

## STRUCTURAL ASPECTS OF STRONG INHIBITION AND ROLE OF SCAFFOLD FOR SERINE PROTEASE INHIBITORS

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**Abstract:** Canonical serine protease inhibitors inhibit their cognate enzymes by binding tightly at the enzyme active site in a substrate-like manner, being cleaved extremely slowly compared to a true substrate. They interact with cognate enzymes through P3-P2 region of the inhibitory loop while the scaffold hardly makes any contact. Neighbouring scaffolding residues like arginine or asparagine shape-up the inhibitory loop and religate the cleaved scissile bond. The specificity of the inhibitor can be altered by mutating the hyper solvent accessible P1 residue without changing loop-scaffold interactions. To understand the loop-scaffold compatibility, we prepared three chimeric proteins ECI<sup>L</sup>-WCI<sup>S</sup>, ETI<sup>L</sup>-WCI<sup>S</sup>, and STI<sup>L</sup>-WCI<sup>S</sup>, where the inhibitory loops of ECI, ETI, and STI were placed on the scaffold of their homologue WCI. Results showed that although ECI<sup>L</sup>-WCI<sup>S</sup> and STI<sup>L</sup>-WCI<sup>S</sup> behave like inhibitors, ETI<sup>L</sup>-WCI<sup>S</sup> behaves like a substrate. Crystal structure of ETI<sup>L</sup>-WCI<sup>S</sup> and its comparison with ETI indicated that three novel scaffolding residues Trp88, Arg74, and Tyr113 in ETI act as barrier to confine the inhibitory loop to canonical conformation. Absence of this barrier in the scaffold of WCI makes the inhibitory loop flexible in ETI<sup>L</sup>-WCI<sup>S</sup> leading to a loss of canonical conformation, explaining its substrate-like behaviour. Furthermore, complex structures of the inhibitors with their cognate enzymes indicate that rigidification of the inhibitory loop at the enzyme active site is necessary for efficient inhibition.

**Keywords:** Serine protease inhibitors; Loop and scaffold; Crystallography; Chimeric inhibitors; Inhibitory loop rigidification.

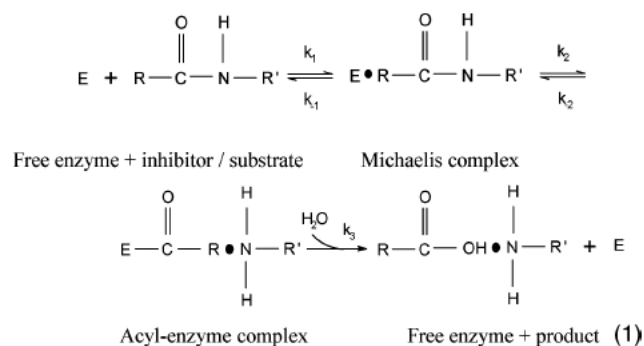
### 1. Canonical Serine Protease Inhibitors: An Overview

Naturally occurring protease inhibitors play key roles in multitude of physiological processes that include regulation of the proteolytic activity of their target proteases, blocking them in emergency cases, or for signalling receptor interactions or clearance. Except for few cases, endogenous inhibitors are mostly found to be proteins. Strong inhibition of an active protease offered by a protein appears to be a paradox. Nevertheless, protein inhibitors of proteolytic enzymes comprise the largest and structurally most diverse group of naturally occurring enzyme inhibitors. Kinetic and thermodynamic parameters obtained from these inhibitors with

their cognate enzyme and the nature of the enzyme-inhibitor complexes are surprisingly diversified. A comprehensive list of 48 inhibitor families has been published in MEROPS (Rawlings *et al.*, 2004) and is available at <http://merops.sanger.ac.uk>. The inhibitors are usually active toward a particular mechanistic class of proteases like serine, cysteine, aspartic or metalloproteases. Among these, number of serine proteases inhibitors isolated and identified so far is extremely large and most intensively studied. The serine protease inhibitors can broadly be divided into three distinctly different types based on the mechanism of inhibition of their target proteases. These are (i) serpins, (ii) non-canonical inhibitors and (iii) standard mechanism canonical inhibitors. The canonical inhibitors are the most prevalent and the most extensively studied example of the three classes of serine protease

inhibitors and will be discussed here. Several classic review papers are available in literature where they introduced the canonical inhibitors, provided a rational nomenclature by grouping them and gave an in depth description of mechanism of inhibition (**Table 1**). The members of one family can be as small as 1.5 kDa (12-14 residues) (Luckett *et al.*, 1999) to as large as 120 kDa having 15 inhibitory domains (Magert *et al.*, 1999). The speciality of the protein inhibitors of serine proteases is that despite the fact that they themselves are proteins they can inhibit their cognate enzymes by binding tightly at the active site of the enzyme in a substrate-like manner (Laskowski, and Kato, 1980; Bode and Huber, 1992). Interestingly, they undergo cleavage extremely slowly (Radisky and Koshland, 2002) compared to an ideal substrate.

