A Novel Double-headed Proteinaceous Inhibitor for Metalloproteinase and Serine Proteinase*

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A novel proteinaceous inhibitor for the metalloproteinase of Streptomyces caesporis has been isolated from the culture supernatant of Streptomyces sp. I-355. It was named ScNPI (Streptomyces caesporis neutral proteinase inhibitor). ScNPI exhibited strong inhibitory activity toward ScNP with a $K_i$ value of 1.6 nM. In addition, ScNPI was capable of inhibiting subtilisin BPN' ($K_i = 1.4$ nM) (EC 3.4.21.62). The $scnpi$ gene consists of two regions, a signal peptide (28 amino acid residues) and a mature region (113 amino acid residues, $M_r = 118.57$). The deduced amino acid sequence of $scnpi$ showed high similarity to those of ScNPI subtilisin inhibitor (SSI) and its homologues. The reactive site of ScNPI for inhibition of subtilisin BPN' was identified to be Met$^{71}$–Tyr$^{72}$ bond by specific cleavage. To identify the reactive site for ScNP, Tyr$^{53}$ and Tyr$^{72}$, which are not conserved among other SSI family inhibitors but are preferable amino acid residues for ScNP, were replaced separately by Ala. The Y33A mutant retained inhibitory activity toward subtilisin BPN' but did not show any inhibitory activity toward ScNP. Moreover, a dimer of ternary complexes among ScNPI, ScNP, and subtilisin BPN' was formed to give the 2:2:2 stoichiometry. These results strongly indicate that ScNPI is a double-headed inhibitor that has individual reactive sites for ScNP and subtilisin BPN'.

Most metalloproteinases were divided into two groups, Gluzincins (HEXXH + E) and Metzincins (HEXXHXXGXXH) based on their zinc ligands (1–3). In 1969, Yokote and Noguchi (4, 5) found a novel zinc metalloproteinase (SeNP)$^1$ in the culture supernatant of Streptomyces caesporosus. ScNP is one of the smallest zinc metalloproteinase with a molecular weight of 14,376 (6). ScNP specifically cleaves the peptide bond at the amino-terminal side of aromatic amino acid residues (7). ScNP has a common zinc ligand motif (HEXXH), but its third zinc ligand is not the conventional Glu or His but an Asp residue (8). Since ScNP carries a Met turn in its structure, which is a feature of Metzincins, it belongs to Metzincins superfamily (3).

Since proteinaceous proteinase inhibitors are very close to the natural substrate, it is very useful for studies of the structure and function of proteinases. Moreover, the studies of inhibitors can lead to efficient drug design that could ultimately lead to novel therapeutic interventions. Many proteinaceous proteinase inhibitors have been found in animals, plants, and microorganisms. However, natural inhibitors for metalloproteinases are very rare. Known examples include Streptomyces metalloproteinase inhibitor (9), Erwinia chrythanthemi inhibitor (10), and tissue inhibitors of matrix metalloproteinases (11–13). The structure-function relationship of these inhibitors has been well characterized.

We have isolated a novel proteinaceous inhibitor for ScNP from a culture supernatant of Streptomyces sp. I-355 and named it Streptomyces caesporis neutral proteinase inhibitor (ScNPI). ScNPI strongly inhibited not only ScNP (metalloproteinase) but also subtilisin BPN' (serine proteinase). Unexpectedly, ScNPI had sequence homology to Streptomyces subtilisin inhibitor (SSI) family (14–19). In order to clarify the function of ScNPI, the reactive sites for ScNP and subtilisin BPN' were identified.

