Structural Basis of Inhibition Revealed by a 1:2 Complex of the Two-headed Tomato Inhibitor-II and Subtilisin Carlsberg*

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Multidomain proteinase inhibitors play critical roles in the defense of plants against predation by a wide range of pests. Despite a wealth of structural information on proteinase-single domain inhibitor interactions, the structural basis of inhibition by multidomain proteinase inhibitors remains poorly understood. Here we report the 2.5-Å resolution crystal structure of the two-headed tomato inhibitor-II (TI-II) in complex with two molecules of subtilisin Carlsberg; it reveals how a multidomain inhibitor from the Potato II family of proteinase inhibitors can bind to and simultaneously inhibit two enzyme molecules within a single ternary complex. The N terminus of TI-II initiates the folding of Domain I (Lys-1 to Cys-15 and Pro-84 to Met-123) and then completes Domain II (Ile-26 to Pro-74) before coming back to complete the rest of Domain I (Pro-84 to Met-123). The two domains of TI-II adopt a similar fold and are arranged in an extended configuration that presents two reactive site loops at the opposite ends of the inhibitor molecule. Each subtilisin molecule interacts with a reactive site loop of TI-II through the standard, canonical binding mode. Remarkably, a significant distortion of the active site of subtilisin is induced by the presence of phenylalanine in the P1 position of reactive site loop II of TI-II. The structure of the TI-II(subtilisin) complex provides a molecular framework for understanding how multiple inhibitory domains in a single Potato II type proteinase inhibitor molecule from the Potato II family act to inhibit proteolytic enzymes.

Proteinaceous serine proteinase inhibitors (PIs)1 from plants were first isolated nearly 65 years ago (1) and are now known to be major constituents of seeds, tubers, and leaves of members of the Solanaceae and Leguminosae families (5–15% of the total protein) (2–4). These PIs are an integral part of the constitutive and inducible defensive mechanisms that protect plants from attacking pests (bacteria, fungi, and insects) (5–7). These defensive mechanisms involve the systemic synthesis of serine PIs that accumulate in distal tissue and can inhibit the digestive trypsin- and chymotrypsin-like enzymes of insects and other related serine proteinases of plant pathogens (8, 9). The inhibitory properties toward serine proteinases of these PIs have already been exploited with varying degrees of success for the production of transgenic plants overexpressing the PIs in an attempt to control pests (9–13). However, a greater understanding of the molecular mechanism of inhibition of these PIs with pest proteinases is required at the structural level to fully harness the potential benefits of these natural PIs to crop protection.

PIs of the Potato II (Pot II) inhibitor family have been isolated from wounded tomato and tobacco leaves (14, 15), green tomatoes (16), potato tubers (17, 18), eggplant fruits (19), paprika seeds (20), and ornamental tobacco flower stigma (21). Pot II PIs can inhibit trypsin, chymotrypsin, subtilisin, oryzin, and elastase (14, 22) and accumulate systemically in potato; TI, tomato proteinase inhibitor; r.m.s.d., root mean square deviation; BBI, Bowman-Birk proteinase inhibitor.

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1 The abbreviations used are: PI, potato proteinase inhibitor; Pot, potato; TI, tomato proteinase inhibitor; r.m.s.d., root mean square deviation; BBI, Bowman-Birk proteinase inhibitor.
repeat, whereas the *Nicotiana alata* PI is a 40.3-kDa precursor consisting of six repeated elements that are proteolytically processed to yield six different PIs (C1 and C2, which are chymotrypsin inhibitors, and T1–T4, which are trypsin and chymotrypsin inhibitors) (21, 33, 34). High sequence identity exists among the Pot II PI sequences, although interesting variations (mostly in the reactive site loops) occur among different species and among different isoforms of the same species (see Fig. 1). These differences in sequence can give rise to a wide range of differences in cognate proteinase specificities. The expression of multiple isoforms of Pot II PIs with differing target proteinase specificities may be particularly important for protecting plants against predators with an arsenal of proteinases having a wide range of substrate specificity. Understanding the structural basis of PI inhibitory specificity is clearly crucial for understanding how variations in PI sequence give rise to different inhibitory specificities.

