

# Thermal stable and oxidation-resistant variant of subtilisin E

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## Abstract

A remarkable thermal stable and oxidation-resistant mutant was obtained using the random mutagenesis PCR technique on the mutant M222A gene of subtilisin E. Sequencing analysis revealed an A was replaced by G at nucleotide 671 of the subtilisin E gene, converting the asparagine codon (AAT) to serine codon (AGT) at position 118. The half-life of M222A/N118S enzyme activity, when heated at 65°C, was approximately 80 min while the half-life of M222A and wild-type subtilisin E were 13 min and 15 min, respectively. This suggested the stability of the M222A/N118S mutant was five times greater than that of the wild-type enzyme. The mutant was also as oxidation resistant as the mutant M222A of subtilisin E. These results indicated the M222A/N118S mutant is both an oxidation-resistant and a heat-stable variant of subtilisin E. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Oxidation-resistant; Random mutagenesis; Subtilisin E; Thermal stability

## 1. Introduction

Subtilisins are a family of alkaline serine endoproteases which are secreted by a variety of *Bacillus* species and widely used in industry, e.g. as biodegradable cleaning agents in laundry detergents. In the view of industrial aspects, subtilisins have been extensively investigated as a promising target for protein engineering (Strausberg et al., 1995; Kidd et al., 1999). The thermal stability of

subtilisin is a very important character for its practical utility. However, wild-type subtilisins, such as subtilisin Carlsberg secreted from *Bacillus licheniformis* and subtilisin E from *Bacillus subtilis* are comparatively stable at room temperature but unstable at higher temperatures. To enhance the thermostability of subtilisin E, Pro239Arg and a disulfide bridge have been introduced into the protein molecule by site-directed mutagenesis (Pantoliano et al., 1987; Takagi et al., 1989, 1990; Takagi, 1993). Unfortunately, only a few mutants with high thermostability have been found at present due to the limited knowledge of the structure–stability relationship of the investigated protein, although thermostability is correlated

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with the increased number of residues involved in charge–charge and aromatic–aromatic interactions. The random mutagenesis *in vitro* is an efficient approach when coupled with an efficient screening of selection procedures to identify colonies expressing variant enzymes with the properties of interest (Strausberg et al., 1995; Sattler et al., 1996; Takagi et al., 1996, 1997, 1998; You and Arnold, 1996). One thermally stable variant of subtilisin BPN', N218S was identified using a simple plate assay procedure to screen for esterase activity on nitrocellulose filters after preincubation at elevated temperature (Bryan et al., 1986). It also exhibited three-fold higher thermostability than wild-type subtilisin E (Wang et al., 1993).

We now report a remarkable thermally stable and oxidation-resistant mutant discovered by a random mutagenesis PCR technique *in vitro* combined with phenotypic screening. The inactivation rate of the mutant is five-fold slower at 65°C than that of wild-type subtilisin E.

## 2. Material and methods

T4 DNA ligase, T7 DNA polymerase of sequencing quality, ATP, dNTPs were purchased from Pharmacia. Restriction enzymes were obtained from New England Biolabs. Wizard *plus* Minipreps DNA purification system and Wizard PCR preps DNA purification system were purchased from Promegam. A synthetic substrate, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (s-AAPF-*p*Na) was from Sigma. All the other reagents used were of the highest grade commercially available.

Plasmid pBY was constructed by inserting a 1.9 kb *Eco*R I/*Sal* I fragment containing subtilisin E gene and its regulatory regions into pBE-2 which is a *B. subtilis*–*Escherichia coli* shuttle vector (Guo et al., 1991). Plasmid pBM and pBH, analogs of pBY, contain the mutation of M222A and M222A/N118S, respectively. All mutations were verified by DNA sequencing using Pharmacia sequencing kit through the dideoxy termination method.