EXPERIMENTAL PROCEDURES

Materials

DEAE-Sepharose fast flow, Sephadex G-75, Mono Q HR 5/5, and Superdex 200-HR-10/30 were purchased from Amersham Pharmacia Biotech. ScNP was purchased from Seikagaku Kogyo, Japan, and purified as described previously (6). Subtilisin BPN' was purchased from Nagase Biochemicals. Thermolysin was kindly donated by Dàiwa Kaisei, Japan. Pseudomonas aeruginosa elastase (20) was kindly donated by Dr. Kumazaki, Hokkaido University, Japan. Vimentin (21) and aldehyde (22) were purified as described previously. BCA (bicinchoninic acid) Protein Assay Kit was purchased from Pierce. MOCA-Ala-Arg-Gly-Tyr-Gln-Gly-Lys(Dnp)-NH$_2$ was kindly synthesized by Prof. Ben M. Dunn and colleagues, University of Florida College of Medicine. Sac- Ala-Ala-Pro-Phe-MCA was purchased from Peptide Institute Inc., Osaka, Japan. Escherichia coli strain JM109, plasmid pUC18, and plasmid pIN-III-OmpA2 (provided by Dr. S. Taguchi, the Institute of Physical and Chemical Research) (23, 24) were used as a host, cloning vector, and expression vector, respectively. Restriction enzymes and DNA-modifying enzymes were purchased from Nippon Gene (Toyama, Japan), New England Biolabs Inc., and Takara Shuzo (Kyoto, Japan). DIG (digoxigenin) DNA Labeling Kit and DIG Nucleic Acid Detection Kit were purchased from Roche Molecular Biochemicals. PCR kit and ABI PRISM$^{	ext{TM}}$ Dye Terminator Cycle Sequencing Ready Reaction Kit were purchased from Perkin-Elmer.

Cultivation of Streptomyces sp. I-355

The mycelia of Streptomyces sp. I-355 were inoculated into 100 ml of a medium that consisted of 2% starch, 4% polypeptide, 0.1% NaCl, 0.1% K$_2$HPO$_4$, 0.1% yeast extract, and 0.05% MgSO$_4$.H$_2$O at pH 7.0 in a 500-ml flask at 30 °C for 72 h with shaking (100 strokes/min). After the
cultivation, mycelia were removed by centrifugation (8,000 rpm, 40 min). The supernatant was adjusted to pH 4 with 1 N HCl and then the supernatant was treated at 80 °C for 5 min to inactivate endogenous proteinases. After the treatment, the supernatant was neutralized with 1 N NaOH and used for the purification of inhibitor.

**Purification of ScNPI**

The precipitate from 80% saturation of ammonium sulfate was collected by centrifugation (15,000 rpm, 20 min) and dissolved in Buffer A and dialyzed against the same buffer. The subsequent purification steps were the same as described for the authentic ScNPI.

**Concentrations of ScNPI and Enzymes**

Molar concentration (as monomer) of ScNPI was measured by BCA Protein Assay Kit using bovine serum albumin as a standard. Concentrations of ScNPI and subtilisin BPN’ were spectrophotometically determined using E₁₀₀,ₐ₅₄₀ at 280 nm values of 15.5 and 11.7, respectively.

**Tricine SDS-PAGE**

Tricine SDS-PAGE was performed by the method of Schagger and Jagow (25) using 16.5% T, 3% C polyacrylamide gel. The following proteins were used as molecular weight standards: bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). After the electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250.

**Gel Filtration on Superdex 200-HR-10/30**

The purified ScNPI was loaded on a column of Superdex 200- HR-10/30 (10 × 300 mm) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, and then the elution was collected at the same buffer at a flow rate of 0.5 ml/min. Bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), chymotrypsinogen A (25.0 kDa), and cytochrome c (12.4 kDa) were used as molecular weight standards.

**Determination of Partial Amino Acid Sequence**

In order to synthesize PCR primers, the partial amino acid sequence of ScNPI was determined. S-Phenyldisetylamine ScNPI was cleaved with 0.1% CNBr in 70% HCOOH and lyophilized. The resulting peptides were separated by Tricine SDS-PAGE. After electrophoresis, the peptides were electrophoretically transferred to polyvinylidene difluoride membrane and subjected to sequence analysis.

**Preparation of Genomic DNA of Streptomyces sp. I-355**

Genomic DNA of Streptomyces sp. I-355 was prepared as previously (26) with slight modification.

**Polymerase Chain Reaction**

PCR was done with Ampli Taq Polymerase Stoffel fragment (Perkin-Elmer). The primers for PCR are shown in Table I.