The consensus model of serine protease-substrate/inhibitor reaction consists of the chemical steps given in equation 1:



The enzyme is represented by E and the N and C terminal fragments, produced after the cleavage of the scissile peptide bond of the inhibitor, are denoted by R and R' respectively.

The main difference between an inhibitor and a substrate is that in case of the inhibitors, the reaction does not proceed beyond the acyl enzyme intermediate formation; rather the backward reaction of the above relation is favoured. Therefore, to be an efficient inhibitor, the newly formed terminals of the cleaved inhibitor should essentially be maintained in close proximity to resynthesize/reigate the scissile bond.

Here we further review serine protease inhibitors taking winged bean chymotrypsin

inhibitor belonging to the Kunitz (STI) family as a model system. Apart from the brief general description, to make the readers familiar with the common jargons, the focus will be on the structural aspects of strong inhibition and role of 'scaffold' in maintaining the inhibitory loop conformation and inhibitory potency.

## 2. Loop and Scaffold of Inhibitors

The serine protease inhibitors can be grouped into at least 20 convergently evolved families (Laskowski, and Kato, 1980) based on their amino acid sequence similarities, topological equivalence of disulphide bond and global fold (**Table 1**). Partial list of these inhibitors is given in tabular form in different reviews but a complete list is available in MEROPS (Rawlings *et al.*, 2004). So far as their structures are concerned they are quite different from each other – having a compact, nearly globular fold (hereafter termed as 'Scaffold') and a convex, solvent exposed, protruding loop emerging from the scaffold that interact with the protease and is called inhibitory loop. During interaction with the protease one of the peptide bond of the inhibitory loop is cleaved which is called the scissile peptide bond. **Fig. 1** shows overall folding of the inhibitor scaffold and their inhibitory loop for some of the representative members.

The scaffold of the inhibitor, excluding the inhibitory loop, is composed of a central hydrophobic core, stabilized by secondary structural elements. The extensive intra-molecular interactions make the above-mentioned folds rigid, stable and highly resistance to proteolysis implying that the framework is useful in supporting the inhibitory epitopes.

## 3. Canonical Conformation of the Inhibitory Loop

The canonical serine protease inhibitors comprise of at least 20 convergently evolved families (Laskowski and Qasim, 2000) that display a strikingly similar backbone conformation around the scissile peptide bond (Bode and Huber, 1992), although the scaffold of these inhibitors differ widely in their folds. The inhibitory loop of the canonical inhibitors is bulged out from the scaffold and is the conserved structural epitope

Table 1  
Families of Serine Protease Inhibitors

Family	Nearest preceding Cys from P1	Nearest following Cys from P1	Three dimensional structure
Antistatin <sup>b</sup>	P <sub>2</sub>	P <sub>3</sub> '	Yes
Arrowhead <sup>c</sup>			
Site1	P <sub>2</sub>	P <sub>45</sub> '	No
Site2		P <sub>13</sub> '	
Ascaris <sup>b</sup>	P <sub>3</sub>	P <sub>2</sub> '	Yes
BB1 <sup>c</sup>	P <sub>3</sub>	P <sub>6</sub> '	Yes
Chelonianin <sup>b</sup>	P <sub>3</sub>	P <sub>6</sub> '	Yes
Ecotin <sup>d</sup>	P <sub>35</sub>	P <sub>3</sub> '	Yes
Grasshopper <sup>b</sup>	P <sub>3</sub>	P <sub>3</sub> '	Yes
Kazal <sup>b</sup>	P <sub>3</sub>	P <sub>6</sub> '	Yes
Kunitz (BPTI) <sup>b</sup>	P <sub>2</sub>	P <sub>15</sub> '	Yes
Kunitz (STI) <sup>c</sup>	P <sub>25</sub>	P <sub>33</sub> '	Yes
Marinostatin <sup>d</sup>			No
Potato I <sup>c</sup>			
Eglin C			Yes
CMII-V	P <sub>42</sub>	P <sub>4</sub> '	
Potato II <sup>c</sup>	P <sub>3</sub>	P <sub>2</sub> '	
Cereal <sup>c</sup>	P <sub>6</sub>	P <sub>10</sub> '	Yes
Rapeseed <sup>c</sup>	P <sub>4</sub>	P <sub>6</sub> '	
Silkworm <sup>b</sup>			
FPI-F	P <sub>3</sub>	P <sub>6</sub> '	
SSI <sup>d</sup>	P <sub>3</sub>	P <sub>28</sub> '	Yes
Squash <sup>c</sup>	P <sub>3</sub>	P <sub>5</sub> '	Yes