### 2.1. Random mutagenesis using modified PCR (Chen and Arnold, 1993)

The unique *Hind*III DNA fragment of pBM encoding mature subtilisin E from amino acid residue 49 to the C-terminus was chosen as the target for random mutagenesis and cloned into pUC19 to obtain plasmid pUA for PCR. The universal M13 forward and reverse primers, 5'-CCC AGT CAC GAC GTT GTA AAA CG-3' and 5'-AGC GGA TAA CAA TTT CAC ACA GG-5' were used as 5' and 3' PCR primers, respectively. PCR was carried out at 94°C for 45 s, 52°C for 1 min, and 70°C for 1 min, and a total of 25 cycles were performed. The size of the amplified DNA fragments were determined by agarose gel electrophoresis and recovered by PCR DNA purification kit.

The PCR generated DNA fragments were digested with *Hind*III and cloned into the *Hind*III fragment of pBM to construct a randomly mutated DNA library.

### 2.2. Screening of thermal stable mutant

The randomly mutated DNA library was transformed to the protease-deficient *B. subtilis* strain DB104 (Kawamura and Doi, 1984) and then plated onto agar plate containing 1% skimmed milk. Clones producing subtilisin determined by the halo formation were inoculated in 3 ml 2 × YT medium with 100 µg ml<sup>-1</sup> ampicillin and incubated at 37°C for 18 h. The supernatants were incubated at 65°C for 30 min, respectively followed by measuring the residual amidase activity and calculating the relative activities. The plasmid of the thermal stable mutant was denoted as pBH and was sequenced by dideoxy termination method as the protocol supplied with Pharmacia sequencing kit.

### 2.3. Purification of the mutant protein

Purification of wild-type and variant subtilisin E was carried out by the following procedure. A recombinant *B. subtilis* carrying the wild-type or mutated subtilisin E was grown at 37°C in 30 ml industrial medium containing 60 g l<sup>-1</sup> cornmeal,

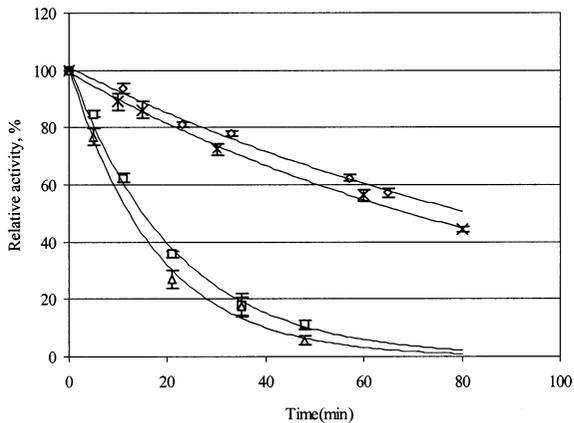


Fig. 1. Thermal stability of subtilisin E and its mutants. □, Wild-type subtilisin E; ×, N118S mutant; ◇, M222A/N118S mutant; △, M222A mutant. The enzymes were dissolved in the solution of 100 mM Tris-HCl, pH 8.0, 5 mM CaCl<sub>2</sub> and incubated at 65°C for the time indicated. The residual amidase activities were measured on the substrate casein 1% at 40°C in 25 mM borate buffer, pH 11. Relative activities are presented as means of four experiments ± S.D.