Few three-dimensional structures are currently available for members of the Potato II inhibitor family, and there is currently only one structure of a complex with a bound proteinase. In 1989, the structure of one of the two domains of PI-II, the chymotrypsin-binding domain (known as PCI-1), was solved to 2.1 Å by x-ray crystallography (35) in complex with *Streptomyces griseus* proteinase B. Remarkably, this structure revealed that the sequence of PCI-1 corresponds to a region that lies across the two repeats of inhibitor II instead of being contained within one repeat. As in other inhibitors, the reactive site loop in PCI-1 adopts the canonical conformation (36) and is constrained by disulphide bridges Cys-3–Cys-40 and Cys-7–Cys-36, which are invariant in the Pot II family (Fig. 1). The NMR structures of single domains of *N. alata* have also been determined (34, 37, 38). In these single-domain inhibitors, the scaffold of the protein is similar to that seen in PCI-1, and the reactive site loops adopt the same canonical conformation with cysteines involved in disulphide bridge formation at P3 and P2’.

Structural information on other families of PIs is mostly restricted to single-domain inhibitors, even though many natural inhibitors have multiple domains. Structures for a multidomain PI bound to a proteinase have only been determined for the two-headed Bowman-Birk inhibitors from mung bean (39), soybean (40), and wheat germ (41) bound to trypsin. The Bowman-Birk inhibitors consist of two domains of roughly 60 amino acids that are related by a nearly perfect 2-fold rotational symmetry axis. Each inhibitory domain binds to and inhibits a separate proteinase molecule in a very similar manner through a reactive-site loop in the standard, canonical mechanism.

For the first time, the three-dimensional x-ray crystallographic structure of a ternary complex of a full-length two-headed inhibitor from the Pot II family, TI-II, bound to two molecules of subtilisin Carlsberg has been solved to 2.5Å resolution. TI-II is a 13.5-kDa potent inhibitor of subtilisin (K<sub>i</sub> = 9 nM) that possesses a remarkable dual specificity toward chymotrypsin (K<sub>i</sub> = 30 nM) and trypsin (K<sub>i</sub> = 80 nM) (14). There is high sequence homology between TI-II and PI-II (86% sequence identity), and both inhibitors contain two repeats, each containing one reactive site loop located near the N terminus. Depending on the species, the N-terminal repeat contains the trypsin-specific region, and the C-terminal repeat contains the
chymotrypsin-specific region. In the TI-II isoform (from *Lycopersicon esculentum* cv. Bonny Best) used for our structural studies, the reactive site loop of the first repeat inhibits trypsin (residues P2-P1-P1′ are TRE) whereas that of the second repeat inhibits chymotrypsin (residues P2-P1-P1′ are TFN). The novel three-dimensional structure of TI-II in complex with a bacterial pest proteinase opens avenues of research previously difficult to undertake because of the lack of structural information on the mode of inhibition of two-domain Pot II inhibitors.