Other protein inhibitors of serine proteases:  
Hirudin family<sup>b</sup>  
Serpins family<sup>b</sup>  
Streptomyces proteinase group peptide family<sup>d</sup>  
Subtilisin type propeptide family<sup>d</sup>  
TAP family<sup>b</sup>

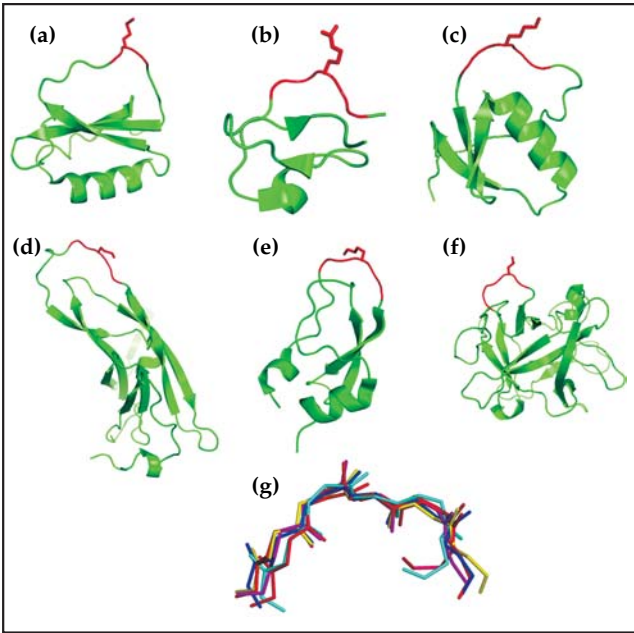


Figure 1: Cartoon representation of some representative family members of serine protease inhibitors (a) Potato inhibitor I (b) Squash seed inhibitor (c) Kazal family (d) Bowman Birk family (e) Kunitz (BPTI) family (f) Kunitz (STI) family. The scaffold is shown in green and the inhibitory loop in red. (g) Superposition of inhibitory loop backbone

with a specific backbone conformation. This convex, protease binding reactive site loop of these inhibitors (irrespective of the families they

belong to) are highly complementary to the active site of the proteases and share a common characteristic conformation around the P3-P3' region either in free inhibitor or in enzyme: inhibitor complex state. In the available crystal structures of free inhibitors and protease-inhibitor complexes, it has been observed that the main-chain conformation of this functional motif resembles that of an ideal substrate (Krowarsch *et al.*, 2003). This characteristic conformation is known as the 'canonical conformation' and these types of inhibitors are termed to represent this specific conformation (Laskowski & Kato, 1980; Bode & Huber, 1992; Jackson & Russell, 2000).

The central position of the reactive site loop is occupied by the solvent exposed scissile peptide bond (P1-P1'), which is hydrolyzed by the protease during complex formation (Fig. 2). Of the residues that come into contact with the protease, the P1 residue plays the major role in the protease-inhibitor association and act as the specificity determinant residue.

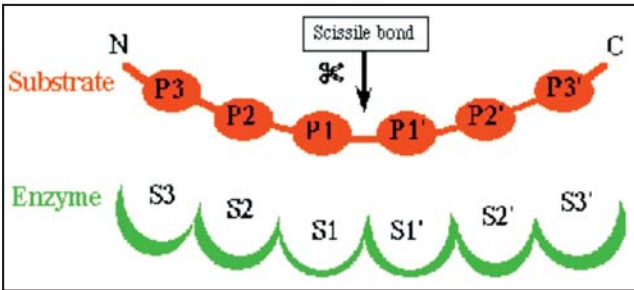


Figure 2: Schechter and Berger (Schechter and Berger, 1967) introduced a universal nomenclature for the protease-protease inhibitor/substrate interactions. According to this convention, the residues of the substrate/inhibitor to the N-terminus of the scissile peptide bond are numbered P1, P2, P3, etc. (the 'non-prime side') whereas those C-terminal to the scissile bond are numbered P1', P2', P3' etc. (the 'prime side'). The equivalent residues at the protease side are numbered as S1, S2, S3, etc. at the N-terminus and S1', S2', S3', etc. at the C-terminus

#### 4. Changing Specificity Through P1 Mutation

The inhibitory loops of serine protease inhibitors have a characteristic conformation (Laskowski & Kato, 1980; Bode & Huber, 1992; Jackson & Russell, 2000), around the P3-P3' (Schechter and Berger, 1967) segment, irrespective of the family they belong to, while the remaining part of the molecule, known as scaffold, has widely different folds in different families of inhibitors. The amino

acid sequence of the inhibitory loop shows high degree of variability except at the P1 recognition site (Laskowski & Kato, 1980) and yet the conformation of the loop is strikingly similar.