**Cloning of the scnpi Gene**

The amplified DNA fragment by PCR using ScNPI-8 as a sense primer and ScNPI-94R as an antisense primer (denaturation at 96 °C for 1 min, annealing at 62 °C for 1.5 min, and extension at 72 °C for 1.5 min, 30 cycles) was labeled with digoxigenin (DIG) and used as a probe for hybridization. The DNA fragments obtained by SaII digestion were ligated into SalI site of pUC18 and transformed into E. coli JM109 cells. DNA sequencing was carried out using ABI PRISM™ T7q Dye Deoxy Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) with a Perkin-Elmer model 373A DNA sequencer.

**Construction of Wild-type and Mutant scnpi Gene for Expression**

The plasmid pScNPI-4, containing the 2.5 kb scnpi gene (in this paper), was digested with SmaI. The resulting 1.0 kb fragment was used as the template for PCR. The mature region of scnpi gene was amplified by PCR (denaturation at 96 °C for 1 min, annealing at 55 °C for 1.5 min, and extension at 72 °C for 1.5 min, 25 cycles) using ER(1) as a sense primer and BH(1) as an antisense primer. The amplified DNA fragment was digested with EcoRI-BamHI and then ligated into EcoRI-BamHI site of plN-III-OmpA2 vector. The resulting plasmid was transformed into E. coli JM109 cells.

Mutant ScNPIs were constructed by PCR using mutagenic primers (Table I). In a first PCR, mutant genes were constructed as separate halves by a combination of primer ER(1) and Y33A(5), primer BH(5), and Y33A(5) with the same PCR condition for the wild-type gene. Another combination was primer ER(1) and Y72A(5), primer BH(5), and Y72A(5). The PCR products were mixed together and used as templates in a second PCR using the flanking sequence primers, ER(5) and BH(5). The amplified fragment was digested with EcoRI-BamHI and then ligated into EcoRI-BamHI site of plN-III-OmpA2 vector. The resulting plasmid was transformed into E. coli JM109 cells.

**Expression and Purification of Recombinant Wild-type and Mutant scnpi Gene**

E. coli JM109 cells harboring recombinant plasmid were inoculated into 100 ml of M9 medium (0.6% Na2HPO4, 0.3% KH2PO4, 1.0% NH4Cl, 0.2% glucose, 0.1% NaHPO4, 0.3% KH2PO4, 1.0% NH4Cl, 0.2% glucose, 0.1 mM MgCl2, 1 mM MgCl2, and 0.2% casamino acid, pH 7.4) containing 50 μg/ml ampicillin and 1 mM isopropyl-1-thio-β-D-galactoside in a 500-ml flask with shaking (110 strokes/min) at 30 °C for 20 h. After the cultivation, cells were centrifuged (6,000 rpm, 20 min) and suspended in 50 mM Tris-HCl, pH 7.5 (10 ml of buffer/1 g of cells). The suspension was sonicated and centrifuged (15,000 rpm, 10 min). Solid ammonium sulfate was slowly added to the supernatant with stirring to give 80% saturation. The precipitate was collected by centrifugation (15,000 rpm, 10 min) and dissolved in Buffer B and dialyzed against the same buffer. The subsequent purification steps were the same as described for the authentic ScNPI.

**Assay for Inhibitory Activity toward ScNP**

0.25 ml of 1.18 μg ScNPI and 0.25 ml of inhibitor solution were incubated at 37 °C for 10 min. Then 1.5 ml of 4/3% Hammersten casein in 0.15 M NaCl, and then the inhibitor was eluted with the same buffer at a flow rate of 30 ml/h. Fractions containing inhibitory activity were pooled. The sample was dissolved in Buffer A and dialyzed against the same buffer. The subsequent purification steps were the same as described for the authentic ScNPI.

**Inhibition Spectra**

In all the reactions, enzyme was preincubated with 10 or 50 μM excess of inhibitor at 37 °C for 10 min.

