*, *18*). Subtilisin is well-known to be inactivated irreversibly through autolysis at elevated temperatures (*4*, *6*, *8*). To determine the autolytic stability of these enzymes, the rate of thermal inactivation was measured at 55–65°C in the presence of Ca^{2+} (Table I). The half-life ($t_{1/2}$) of the mutants was found to be 2–4 times longer than that of the wild-type enzyme. When the enzymes were treated with 5 mM DTT for reduction, the mutants exhibited a half-life nearly equivalent to that of the wild-type enzyme. In addition, the relative activity toward AAPF *versus* assay temperature was investigated (Fig. 3). The S161C mutant subtilisin E showed an optimum temperature of 55°C, which was 5°C higher than that observed for the wild-type enzyme. Under reducing conditions, however, the temperature pattern of the mutant was found to decrease to that of the wild-type enzyme.

DISCUSSION

We previously examined the effect of novel disulfide bonds engineered in subtilisin E on the basis of structural comparison with a thermophilic serine protease (*8*, *9*). When Ser161 alone in subtilisin E was replaced with Cys, the rate of thermal inactivation ($t_{1/2}$) increased relative to that of the wild-type enzyme (Takagi, H., unpublished observations). Independently, Zhao and Arnold (*11*) used directed evolution to convert subtilisin E into an enzyme functionally equivalent to its thermophilic homolog, thermitase, and isolated the S161C variant after random mutagenesis by error-prone PCR and *in vitro* recombination by the staggered extension process (*19*). However, the mechanism by which amino acid substitution S161C enhances the thermostability has not been elucidated. It is difficult to speculate stabilizing mutation on the basis of structural comparisons of subtilisins from mesophilic and thermophilic organisms, because mutation S161C only exists in a mesophilic enzyme from *Drosophila melanogaster* (*11*).

We showed here that under oxidizing conditions two forms of the S161C enzyme with apparent molecular weights of 28 kDa and ~100 kDa were detected on SDS-PAGE (Fig. 1), and two peaks corresponding to molecular masses of 27,879 Da and 55,838 Da were detected on mass spectrometry (Fig. 2). There is no evidence, however, that a portion of the S161C enzyme exists in a dimeric form in solution. The ~100-kDa protein detected on SDS-PAGE may represent an oligomeric protein, instead of a dimeric structure. Although the results of mass spectrometry indicate that a small amount of the S161C enzyme forms a dimeric protein, probably due to an intermolecular disulfide bond, one cannot judge whether or not the monomeric or dimeric proteins associate with one another to form oligomeric proteins in solution. Further, based on the detection of the free SH content with Ellman's reagent, the SH modification of a free Cys residue was supposed to occur in the monomeric protein (data not shown).

From the X-ray crystal structure of subtilisin E, it is known that two Ser residues at positions 161 and 194 are located in flexible and variable loops on the protein's surface. Hu *et al.* (*20*) reported that the conserved Ser49 on the surface of subtilisin E was replaced by Cys and that the mutant formed a disulfide-linked dimer, prosubtilisin, spontaneously *in vivo*, thereby trapping a folding intermediate with no activity. The expressed protein was secreted into an oxidizing environment, the periplasmic space of *E. coli*. After purification and reduction with DTT, the resulting prosubtilisin monomer was found to be capable of undergoing autoprocessing *in vitro* in renaturing medium (*20*). In contrast, it would appear in our case that SH modification of the surface Cys residue with small SH compounds such as glutathione rather than oligomer formation *via* Cys161 or Cys194 easily takes place in the culture medium of *B. subtilis* and in the periplasmic space of *E. coli*, both being oxidizing environments.

The S161C and S194C mutant enzymes possess nearly identical protease activity to that of the wild-type subtilisin E. This is not unexpected, since these residues are presumed to be on the surface of subtilisin E and to be far from the catalytic triad. Both single-Cys mutant subtilisins E were found to be rather resistant to thermal autolysis under oxidizing conditions (Table I). With regard to position 194, the replacement of Ser by Pro, not Cys, has already been shown to have a small stabilizing effect, probably through a reduction in the entropy of the flexible loop (*11*).

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

In general, the surface structure of a protein is mainly involved in the initial stage of unfolding on thermal inactivation (*21*). Here, the question arises as to why both single-Cys mutant enzymes enhance the thermostability under oxidizing conditions. Although pure forms were not obtained from the mixed solutions of monomeric and oligomeric forms for their characterization, we now consider the following two possible mechanisms: (i) the SH modification of the surface Cys residue on the binding of small SH compounds such as glutathione reduces thermal unfolding or autolysis, and inhibits the oxidative formation of cysteine sulfonate, which may cause protein denaturation or inactivation, and (ii) the oligomerization including an intermolecular disulfide bond occurs between the monomeric and/or dimeric proteins, whereby unfolded monomer forms are unable to gain access to the substrate-binding pocket due to steric repulsion.