In complexes of chymotrypsin-like serine proteinases, it has been observed that P1 side chain makes most of the interactions with the S1 specificity pocket and these interactions are energetically most important. They are therefore the primary determinants of specificity of a given inhibitor for a particular proteinase (e. g., Arg and Lys residues at P1 confer trypsin-like specificity; Leu, Met, Phe at P1 confer chymotrypsin-like specificity). Therefore, substitution of the P1 residue particularly influences specificity towards cognate proteinases.

Change in specificity, caused by the mutations at P1 site, of the serine protease inhibitors of different families is reported in the literature (Helland *et al.*, 1999; Heinz *et al.*, 1992; Wang *et al.*, 2001). A single mutation (Leu65→Arg) at P1 converted WCI, a specific inhibitor of chymotrypsin, to a strong inhibitor of trypsin (having no chymotrypsin inhibitory activity). L65R inhibits trypsin with an association constant of  $4.8 \times 10^{10} \text{ M}^{-1}$  that is comparable to other potent trypsin inhibitors of the family. The crystal structure ( $2.15 \text{ \AA}$ ) of this mutant (L65R) showed that its reactive site loop conformation deviates from that of WCI and adopts a structure similar to that of *Erythrina caffra* trypsin inhibitor belonging to the same family (Fig. 3). A critical analysis of the amino acid sequence of the reactive site loop (P3–P3') of WCI, ETI and STI shows that except at P1, the residue type of ETI resembles WCI, whereas those of STI differ significantly from WCI at P3, P1, P1' and P2' positions (Khamrui *et al.*, 2005). Among these differences, Pro (with a fixed  $\phi/\psi$  value) at P3 and Ile at P1' are conserved for the inhibitors, belonging to the branch of STI whereas the inhibitors belonging to ETI type possess Arg/Gln at P3 and Ser at P1'. Leu→Arg mutation at P1 of WCI abolishes the only difference in residue type between WCI and ETI but the difference between mutant L65R and STI still remains in three other positions which may be considered as a reason for the conformational conversion of L65R towards ETI, but not STI.

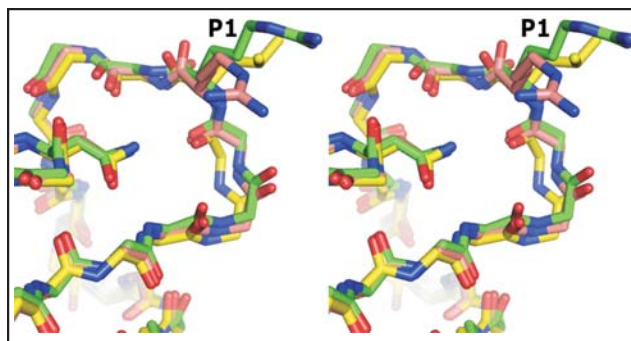


Figure 3: The stereoscopic presentation of the superposition of the scaffolding residue Asn14 along with the reactive site loop (P4–P2') of WCI (Yellow) and ETI (Green) on that of L65R (Salmon)

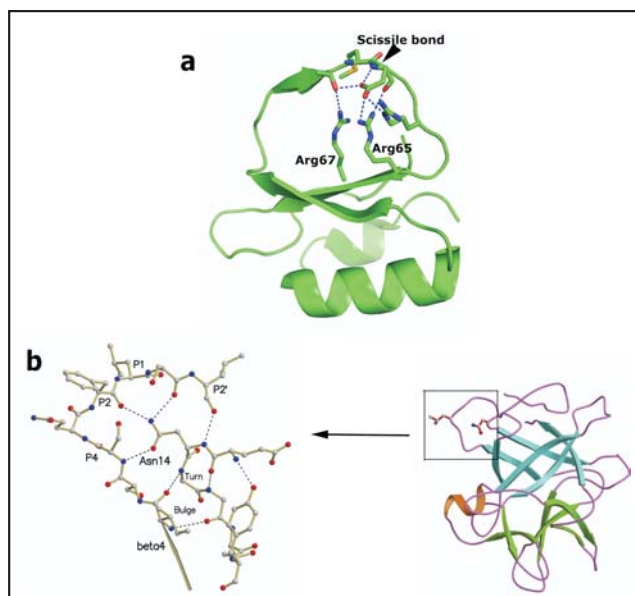
## 5. Origin of Strong Inhibition and Relegation of Scissile Peptide bond

What are the structural elements that make the serine protease inhibitors to offer strong inhibition compared to a substrate (see Equation 1)? Reasons postulated for the inhibitors' poor reactivity include (i) extreme rigidity of the complex preventing productive nucleophilic attack, (ii) poor orientation of the reacting groups resulting formation of nonproductive complex, and (iii) positioning of the leaving group H<sub>2</sub>N-R<sub>2</sub> in the acyl–enzyme complex favors the back reaction toward the Michaelis complex. Incubation of CI2 with subtilisin showed that an equilibrium between the Michaelis complex and the acyl–enzyme is quickly established. The rapidity with which equilibrium is reached indicates the absence of a large energy barrier to acylation and disproves the hypotheses that either rigidity or poor orientation prevents productive nucleophilic attack.

