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Contribution of salt bridge in the protease inhibitor SSI (*Streptomyces* subtilisin inhibitor) to its inhibitory action

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Abstract

The tertiary structure of proteinaceous protease inhibitors is considered to be maintained by various interactions in the molecule that prevent degradation by protease. In this study, the Arg^{29} of *Streptomyces* subtilisin inhibitor (SSI) forming a salt bridge with the carboxyl group of carboxyl-terminal Phe^{113} was replaced with Ala, Met or Lys by cassette mutagenesis to clarify the role of Arg^{29} in the function of SSI. The inhibitory activity of each mutated SSI decreased with increasing incubation time after mixing with subtilisin, indicating that the SSI was changed into a temporary inhibitor upon mutation. This decrease was shown by SDS polyacrylamide gel electrophoresis to be due to cooperative degradation of the mutated SSI by subtilisin. In addition, the denaturation temperature of the Ala or Met mutant was decreased by ten degrees and that of the Lys mutant by 1.5 degrees, suggesting that the destabilization of SSI may be related to its temporary inhibition. Thus, interaction in the protease inhibitor molecule for maintaining the tertiary structure, such as that of Arg^{29} in SSI, was shown to be required for the inhibitory action.

Key words: Protease inhibitor; Protein engineering; Electrostatic interaction; Temporary inhibition

1. Introduction

Structure–function relationship studies on serine protease inhibitors including *Streptomyces* subtilisin inhibitor (SSI) have shown that the local structure around the reactive site resembles that of the substrate, and thus the amino acid residue at the P1 site of the inhibitor determines the specificity toward protease [1–6]. Previously, we have succeeded in alterating the inhibitory specificity of SSI by replacing the amino acid residue at the P1 site [6]. Protein protease inhibitors of relatively small size, such as SSI and bovine pancreatic trypsin inhibitor, inhibit protease by forming a stable Michaelis complex without cleavage of the peptide bond at the reactive site [1,2]. However, it is not clear why protease inhibitors having a substrate-like structure are not degraded by cognate protease. In an attempt to address this issue, we have been investigating the structural features of SSI that are necessary for its inhibitory action.

In this study, we focused on the salt bridge between the positive charge of the Arg^{29} guanidino group and the negative charge of the carboxyl-terminal Phe^{113} carboxyl group [7] for the following reasons. Sakai et al. have reported that SSI is converted to a temporary inhibitor when the four carboxyl-terminal amino acid residues (Val-Phe-Thr-Phe) in the hydrophobic core are released from SSI by treatment with carboxypeptidase A [8]. The release of these four residues includes the loss of the salt bridge between the terminal carboxylate and the guanidino group of Arg^{29}. Therefore, it is important to clarify whether the temporary inhibition exhibited by SSI lacking these four residues is due to the loss of the salt bridge or the release of the four residues in the hydrophobic core. In addition, the Arg^{29} residue is conserved in all members of the SSI family of protease inhibitors isolated from *Streptomyces* [9,10], and its importance for the function of SSI as an inhibitor has been suggested.

Therefore, we substituted Arg^{29} with other amino acids to destroy the salt bridge between Arg^{29} and the carboxyl terminus, and examined the effects of these substitutions on the inhibitory action and stability of SSI.

2. Materials and methods

2.1 Materials

Restriction enzymes and DNA-modifying enzymes were purchased from Toyobo and Boehringer Mannheim. Subtilisin BPN′ and casein (Hammersten) were obtained from Nagase Biochemicals and Merck, respectively. All other chemicals were of the best grade available for biochemical research.

