Contribution of Conserved Asn Residues to the Inhibitory Activities of Kunitz-Type Protease Inhibitors from Plants

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Plant Kunitz-type protease inhibitors contain a conserved Asn residue in the N-terminal region. To investigate the role of Asn residue in protease inhibitory activities, Erythrina variegata trypsin inhibitor a (ETIa), E. variegata chymotrypsin inhibitor (ECI), and their mutants, ETIa-N12A and ECI-N13A, were used. Both mutants exhibit weaker inhibitory activities toward their cognate proteases than the wild-type proteins and were readily cleaved at reactive sites. Furthermore, kinetic analysis of the interactions of the mutated proteins with their cognate proteases by surface plasmon resonance (SPR) measurement indicated that replacements of the Asn residue mainly affected dissociation rate constants. The conserved Asn residues of Kunitz-type inhibitors play an important role in exhibiting effective inhibitory activity by stabilizing the structures of the primary binding loop and protease-inhibitor complex.

Key words: Erythrina variegata; hydrogen bonds; Kunitz-type protease inhibitor; primary binding loop; surface plasmon resonance

Protein structures of proteinaceous serine protease inhibitors have been studied extensively to address the enigma that they bind tightly to the active site of the target protease and yet are not hydrolyzed by the protease. It has been found that intramolecular interactions, e.g., disulfide bond, hydrogen bond, and hydrophobic interaction, are involved in stabilization of the primary binding loop (the so-called reactive site loop) structure, which forms a stable complex with a cognate protease, thereby exhibiting inhibitory activity toward them.1,2

The plant Kunitz-type protease inhibitors from plants, such as soybean trypsin inhibitor STI, have molecular masses of approximately 20 kDa and consist of about 180 amino acid residues with two disulfide bonds.3 Since one disulfide bond (Cys33–Cys77 in STI numbering) is in close vicinity to the reactive site, it was initially thought to be a structural element that stabilizes the reactive site loop. It was, however, reported that chemical modification and substitution of the disulfide bonds in Erythrina caffra trypsin inhibitor did not affect inhibitory potency toward trypsin.3,4 These results indicated that disulfide bonds in Kunitz-type protease inhibitor are not necessarily involved in stabilization of the reactive site loop. Meanwhile, crystallographic studies of Kunitz-type protease inhibitors showed that the reactive site loop is supported by a hydrogen bond network between the loop and the N-terminal region.4–7 In particular, the conserved Asn residue (Fig. 1A) in N-terminal region was suggested to play an important role in the stabilization of the primary binding loop via hydrogen bonds.8

To evaluate the involvement of the conserved Asn residue in Kunitz-type protease inhibitors in their inhibitory activities, trypsin inhibitor ETIa (172 amino acids) and chymotrypsin inhibitor ECI (179 amino acids) from Erythrina variegata seeds,9 and their mutants, ETIa-N12A and ECI-N13A, in which the Asn12 in ETIa and Asn13 in ECI were substituted with Ala, were used.

ETIa and ECI were overproduced in the Escherichia coli BL21(DE3) strain using the expression vector pET-22b, and purified as described previously.10,11 Site-directed mutagenesis was done by the unique site elimination method developed by Deng and Nickoloff.12 The mutated genes were expressed in E. coli and purified by the same procedures as described for wild-type ETIa and ECI.10,11 The inhibitory activities of the proteins toward trypsin and chymotrypsin

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Abbreviations: ETIa, Erythrina variegata trypsin inhibitor a; ECI, E. variegata chymotrypsin inhibitor; RU, resonance units; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPR, surface plasmon resonance
were assayed with casein as the substrate and the inhibitory constants \((K_i)\) were calculated from the inhibitory profile obtained by the method of Henderson,\(^{13}\) as described previously.\(^{11}\) Real time analyses of interactions of inhibitors and cognate proteases were performed using BIAcore\(^{TM}\), as described in our previous paper.\(^{11,14}\)