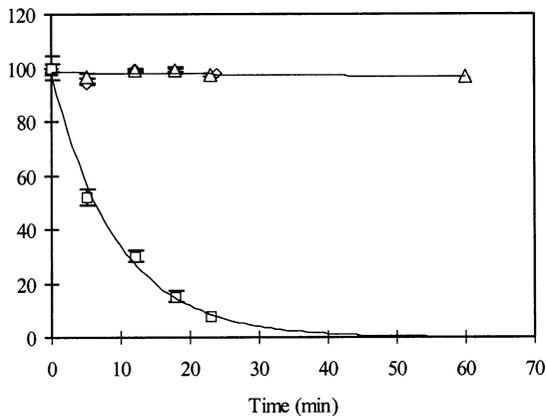


Fig. 2. Effects of hydrogen peroxide on the activity of subtilisin E and its mutants. □, Wild-type subtilisin E; ◇, M222A/N118S mutant; △, M222A mutant. The enzymes were dissolved in the solution of 100 mM Tris-HCl, pH 8.0, 5 mM CaCl<sub>2</sub>, 1 M H<sub>2</sub>O<sub>2</sub> at the concentration of 1 mg ml<sup>-1</sup>, and incubated at 37°C for the time indicated. The residual initial activities were measured over time by mixing not more than 50 µl of the enzyme solution into 1.5 ml 0.1 mM s-AAPF pNa substrate solution of 100 mM Tris-HCl, pH 8.0, 5 mM CaCl<sub>2</sub> at 37°C. Relative activities are presented as means of four experiments ± S.D.

40 g l<sup>-1</sup> bean cake, 4 g l<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>·3H<sub>2</sub>O, 0.3 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 1 g l<sup>-1</sup> Na<sub>2</sub>CO<sub>3</sub> and kanamycin (final concentration 50 µg ml<sup>-1</sup>) for approximately 48 h. Subtilisin produced and excreted into the medium was recovered by 30–65% ammonium sulfate fraction and then dialyzed against 100 mM sodium phosphate buffer, pH 6.0, containing 5 mM CaCl<sub>2</sub>. The enzyme was then purified by DEAE Sephadex A-25 column chromatography and cation exchange column Hiload 26/10S sepharose HP on FPLC system with a gradient of 0–0.4 M NaCl in 100 mM sodium phosphate buffer, pH 6.0 followed by dialysis against 100 mM sodium phosphate buffer, pH 6.0, containing 5 mM CaCl<sub>2</sub> at 4°C overnight. The purity of recovered enzyme was checked by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis.

#### 2.4. Enzyme kinetics

Subtilisin amidase activities were measured on the substrate s-AAPF-pNa at 37°C in 100 mM Tris-HCl, pH 8.0, containing 5 mM CaCl<sub>2</sub>. Initial rates of hydrolysis of s-AAPF-pNa, and specific activities were determined as described (Estell et al., 1985). Data were fitted to the Michaelis-Menten equation by non-linear regression.

#### 2.5. Thermal stability of the enzymes

Enzyme stability at 65°C were measured. The enzymes resolved in 100 mM Tris-HCl, pH 8.0, containing 5 mM CaCl<sub>2</sub> were incubated at 65°C and withdrawn at various time intervals (Fig. 1). The residual amidase activities were measured on the substrate casein 1% at 40°C in 25 mM borate buffer, pH 11.

#### 2.6. Effect of H<sub>2</sub>O<sub>2</sub> on the activity of mutants and wild-type subtilisin E

Enzyme dissolved in 100 mM Tris-HCl, pH 8.0, 5 mM CaCl<sub>2</sub> and 1 M H<sub>2</sub>O<sub>2</sub> at a concentration of 1 g l<sup>-1</sup> was incubated at 37°C for the time indicated (Fig. 2). The residual initial activity was measured at 37°C on the substrate s-AAPF-pNa and compared with the enzyme untreated with H<sub>2</sub>O<sub>2</sub>.

Table 1  
Kinetic constants for hydrolysis of s-AAPF-pNa by wild-type and variant subtilisin E<sup>a</sup>

Enzyme	$k_m$ mM <sup>-1</sup>	$k_{cat}$ s <sup>-1</sup>	$k_{cat}/k_m$ s <sup>-1</sup> mM <sup>-1</sup>
Wild type	0.43 ± 0.06	29.6 ± 1.68	68.89 ± 1.48
M222A	0.56 ± 0.03	14.8 ± 0.14	26.51 ± 1.40
N118S	0.52 ± 0.04	40.8 ± 0.26	78.13 ± 4.34
M222A, N118S	0.91 ± 0.04	31.2 ± 0.28	34.35 ± 1.47

<sup>a</sup> Values are given as means of four experiments ± S.D.

