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Letter to the Editor: Backbone ^{1}H, ^{13}C, and ^{15}N resonance assignments of *Streptomyces* subtilisin inhibitor

Hiroaki Sasakawa[a], Atsuo Tamura[a], Kazuyuki Akasaka[a,*], Seiichi Taguchi[b], Yoko Miyake[c] & Masatsune Kainosho[c]

[a]*Graduate School of Science and Technology, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan;* [b]*Department of Biological Science and Technology, Science University of Tokyo, Noda, Chiba 278, Japan;* [c]*Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Hachioji, Tokyo 192, Japan*

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Biological context

Streptomyces subtilisin inhibitor (SSI) is a dimeric 23 kDa protease inhibitor isolated from *Streptomyces albogriseolus* (Murao et al., 1973; Hiromi et al., 1985). The crystal structure of SSI retains a unique dimeric structure with two identical subunits associated through the β-sheet, each of the subunits consisting of 113 amino acids with two disulfide bridges (Mitsui et al., 1979). Complete assignments of $^{1}H^{N}$, ^{15}N and ^{13}C NMR signals have been hampered by the lack of an appropriate expression system for uniform isotope labeling and the self-association of the molecules in a relatively low concentration range. In the present work, we have succeeded in constructing an efficient expression system that can produce a sufficient amount of soluble SSI labeled with ^{15}N and/or ^{13}C nuclei. The resultant protein has been used for 3D ^{13}C/^{15}N heteronuclear NMR measurements, which allowed nearly complete signal assignments of the backbone atoms.

Methods and results

The solubility of SSI produced from the secretory expression vector for the SSI gene, pOS1t2 (Taguchi et al., 1993), turned out to be too low for complete signal assignments by heteronuclear 3D NMR measurements. The reason for this low solubility was considered to be the hydrophobicity of the Phe residue in the N-terminal region, which was inevitably inserted when the plasmid of the SSI was constructed. To improve the solubility of the recombinant SSI, gene engineering was performed in the region encoding the SSI N-terminus for truncation of the Phe residue. pOS1t2 was digested with *Eco*RI and *Hin*dIII to obtain the DNA fragment containing the SSI-mature-portion-encoding region. The isolated *Eco*RI/*Hin*dIII DNA fragment was inserted into the same sites of the secretion plasmid vector, pIN-III-*ompA*-3 (Ghrayeb et al., 1984). The resultant plasmid, pOS2t2, was digested with *Eco*RI and the 5′-protruding 4 nucleotides (AATT) were deleted with mung bean nuclease, followed by self-ligation. *E. coli* JM109 was transformed to a wild-type pOS2t2 with the ligated sample after *Eco*RI digestion to eliminate undesired derivatives such as the regenerated form. Six colonies exhibiting inhibitor activity were found to have the desired nucleotide sequence with a disrupted *Eco*RI site. One of these isogenic plasmids was designated to pOST. The newly obtained recombinant SSI showed a much higher solubility and practically the same ^{1}H-NMR spectrum with the same inhibitory activity against subtilisin as the wild-type SSI.

SSI, uniformly labeled with^{15}N or with ^{13}C/^{15}N isotopes was produced by cultivating *E. coli* JM109 carrying pOST plasmid in an M9 medium with $(^{15}NH_4)_2SO_4$ and ^{13}C-D-glucose as the only nitrogen and carbon sources, respectively. For NMR measurements, lyophilized SSI was dissolved to a concentration of 1 mM in 95% $^{1}H_2O$/5% $^{2}H_2O$ containing 25 mM phosphate buffer, pH 6.3. For sequential assignment, HSQC, CT-HNCO, CT-HNCA and CT-

*To whom correspondence should be addressed.

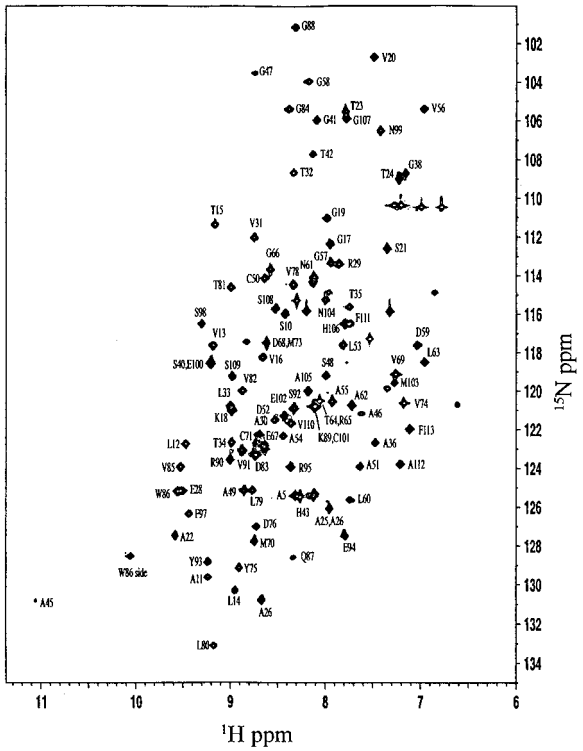

Figure 1. The amide region of the $^{1}H/^{15}N$ HSQC spectrum of ^{15}N-uniformly labeled SSI (1 mM) at pH 6.3 and 303 K. Assignment of cross peaks is shown by the one-letter code for amino acids.

HN(CO)CA experiments (Bodenhausen and Ruben, 1980; Kay et al., 1990, 1994) were performed on a Bruker DMX-750 spectrometer equipped with a triple resonance inverse probe with a field gradient unit. To identify the starting position of the backbone signal assignment, various specifically labeled SSI samples were obtained by cultivating *Streptomyces albogriseolus* S-3253 (Hiromi et al., 1985) in a medium containing various labeled amino acids (Kainosho and Tsuji, 1982). These amino acids were either chemically synthsized from $Na^{13}CN$ or produced by fermentation of microorganisms with ^{15}N or ^{13}C sources. Some amide nitrogen and amide proton signals were identified using CT-HNCO or other NMR experiments by referring to previously assigned carbonyl carbon signals by the $^{13}C/^{15}N$-double labeling technique (Kainosho and Tsuji, 1982).

Extent of assignments and data deposition

The assignments of the $^{1}H^{N}$ and ^{15}N chemical shifts of SSI are shown in Figure 1. NMR signals of 9 residues in the N-terminus (Gly-Ala-Pro-Ser-Ala-Leu-Tyr-Ala-Pro) were not observed due probably to the truncation of the segment (Hiromi et al., 1985). Other proline residues include Pro27, 37, 39, 44, 72, and 77. The backbone resonance assignments, including $^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$ and ^{13}CO chemical shifts, of SSI have been deposited in the BioMagResBank database (accession number: 4331).

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