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2.2. Cassette mutagenesis of the SSI gene

We constructed a new plasmid for codon substitution of Arg^{29} (CGC). A *Sma*I-*Aat*II fragment (210 bp) corresponding to the coding region of Pro(-2)-Val^{69} of SSI was isolated from an SSI-encoding plasmid, pSI52, and inserted into the *Sma*I-*Aat*II site of pUC18. The resulting plasmid pSISA containing the Arg^{29} codon was used for cassette mutation. Fig. 1 shows a schematic representation of cassette mutagenesis. pSISA was digested with *Bst*EII and *Ava*I. The *Ava*I-*Bst*EII small fragment was then digested with *Hpa*II to remove the region containing Arg^{29}. The two synthetic oligonucleotides shown in Fig. 1 were designed as a mixture of twelve species in order to introduce Lys, Met and Ala into the position of Arg^{29}. The *Ava*I-*Bst*EII large fragment of pSISA, the *Ava*I-*Hpa*II small fragment of pSISA, and the annealed oligonucleotides were ligated, and the resulting DNA was transferred into *Escherichia coli* JM109. Screening of *E. coli* harboring the mutated pSISA was carried out by colony hybridization using the following synthetic oligonucleotides:

```
        ***
5'ACCGGAAAAGGCGGTCA3' for Lys,
        ***
5'ACCGGAAATGGCGGTCA3' for Met,
        ***
5'ACCGGAAGCCGCGGTCA3' for Ala
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(asterisks indicate the codon at position 29). The mutations were verified by dideoxy sequencing of the plasmid using a 'reverse' primer.

2.3. Expression of the mutated SSI gene and purification of mutated SSIs

Mutated pSISA was digested with *Sma*I and *Aat*II, and the small fragment containing the SSI gene was inserted into the *Sma*I-*Aat*II site of pSIML containing a minimal fragment of the SSI gene sufficient for its expression [11]. The mutated SSI gene was ligated with streptomycete plasmid pIJ702 at the *Sac*I-*Sph*I sites. *Streptomyces lividans* 66 was transformed with the resulting plasmid using thiopeptin as an antibiotic marker. The secreted mutated SSIs were purified from the culture broth of *S. lividans* 66 by ammonium sulfate precipitation and three steps of chromatography: ion-exchange chromatography on DE-32 and gel-filtration on Sephacryl S-200, as described previously [6], and hydrophobic chromatography on Phenyl-Sepharose. The concentrations of SSIs were estimated from the molar absorbance coefficient of wild-type SSI [12].

2.4. Other analytical methods

Measurements of the inhibitory activity of mutated SSIs toward subtilisin BPN′ and its dependency on incubation time, circular dichroism measurements and SDS polyacrylamide gel electrophoresis were carried out by the same procedures as those described previously [6,11,13]. Details of these measurements are given in the figure legends.

3. Results

3.1. Inhibition of subtilisin BPN′ by the mutated SSIs

