Appl Microbiol Biotechnol (1994) 41:239–244

ORIGINAL PAPER

T. Tange · S. Taguchi · S. Kojima
K. Miura · H. Momose

Improvement of a useful enzyme (subtilisin BPN′) by an experimental evolution system

Received: 2 August 1993/Revised: 8 November 1993/Accepted: 23 November 1993

Abstract In order to improve a natural enzyme so as to fit industrial purposes, we have applied experimental evolution techniques comprised of successive in vitro random mutagenesis and efficient screening systems. Subtilisin BPN′, a useful alkaline serine protease, was used as the model enzyme, and the gene was cloned to an *Escherichia coli* host-vector system. Primary mutants with reduced activities of below 80% of that of the wild type were first derived by hydroxylamine mutagenesis directly applied to subtilisin gene DNA, followed by screening of clear-zone non-forming transformant colonies cultured at room temperature on plates containing skim-milk. Then, secondary mutants were derived from each primary mutant by the same mutagenic procedure, but screened by detecting transformant colonies incubated at 10°C with clear zones that were greater in size than that of the wild type. One such secondary mutant, 12–12, derived from a primary mutant with 80% activity, was found to gain 150% activity (k_{cat}/K_m value) of the wild-type when the mutant subtilisin gene was subcloned to a *Bacillus subtilis* host-vector system, expressed to form secretory mutant enzyme in the medium, and the activity measured using *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide as the substrate. When *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Leu-*p*-nitroanilide was used, 180% activity was gained. Genetic analysis revealed that the primary and secondary mutations corresponded to D197N and G131D, respectively. The activity variations found in these mutant subtilisins were discussed in terms of Ca^{2+}-binding ability. The thermostability was also found to be related to the activity.

T. Tange · S. Taguchi (✉) · H. Momose
Department of Biological Science and Technology,
Science University of Tokyo, Noda, Chiba 278, Japan.
FAX: 0471-25-1841

S. Kojima · K. Miura
Institute for Biomolecular Science,
Gakushuin University,
Mejiro, Tokyo 171, Japan

Introduction

For future biotechnological applications, it is important to intentionally re-evolve an enzyme of interest that has naturally evolved only for the benefit of its producing organism, such that the re-evolved enzyme fits the conditions of man-made bioreactor systems, e.g., components of the reaction solution, pH and temperature.

As a powerful methodology for approaching the above purpose, protein engineering (Ulmer 1983) has been extensively applied to alter a variety of proteins through site-directed mutagenesis. Actually, Takagi et al. (1992), including one of the present authors, previously succeeded in enhancing the thermal stability of subtilisin, an industrially important alkaline serine protease, by introducing a disulphide bond to specific site of the protein molecule. However, the application of protein engineering has a disadvantage in that it is limited only to proteins the structure-function relationship of which has been investigated in detail, so as to allow us to design the altered proteins.

In this paper, as another approach to enzyme improvement, we describe the construction of an efficient screening system of evolved subtilisins using programmed random mutagenesis and host-vectors of both *Escherichia coli* K12 (for evolvant screening) and *Bacillus subtilis* ISW 1214 (for enzyme preparation). Also we describe the analytical results of an evolved subtilisin obtained by this system, which brought about a 1.5-fold increase in activity when measured using culture supernatant as the enzyme source and several synthetic peptides as the substrate.

Materials and methods

Materials

The Klenow fragment of DNA polymerase I, T4 DNA ligase, T4 polynucleotide kinase, and restriction enzymes were obtained from Boehringer Mannheim, Takara Shuzo (Kyoto Japan), Nippon Gene or Toyobo. Subtilisin BPN′ was kindly supplied by Nagase Biochemicals. Synthetic substrates, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (AAPF), *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Leu-*p*-nitroanilide (AAPL) and *N*-benzoyl-L-Phe-L-Val-L-Arg-*p*-nitroanilide (FVR) were purchased from Sigma. DEAE-cellulose was the product of Wako Pure Chemicals. All other chemicals were of reagent grade and were used without further purification.

Expression systems

E. coli JM109 (Yanisch-Perron et al. 1985) was used for the screening of mutant subtilisins on skim-milk plates containing 2% skim-milk, 1% lactose, 1% yeast extract and 50 μg/ml of ampicillin. *B. subtilis* ISW1214 (Ikawa et al. 1981) was used for the production of wild-type and mutant subtilisins in liquid LB medium (Sambrook et al. 1989) containing 20 μg/ml of tetracycline.

