38. *Partial Alteration of a Protein Streptomyces Subtilisin Inhibitor by Site-Directed Mutagenesis*

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A protease inhibitor produced by *Streptomyces albogriseolus* was isolated by Murao and Sato¹⁾ in 1971. It is a protein consisted of 110 amino acids. Since it inhibits a protease subtilisin specifically, it is called as *Streptomyces subtilisin inhibitor*, SSI. Its tertiary structure as well as primary structure has been studied well by means of various physical techniques, which includes X-ray analysis on its crystal. Documents of the research on SSI carried out by cooperation of Japanese investigators were well summarized in a book, "Protein Protease Inhibitor—The Case of *Streptomyces Subtilisin Inhibitor* (SSI)"²⁾ issued by Publisher Elsevier in 1985. Standing on these precise studies on the structure and the function, we selected SSI as a target for protein engineering.

Cloning of SSI gene. First of all we started from cloning of the SSI gene. DNA was isolated from *S. albogriseolus* by Saito-Miura's method which used pH 9-phenol for efficient extraction of DNA and ribonucleases for complete elimination of RNA.³⁾ *Streptomyces* DNA was digested with restriction endonucleases SalI and Bgl II. DNA fragments were combined with *E. coli* plasmid pBR322, which was treated with the restriction endonucleases, SalI and BamHI, and linked with DNA repairing enzymes. The recombinant plasmids were incorporated into *E. coli*. SSI gene was searched by annealing with the probes, 26 and 29 base long, respectively, which were synthesized chemically, incorporating inosinic acid into third letter position of a codon to read degenerated bases. pSI30 containing SSI gene was cloned. The SSI gene was once more cloned by the plasmid pUC18. Thus, pSI51 and pSI52 with opposite orientation of SSI gene were obtained.

Structure of SSI gene. Nucleotide sequence of the SSI gene was analysed mainly by the "dideoxy" method. The corresponding amino acid sequence was the same to the sequence obtained by Ikenaka *et al.*⁴⁾ with the analysis of amino acids (Fig. 1). The initiation codon ATG precedes 31 amino acid codons upstream of N terminal of SSI. These 31 amino acids would correspond to the signal peptide. In the used clone, there were 583 bases upstream of SSI gene, which were considered as sufficient length for expression of the gene. The base sequence of SSI gene was quite rich in GC content (73%) as whole DNA of *Streptomyces*. The GC content of third letter in codons reached 96%.

Expression of SSI gene. The cloned SSI gene was incorporated in a *Streptomyces* multicopy plasmid (pIJ702) and transferred into *Streptomyces lividans*. SSI was well expressed in this system and secreted outside of the bacterium cell. It was purified as a single protein, which reacted with the antibody produced by SSI. The size of this SSI expressed in *S. lividans* was 3 amino acids long to upstream of the N-terminal amino acid of the ordinary SSI, which was

Nucleotide Sequence of SSI gene

AAG,AAA,CGA,AGC,GGG,AGG,ATG,CAC,ACA,ATG,CGG,AAC,ACC,GGC,GCT,GGG,CCA,TCA,CCC,TCG
 MET-Arg-Asn-Thr-Gly-Ala-Gly-Pro-Ser-Pro-Ser
 -20 GTC,TCA,CGG,CCA,CCG,CCG,TCT,GGC,GCC,CCC,CTC,TCC,GGG,GGC,GGC,CTC,GGC,GGC,CCG,GGG -1
 Val-Ser-Arg-Pro-Pro-Pro-Ser-Ala-Ala-Pro-Leu-Ser-Gly-Ala-Ala-Leu-Ala-Ala-Pro-Gly
 1 GAT,GGC,CCG,TCC,GGC,CTC,TAC,GGC,CCC,TCC,GGC,CTG,GTG,CTG,ACC,GTG,GGC,AAG,GGC,GTG 20
 Asp-Ala-Pro-Ser-Ala-Leu-Tyr-Ala-Pro-Ser-Ala-Leu-Val-Leu-Thr-Val-Gly-Lys-Gly-Val
 AGC,GGC,ACG,ACC,GGC,GCA,CCG,GAA,CGC,GGC,GGC,CTG,ACC,CTG,ACC,TGT,GCT,CCG,GGC,CCG,TCG 40
 Ser-Ala-Thr-Thr-Ala-Ala-Pro-Glu-Arg-Ala-Val-Thr-Leu-Thr-Cys-Ala-Pro-Gly-Pro-Ser
 GGC,ACC,CAC,CCG,GGC,GGC,GGC,TCG,GGC,TGC,GGC,GAC,CTG,GGC,GGC,GGC,GGC,GGC,GAC,CTG 60
 Gly-Thr-His-Pro-Ala-Ala-Gly-Ser-Ala-Cys-Ala-Asp-Leu-Ala-Ala-Val-Gly-Gly-Asp-Leu
 AAC,GGC,CTG,ACG,CGG,GGC,GAG,GAC,GTG,ATG,TGC,CCG,ATG,GTG,TAC,GAC,CCG,GTG,CTG,CTC 80
 Asn-Ala-Leu-Thr-Arg-Gly-Glu-Asp-Val-Met-Cys-Pro-Met-Val-Tyr-Asp-Pro-Val-Leu-Leu
 ACC,GTG,GAC,GGC,GGC,TGG,CAG,GGC,AAG,CGG,GGC,GGC,TCC,TAC,GAG,CGC,GGC,GGC,GGC,GGC,GGC 100
 Thr-Val-Asp-Gly-Val-Trp-Gln-Gly-Lys-Arg-Val-Ser-Tyr-Gly-Arg-Val-Phe-Ser-Asn-Glu
 TGC,GAG,ATG,AAC,GGC,CAC,GGC,TCG,AGC,GGC,GGC,GGC,GGC,GGC,GGC,GGC,GGC,GGC,GGC,GGC 110
 Cys-Glu-Met-Asn-Ala-His-Gly-Ser-Ser-Val-Phe-Ala-Phe-xxx- 113

Fig. 1. Primary structure of SSI and its gene.

Fig. 2. A schematic drawing of SSI by T. Komiyama.²⁾

Fig. 3. Inhibition of subtilisin (left) and trypsin (right) by wild type SSI (Met73) and mutant SSI (Lys73).

expressed in *S. albogriseolus*.

Replacement of an amino acid by gene engineering. 73 Met in SSI is known as the reaction site to subtilisin (Fig. 2). The nucleotide sequence of ATG corresponding to Met at the position 73 was converted into AAG corresponding to Lys by gene engineering. The altered gene was also expressed well in *S. lividans*. Inhibition for subtilisin of the 73 Lys-SSI was almost the same to the wild type SSI. Although the wild type of SSI does not inhibit trypsin, the mutant 73 Lys-SSI inhibits trypsin (Fig. 3). K_i value for trypsin was 1×10^{-4} with 73 Met-SSI, but 1×10^{-8} to 4×10^{-9} with 73 Lys-SSI. Similar change was also observed for lysyl endopeptidase. The mutant SSI substituted 73 Met to Arg inhibited trypsin as well as subtilisin, but not lysyl endopeptidase.

Functional specificity of SSI was changed by substitution of one amino acid in the reaction site for a protease.

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