**EXPERIMENTAL PROCEDURES**

Purification and Crystallization—Tomato inhibitor-II was prepared from transgenic tomato plants that overexpressed a prosystemin transgene, which resulted in the synthesis and accumulation of high levels of TI-II in the leaves (~1 mg/ml leaf juice). Leaves from 1000 young tomato plants were collected (~800 g of leaf tissue) and blended with 1600 ml of a buffer containing 0.01 M sodium citrate, 0.5 M sodium chloride, and 0.7% sodium hydrosulfite, pH 4.3. The homogenate was expressed through eight layers of cheesecloth by hand squeezing, and the debris retained in the cloth was resuspended in 400 ml of buffer, and the mixture was expressed. The combined expressed liquid was clarified by centrifugation at 12,000 × g for 20 min. Proteins in the supernatant liquid were precipitated by adding solid ammonium sulfate to 80% saturation and stirred at 4 °C for 2 h. The precipitate was recovered by centrifugation as above. The pellet was solubilized in 600 ml of water, and the remaining debris was removed by centrifugation. The resulting solution was placed in a flask and immersed in a boiling water bath with stirring until the temperature of the liquid was 70 °C. The contents of the flask were then cooled rapidly in an ice bath to room temperature, and the precipitated proteins were repelleted by centrifugation. The solution was dialyzed overnight at 4 °C against 0.01 M Tris, 0.10 M KCl buffer, pH 8.1. The retentate was passed through a 3 × 12-cm column containing chymotrypsin-Sepharose CL4B affinity resin (42) that had been equilibrated with the buffer. The column was then washed with 3 bed volumes of buffer and then with 8 M urea, pH 3, to elute the inhibitor proteins bound to chymotrypsin. The eluate was dialyzed against several changes of 50 mM ammonium carbonate and lyophilized and stored. The preparation yielded about 80 mg of dry material containing about 24 mg of TI-II and 14 mg of tomato inhibitor-I. TI-II was purified to homogeneity using reverse phase high pressure liquid chromatography. A Vydac (Hesperia, CA) 218TP510 C18 semi-preparative column (10 × 250 mm, 5 μm, 300 Å) was employed using a 90-min gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. TI-II eluted as a single peak at 53 min. The yields of inhibitor from this step were nearly quantitative. The TI-II protein obtained was

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**Fig. 2.** A, structure of the TI-II (subtilisin)2 complex. Subtilisin molecules are drawn in yellow, and the two domains of TI-II are drawn in red and blue. B, stereoscopic view of the structure of TI-II. In the Cα trace of TI-II, cysteine residue side chains and disulfide bonds are drawn in yellow and given residue numbers. Residues 74 to 85 and 117 to 123 are missing from the final model, because these portions of the structure were not defined in electron density maps. The approximate location of these residues is designated by magenta dots.
Structural Basis for Inhibition by TI-II

RESULTS AND DISCUSSION

Structure of the TI-II(Stabilisin)_2 Complex—The structure of the TI-II(stabilisin)_2 complex was determined by the molecular replacement technique to 2.5-Å resolution, using the coordinates of subtilisin Carlsberg (54) (PDB code 1SCN) as the search model. There are two molecules of subtilisin bound to one molecule of the two-headed inhibitor TI-II (see Fig. 2; refer to Table I for crystallographic parameters). The two subtilisin molecules are bound at opposite ends of the elongated inhibitor molecule, forming a 673-residue ternary complex with dimensions of roughly 100 × 50 × 40 Å.

TI-II has a novel two-domain structure; each domain adopts the fold described previously in the single-domain Pot II inhibitors (34, 35, 37, 38). Each domain contains only a small amount of regular secondary structure in the form of a three-stranded antiparallel β-sheet, as well as a series of stretches of polypeptide chain interconnected by four disulphide bonds. As first predicted after the determination of the structure of the single-domain Pot II inhibitor PCI-I, the sequence repeats present in TI-II do not correspond to individual structural domains (Fig. 1B(35). Instead, Domain I consists of the first 15 residues and residues 86 to 116, whereas Domain II consists of residues 16–73. Even though Domain I is composed of two non-contiguous polypeptide sequences, it adopts the same fold as Domain II (r.m.s.d. = 0.86 Å, 41 Cα atom pairs; see Fig. 3A). The β-sheet scaffold of Domain I is similar to that seen in the single-domain C2 inhibitor from N. alata (r.m.s.d. = 0.99 Å, 19 Cα atom pairs; see Fig. 3B). Both Domain I and C2 consist of two noncontiguous segments of polypeptide connected through disulphide bonds, giving rise to a “clasped bracelet” fold that is thought to be characteristic of inhibitors of the Pot II family (34). Although Domain I shares the same folding topology and aligns very well with C2 in the β-sheet region, the rest of the domain, including the reactive site loops, differs from C2 substantially in conformation. These differences in conformation between Domain I and C2 reflect intrinsic structural differences between TI-II and C2, as well as the inability of NMR structure calculations to define precisely the conformation of residues A1 to A9 and B1 to B12 of C2 (see Table I in Ref. 34).