Construction of plasmid vectors

The subtilisin BPN′ gene including the promoter region was finally cloned to the *Eco*RI-*Hin*dIII sites of pUC18 (Masuda-Momma et al. 1993), during which process 526-bp nucleotides were deleted by *Nae*I and *Bal*31 digestion from the upstream region of the subtilisin promoter so as to stimulate the expression due to the *lac* promoter of the vector.

When *E. coli* JM109 was transformed with the recombinant vector was cultured on the skim-milk plate at 37°C overnight to form transformant colonies, detectable clear zones, caused by the proteolysis of skim-milk, appeared around these colonies after a further 2 days of incubation at 10°C. For the production of mutant subtilisins, the mutated subtilisin genes were subcloned to the *Eco*RI-*Hin*dIII sites of pHY300PLK (Ishiwa and Shibahara 1985), a shuttle vector between *E. coli* and *B. subtilis*.

In-vitro random mutagenesis

The recombinant pUC18 plasmid harbouring the subtilisin gene was treated with 0.8 M hydroxylamine at 65°C for 2 h in 0.1 M phosphate buffer, pH 6.0, containing 1 mM ethylenediaminetetraacetic acid (EDTA) (Kajiyama and Nakano 1991). The mutagen-treated plasmid DNA was precipitated with ethanol and re-dissolved in 10 mM TRIS-HCl buffer, pH 8.0, containing 1 mM EDTA.

DNA sequencing

The analysis of the mutated positions in the subtilisin gene was carried out by the method of dideoxynucleotide-chain termination (Morinaga et al. 1984) using a BcaBEST Sequencing Kit purchased from Takara Shuzo. Five sequencing primers were synthesized by the solid-phase phosphoamidite method with an Applied Biosystems 381A DNA synthesizer (Taguchi et al. 1992).

Isolation and purification of subtilisin

A recombinant *B. subtilis* carrying the wild-type or mutated subtilisin gene was grown in 100 ml LB medium containing tetracycline (final concentration, 20 μg/ml) aerobically at 37°C for 24 h. Subtilisin produced and excreted into the medium was recovered by ammonium sulphate precipitation (40% saturation), followed by dialysis against 20 mM TRIS-HCl buffer, pH 6.3, for 2 days. The enzymes were purified by applying ion-exchange chromatography on DEAE-cellulose, eluted by 20 mM TRIS-HCl buffer. The pass-through fraction was pooled at 4°C, followed by addition of a four fold volume of acetone to the void fraction containing subtilisin. The purity of recovered samples was checked by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis.

Measurement of subtilisin activity

Wild-type and mutant subtilisin activities were assayed at 25°C by monitoring at 410 nm the release of *p*-nitroaniline due to enzymatic hydrolysis of AAPF (0.02–0.8 mM), AAPL (0.1–0.8 mM), or FVR (0.02–0.2 mM) in 100 mM TRIS-HCl buffer (pH 8.6, containing 2 mM $CaCl_2$ (Ikemura et al. 1987). Five microlitres (for AAPF and AAPL) or 100 μl from 5 ml of culture supernatant samples, or 5 μl of 6–9 nM partially purified samples were combined rapidly with the above substrate solution to give a 1 ml final reaction volume. To correct the value of apparent specific activity and the kinetic constant, K_m, the enzyme concentration in each culture supernatant was estimated by densitometric measurement of the stained bands on SDS polyacrylamide gel using a microdensitometer (CS-9000, Shimadzu). Partially purified subtilisin concentrations were determined spectrophotometrically using an absorbance coefficient, $E^{1\%}_{280\,nm} = 11.7$ with a spectrophotometer (U-2000, Hitachi) (Matsubara et al. 1965).

Thermal stability of subtilisin. One millilitre of 24-h-old culture supernatant was heated at 60°C, and 50 μl of each sample was withdrawn at various time intervals and immediately cooled on ice. Measurement of the residual subtilisin activity was the same as described before, using AAPF as the substrate (Takagi et al. 1992).

Results

Screening of evolved subtilisins with increased activity

As a strategy for obtaining evolved subtilisins with increased activity, the following two-step mutagenesis was applied: the primary mutation was carried out to obtain negative mutant subtilisins with reduced or no proteolytic activity, and then the secondary mutation was carried out to obtain positive mutant subtilisins that re-acquired their activity by intragenic suppression.