Then, the obvious question is, why does deacylation proceed so slowly? Fersht and coworkers (Longstaff *et al.*, 1990; Shaw *et al.*, 1995) suggested that the leaving group amine is poised for nucleophilic attack on the acyl–enzyme, resulting in the back reaction illustrated by  $k_{-2}$  in Equation 1. So what are the structural elements present in the inhibitors that favored the back reaction?

In CI2 of the potato inhibitor-1 family, Arg65 and Arg67 extend in a parallel fashion from the protein scaffold to form hydrogen bonds with the binding loop (Radisky and Koshland, 2002) and act as a spacer (Fig. 4). Radisky *et al.* showed that





**Figure 4:** (a) Hydrogen bonding network by Arg65 and Arg67 in CI2; (b) Interactions of scaffolding residue Asn14 with the inhibitory loop in WCI

mutations of these arginines and a few loop residues that interact with Arg65 and Arg67 render the binding loop less resistant toward proteolysis, and from this study they proposed a role for these parallel spacer arginines as well as for other scaffolding residues in keeping the leaving group in place that prevents hydrolysis and favors relegation (Radisky *et al.*, 2003, 2004, 2005). However, they correctly pointed out that the pervasiveness of their proposed mechanism needs to be experimentally explored with enzymes and inhibitors beyond subtilisin BPN and CI2 (Radisky *et al.*, 2005). It is also noted that out of 18 serine protease inhibitor families, parallel spacer arginines are present only in the PI-1 family. Hence, to propose a more generalized mechanism of inhibition for serine protease inhibitors, it is desirable to extend this investigation to other families of inhibitors.

The inhibitors of the Kunitz family have a characteristic b-trefoil fold, and they mostly inhibit chymotrypsin clan enzymes. Structural studies on Kunitz (STI) inhibitors revealed that a conserved scaffolding Asn14, through a network of hydrogen bonds (Fig. 4), maintains the conformation of the reactive site loop (Onesti *et al.*, 1991; Meester *et al.*, 1998; Dattagupta *et al.*, 1999). We investigated the specific role of this spacer Asn in the prevention of proteolysis

through structural and biochemical studies on the mutants where Asn14 of WCI was replaced by Gly, Ala, Thr, Leu, and Gln (Dasgupta *et al.*, 2006). Structural and biochemical studies on the mutants indicate that a residue having no side chain or b-branching at the 14th position creates deformation and insufficient protrusion of the binding loop, and as a result N14G and N14T lose the ability to recognize proteases. Although the reactive site loop conformation of N14A and N14Q are almost identical to WCI, results of residual enzymatic activity and limited proteolysis present N14A as a substrate, indicating that the methyl group of Ala14 is not suitable to capture the cleaved parts together for relegation (Fig. 5a, b). The poor inhibitory power of N14L points toward the chemical incompatibility of Leu at the 14th position, although its size is the same as Asn; on the other hand, slight loss of inhibitory potency of N14Q is attributed to the inappropriate placement of the Gln14 polar head, caused by the strained accommodation of its bigger side chain (Fig. 5a, b). These observations collectively allow us to conclude that the side chain of spacer Asn fits snugly into the concave space of the reactive site loop cavity and its ND2 atom forms hydrogen bonds with the P2 and P1 carbonyl O at either side of the scissile bond holding the cleaved products together for relegation (Fig. 5c).

