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Review

Complementation of intramolecular interactions for structural–functional stability of plant serine proteinase inhibitors

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ABSTRACT

Background: Plant protease inhibitors (PIs) constitute a diverse group of proteins capable of inhibiting proteases. Among PIs, serine PIs (SPIs) display stability and conformational restrictions of the reactive site loop by virtue of their compact size, and by the presence of disulfide bonds, hydrogen bonds, and other weak interactions.
Scope of review: The significance of various intramolecular interactions contributing to protein folding mechanism and their role in overall stability and activity of SPIs is discussed here. Furthermore, we have reviewed the effect of variation or manipulation of these interactions on the activity/stability of SPIs.
Major conclusions: The selective gain or loss of disulfide bond(s) in SPIs can be associated with their functional differentiation, which is likely to be compensated by non-covalent interactions (hydrogen bonding or electrostatic interactions). Thus, these intramolecular interactions are collectively responsible for the functional activity of SPIs, through the maintenance of scaffold framework, conformational rigidity and shape complementarities of reactive site loop.
General significance: Structural insight of these interactions will provide an in-depth understanding of kinetic and thermodynamic parameters involved in the folding and stability mechanisms of SPIs. These features can be explored for engineering canonical SPIs for optimizing their overall stability and functionality for various applications.

1. Introduction

A protease inhibitor (PI) is a molecule that inhibits the proteolytic activity of a protease by binding and thereby blocking its active site. Plant PIs have been considered as defense molecules that protect plants from the attack of pests and pathogens by an antibiosis mechanism, which are also constituent of a storage protein in seeds and tubers [1,2]. Due to their small size, PIs have evolved and developed the various inhibitory mechanisms in response to a wide range of proteases and have been generally classified into families, such as serine, cysteine, aspartic and metallo PIs based on target protease(s) [3–6]. They have been reported to have co-evolved with insect gut proteases [4]. In this review our focus is on the plant serine PIs (SPIs), especially disulfide bond modification and other complementary intramolecular interactions involved in folding and reaction site loop (RSL) stability, which might have facilitated co-evolution of inhibitors with the target insect gut proteases.

The functional characterization of SPIs present in many plant families has been discussed extensively in the literature. SPIs are further classified into different types, for example Kunitz-type (KSTI, MI: I03), Bowman-Birk (BBI; MI: I12), potato type I and II (Pin-I, MI: I13 and Pin-II, MI: I20), and squash inhibitors (MI: I55) [2,5,7]. The reactive site loop of SPIs has a common characteristic conformation irrespective of the type, while the remaining part of the molecule, known as the scaffold, has different folds in various inhibitor types [8]. SPIs are tight binding inhibitors with competitive and reversible interactions. Several reports on crystallographic and NMR structures of SPIs in free and protease bound form have illustrated the characteristic features of standard mechanism of inhibition [9–12]. The tight binding of SPIs with the enzyme (low K_m) is achieved through maintaining both stable conformation of RSL, which protrudes from the protein scaffold, and structural complementarities to the substrate-binding site of proteases, thus leading to very slow hydrolysis (low K_{cat}) [2,8,13]. The P_1 residue of the RSL makes contacts with the S_1 pocket of proteases by forming scissile bond, which is thought to be involved in determining the inhibitory specificity [8–12]. Furthermore, additional interactions such as hydrogen bonding by residues that surround the scissile peptide bond and van der Waals contacts at the interface results in tight binding and slow hydrolysis of SPIs. The strength of the protease–PI interaction is determined by the compatibility of all the interfacial amino acid residues (P_4–P_4'); these residues also direct the RSL of inhibitor towards the active site of proteases [13,15]. The disulfide bonds restrict any distortion of the P_1–P_1' peptide bond and together these interactions increase the activation-energy barrier for hydrolysis [8,11,13–15].

Abbreviations: SPI, Serine proteinase inhibitor; PI, Proteinase inhibitor; RSL, Reactive site loop; MI, MEROPS Database ID

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SPIs follow a two-state model of protein folding, where the SPI can occupy only one of the two states: the unfolded or the folded [16]. In general SPI folding is dominated by tradeoff between loss of configurational entropy (thermodynamically unfavorable) and gain in attractive (thermodynamically favorable) interactions [16]. Furthermore, most of the SPI types are disulfide rich and follow the oxidative folding pathway [17], while the remaining ones follow either a folding code (folding according to an amino acid sequence and intrinsic properties of protein) or intermediary folding reactions to attain a metastable, native conformation [18].

SPIs have phenomenal structural stability in different environments and conditions due to their compact size, the cyclization of the N- and C-termini, and the high density of the intramolecular disulfide and hydrogen bonds [15]. The contribution to structural stability of SPIs is from hydrophobic interactions in the core of the protein and owing to charged amino acids occupying solvent-exposed surface. Stability also arises from the formation of intramolecular hydrogen bonds by hydrophilic and charged side chains [19,20]. However, the strength of H-bonds depends on their environment; H-bonds enveloped in a hydrophobic core contribute more to the SPI stability than do surface-exposed H-bonds [20,21].

Here, the importance and interplay of various intramolecular interactions in SPIs' stability/activity are reviewed. The factors that account for the underlying differences in the folding mechanisms, functional implications and evolution of SPI variants, are also addressed. In addition, we discuss recent application oriented developments in the field; such as the mechanisms for engineering canonical SPIs in order to optimize protease–PI contacts during interaction.