To raise the efficiency of mutant isolation, in-vitro random mutagenesis was applied directly to the subtilisin BPN′ gene cloned into plasmid vector pUC18. Hydoxylamine was used as the chemical mutagen (Kajiyama and Nakano 1991) in the present study. The mutagenized plasmids including heterogeneous mutant subtilisin genes were introduced into *E. coli* JM109 host cells, and ampicillin-resistant transformant colonies

were formed at room temperature on plates containing lactose added both as the carbon source for growth and as the inducer for subtilisin gene expression, and also containing skim-milk as well as ampicillin.

The subtilisin mutants with reduced activities (the primary mutants) were easily screened by detecting colonies with no clear zone. As the control, transformant colonies with plasmids containing the wild-type subtilisin gene formed apparent clear zones caused by proteolysis of skim-milk. An experiment was performed to obtain the primary mutants according to this screening system, and forty-seven clear-zone non-forming mutants were detected and picked up from a total of 293 ampicillin-resistant transformant colonies.

The screening of the secondary mutants (evolvants) that reacquired the proteolytic activity was carried out as follows: the primary mutant plasmids were prepared from each clear-zone non-forming transformant, and again subjected to the same procedure of in-vitro random mutagenesis as that for the primary mutant derivation. After introducing the secondarily mutagenized plasmids into the same host cells, the ampicillin-resistant transformant colonies were formed on the same plates by overnight incubation at 37°C, followed by further incubation at 10°C, an incubation programme different from the primary one. As a result, thirty-one transformant colonies with clear zones, which were equal to or greater than that of the wild type in size, were screened and picked up from a total of approximately 8,500 colonies.

In order to select evolvants with increased subtilisin activities, the subtilisin genes of the thirty-one clear zone formers were subcloned from *E. coli* to the *B. subtilis* host-vector system, respectively, and the expressed mutant subtilisin in the culture supernatant of each *B. subtilis* transformant was assayed for its specific activity using AAPF as the substrate. The background proteolytic activity of the original *B. subtilis* host cell was negligible so long as the assay system was used. As a result, five mutants were finally screened as evolvants with increased activities.

Genetic and enzymatic analysis of an evolved subtilisin

B. subtilis mutant 12-12, one of the five evolvants with increased subtilisin activity, was investigated in detail to analyse the effect of primary and secondary mutations on enzyme activity. DNA sequencing revealed that the primary mutation (GAT → AAT) corresponded to the amino acid change of Asp → Asn at position 197 (D197N), and the secondary mutation (GGT → GAT) corresponded to that of Gly → Asp at 131 (G131D). It was found that the secondary mutagenesis provoked another amino acid change of Thr → Ile, at the same time, at − 28 (T-28I in the pro-region), probably due to highly efficient mutagenesis with hydroxylamine. Although the pro-region was thought to be responsible for the folding process of the matured subtilisin (Kobayashi and Inouye 1992), the present amino acid change, T-28I, was judged not to affect the activity of subtilisin BPN′ (data not shown).

We then constructed all the combination of single (T-28I, G131D, D197N) and double (T-28I + G131D, G131D + D197N, D197N + T-28I) mutants from the triple (T-28I + G131D + D197D) mutant. After each mutant subtilisin was expressed using the *B. subtilis* host-vector system, the k_{cat} and K_m values were measured with culture supernatant (secreted intact subtilisin) and with a partially purified enzyme preparation using AAPF as the substrate.

Table 1 shows the results with wild-type, G131D, D197N and T-28I + G131D + D197N mutants when culture supernatant was used as the enzyme source. It was found that the primary mutation certainly reduced the k_{cat}/K_m value, but only to approximately 80% of that of the wild-type. The secondary mutation increased the activity, as expected, up to 120% in the triple mutant and 160% in the single mutant (G131D only), respectively. Table 1 also shows that when a partially purified subtilisin was used, the increase in enzyme activity by the secondary mutation was slightly lower than that when the intact subtilisin was used. This suggested that the secondary mutation might give a somewhat thermolabile character to the enzyme,

Table 1. Kinetic parameters of subtilisin for hydrolysis of *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide k_{cat} Catalytic constant, K_m Michaelis constant