**Table I**

| Primer | Sequence |
|--------|----------|
| ScNPI-8 | 5’-ATGGGTGTCTACGCGGATCACGAGG-3’ |
| ScNPI-94R | 5’-AGGGTTGCTTCCGCCCCGACCG-3’ |
| ER (+) | 5’-CGGCGATTCACCGCAGCAGCGGATCCGGG-3’ |
| BH (–) | 5’-CGGCGATTCACCGCAGCAGCGGATCCGGG-3’ |
| Y33A (+) | 5’-CAGCGTGGCGCTACGCGCCCCAGG-3’ |
| Y33A (–) | 5’-GCGGATTGCGGGCGAATCAGGTTG-3’ |
| Y72A (+) | 5’-GGCCGATGCGCCCGTACGGGACG-3’ |
| Y72A (–) | 5’-GGGGTCGAAGCCGATCGCCGGA-3’ |

*E. coli* JM109 cells were transformed by small. The resulting 1.0 kb fragment was used as the template for PCR. The mature region of scnpi gene was amplified by PCR (denaturation at 96 °C for 1 min, annealing at 55 °C for 1.5 min, and extension at 72 °C for 1.5 min, 25 cycles) using ER(1) as a sense primer and BH(1) as an antisense primer. The amplified DNA fragment was digested with EcoRI-BamHI and then ligated into EcoRI-BamHI site of plN-III-OmpA2 vector. The resulting plasmid was transformed into E. coli JM109 cells.

Mutant ScNPIs were constructed by PCR using mutagenic primers (Table I). In a first PCR, mutant genes were constructed as separate halves by a combination of primer ER(1) and Y33A(5), primer BH(5), and Y33A(5) with the same PCR condition for the wild-type gene. Another combination was primer ER(1) and Y72A(5), primer BH(5), and Y72A(5). The PCR products were mixed together and used as templates in a second PCR using the flanking sequence primers, ER(5) and BH(5). The amplified fragment was digested with EcoRI-BamHI and then ligated into EcoRI-BamHI site of plN-III-OmpA2 vector. The resulting plasmid was transformed into E. coli JM109 cells. The mutation site was confirmed by DNA sequence using ER(+) as a sequencing primer.
Novel Double-headed Proteinaceous Inhibitor

KINETIC ANALYSIS

Kinetic analysis for ScNP was performed by using a newly designed fluorogenic substrate, MOCAc-Ala-Arg-Tyr-Gly-Gly-Lys(Dnp)-NH₂. All of the reactions were carried out in 50 mM TES-NaOH, pH 7.0, containing 10 mM CaCl₂ and 0.01% Triton X-100 (n/v) (Buffer C) at 25 °C. Fluorescence intensity was measured with a Hitachi F-2000 spectrophotometer at λex 380 nm and λem 460 nm.

The effects of ScNPI on several proteinase activities were investigated. Toward metalloproteinases, ScNPI also inhibited subtilisin BPN’ and trypsin, and chymotrypsin was demonstrated by gel filtration on Superdex 200-HR-10/30 (10 \times 300 mm). ScNP (1 nmol) was incubated with ScNP (final concentration of 5.9 nM) and various concentrations of ScNPI (final concentration of 0.5–2.09 nM) in 980 μl of 50 mM Tris-HCl, pH 8.0, containing 10 mM CaCl₂ and 0.01% Triton X-100 (n/v). Initial velocity was calculated from the increase of fluorescence intensity for 1 min caused by the release of MOCAc-Ala-Arg-Pro-Phe-MCA in 50 mM NaCl buffer, pH 7.5, and incubated at 37 °C for 10 min. Then an equal volume of 0.5 mM glycine HCl, pH 2.5, was added to the mixture and incubated at 4 °C for 60 min.

CD SPECTRA

Complex formation between ScNPI and ScNP or subtilisin BPN’ was demonstrated by gel filtration on Superdex 200-HR-10/30 (10 \times 300 mm) and an equal volume of 0.5 M glycine HCl, pH 7.5, was added to the mixture and incubated at 37 °C for 20 min. The reaction was stopped by the addition of 800 μl of 15% acetic acid. Fluorescence intensity was measured with a Hitachi F-2000 spectrophotometer at λex 380 nm and λem 460 nm.

The results were determined from a Lineweaver-Burk plot. The kinetic parameters were determined from a Lineweaver-Burk plot.