2. Oxidative folding and the indispensability of spatial disulfide bonds

Intramolecular disulfide bonds (S–S) are important for the proper folding of SPIs [22,23]. The process that is responsible for the formation of disulfide bonds between the cysteine residues in proteins due to redox reaction is called oxidative folding (Fig. 1). It consists of oxidation, reduction, and isomerization [17]. In general and especially in SPI this process is affected by many factors such as the concentration of thiolate ions; the proximity, reactivity, and accessibility of thiol groups; and the existence of disulfide bonds. The proximity of two reactive thiol groups is determined by the thermodynamic interaction of the loop, whereas the reactivity of the thiol group is affected largely by the local electrostatic environment [16,17]. Although, the disulfide bonds are considered as chemically inert, but recent findings show how deleting or rearranging disulfide bond affects the functionality of BBI and Pin-II type inhibitors, an indication of their indispensability [16].

A decrease in entropy by the formation of disulfide bonds favors the folded state over the unfolded state; the folded state helps to stabilize the native conformation of SPI. The disulfide bonds protect the protein from cellular damage and maintain its integrity [24]. SPIs display the presence of both highly conserved cysteine residues at specific positions and a conserved disulfide-bonding pattern (Fig. 2a–d). All SPI types have a characteristic protein fold and conserved disulfide bonds as listed in Table 1. The Kunitz-type inhibitor contains a three-fold β-trefoil, accompanied by twelve anti-parallel β-strands connected with thirteen relatively long loops with RSLs in between β-strands 4 and 5 [22,25]. However, the RSLs are not always location specific. Some Kunitz-type inhibitors such as the arrowhead protease inhibitor from *Sagittariasa*

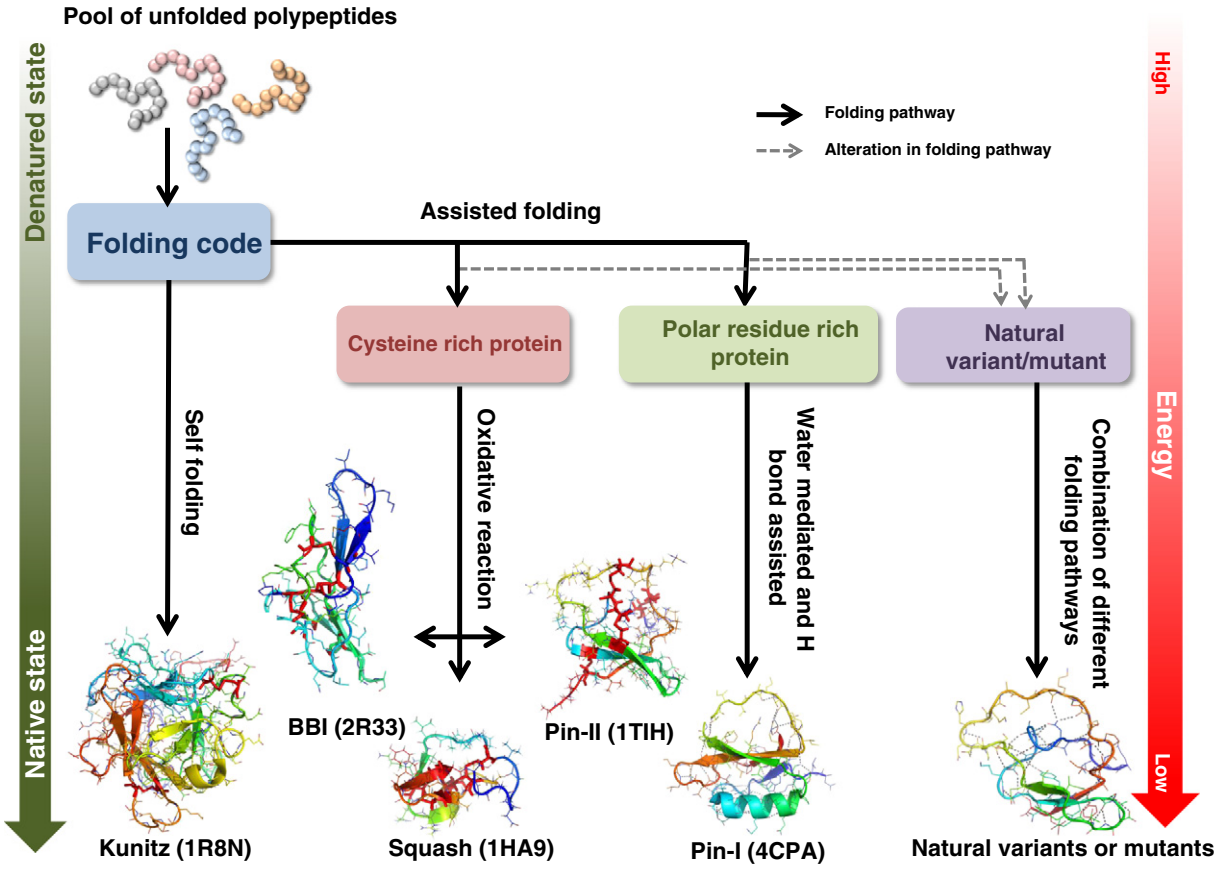

Fig. 1. General scheme and competing reactions of SPIs folding. General scheme of SPIs explore as they move towards the native state by following self or assisted folding. The free-energy of molecule follows the favorable downward path. Various SPI molecules fold in either of pathway depending on their composition. In self-folding pathway of Kunitz-type inhibitors reached free energy minima by following folding code. BBI, Pin-II and squash inhibitors are cysteine rich proteins thus they follow oxidative reaction assisted folding (formation of disulfide bond). On contrary, Pin-I inhibitors, rich in polar residues are more likely to use hydrogen bonding and water mediated assistance for protein folding. Variation or manipulations in this assisted folding pathway might result in emergence of compensatory folding mechanism.