The inhibitory profiles of the mutated SSIs, in which the Arg^{29} residue was converted to Lys, Met or Ala, toward subtilisin BPN′ are shown in Fig. 2, together with that of the wild-type SSI. When the molar ratio of SSI to subtilisin was larger than one, the inhibitory activity of wild-type SSI did not decrease with incubation time after mixing of SSI with protease. However, under the same conditions, the inhibitory activity of each mutated SSI was found to decrease with increasing incubation time. This is the characteristic of temporary inhibition. Among the three mutant forms of SSI, the rate of decrease in the inhibitory activity was fastest for the Ala mutant, and slowest for the Lys mutant. These results indicate that Arg^{29} is required for the inhibitory action

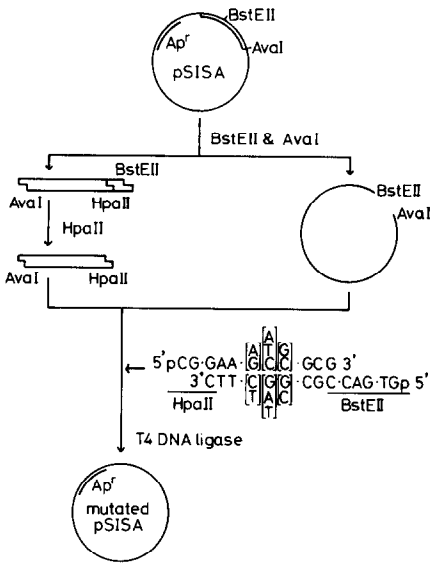

Fig. 1. Schematic representation of plasmid construction for cassette mutagenesis of Arg^{29}. The *Ava*I-*Bst*EII large fragment and *Ava*I-*Hpa*II small fragment were isolated from the plasmid pSISA, which contains the amino-terminal half of the SSI gene, and then ligated with annealed synthetic oligonucleotides, which were designed so as to introduce Ala, Met and Lys into position 29 of SSI. The mutated gene containing each mutation was then screened by colony hybridization.

of SSI, and that substitution with other residues converts SSI to a temporary inhibitor.

3.2. SDS-polyacrylamide gel electrophoretic analysis of subtilisin-mutated SSI complex

In order to examine whether the temporary inhibition produced by these mutated SSIs was due to their degradation, SDS polyacrylamide gel electrophoresis was carried out. Fig. 3 shows the electrophoretic patterns of mixtures of subtilisin and the mutated SSIs after various incubation times. The bands of the mutated SSIs were found to fade with increasing incubation time after mixing with subtilisin. Initially, the amino-terminal region (to Leu^6) of SSI was degraded, and this species appeared as a faster-migrating band on SDS-PAGE. Amino-terminal shortening, which is also observed for wild-type SSI, was shown not to affect the inhibitory property of the SSIs, and thus was not directly related to temporary inhibition. Then, the shortened SSI was additionally degraded by subtilisin without any intermediate species, suggesting that the degradation was cooperative. Among the three mutants, the degradation rate of the Ala mutant was the fastest, and that of the Lys mutant the slowest. This order was the same as that for the inhibitory activity.

3.3. Thermal stability of mutated SSIs measured by circular dichroism (CD)

The CD spectra of the mutant SSIs at room temperature were the same as that of the wild-type SSI (data not shown), indicating the correct folding of each mutant. Then, the thermostability of the mutant SSIs was investi-

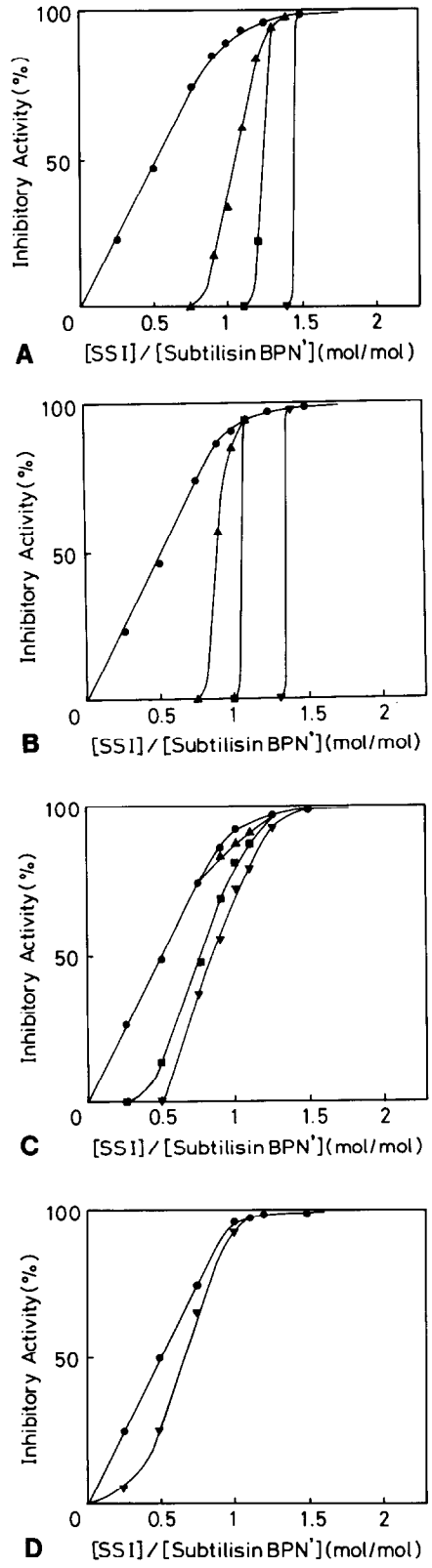

Fig. 2. Time dependence of inhibitory activities of SSI(29A) (A), SSI(29M) (B), SSI(29K) (C) and wild-type SSI (D) toward caseinolytic activity of subtilisin BPN'. At 0 min (●), 10 min (▲), 30 min (■) and 60 min (▼) of incubation at 37°C after mixing subtilisin (364 nM) with various amounts of SSI, an aliquot (100 μl) containing 4 μg of subtilisin was withdrawn, and the inhibitory activity was measured using 1% casein as a substrate.