Purification and Some Characteristics of ScNP—ScNP was purified from a culture supernatant of Streptomyces sp. I-355 to electrophoretic homogeneity by three steps of column chromatography: DEAE-Sepharose fast flow, Sephadex G-75, and Mono Q. About 10 mg of the purified ScNP was obtained from 2 liters of the culture supernatant (data not shown). The specific inhibitory activity of purified ScNP was 340 IU/mg. The purified ScNP showed a single protein band on Tricine SDS-PAGE with a molecular weight of 11,000 (Fig. 3). The molecular weight in the native state was also estimated to be 20,000 by gel filtration (data not shown), indicating that the inhibitor exists as dimers.

Inhibition Spectra—The effects of ScNP on several proteinase activities were investigated. Toward metalloproteinases, ScNP strongly inhibited ScNP and slightly inhibited aminopeptidase B. ScNP did not show any inhibitory activity toward thermolysin, Pseudomonas elastase, and alamethicin. In addition, ScNP also inhibited subtilisin BPN’, trypsin, and chymotrypsin belonging to serine proteinase. ScNP did not inhibit cysteine and aspartic proteinase (Table II).

Limited Proteolysis of ScNPI by Subtilisin BPN’

Limited proteolysis by subtilisin BPN’ was performed according to the method of Hiromi et al. (14) with slight modification. ScNPI (1 nmol) and subtilisin BPN’ (0.65 nmol) were mixed in 50 μl of 50 mM Tris-HCl, pH 7.5, and incubated at 25 °C for 10 min. Then an equal volume of ice-chilled 0.5 M glycine HCl, pH 2.5, was added to the mixture. Immediately, proteins were precipitated with trichloroacetic acid at a final concentration of 15%. The precipitate was subjected to Tricine SDS-PAGE. In the case of ScNP, ScNPI (1 nmol) was incubated with ScNP (1 nmol) in 100 μl of 50 mM Tris-HCl, pH 7.5, at 37 °C for 60 min. Then an equal volume of 0.5 M glycine HCl, pH 2.5, was added to the mixture and incubated at 4 °C for 60 min.
**TABLE II**

| Proteinase | Type     | Substrate (pH) | Molar ratio | Inhibitory activity |
|------------|----------|----------------|-------------|---------------------|
| ScNP       | Metallo  | Casein (7.5)   | (I/E)       | %                   |
| Thermolysin| Metallo  | Casein (7.5)   | 10          | 100                 |
| Vibrylase  | Metallo  | Casein (7.5)   | 50          | 0                   |
| P. elastase| Metallo  | Casein (7.5)   | 50          | 0                   |
| Almelysin  | Metallo  | Casein (7.5)   | 50          | 0                   |
| Subtilisin BPN I | Serine | Casein (9.5)   | 10          | 100                 |
| Trypsin    | Serine   | Bz-Arg-MCA (8.0)| 10          | 42.1                |
| Chymotrypsin| Serine   | Suc-Ala-Ala-Pro-Phe-MCA (8.0)| 50 | 89.5                |
| Cathepsin B | Cysteine | Z-Phe-Arg-MCA (6.0)| 50 | 54.2                |
| Pepsin     | Aspartic | Casein (3.0)   | 50          | 50                  |
|            |          |                | (I/E)       | %                   |

**Expression and Purification of Recombinant ScNPIs**—A high level expression system for ScNPI was constructed using pIN-III-OmpA2 vector. All the activity of the ScNPIs was found in the bacterial sonicate. Recombinant ScNPIs were purified as described under “Experimental Procedures.” About 10–15 mg of the various ScNPIs were purified to homogeneity in Tricine SDS-PAGE from 1 liter of the culture (Fig. 3). Recombinant ScNPIs contained three extra amino acid residues, Ala-Glu-Phe derived from EcoRI site at the amino terminus. Except for Y33A mutant, specific inhibitory activity of the purified recombinant ScNPIs was 440 IU/mg.

**Limited Proteolysis**—Since ScNPI was found to be a member of the SSI family, the reactive site for subtilisin BPN’ was identified. The reactive site peptide bond of ScNPI was specifically cleaved by subtilisin BPN’ under acidic conditions. The reaction mixture was subjected to Tricine SDS-PAGE in the presence of 2-mercaptoethanol. Two protein bands with molecular weights of 6,500 and 4,000 were observed (Fig. 4) and subjected to NH2-terminal and COOH-terminal amino acid sequence analysis. ScNPI was specifically cleaved at the Met71-Tyr72 peptide bond. Therefore it was concluded that the Met71-Tyr72 bond of ScNPI was the reactive site for subtilisin BPN’. However, ScNPI did not undergo degradation by ScNP even after prolonged incubation.