Enzyme source	Mutation	$k_{cat}(s^{-1})$	$K_m(\mu M)$	k_{cat}/K_m ($10^5\ s^{-1}\ M^{-1}$)	Relative value to wide-type
Culture supernatant	Wide-type	45 (± 2)	126 (± 11)	3.6	1.00
	G131D	56 (± 3)	97 (± 6)	5.8	1.57
	D197N	39 (± 3)	131 (± 5)	3.0	0.83
	T-28I + G131D + D197N	51 (± 4)	122 (± 13)	4.2	1.17
Partially purified subtilisin	Wild-type	41 (± 2)	118 (± 5)	3.5	1.00
	G131D	55 (± 5)	109 (± 5)	5.0	1.43
	D197N	34 (± 2)	143 (± 7)	2.4	0.69
	T-28I + G131D + D197N	47 (± 4)	131 (± 5)	3.6	1.03

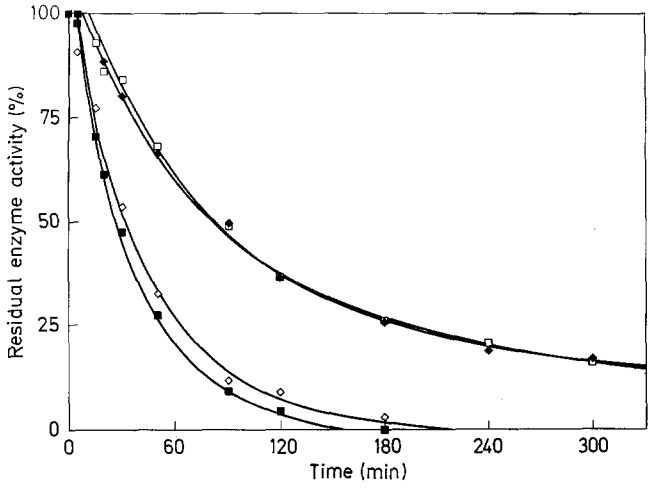

Fig. 1. Thermostability of wild-type and mutant subtilisins. Residual enzyme activity against 60°C in each transformant was assayed at 25°C by the addition of culture supernatant taken up at various time intervals into the 100 mM TRIS-HCl buffer, pH 8.6, containing 2 mM $CaCl_2$ as described in Materials and methods: □, wild type; ◆, G131D; ■, D197N; ◇, T-28I + G131D + D197N

together with the activity increase. Therefore, we tested the thermostability of G131D mutant subtilisin, as well as wild-type, D197N, T-28I + G131D + D197N mutant subtilisins, using culture supernatant as the enzyme source.

Figure 1 shows that the half-life of G131D enzyme activity, when heated at 60°C, was practically the same as that of the wild-type enzyme (approximately 90 min). In contrast, the primary mutation, D197N, had an apparently reduced half-life (approximately 30 min).

The difference in substrate specificity of each mutant subtilisin was examined using AAPL and FVR as substrates (data not shown). In the case of mutant G131D, the k_{cat}/K_m value toward AAPL was 1.81-fold greater than that of the wild type. In contrast, toward FVR, the specific activity decreased drastically to 25% of that of the wild type. Almost the same decrease (24%) was obtained in T-281 + G131D + D197N. Therefore, the mutation at position 131 is considered to be responsible not only for the activity increase, but also for the substrate specificity.

Discussion

In the present study, we applied localized in-vitro random mutagenesis to increase the enzyme activity via multi-step mutations, including an activity loss by the primary mutation, and an activity recovery by the secondary mutation of intragenic suppression-type, which, in turn, gives an activity increase to the enzyme. The present results verified that our system, the combination of the use of *E. coli-B. subtilis* host-vectors, multi-step random mutagenesis and the screening procedure of the mutants, actually functioned to obtain improved mutant enzymes (evolvants) efficiently. The frequencies of the primary mutants (16% of the total transformants), the secondary mutants (0.3–0.4% of the total transformants) and the activity-elevated evolvants (16% of the total secondary mutants) were estimated as high efficiencies, enough to be a practical procedure for enzyme improvement.

The primary mutation seems crucial in the sense that each mutational event determines both the possibility and character of the secondary mutation. Indeed, we observed that some primary mutants gave secondary mutants with reverted activity and others not. The use of the *E. coli* host-vector system linked with our plate screening system enabled us to detect clear-zone non-forming colonies that contained a primary mutant still possessing 80% subtilisin activity of the wild type (Table 1). This implies that, under the experimental conditions, we could detect mutants with lower activities of at least 80% to 0% as "negative", since the expression-secretion level of subtilisin in *E. coli* is adequately low compared with that in *B. subtilis*. This wide-range detection system helped in obtaining primary mutants with the potentiality of evolving to activity-increased secondary mutants.