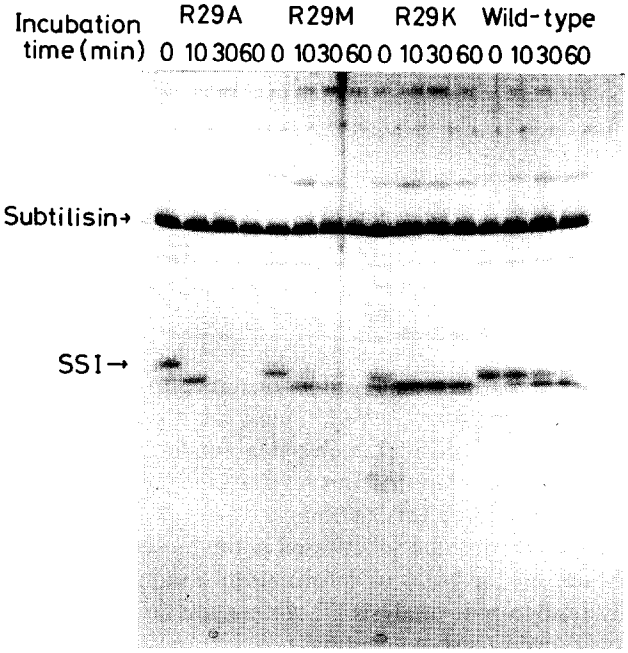

Fig. 3. SDS polyacrylamide gel electrophoresis of mixtures of mutant SSIs and subtilisin BPN' after incubation for various times. Equimolar amounts of subtilisin and each mutated SSI were mixed and incubated at 37°C in 0.1 M borate buffer (pH 9.5). At each time, a 100-μl aliquot containing 4 μg of subtilisin, was withdrawn. Proteins were precipitated with trichloroacetic acid at a final concentration of 6%, and then subjected to SDS-polyacrylamide gel electrophoresis (gel concentration 18.8%).

gated by carrying out measurements at increasing temperature. CD intensity (218 nm) at various temperatures was plotted as a value relative to that at 30°C (Fig. 4). $T_{1/2}$ (defined as the temperature at which the midpoint of decreasing intensity occurs) of wild-type SSI was 78°C, and conversion of Arg^{29} to Lys lowered $T_{1/2}$ by 1.5°C (76.5°C). $T_{1/2}$ of mutated SSIs in which Arg^{29} was substituted with Met or Ala was 68°C, indicating a 10°C decrease in thermal stability. These observations suggest that the positive charge at position 29 is also required for

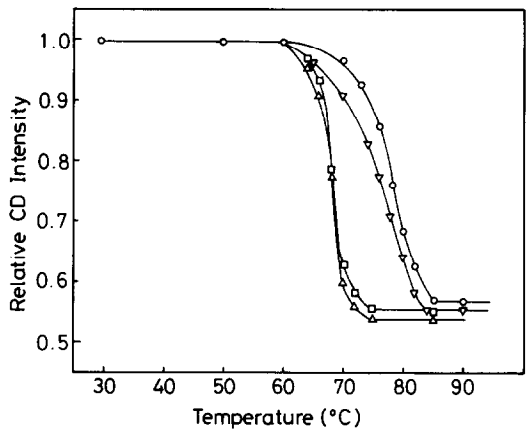

Fig. 4. Temperature dependency of CD intensity of mutated (△ = Ala, □ = Met, ▽ = Lys) and wild-type SSIs (○) at 218 nm. CD intensities were measured at a concentration of 100 μg/ml in 50 mM phosphate buffer (pH 7.0) and are represented as values relative to that at 30°C.

the stability of SSI through interaction with the negative charge at the carboxyl-terminus.

4. Discussion

Protein protease inhibitors have the unique property of resisting degradation by proteases despite having a structure around their reactive site which is similar to that of a substrate. Therefore, it is likely that the molecular structure of protease inhibitors includes some characteristic features that confer resistance to degradation. We have therefore been searching for the structural requirements of SSI that enable it to function as an inhibitor, in expectation that this help to elucidate the mechanism of the inhibitory action.

Arg^{29}, the residue substituted in the present study, was chosen on the basis of information obtained by X-ray crystallographic analysis [7], an enzymatic modification study using carboxypeptidase A [8] and its degree of conservation in closely related protease inhibitors [9,10]. The Arg residue at position 29 was converted to Lys, Met or Ala. These residues were chosen in order to investigate the role of the positive charge and hydrophobic methylenes in the side chain of Arg.

Replacement of Arg^{29} with Ala or Met resulted in conversion of SSI to a temporary inhibitor. Thus, the positive charge of Arg^{29} was found to be very important for the inhibitory action of SSI. Among the two SSI mutants (with Ala or Met at position 29), the rate of decrease of the inhibitory activity of the Ala mutant was faster than that of the Met mutant, indicating that hydrophobic methylenes also contribute to structural maintenance of the SSI molecule, since X-ray crystallographic analysis has shown that the side chain of Arg^{29} is buried in the hydrophobic core. In addition, the temporary inhibition shown by even the Lys mutant carrying a positive charge demonstrates that the length of the side chain at position 29 is important for electrostatic interaction with a carboxyl group at the carboxyl-terminus, although the positive charge is more important.