**Kinetic Analysis**—The inhibition constant (Ki) of ScNPIs was calculated from a Dixon plot analysis. Wild-type ScNPI (Ki = 8.7 × 10^{-3} mol) and the Y72A mutant (Ki = 1.0 × 10^{-3} mol) had approximately a 2-fold lower Ki value than that of naturally occurring ScNP (Ki = 1.6 × 10^{-3} mol). However, the Y33A mutant did not show any inhibitory activity toward ScNP. In contrast, all of the ScNPIs inhibited subtilisin BPN’ with a Ki of about 2 × 10^{-3} mol (Table III).

To confirm their structural identity, the CD spectra were taken for authentic ScNP and recombinant ScNPIs (data not shown). The CD spectral patterns of these proteins were identical. These results strongly indicated that the Tyr29 residue has a very important role for inhibitory activity toward ScNP.

**Complex Formation**—The interaction of ScNPI with its target enzymes was analyzed by gel filtration on Superdex 200-HR-10/30 (Fig. 5). When ScNPI was incubated with 1 molar equivalent of ScNP or subtilisin BPN’ before application to the column, complete complex formation with each of the target enzymes could be demonstrated. These results indicated that ScNPI binds to each enzyme with E2I2 stoichiometry.

In addition, the ternary complex of ScNPI, ScNP, and subtilisin BPN’ was also analyzed. After incubation, the peaks corresponding to the inhibitor and enzymes were significantly

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**Fig. 1. Nucleotide sequence and the deduced amino acid sequence of scnpi.** The potential SD sequence is indicated by a box. The putative transcription terminator is shown by double underline. The termination codon is indicated by an asterisk. In the protein sequence, the mature region of ScNPI is underlined.
reduced. Moreover, a newly generated peak that was independent from that of ScNPI-ScNP and ScNPI-subtilisin BPN\textsuperscript{9} complex appeared. It was found that ScNPI binds to its target enzymes at different sites of the molecule and forms a complex with E\textsubscript{2}I\textsubscript{2}I\textsubscript{2}' stoichiometry.

**DISCUSSION**

We found a novel proteinaceous ScNP inhibitor from a culture supernatant of *Streptomyces* sp. I-355 (ScNPI). Unexpectedly, ScNPI was found to be a member of the SSI family isolated from various species of *Streptomyces*. However, ScNPI strongly inhibited ScNP with a \( K_i \) of 1.6 nM, and weakly inhibited vimelysin which is an alcohol-resistant metalloproteinase from *Vibrio* sp. T1800. ScNPI did not show any inhibitory activity toward other metalloproteinases such as thermolysin. In addition, ScNPI was capable of inhibiting subtilisin BPN\textsuperscript{9} (\( K_i = 1.4 \) nM), trypsin, and chymotrypsin belonging to the serine proteinase family as has been observed for the SSI family inhibitors (Table II and Table III). ScNPI was a homodimeric protein and interacted with ScNP or subtilisin BPN\textsuperscript{9} by forming an E\textsubscript{2}I\textsubscript{2} complex (Fig. 5). To elucidate the reaction mechanism why ScNPI inhibits both metalloproteinase and serine proteinase with an almost identical \( K_i \) value, we cloned and characterized the gene encoding ScNPI.

The deduced amino acid sequence of *scnpi* showed high similarity to those of SSI family inhibitors (identity was between 35 and 50%). However, conserved residues among the SSI family inhibitors such as Pro\textsubscript{38}, Ala\textsubscript{52}, and Trp\textsubscript{86} (residue number in SSI) were substituted by Tyr, Asp, and Leu, respectively, in the amino acid sequence of ScNPI (Fig. 2). In addition, it was reported that the substitution of Trp\textsubscript{86} to His resulted in temporary inhibition (29). Thus, ScNPI exhibits very unique features.