### 3. Results

#### 3.1. Random mutagenesis and isolation of N118S/M222A mutant

The subtilisin E gene containing the M222A mutation is located in the 1.9 kb fragment of pBM digested by *EcoR* I/*Sal* I. The *Hind*III fragment of pBM, approximately 0.8 kb, coding mature subtilisin E from amino acid residue 49 to the C-terminus was cloned into pUC19 and the resulted recombinant plasmid pUA was used for random mutagenesis by PCR. Thirty-six clones producing active subtilisin were selected by the halo formation on skimmed plate and grown at 37°C in 3 ml of 2 × YT medium for 18 h. The supernatants were assayed for proteolytic activity on the specific substrate s-AAPF-pNa after being incubated at 65°C for 30 min. Only one clone exhibited significantly more stability than the wild-type subtilisin E at 65°C. A at 671 in the coding region was changed to G leading to the replacement of N118 (AAT) by S (AGT) according to the sequencing analysis. This further evidenced random mutagenesis is an essential approach to discover desirable mutants.

#### 3.2. Effect of the mutation on amidase activity and thermal stability

$k_m$  and  $k_{cat}$  values were determined from initial rate measurements for the hydrolysis of pNa over a substrate s-AAPF-pNa. Enzyme concentrations were determined spectrophotometrically  $E_{280}^{0.1\%} = 1.17$  (Estell et al., 1985) to permit calculation of  $k_{cat}$  from the relationship of  $k_{cat} = V_{max}/[enzyme]$ .

Table 1 shows that the N118S mutation increased  $k_{cat}/k_m$  value slightly of the mutant M222A although the  $k_{cat}/k_m$  value of M222A/N118S mutant is 50% of that of the wild-type subtilisin. We tested the thermostability of the M222A/N118S mutant, as well as the M222A mutant and wild-type subtilisin E. Fig. 1 shows the half-life of M222A/N118S enzyme activity, when heated at 65°C, was approximately 80 min while the half-life of the M222A and the wild-type subtilisin E were 13 min and 15 min, respectively. The half-life of the M222A/N118S mutant was six times greater than that of M222A and five-fold that of wild-type subtilisin E. Therefore, the N118S mutation increased the heat stability of the M222A mutant while slightly increasing the activity of mutant M222A. On the other hand, the M222A/N118S mutant was also as oxidation-resistant as the M222A mutant as shown by Fig. 2. All these results indicated the M222A/N118S mutant showed a significant increase in both thermo and oxidative stabilities.

In order to probe the effect of N118S mutation on the thermostability of subtilisin E, N118S mutation was introduced into the wild-type subtilisin E with the primer, 5'-ATT TCC AAC TCT ATG GAT GTT-3' (underline indicates altered codon). The half-life of N118S at 65°C is about 68 min which is a little unstable compared to the M222A/N118S mutant but is more stable than wild-type subtilisin E and M222A mutant. At the same time, N118S mutation also improved the catalytic efficiency ( $k_{cat}/k_m$ ) of subtilisin E slightly. Table 1 shows the catalytic efficiency of N118S mutant is 2.2 times that of M222A/N118S mutant while the catalytic efficiency of wild-type subtilisin E is 2.6 times that of M222A mutant. These results indicated N118S mutation can improve the heat-stability of subtilisin E while M222A mutation is deleterious to catalytic activity although it was found to improve the oxidative stability of subtilisin.