The arrangement of the two domains in TI-II gives rise to an extended structure with overall dimensions of ~50 × 25 × 15 Å. The arrangement of the two domains differs substantially from an earlier prediction based on the structure of PCI-I, in which a pseudo 2-fold rotation axis relates the two domains across a continuous six-stranded antiparallel β-sheet (35). Instead, the three-stranded β-sheet of Domain I is packed against the C terminus of the reactive site loop of Domain II. The arrangement of the two domains in TI-II also differs substantially from that proposed for the six-domain inhibitor precursor.
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Fig. 3. A, stereoscopic view of a least-squares superposition of TI-II domains I and II. Cyan atoms of Domains I and II that align within 3.5 Å of each other are drawn in red and blue, respectively, whereas magenta atoms that do not align are drawn in magenta and cyan, respectively. The N- and C-terminal residues of the polypeptide segments in each domain are labeled. B, stereoscopic view of a least-squares superposition of TI-II Domain I and the C2 inhibitor from N. alata (34). Cyan atoms of Domain I and C2 that align within 3.5 Å of each other are drawn in red and blue, respectively, whereas magenta atoms that do not align are drawn in magenta and cyan, respectively. The N- and C-terminal residues of polypeptide segments in each domain are labeled.

TABLE II
Main chain torsion angles of reactive site loops

|         | P4 | P3 | P2 | P1 | P1' | P2' | P3' |
|---------|----|----|----|----|-----|-----|-----|
| TI-II (D1) | −120 | 147 | −126 | 158 | −62 | 132 | −85 | 36 |
| TI-II (D2) | −84 | 136 | −134 | 152 | −67 | 145 | −97 | 49 |
| PCI-I | −82 | 132 | −137 | 152 | −65 | 151 | −115 | 51 |
| OMTKY3a | −158 | 158 | −126 | 147 | −69 | 162 | −119 | 45 |
| OMTKY3b | −129 | 136 | −131 | 150 | −68 | 160 | −107 | 32 |
| CI-2 | −93 | 140 | −133 | 166 | −64 | 147 | −103 | 34 |
| Eglin-C | −76 | 138 | −143 | 165 | −65 | 151 | −112 | 42 |
| BPTI | −78 | 175 | −77 | −30 | −70 | 156 | −117 | 39 |
| sBBI | −121 | 134 | −139 | 145 | −71 | 156 | −95 | 45 |

a Ovomucoid third domain bound to Streptomyces griseus proteinase B.

b Ovomucoid third domain bound to α-chymotrypsin.

c Soybean Bowman-Birk inhibitor bound to bovine trypsin (40).

suggests how domains may be oriented relative to each other in other multidomain Pot II inhibitors, such as the six-domain inhibitor from N. alata (21).

Enzyme-Inhibitor Interactions—Standard mechanism, canonical proteinaceous inhibitors of serine proteases from at least 18 nonhomologous families (55) bind to the active sites of proteases in a substrate-like manner (56) through the reactive site loops (57). According to the nomenclature of Schechter and Berger (58), where P1 is the residue N-terminal to the scissile bond and P1' is the residue C-terminal to the scissile bond, residues P4 to P2' of each inhibitor almost always interact with binding pockets S4 to S2' on the protease. The reactive site loop adopts an approximate extended conformation and forms a distorted antiparallel β-strand pair with residues near the active site of the protease (59).

from N. alata based on the structure of the single-domain C2 inhibitor (34).

The interdomain interface in TI-II consists of a small cluster of highly conserved hydrophobic residues (I1e-14, Pro-16, Tyr-98, Phe-100, and Phe-106 from Domain I and Tyr-34, Pro-54, and Lys-55 from Domain II). Although this interface is quite small (buried surface area of 487 Å²), it appears to form a stable packing arrangement between the two domains. Preliminary data from a crystal form of the unbound inhibitor containing four copies of TI-II in the asymmetric unit indicate that the orientation of the two domains in TI-II observed in the TI-II(subtilisin), complex is similar to that seen for the unbound inhibitor. The arrangement of domains observed in TI-II also suggests how domains may be oriented relative to each other in other multidomain Pot II inhibitors, such as the six-domain inhibitor from N. alata (21).
Each reactive site loop in TI-II interacts with a separate molecule of subtilisin in the standard, canonical manner as observed in other proteinase-inhibitor complexes. Main chain torsional angles in the reactive site loops are highly similar to those seen in other PIs (Table II). The conformations adopted by the reactive site loops of each domain in TI-II are very similar with an r.m.s.d. of 0.49 Å for the 32 main chain atoms of the residues at positions P5 to P3’ (Fig. 4A). As in other