The altered proteins thus evolved would be quite different from those generated by theoretically designed site-directed mutagenesis. Strain 12–12, one of the secondary mutants isolated, provided us with such an example: the amino acid residue changed by the secondary mutation (G131D) is located at the *N*-terminal region of an α-helix (Fig. 2), near but on the reverse side of the substrate binding area. The amino acid change of that position for elevated activity was hard to predict theoretically. The reason for the slight reduction in activities found in Table 1 caused by the purification procedure used in the study remains unclear. The possibility is that subtilisin is somewhat sensitive to the purification, or somewhat fragile without some element contained in the culture supernatant. Previously, Wells et al. (1987) reported that the autolysis of subtilisin may be restrained by the presence of peptides in the culture supernatant.

The amino acid residue changed by the primary mutation (D197N) is located also on the reverse side of the substrate binding area (Fig. 2). Judging from the fact that the change of aspartic acid to asparagine reduced both activity and thermostability, the negatively charged Asp-197 might interact electrostatically with the positively charged Arg-247 to maintain the active subtilisin structure. In addition, Asp-197 has been reported by Pantoliano et al. (1988) to form a weak Ca^{2+}-binding site (designated the Ca B site) with a carbonyl oxygen of Pro-172 and Rollence et al. (1988) also have found that the Ca^{2+}-concentration-dependent half-life for thermal inactivation of D197E variant, prepared by site-directed mutagenesis, decreased dramatically.

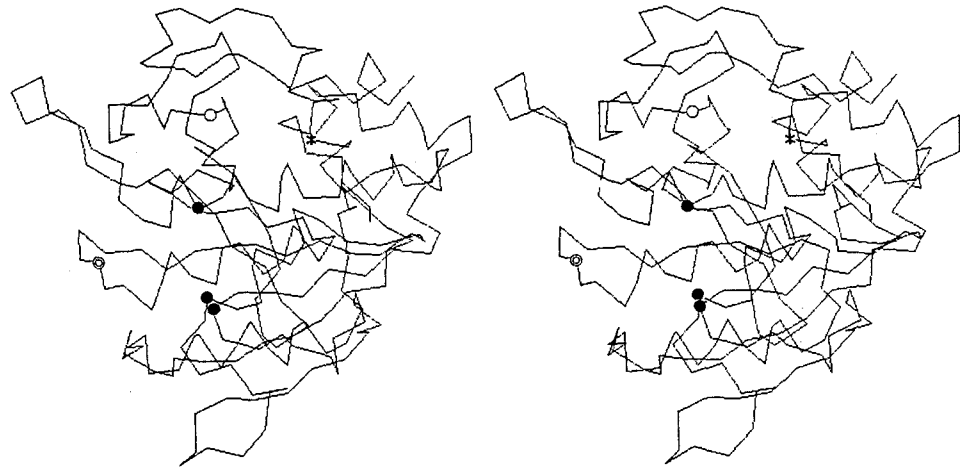

Fig. 2. Sterographic view of subtilisin BPN'. Molecular graphic software GDPS (Graphic Display of Protein Structure) 98, developed by Dr. K. Iso, was used. The *three closed circles* indicate the catalytic triad (Ser-221, His-64, Asp-32 from *upper* to *lower circles*). Asp-197 (*single open circle*) was changed to Asn by the primary mutation that decreased the enzyme activity and thermostability. The *asterisk* indicates Arg-247, which might interact with Asp-197 but not with Asn-197. Gly-131 (*double open circle*) was changed to Asp by the secondary mutation which increased the enzyme activity

The decrease in activity of subtilisin by the D197N substitution may thus possibly be related to the reduction of thermostability caused by loss of the negative charge that interacts with Ca^{2+}.

It is noteworthy that the secondary suppressor-type mutation (Gly → Asp) at position 131, which was found to be about 13 Å apart from the weak Ca^{2+}-binding site, was the same one as the mutant had, which was obtained by single random mutagenesis (Rollence et al. 1988) and acquired a longer half-life against thermal inactivation at 65°C and also higher Ca^{2+}-binding affinity. The weak Ca^{2+}-binding site, once lost by the D197N mutation, may possibly be regenerated between the carbonyl oxygen of Pro-172 and the negative charge of Asp-131. Therefore, it remains a possibility that the increase in activity is also related to the weak Ca^{2+}-binding site, although details of the direct relationship of activity and thermal stability are at present unclear. However, these results suggest that the primary mutation (activity loss) is potentially linked with the secondary mutation (activity gain).