### 4. Discussion

Random mutagenesis by PCR is widely used in enzyme engineering. In this study, a remarkably

heat-stable mutant N118S/M222A was discovered using random mutagenesis combined with screening after incubation at 65°C for 30 min. Sequencing analysis revealed only an A to G substitution in the encoding region that converted the asparagine codon, AAT, to the serine codon AGT and an A to G substitution in the non-encoding region that converted the *Pst* I site (CTGCAG → CTGCGG). The ratio of dATG/dGTG reduced to 1/10 in PCR conditions increased the possibility of A → G substitution or T → C (due to the base substitution occurring on the complementary strand). Thus, altering the ratio of dNTPs can introduce desired base substitutions into genes and significantly increase the frequency and range of possible amino acid substitutions.

Subtilisin is easily inactivated by the oxidation of M222 residue to methionine. The substitution of M222 by nonoxidizable amino acid (i.e. S, A, and L) can improve the oxidative stability of subtilisin (Estell et al., 1985). Fig. 2 indicated M222A and M222A/N118S mutants were resistant to inactivation by 1 M H<sub>2</sub>O<sub>2</sub> and also, the N118S mutation did not have an effect on the oxidative stability of M222A. Therefore M222A/N118S mutant is both an oxidation-resistant and a heat-stable variant of subtilisin E. This suggests the N118S mutation might give a heat stable character to the enzyme, together with the higher

activity.

The N218S mutation was found to increase thermostability of subtilisin BPN' (Bryan et al., 1986). It also increased the thermostability of subtilisin E (Wang et al., 1993). This coincided with the prediction that reducing the N-G sequence will improve the heat stability (International patent no. W 087104461). In the case of N118S, however, N118 was followed by M instead of G. Why did the mutation N118S lead to a more heat-stable enzyme?

The residue N118 is located in a surface region of the subtilisin E molecule, quite far from the catalytic triad (H64, S221 and D32). There are no ordered water molecules or inter-residual contacts around the side chain of residue N118. But the peptide segment around residue Asn118 is packed tightly. In addition to a number of van der Waals' contacts, there are at least four hydrogen bonds between the segment N117 and M119 and the segment W113 and I115, stabilizing the main-chain conformation. The mutation of the N118S should effect the amidase activity insignificantly and have little effect on the local structure around 118 position. But in the case of mutant N118S of subtilisin E, a water molecule between the side chains of S118 and K27 was found using computer simulation with QUANTA (Chu et al., 1995). The water molecule might bind to three adjacent residues, S118, K27 and D120 in the mutant through hydrogen bonds (Fig. 3). Thus, N118 to S mutation modifies intramolecular interactions through changes of its side chain and surrounding side chains. It is probable that through a water molecule, the side chain of S118 is hydrogen bonded to K27, a residue which is a long distance from S118 in the sequence, and should contribute to the stability of subtilisin E. Many experiments to improve the stability of protein have been accumulated, including increasing the number of hydrogen bonds. This was evidenced by the fact S improved the thermal stability of subtilisin BPN' by N218S mutation through the forming of hydrogen bond (Gilliland et al., 1996). Accordingly, the supposed hydrogen bonds formed between the water molecule and S118 residue as well as its surrounding residues should increase the heat stability of M222A/N118S mutant.

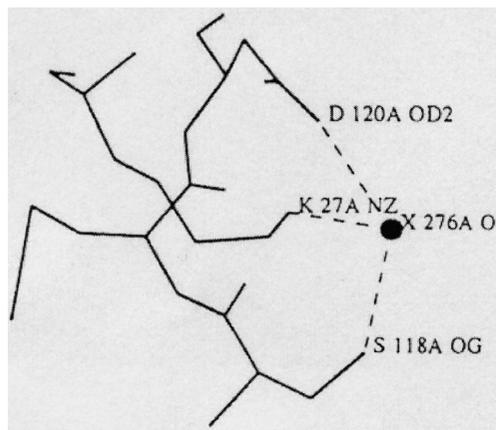


Fig. 3. Local structure obtained after energy minimization of the N118S/M222A mutant of subtilisin E. It is constructed by Ser118, Lys27, Asp120 and a water molecule (X276) hydrogen bonded to them (Chu et al., 1995).