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**Fig. 4. Conformations of the reactive site loops.** A, stereoscopic view of a superposition of the reactive site loops of both domains. Conformation of the reactive site loops of Domain I (B) and Domain II (C) bound to subtilisin are shown. The solvent-accessible surface of the subtilisin molecules are drawn with negatively charged residues colored red, positively charged residues colored blue, and hydrophobic residues colored magenta.
FIG. 5. Interactions between subtilisin and TI-II. Shown is a stylized representation of the interactions between subtilisin and the reactive site loops of Domain I (A) and Domain II (B). Residues of subtilisin making van der Waals interactions with the reactive site loops of TI-II are shown as blue circles and, in some instances, as parallel lines. Also shown is a stereoscopic view of the interactions between subtilisin and the reactive site loops of Domain I (C) and Domain II (D). Inhibitor is green, and subtilisin is black.
members of the Pot II family, disulfide bonds formed by cysteine residues at the P3 and P2/H1 positions (Cys-3 and Cys-7 in Domain I and Cys-60 and Cys-64 in Domain II) help to hold the reactive site loop in a relatively rigid conformation that likely helps to prevent proteolytic cleavage of the inhibitor upon interaction with proteinases. Additional features of the structure of TI-II that contribute to the stability of both reactive site loops is the presence of a hydrogen bond between the side chains of the P2 and P1/H residues (Thr-4 and Glu-6 in Domain I and Thr-61 and Asn-63 in Domain II), which is also seen in many other PIs (60). In addition, the N\textsuperscript{62} atom of the side chain of Asn-29 donates hydrogen bonds to the main chain carbonyl oxygen atoms of Thr-61 and Asn-63 at the P2 and P1/H positions of the reactive site loop of Domain II, and the main chain carbonyl oxygen atom of Ile-28 accepts a hydrogen bond from the main chain amide nitrogen atom of Asp-65. The reactive site loop in Domain I is less well stabilized, with a van der Waals contact between the side chains of Thr-89 and Glu-6 replacing the hydrogen bonds donated by Asn-29 in Domain II.

Residues in the P5 to P2/H positions and the P6 to P2/H positions of the reactive site loops from Domains I and II, respectively, interact with the substrate-binding clefts of separate subtilisin molecules (Fig. 4, B and C). Specific interactions between the reactive site loops of TI-II and subtilisin are illustrated schematically in Fig. 5. A number of the inhibitor-enzyme contacts consist of van der Waals interactions, with the P1 residues contributing the largest number of contacts. In addition, the polypeptide backbone of the reactive site loops of TI-II forms a number of hydrogen bonds with the polypeptide backbone of subtilisin that are characteristic of standard, canonical inhibitor-proteinase complexes (Fig. 5, A and B).

Subtilisin has a broad substrate promiscuity compared with most other serine proteinases, mostly because of the relatively shallow S1 specificity pocket. The highly accommodating nature of the S1 pocket is dramatically illustrated by the binding of two large P1 residues, arginine and phenylalanine, in the TI-II complex. Although the binding mode of the reactive site loop in both domains is similar to that seen in other proteinase-inhibitor complexes (Table II), there are some notable differences. The arrangement of the active site residues in the subtilisin molecule bound to Domain I is very similar to that seen in subtilisin Carlsberg and subtilisin Novo BPN\textsuperscript{'} bound to eglin-C (61–63), chymotrypsin inhibitor-2 (62), and Streptomyces subtilisin inhibitor (64, 65). In the subtilisin molecule bound to Domain II, however, the active-site Ser-221 side chain lies closer to the carbonyl group of the P1 residue of the inhibitor than in other proteinase-inhibitor complexes. As a result, the active-site Ser-221 residue adopts a rotamer ($\chi_1 = -153^\circ$) in the molecule of subtilisin bound to Domain II that differs from the rotamer ($\chi_1 = -99^\circ$) seen in the molecule of subtilisin bound to Domain I, as well as in other subtilisin-inhibitor complexes (Fig. 6).