**Table 2**  
The Sequences of the Reactive Sites for WCI, ETI and STI

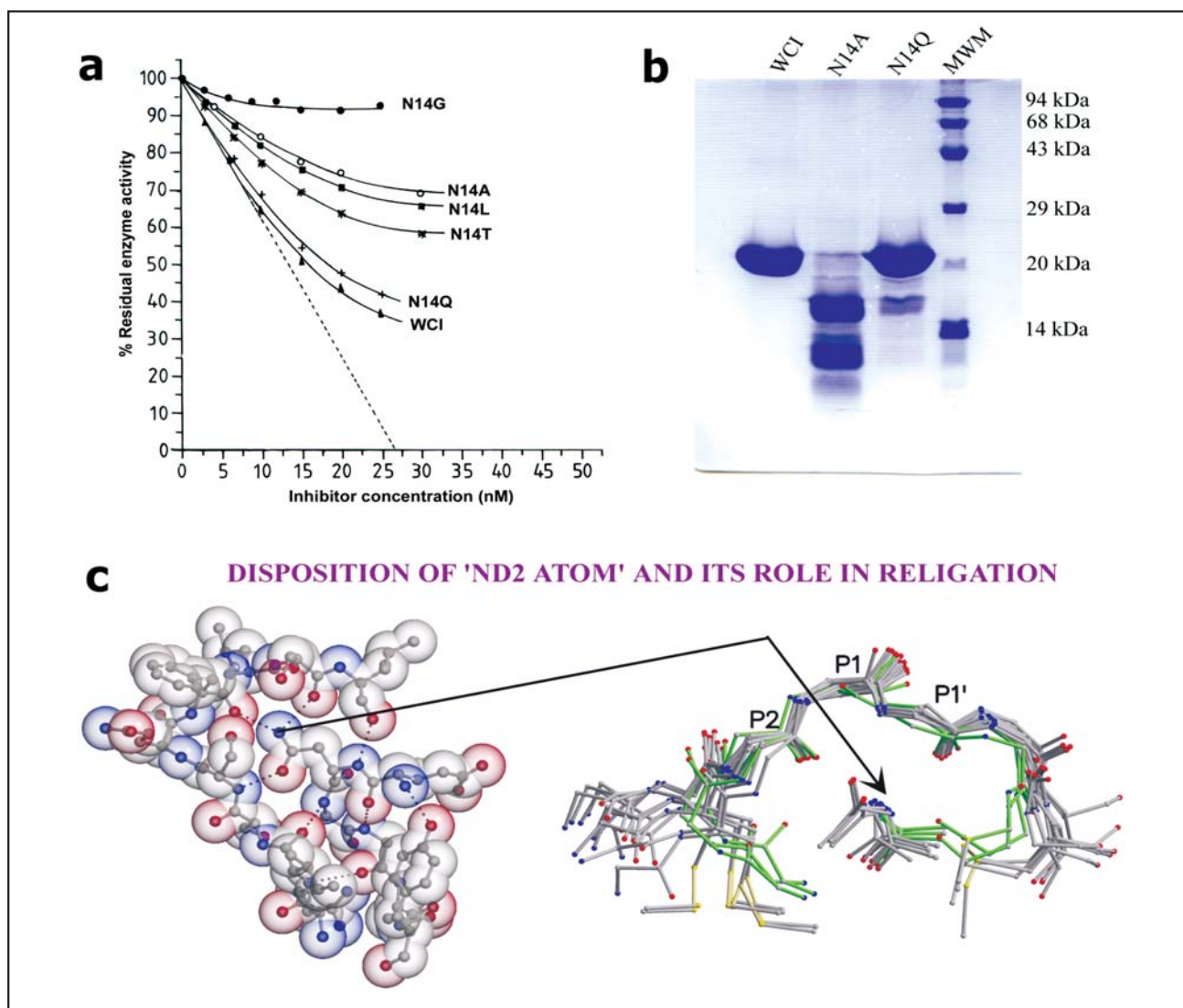
	P3	P2	P1	P1'	P2'	P3'
WCI	Q	F	L	S	L	F
ETI	R	L	R	S	A	F
STI	P	Y	R	I	R	F

Through a database analysis, we identified such spacer Asn necessary for relegation of the scissile peptide bond in five other families of SPIs, that is, Kazal, SSI, Ecotin, Potato inhibitor-2, and Grasshopper. The inhibitors of these families, listed in Table 3 widely differ in their size (ranging from 30 to 200 amino acids, approximately), sequence, overall folding and disulfide bridge pattern. However, when the inhibitory loops of these inhibitors were superposed, based only on their P2 and P1' carbonyl groups, we see that although the side

chain of Asn of different inhibitors approaches from different directions, the ND2 atoms of these Asn residues cluster tightly at a particular position so that it can form hydrogen bonds with P2 and P1' carbonyl O (Fig. 5c). The conserved nature of these Asn residues, their space availability and strikingly similar disposition of their ND2 atoms around scissile bond (as in WCI) lead us to speculate that in all these five families of inhibitors the spacer Asn plays a similar role in the relegation mechanism.

## 6. Loop Scaffold Compatibility: Tale of Three Chimeric Proteins

Spacer Asn or Arg of SPIs relegates cleaved scissile peptide bond to offer strong inhibition. However, several designed 'mini-proteins' having 'trimmed scaffold' with spacers and the inhibitory loop behave like substrate. A cyclic peptide, analogous to the binding loop of CI2 (Fig. 6a) having the Arg65 and Arg67 for relegation, was found to be a poor inhibitor, suggesting the role of scaffold beyond spacers.