Based on the sequence homology around the reactive site for subtilisin, we assumed that ScNPI inhibited subtilisin BPN\textsuperscript{9} through interaction at the Met\textsuperscript{71}-Tyr\textsuperscript{72} bond. In addition, based on the substrate specificity of ScNP, Tyr\textsuperscript{72} was a preferred residue at the reactive site for ScNP, so the Met\textsuperscript{71}-Tyr\textsuperscript{72} bond was hypothesized to be a reactive site for both enzymes (Fig. 2).
For the details see “Experimental Procedures.”

Enzyme(s) and ScNPI (each 1.2 nmol) were incubated and analyzed by gel filtration on Superdex 200-HR-10/30.

Thus, the reactive site for ScNP could not be identified by CD spectra, it was assumed that the substitution of Tyr33 for Ala had no influence on the overall structure of ScNPI. These results strongly indicated that the Tyr33 plays an important role on inhibitory activity toward ScNP. The reactive sites of some metalloproteinase inhibitors have been identified (31, 33, 34). These inhibitors interact with the target enzyme in almost the same mechanism. The P1 residue (mainly carbonyl oxygen) interacts with catalytic zinc ion of the enzyme, and the side chain of the P1’ residue occupies S1’ pocket of the enzyme (33–35). It was supposed that ScNPI interacted with ScNP in the same manner, so the reactive site for ScNP was identified to be the Ala32-Tyr33 bond.

The identification of the reactive site of ScNPI suggested that ScNPI was a double-headed inhibitor. To clarify this suggestion further, the formation of ternary complex among ScNPI, ScNP, and subtilisin BPN’ was tested. When ScNPI was incubated with 1 molar equivalent of ScNP and subtilisin BPN’, a newly generated peak that was different from that of the ScNPI-ScNP and ScNPI-subtilisin BPN’ complex was detected by the gel filtration (Fig. 5).

In the case of SSI, it was reported to be a dimer (Mr, 23,000) composed of identical subunits and inhibited subtilisin BPN’ by forming a tightly bound inhibitor-proteinase complex in a molar ratio of 2:2 (36). The amino acid sequence of ScNPI showed a homology to the SSI family, and it formed E2I2 complex with ScNP or subtilisin BPN’. Based on the characteristics of ScNPI, therefore, the result of the gel filtration described above suggested that ScNPI formed a dimer of ternary complexes to give the 2:2:2 stoichiometry. In conclusion, ScNPI is a double-headed inhibitor that has quite different reactive sites for ScNP or subtilisin BPN’.

Here we assumed the location of the reactive sites for ScNP and subtilisin BPN’ based on the structure of the SSI subunit (28) (Fig. 6). It was presumed that the reactive sites for subtilisin BPN’ and ScNP existed at reactive site loop and reactive site loop of the P1 side chain of the P1’ residue, respectively. These new reactive sites were located at the opposite site of the molecule. This presumption agreed closely with the result that ScNPI was a double-headed inhibitor with quite different reactive sites.

The presence of a disulfide bridge in the reactive site is one of the common features of proteinaceous proteinase inhibitors obeying the standard mechanism (30). As in the case of ScNPI, it was suggested that there was a disulfide bridge around the
reactive site for ScNP (Fig. 6). As mentioned above, ScNP was extremely stable against proteolytic attack by ScNP. According to the structural model in Fig. 6, β-turn in the reactive site for ScNP is a very short loop connecting the two anti-parallel β-strands (βα and ββ strand). These β-strands seem to form an anti-parallel β-sheet and make the structure of the inhibitor more stable. This anti-parallel β-sheet probably contributed further to the rigidity of the reactive site, and so kcat value on the hydrolysis of Ala32-Tyr33 bond by ScNP is assumed to be negligibly small.

It was reported that ragi bifunctional inhibitor from ragi grain, which inhibits both α-amylase and trypsin, is a double-headed inhibitor (37–39). However, ragi bifunctional inhibitor formed a ternary complex with the enzymes by 1:1:1 stoichiometry, and the reactive site for α-amylase has not been identified (38, 39). We believe that ScNP is a novel inhibitor in that (i) ScNP is a double-headed inhibitor that can strongly inhibit both metalloproteinase and serine proteinase, and (ii) the reactive sites for each enzyme were clearly identified at different positions located at the opposite sides of the ScNP molecule.

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