To elucidate the mechanism further, attempts *in vitro* to separate the higher molecular mass protein from the monomer by means of gel filtration or ion-exchange column chromatography or to increase its content through chemical oxidization are currently in progress. If the oligomeric (or dimeric) proteins were not linked through the covalent bonds such as disulfide bonds, it might not be possible to obtain the pure forms in the equilibrium system. Also, it would be of interest to determine the tertiary structure for an understanding of the stabilization mechanism of the single-Cys mutant subtilisin E under oxidizing conditions.

We wish to thank Ryoka Systems (Chiba) for their help in the computer modeling. The technical assistance of Dr. T. Hibi in the mass spectrometry is greatly appreciated.

REFERENCES

1. Wells, J.A. and Estell, D.A. (1988) Subtilisin—an enzyme designed to be engineered. *Trends Biochem. Sci.* **13**, 291–297
2. Takagi, H. (1993) Protein engineering on subtilisin. *Int. J. Biochem.* **25**, 307–312
3. Siezen, R.J. and Leunissen, J.A.M. (1997) Subtilases: the superfamily of subtilisin-like serine proteases. *Protein Sci.* **6**, 501–523
4. Wells, J.A. and Powers, D.B. (1986) *In vivo* formation and stability of engineered disulfide bonds in subtilisin. *J. Biol. Chem.* **261**, 6564–6570
5. Pantoliano, M.W., Ladner, R.C., Bryan, P.N., Rollence, M.L., Wood, J.F., and Poulos, T.L. (1987) Protein engineering of subtilisin BPN′: enhanced stabilization through the introduction of two cysteines to form a disulfide bond. *Biochemistry* **26**, 2077–2082
6. Mitchinson, C. and Wells, J.A. (1989) Protein engineering of disulfide bonds in subtilisin BPN′. *Biochemistry* **28**, 4807–4815
7. Katz, B. and Kossiakof, A.A. (1990) Crystal structures of subtilisin BPN′ variants containing disulfide bonds and cavities: concerted structural rearrangements induced by mutagenesis. *Proteins Struct. Funct. Genet.* **7**, 343–357
8. Takagi, H., Takahashi, T., Momose, H., Inouye, M., Maeda, Y., Matsuzawa, H., and Ohta, T. (1990) 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. *J. Biol. Chem.* **265**, 6874–6878
9. Takagi, H., Hirai, K., Maeda, Y., Matsuzawa, H., and Nakamori, S.J. (2000) Engineering subtilisin E for enhanced stability and activity in polar organic solvents. *J. Biochem.* **127**, 617–625
10. Matsuzawa, H., Tokugawa, K., Hamaoki, M., Mizoguchi, M., Taguchi, H., Terada, I., Kwon, S.T., and Ohta, T. (1988) Purification and characterization of aqualysin I (a thermophilic alkaline serine protease) produced by *Thermus aquaticus* YT-1. *Eur. J. Biochem.* **171**, 441–447
11. Zhao, H. and Arnold, F.H. (1999) Directed evolution converts subtilisin E into a functional equivalent of thermitase. *Protein Eng.* **12**, 47–53
12. Ikemura, H., Takagi, H., and Inouye, M. (1987) Requirement of pro-sequence for the production of active subtilisin E in *Escherichia coli*. *J. Biol. Chem.* **262**, 7859–7864
13. Ghyayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y., and Inouye, M. (1984) Secretion cloning vectors in *Escherichia coli*. *EMBO J.* **3**, 2437–2442
14. Ohse, M., Takahashi, K., Kadowaki, Y., and Kusaoke, H. (1995) Effects of plasmid DNA sizes and several other factors on transformation of *Bacillus subtilis* ISW1214 with plasmid DNA by electroporation. *Biosci. Biotechnol. Biochem.* **59**, 1433–1437
15. Takagi, H., Morinaga, Y., Ikemura, H., and Inouye, M. (1988) Mutant subtilisin E with enhanced protease activity obtained by site-directed mutagenesis. *J. Biol. Chem.* **262**, 19592–19596
16. Jain, S.C., Shinde, U., Li, Y., Inouye, M., and Berman, H.M. (1998) The crystal structure of an autoprocessed Ser221Cys-subtilisin E-propeptide complex at 2.0 Å resolution. *J. Mol. Biol.* **284**, 137–144
17. Ellman, G.L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70–77
18. Daniel, R.M., Cowan, D.A., Morgan, H.W., and Curran, M.P. (1982) A correlation between protein thermostability and resistance to proteolysis. *Biochem. J.* **207**, 641–644
19. Zhao, H., Giver, L., Shao, Z., Affholter, J.A., and Arnold, F.H. (1998) Molecular evolution by staggered extension process (StEP) in vitro recombination. *Nat. Biotechnol.* **16**, 258–262
20. Hu, Z., Zhu, X., Jordan, F., and Inouye, M. (1994) A covalent trapped folding intermediate of subtilisin E: spontaneous dimerization of a prosubtilisin E Ser49Cys mutant *in vivo* and its autoprocessing *in vitro*. *Biochemistry* **33**, 562–569
21. Jackson, S.E. and Fersht, A.R. (1991) Folding of chymotrypsin inhibitor 2. 2. Influence of proline isomerization on the folding kinetics and thermodynamic characterization of the transition state of folding. *Biochemistry* **30**, 10436–10443

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012