Furthermore, the difference in substrate specificity was observed in mutant G131D (data not shown). Activity toward Arg substrate was clearly decreased by the G131D mutation, compared with the Phe or Leu substrate. As the amino acid residue at position 131 is located near the substrate binding site containing Glu-156, the determinant toward the Arg substrate (Takeuchi et al. 1991), Asp at position 131 may change the electrostatic environment around Glu-156, leading to the reduction in activity toward the Arg substrate.

Acknowledgements We wish to thank Yoshitaka Arai and Shinji Kameyama of our laboratory for their excellent experimental assistance. This work was supported, in part, by a grant-in-aid (no. 04660126) from the Ministry of Education, Science and Culture of Japan.

References

Ikawa S, Shibata T, Matsumoto K, Iijima T, Saito H, Ando T (1981) Chromosomal loci of genes controlling site-specific restriction endonucleases of *Bacillus subtilis*. Mol Gen Genet 183:1–6

Ikemura H, Takagi H, Inouye M (1987) Requirement of pro-sequence for the production of active subtilisin E in *Escherichia coli*. J Biol Chem 262:7859–7864

Ishiwa H, Shibahara H (1985) New shuttle vectors for *Escherichia coli* and *Bacillus subtilis*. II plasmid pHY300PLK, a multi purpose cloning vector with a polylinker, derived from pHY460. Jpn J Genet 60:235–243

Kajiyama N, Nakano E (1991) Isolation and characterization of mutants of firefly luciferase which produce different colors of light. Protein Eng 4:691–693

Kobayashi T, Inouye M (1992) Functional analysis of the intramolecular chaperone mutational hot spots in the subtilisin propeptide and a second-site suppressor mutation within the subtilisin molecule. J Mol Biol 226:931–933

Masuda-Mommma K, Shimakawa T, Inouye K, Hiromi T, Kojima S, Kumagai I, Miura K, Tonomura B (1993) Identification of amino acid residues responsible for the changes of absorption and fluorescence spectra on the binding of subtilisin BPN' and *Streptomyces* subtilisin inhibitor. J Biochem 114:906–911

Matsubara H, Kasper CB, Brown DM, Smith EL (1965) Subtilisin BPN'. I. Physical properties and amino acid composition. J Biol Chem 240:1125–1130

Morinaga Y, Franceschini T, Inouye S, Inouye M (1984) Improvement of oligonucleotide-directed site-specific mutagenesis using double-stranded plasmid DNA. Bio/Technology 2:636–639

Pantoliano MW, Whitlow M, Wood JF, Rollence ML, Finzel BC, Gilliland GL, Poulos TL, Bryan PN (1988) The engineering of binding affinity at metal ion binding sites for the stabilization of proteins: subtilisin as a test case. Biochemistry 27:8311–8317

Rollence ML, Filpula D, Pantoliano MW, Bryan PN (1988) Engineering thermostability in subtilisin BPN' by in vitro mutagenesis. CRC Crit Rev Biotechnol 8:217–224

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Taguchi S, Maeno M, Momose H (1992) Extracellular production system of heterologous peptide driven by a secretory protease inhibitor of *Streptomyces*. Appl Microbiol Biotechnol 36:749–753

Takagi H, Takahashi T, Momose H, Inouye M, Maeda Y, Matsuzawa H, Ohta T (1992) Enhancement of the thermostability of subtilisin E by introduction of disulfide bond engineered on the basis of structural comparison with a thermophilic serine protease. J Biol Chem 89:4407–4411

Takeuchi Y, Satow Y, Nakamura KT, Mitsui Y (1991) Refined crystal structure of the complex of subtilisin BPN′ and *Streptomyces* subtilisin inhibitor at 1.8Å resolution. J Mol Biol 221:309–325

Ulmer KM (1983) Protein engineering. Science 219:666–671

Wells LA, Powers DB, Bott RR, Graycar TP, Estell DA (1987) Designing substrate specificity by protein engineering of electrostatic interactions. Proc Natl Acad Sci USA 84:1219–1223

Yanisch-Perron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33:103–119