**Figure 5:** (a) Inhibition of esterase activity of chymotrypsin by WCI and its mutants were measured. Chymotrypsin (50 nM) was incubated at 25 °C for (a) 3 min and (b) 15 min with varying amounts (2-30 nM) of inhibitors and assayed for the residual enzyme activity at A256, taking 200 nM BTEE as substrate. (b) 15% SDS-PAGE showing the proteolysis products of WCI, N14A, and N14Q by chymotrypsin in 100:1 mixtures of inhibitor and enzyme, incubated for 6 h at 25 °C. (c) Reactive site loop of WCI and Asn14 shown in space filling model (left side) and Superposition of the reactive site loops (P4-P3c) and spacer asparagines of fourteen inhibitors, belonging to different families of serine protease inhibitors are represented stereoscopically. The inhibitors of Kunitz (STI) family, i.e., 1EYL, 1TIE and 1AVW are shown in different stick color (green) from the rest of the inhibitors (right side). [Adopted from Dasgupta *et al.*, 2006]

Table 3  
Families of Serine Protease Inhibitors having Spacer ASN

family name/ MEROP ID (34)	coordinates taken	P1–P1'	Asn position	nearest preceding Cys from P1	nearest following Cys from P1	ref
Kunitz (STI)/13	1EYL	65–66	14	P25	P23'	13
	1TIE	63–64	12			10
	1AVW	63–64	13			6
Kazal/11	2OVO	18–19	33	P3	P6'	26
	1HPT	18–19	33			27
	1YU6	18–19	33			28
	1TBR	10–11	25			29
ecotin/111	1IFG	84–85	51	P35	P3'	30
SSI/116	2SIC	73–74	99	P3	P28'	31
grasshopper/119	1GL1	30–31	15	P3	P3'	32
	1YR4	29–30	15			33
potato II/120	4SGB	38–39	5	P3	P2'	34
	1PJU	62–63	29			35

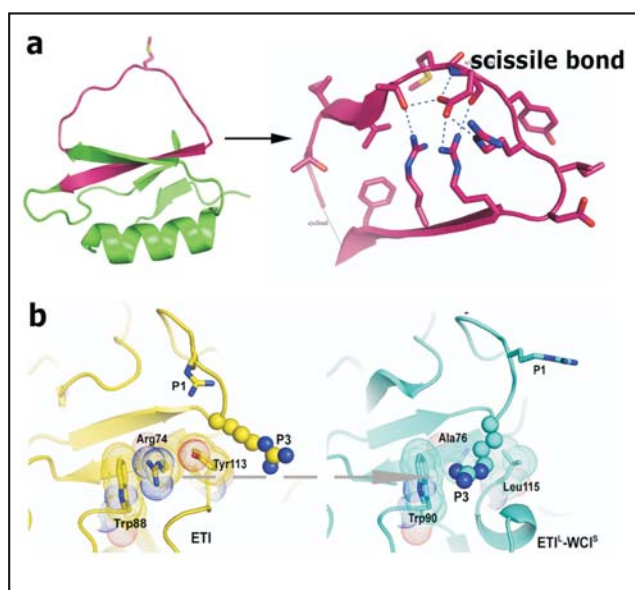


Figure 6: (a) Cartoon diagram of CI2 (green and magenta) and a cyclic peptide, analogous to the binding loop of CI2, having the Arg65 and Arg67 for relegation (b) Orientation of P3 Arg in the presence of scaffolding barrier in ETI (yellow), and the movement of P3 Arg in the absence of such a scaffolding barrier in ETI<sup>L</sup>-WCI<sup>S</sup> (cyan). Arrow indicates the loss of barrier from ETI to ETI<sup>L</sup>-WCI<sup>S</sup>

Chimeric inhibitors having the reactive site loop of one inhibitor on the scaffold of the other could be a good model to understand the extent of loop-scaffold compatibility and hence the role of the scaffold in the inhibitory process. As different families of inhibitors have different folds, it would be logical to restrict this loop-scaffold swapping experiment within one family of inhibitors. We, therefore, took four representative members of Kunitz (STI) family, two of which are chymotrypsin inhibitors, namely, WCI (Dattagupta *et al.*, 1996) and

*Erythrina variegata* chymotrypsin inhibitor (Iwanaga *et al.*, 1999) and other two are trypsin inhibitors, ETI (Onesti *et al.*, 1991) and STI (Song and Suh, 1998). All of them possess common fold of scaffolds including a conserved Asn, required for relegation.

We prepared three chimeric proteins ECI<sup>L</sup>-WCI<sup>S</sup>, ETI<sup>L</sup>-WCI<sup>S</sup>, and STI<sup>L</sup>-WCI<sup>S</sup>, where the inhibitory loop (L) of ECI, ETI, and STI is placed on the scaffold (S) of their homolog WCI. Results show that although ECI<sup>L</sup>-WCI<sup>S</sup> and STI<sup>L</sup>-WCI<sup>S</sup> behave like good inhibitors, ETI<sup>L</sup>-WCI<sup>S</sup> behaves like a substrate. That means a set of loop residues (SRLRSAFI), offering strong trypsin inhibition in ETI, act as a substrate when they seat on the scaffold of WCI. Crystal structure of ETI<sup>L</sup>-WCI<sup>S</sup> shows that the inhibitory loop is of noncanonical conformation. We identified three novel scaffolding residues Trp88, Arg74, and Tyr113 in ETI that act as barrier to confine the inhibitory loop to canonical conformation (Fig. 6b). Absence of this barrier in the scaffold of WCI makes the inhibitory loop flexible in ETI<sup>L</sup>-WCI<sup>S</sup> leading to a loss of canonical conformation, explaining its substrate-like behavior. Incorporation of this barrier back in ETI<sup>L</sup>-WCI<sup>S</sup> through mutations increases its inhibitory power, supporting our proposition (Khamrui *et al.*, 2010).