The fact that these position-29 mutants had inhibitory activity as strong as that of the wild type suggested that the structural change around their reactive site was small. However, since structural fluctuation may occur in the mutated SSI molecule, possibly due to reduction of the interaction with the carboxyl group at the carboxyl terminus by replacement of the Arg^{29} residue, mutated SSI was degraded by subtilisin. Furthermore, structural instability of the position-29 mutants was shown indirectly by the reduction of their thermal stability in terms of CD intensity at 218 nm. In other words, these mutants become easily denatured due to the loss of the electrostatic interaction between Arg^{29} and the carboxyl-terminus. Thus, it seems likely that this destabilization of SSIs is closely related to their temporary inhibition. Analysis of the other protease-sensitive SSI mutants also suggests a high correlation between destabilization and temporary inhibition [11,13,14].

Two possibilities can be considered for the mechanism of temporary inhibition of mutated SSIs. One is that the denatured fraction, which is extremely small in the wild type, is increased by reduction of stability occurring upon mutation, and thus degradation of the mutant SSI proceeds via proteolytic attack toward the denatured form. This mechanism has been proven for the $Trp^{86} \rightarrow$ His mutant of SSI; Trp^{86}, which is located distant from the reactive site, was shown to contribute to the stability and function of SSI by reducing the denatured fraction [14]. This situation seems to resemble that in the present mutations. The other possible mechanism is that digestion occurs when the mutated SSI is in a complex with protease. In this case, the reactive site is digested initially, and a specific intermediate of SSI with only the reactive site cleaved is detected. This phenomenon has been observed in mutant SSIs in which the disulfide bridge near the reactive site [13] or two amino acid residues in the flexible loop near the reactive site were deleted [11]. Therefore the structure of protease inhibitors enables them to resist proteolysis after binding with protease.

With regard to the position-29 mutants, the former mechanism seems more likely, because: (i) the conformational change around the reactive site is small, as in the case of $Trp^{86} \rightarrow$ His, because the binding activity of the mutants did not change markedly, (ii) specific intermediates such as reactive-site-cleaved species were not detected, and (iii) Arg^{29} is located relatively distant from the reactive site of SSI.

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