Figure 2A shows inhibitory profiles of ETIa and ETIa-N12A toward trypsin. ETIa-N12A exhibited remarkably weaker inhibitory activity toward trypsin than wild-type ETIa when their inhibitory activities were assayed after incubation with trypsin for 10 min. The \(K_i\) value \((5.2 \times 10^{-6} \text{ M})\) of ETIa-N12A was approximately 260-fold higher than that of ETIa \((2.0 \times 10^{-8} \text{ M})\). Furthermore, the inhibitory activity of ETIa-N12A gradually decreased with increasing incubation time after mixing with trypsin (Fig. 2A). On the other hand, the replacement of Asn13 in ECI with Ala also resulted in a decrease of inhibitory activity toward chymotrypsin; the \(K_i\) value \((3.2 \times 10^{-7} \text{ M})\) of this mutant was about 3.3-fold higher than that of ECI \((9.8 \times 10^{-8} \text{ M})\). In contrast to ETIa-N12A, no decrease in the inhibitory activity of ECI-N13A toward chymotrypsin was found with increasing incubation time after mixing with chymotrypsin (Fig. 2B). These results indicate that the conserved Asn residues of Kunitz-type protease inhibitors played an important role in inhibitory activities toward their cognate proteases. In respect to ETIa, the substitution of this residue may convert the inhibitor to a temporary inhibitor.
To confirm this assumption, ETIa-N12A and ECI-N13A were incubated with trypsin and chymotrypsin respectively for various times, and the incubated mixtures were subjected to SDS–PAGE in the presence of 2-mercaptoethanol. The wild-type ETIa and ECI were hardly digested with cognate proteases even when they were incubated for more than 2 h (Fig. 3A and 3B). In contrast, ETIa-N12A and ECI-N13A were digested gradually and two novel fragments were generated in each case, as shown in Fig. 3C and D. N-terminal sequence analysis indicated that both of these two mutant proteins were cleaved at the reactive site, that is, between Arg63 (P1) and Ser64 (P1') in ETIa and between Leu64 (P1) and Ser65 (P1') in ECI.9,15) These results indicate that the conformation of the primary binding loops in ETIa and ECI was changed by the substitution of conserved Asn residues in ETIa and ECI, increasing their susceptibility toward their cognate proteases.

Further to investigate the contribution of Asn residue, the kinetics of the interaction of ETIa-N12A and ECI-N13A with trypsin and chymotrypsin respectively were analyzed by surface plasmon resonance (SPR) with the BIACore™ system. The association rate constants (k ΔG) and the dissociation rate constants (k ΔG) of ETIa and ETIa-N12A were calculated to be $3.5 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ and $2.5 \times 10^{-4} \text{s}^{-1}$, and $2.6 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ and $4.4 \times 10^{-4} \text{s}^{-1}$, respectively. On the other hand, the association rate constants and the dissociation rate constants of ECI and ECI-N13A were calculated to be $4.4 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ and $4.7 \times 10^{-4} \text{s}^{-1}$, and $4.4 \times 10^5 \text{M}^{-1} \text{s}^{-1}$ and $8.7 \times 10^{-4} \text{s}^{-1}$, respectively. These results indicated that the decreased inhibitory activities of ETIa-N12A and ECI-N13A toward trypsin and chymotrypsin respectively were due almost entirely to increases in their dissociation rate constants (k ΔG), suggesting that the substitutions of conserved Asn residue caused the decrease in the stability of the inhibitor-protease complex.

Our results indicate that the conserved Asn residues in Kunitz-type protease inhibitors stabilize the primary binding loop structure by hydrogen bonds and contribute to inhibitory potency toward cognate proteases by forming the stable inhibitor-protease complex. This agrees with recent studies on the winged bean chymotrypsin inhibitor WCI-2. WCI-2 is a Kunitz-type protease inhibitor and has a high sequence similarity with ECI, including around the reactive site (Fig. 1B). Ravichandran et al. constructed two WCI-2 mutants in which the conserved Asn14 of WCI-2 (Fig. 1A) was substituted for Lys or Asp residue, and they determined the crystal structures of the mutants. These mutations altered the hydrogen-bonding patterns between the side and main chains around the primary binding loops compared with that of wild-type WCI-2. Furthermore, the number of water molecules involved in the formation of the hydrogen bond network of the primary binding loop decreased. Consequently, the mutants...
showed a 2-fold increase in $K_i$ values as compared with the wild type.\(^6\) In the case of ECI, the substitution of conserved Asn residue to Ala also showed a decrease in inhibitory activity, but there is no hydrogen-bonding donor or acceptor in the side chain of the Ala residue of inhibitory activity, but there is no hydrogen-bonding conserved Asn residue to Ala also showed a decrease in effective inhibitory activity toward chymotrypsin. Additional structural factors such as hydrophobic interactions in ECI cannot prevent the cognate protease cleaving the reactive site of the inhibitor readily if the conserved Asn residue was replaced (Fig. 3). However, unlike ETIa-N12A, the additional structural factors may function to maintain the structure of ECI-N13A after cleavage of the primary binding loop and to exhibit effective inhibitory activity toward chymotrypsin.

References