## 7. Crystal Structure of Inhibitor/protease Complex: Rigidification of Inhibitory Loop

A common structural theme of the serine protease inhibition is that these inhibitors bind to the target proteases in a substrate like manner and forms a stable complex. The  $k_{cat}/K_M$  index for the reactive



site peptide bond hydrolysis lies in the range of  $10^6 \text{ M}^{-1}\text{S}^{-1}$ , which suggests that the inhibitors are good substrates (Buczek *et al.*, 2002), yet they undergo a very slow hydrolysis (by a factor of  $10^6$ - $10^{10}$ ) (Radisky & Khoshland, 2002). Crystal structures of canonical serine protease inhibitors in complex with their cognate enzyme shows that P3-P2 region of the inhibitory loop makes majority of the interactions with protease while the rest of the inhibitor hardly makes any contact as observed in L65R:trypsin complex (**Fig. 7a**). Side chain of P1 residue is the specificity determinant and interacts with the S1 subsite of the enzyme while the main-chain of P3-P2 segment makes more-or-less defined interactions at the active site. In case of trypsin inhibitor, the P1 Arg residue interacts with Asp189 of the enzyme and makes salt bridge interaction (**Fig. 7b**) while the hydrophobic residue in chymotrypsin inhibitors interacts with the wall of the active site through Met192 of the enzyme. The P3-P1 segment of the inhibitor forms a short anti parallel  $\beta$ -sheet with the 214-216 residues of the chymotrypsin like enzymes. The catalytic Ser195 OG makes 'sub-van-der-Waal' contact

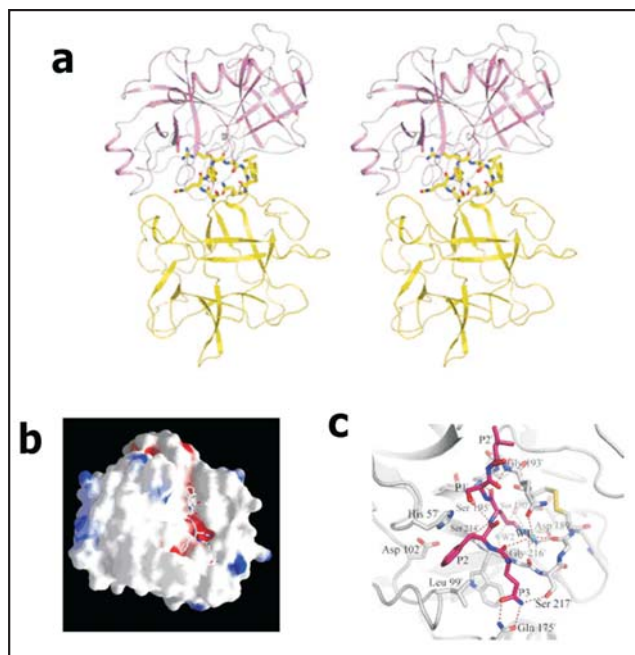
with the carbonyl 'C' of P1 indicating that it is poised to make a nucleophilic attack. The scissile carbonyl O points towards the oxyanion hole, formed by the main chain amides of Gly193 and Ser195 (**Fig. 7c**), and get stabilized by forming hydrogen bonds with the aforesaid residues. Extensive hydrophilic interactions at the enzyme inhibitor interface reduces the thermal motion of the inhibitory loop as reflected by the dramatic reduction of temperature factor of the inhibitory loop when compared with the free inhibitor and complexed inhibitor.

### Abbreviations

WCI, winged bean chymotrypsin inhibitor; STI, soybean trypsin inhibitor; ETI, *Erythrina caffra* trypsin inhibitor; CI2, chymotrypsin inhibitor-2; PI-1, potato inhibitor-1; SPI, serine protease inhibitor; ECI, *Erythrina variegata* chymotrypsin inhibitor.

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**Figure 7:** (a) Stereoscopic representation of the L65R:BPT complex. The L65R molecule is shown in yellow and the trypsin molecule in magenta. The reactive site loop of L65R is represented as stick (b) surface representation of BPT and the P3-P2' region (as stick) of the inhibitor (L65R) bound to it (c) The interactions made by the reactive site loop (P3-P3') residues of LR with trypsin in L65R:BPT



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