The surface area of the inhibitor that is buried upon complex formation is 611 Å\textsuperscript{2} for Domain I and 970 Å\textsuperscript{2} for Domain II.

Fig. 6. Stereoscopic view of simulated annealing omit maps for the active site regions of subtilisin bound to Domain I (A) and Domain II (B). Electron density maps were calculated after omitting subtilisin residues 64, 152, and 221, as well as the P1 residue from the inhibitor, and refining the structure by the simulated annealing protocol, starting at 1000 K (47).
Each domain of TI-II appears to bind the proteinase independently of the other domain, with the exception of a small number of additional contacts outside of the reactive site loops (Table III). The reactive site loops of Domains I and II contribute 547 Å² and 611 Å² of buried surface area respectively, accounting for 90 and 62% of each domain’s binding interface with subtilisin. Preliminary modeling studies in which other proteinases are docked onto the reactive site loops of TI-II suggest that TI-II may contact other proteinases at a wider range of sites outside of the reactive site loop region. For subtilisin and likely also for other proteinases, the two molecules of proteinase bound to the inhibitor are separated by a large distance and do not contact each other.

The structure of the TI-II(subtilisin)₂ ternary complex reveals for the first time the structural basis of inhibition by a multidomain Pot II family inhibitor and shows a number of interesting similarities and differences with ternary complexes formed between the structurally unrelated Bowman-Birk family of proteinase inhibitors (BBIs) and trypsin (39–41). Like TI-II, the structurally simpler BBIs also contain two domains, each of which presents a reactive-site loop at the extremities of the inhibitor molecule. Also as seen in TI-II, a single BBI molecule can bind two proteinase molecules simultaneously. The fold of each domain and the arrangement of the two inhibitory domains of the BBIs differ strikingly from TI-II, however. The BBIs consist of two domains that are related by an approximate 2-fold rotational symmetry axis, and each domain interacts in a very similar manner with target proteinase. Interactions between the BBIs and proteinases are almost completely restricted to the reactive-site loop, with buried surface areas of ~650–700 Å². These values are similar to that seen for Domain I in TI-II but significantly smaller than that seen for Domain II (970 Å²), where non-reactive site loop interactions account for roughly one-third of the inhibitor-proteinase interface.

Secondary contacts not involving the reactive-site loop in Domain II of two-domain Pot II family inhibitors may be of critical importance to determining proteinase inhibition specificity, as suggested by the results of site-directed mutagenesis studies on PI-II from potato (66). In PI-II, the capacity for trypsin inhibition in Domain II could not be transferred to Domain I by mutation of the P1 residue, the P2-P1-P1’ sequence, or even the entire stretch of sequence from P2 to P10. These results underline the importance of regions of the inhibitor apart from the reactive site loop in determining inhibitor specificity. The determination of the structure of the TI-II(subtilisin)₂ complex provides an important step toward understanding the roles of secondary binding surfaces as well as reactive site loops in the mechanism of inhibition in the Pot II family of proteinase inhibitors.

CONCLUSION

The 1:2 complex of TI-II bound to subtilisin Carlsberg reveals how multidomain Pot II inhibitors can simultaneously inhibit several proteinases within a single complex. Each domain of TI-II interacts with a single proteinase molecule primarily through the reactive site loop but also through secondary contacting regions mostly within the same domain. The extended conformation adopted by TI-II enables this inhibitor to form 1:2 complexes without encountering steric clashes between the proteinase molecules. This represents an initial step toward understanding how a large number of multidomain Pot II inhibitors utilize multiple inhibitory domains to defend plants against predation by a wide variety of pests.

When questions concerning the exact mechanism of inhibition are better answered, engineering better inhibitors for the purpose of controlling pests in economically important crops becomes more feasible. The structure of the 1:2 complex of TI-II bound to subtilisin provides a structural framework for the design of multidomain inhibitors carrying different specificities within a single polypeptide.

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