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## References

- Bryan, P.N., Rollence, M.L., Pantoliano, M.W., et al., 1986. Protease of enhanced stability: characterization of a thermostable variant of subtilisin. *Proteins: Struct. Funct. Genet.* 1, 326–334.
- Chen, K.Q., Arnold, F.H., 1993. Tuning the activity of an enzyme for unusual environments: sequential random mutagenesis of subtilisin E for catalysis of dimethylformamide. *Proc. Natl. Acad. Sci. USA* 90, 5618–5622.
- Chu, N., Cao, Y., Bi, R.C., 1995. The 2 Å crystal structure of subtilisin E with PMSF inhibitor. *Protein Eng.* 8, 211–215.
- Estell, D.A., Graycar, T.P., Wells, J.A., 1985. Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation. *J. Biol. Chem.* 260, 6518–6521.
- Gilliland, G.L., Gallagher, D.T., Alexander, P., Bryan, P., 1996. Crystal structure analysis of subtilisin BPN' mutants engineered for studying thermal stability. *Adv. Exp. Med. Biol.* 379, 159–169.
- Guo, X.H., Xiong, Z., Zhou, M., Jia, S.F., 1991. The construction shuttle vectors of *Bacillus subtilis*–*Escherichia coli*. *Chin. J. Biotechnol.* 7, 224–229.
- Kawamura, H., Doi, R.H., 1984. Construction of a *Bacillus subtilis* double mutant deficient in extracellular alkaline and neutral proteases. *J. Bacteriol.* 160, 442–444.
- Kidd, R.D., Sears, P., Huang, D.H., Witte, K., Wong, C.H., Farber, G.K., 1999. Breaking the low barrier hydrogen bond in a serine protease. *Protein Sci.* 8, 410–417.
- Pantoliano, M.W., Ladner, R.C., Bryan, P.N., Rollence, M.L., Wood, L.R., 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.
- Sattler, A., Kanka, S., Kaurer, K.H., Riesner, D., 1996. Thermostable variants of subtilisin selected by temperature-gradient gel electrophoresis. *Electrophoresis* 17, 784–792.
- Strausberg, S.L., Alexander, P.A., Gallagher, D.T., Gilliland, G.L., Barnett, B.L., Bryan, P.N., 1995. Directed evolution of a subtilisin with calcium-independent stability. *Biotechnology* 13, 669–673.
- Takagi, H., Morinaga, Y., Ikemura, H., Inouye, M., 1989. The role of pro-239 in the catalysis and heat stability of subtilisin E. *J. Biochem.* 105, 953–956.
- Takagi, H., Takahashi, T., Momose, H., et al., 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.
- Takagi, H., 1993. Protein engineering on subtilisin. *Int. J. Biochem.* 25, 307–312.
- Takagi, H., Maeda, T., Ohtsu, I., Tsai, Y.C., Nakamori, S., 1996. Restriction of substrate specificity of subtilisin E by introduction of a side chain into a conserved glycine residue. *FEBS Lett.* 395, 127–132.
- Takagi, H., Ohtsu, I., Nakamori, S., 1997. Construction of novel subtilisin E with high specificity, activity and productivity through multiple amino acid substitutions. *Protein Eng.* 10, 985–989.
- Takagi, H., Yamamoto, M., Ohtsu, E., Nakamori, S., 1998. Random mutagenesis into the conserved Gly154 of subtilisin E: isolation and characterization of the revertant enzymes. *Protein Eng.* 11, 1205–1210.
- You, L., Arnold, F.H., 1996. Directed evolution of subtilisin E in *Bacillus subtilis* to enhance total activity in aqueous dimethylformamide. *Protein Eng.* 9, 77–83.
- Wang, X.S., Wang, P.Z., Kong, L.Y., Ruang, H.J., 1993. Thermal stability improvement of subtilisin E with protein engineering. *Chin. J. Biochem. Biophys.* 25, 51–61.