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Minireview

Structural Features and Molecular Evolution of Bowman-Birk Protease Inhibitors and Their Potential Application

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Abstract The Bowman-Birk inhibitors (BBIs) are well-studied serine protease inhibitors that are abundant in dicotyledonous and monocotyledonous plants. BBIs from dicots usually have a molecular weight of 8k and are double-headed with two reactive sites, whereas those from monocots can be divided into two classes, one approximately 8 kDa in size with one reactive site (another reactive site was lost) and the other approximately 16 kDa in size with two reactive sites. The reactive site is located at unique exposed surfaces formed by a disulfide-linked β-sheet loop that is highly conserved, rigid and mostly composed of nine residues. The structural features and molecular evolution of inhibitors are described, focusing on the conserved disulfide bridges. The sunflower trypsin inhibitor-1 (SFTI-1), with 14 amino acid residues, is a recently discovered bicyclic inhibitor, and is the most small and potent naturally occurring Bowman-Birk inhibitor. Recently, BBIs have become a hot topic because of their potential applications. BBIs are now used for defense against pathogens and insects in transgenic plants, which has advantages over using toxic and polluting insecticides. BBIs could also be applied in the prevention of cancer, Dengue fever, and inflammatory and allergic disorders, because of their inhibitory activity with respect to the serine proteases that play a pivotal role in the development and pathogenesis of these diseases. The canonical nine-residue loop of BBIs/STFI-1 provides an ideal template for drug design of specific inhibitors to target their respective proteases.

Key words Bowman-Birk protease inhibitor; sunflower trypsin inhibitor-1 (SFTI-1); molecular evolution; drug design

Proteases and their specific inhibitors are ubiquitously distributed in the animal, plant and microorganism kingdoms, and play key regulatory roles in many biological processes, including the blood coagulation system, the complement cascade, apoptosis and the hormone processing pathways [1]. Naturally occurring protease inhibitors are essential for regulating the activity of their corresponding proteases within these pathways. Plants contain a variety of serine protease inhibitors, which can be divided into at least 12 families [2,3]. One well-studied family of plant serine protease inhibitors, the Bowman-Birk inhibitors (BBI), are named after the workers who first isolated [4] and characterized [5] a member of this family from soybean—now perhaps the most well studied member of the BBI family, and often referred to as a "classical BBI". More BBI proteins were later found in other leguminous plants [6,7] and in the Poaceae [8]. Until now, all identified BBIs have been found only in Fabaceae and Poaceae. Their synthesis may be induced by infection or wounding, particularly in vegetative tissues [3], suggesting that they play a role in plant defense, conferring a broad spectrum of resistance against pests and pathogens. Recently, a cyclic 14-amino-acid sunflower trypsin inhibitor-1 (SFTI-1) has been characterized, showing high similarity to the reactive site loop of the Bowman-Birk inhibitors, which is the most potent and small BBI yet discovered [9]. BBIs are stable at cooking temperatures and also towards acidic pH values in the digestive systems of humans and animals,

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most probably due to their large number of disulfide bonds (seven bonds out of about 70 amino acid residues) and the polar interactions between sub-domains [10,11]. The potential applications of BBIs have now attracted much attention. BBIs are already used to defend against insects in transgenic plants, and they also have prospects in the prevention of cancer, Dengue fever, and inflammatory and allergic disorders [12–14], especially after the finding of SFTI-1, which provides an ideal platform for the design of small peptic pharmaceuticals.

Structural Features

Many BBI sequences have been determined from both monocotyledonous and dicotyledonous seeds. The covalent structure of soybean BBI was first elucidated by Odani and Ikenaka [15] and is shown in **Fig. 1**. Bowman-Birk protease inhibitors are double-headed serine protease inhibitors with a network of highly conserved disulfide bridges. They are classified according to their structural features and inhibitory characteristics. BBIs from dicots usually have a molecular weight of approximately 8k and are double-headed (two reactive sites separately distributed in two homologous active loops) [16], while those from monocots can be divided into two classes, one approximately 8 kDa in size with one reactive site (another reactive site has been lost) and the other approximately 16 kDa in size with two reactive sites [8,17–19]. BBIs from dicots are found in the seeds of legumes in particular [2]. Characteristically, they are single polypeptides and comprise a binary arrangement of two sub-domains with a conserved array of seven disulfide bridges, which play a prominent role in the stabilization of their reactive site configuration [16,20,21]. These inhibitors interact simultaneously and independently with two (not necessarily identical) molecules of protease. They have two tandem homologous regions comprising a consensus motif of three β-strands. In some, it has been demonstrated that splitting the homologous regions by partial peptic digestion yields two active fragments [15,22,23]. Chi and coworkers first proved these features by crystallization of the mung bean inhibitor itself and the complexes of the inhibitor with trypsin at a molar ratio of 1:1 or 1:2, respectively [20,23–25]. The 16 kDa monocotyledon BBIs have two reactive sites, each located at the N-terminal part of the 8 kDa BBI region, and these two regions have a high intra-molecular identity.

Prakash *et al*. aligned 27 domains in BBIs from both monocots and dicots, showing that disulfide bridges occur mostly between two highly conserved cysteine residues (e.g. C_1-C_{14}, C_2-C_6, C_3-C_{13}, C_4-C_5, C_7-C_9, C_8-C_{12}, C_{10}-C_{11}; cysteines are numbered 1–14 in the order of appearance along the sequence) [19]. Dicot BBIs have 14 cysteines, whereas the monocot BBIs with a similar size have 10 cysteines (except ORYSAN, which lost two more cysteines, Cys 4 and 5). The cysteines lost in monocot BBIs correspond to C_3-C_{13} and C_{10}-C_{11} of dicot BBIs, as shown in **Fig. 2**.

Compared with monocot inhibitors, dicot BBIs show a broader specificity for the enzymes inhibited. The first reactive site of most dicot inhibitors inhibits trypsin, whereas the second one inhibits trypsin, chymotrypsin and elastase. The P_1 residue of the first site in most of the dicot inhibitors is Lys/Arg, except in soybean inhibitor C-

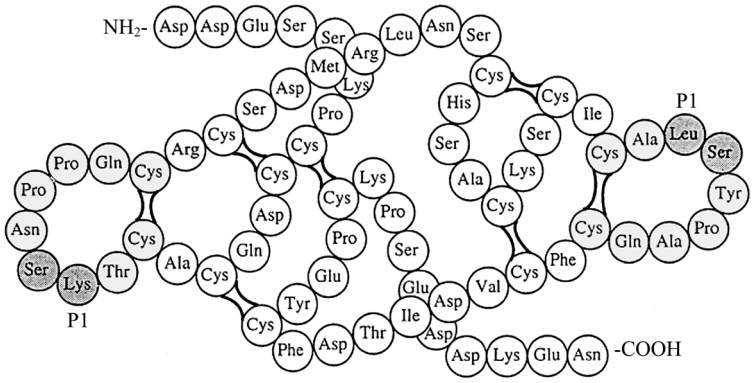

Fig. 1 Primary structure of soybean Bowman-Birk inhibitor

The primary structure of the soybean Bowman-Birk inhibitor protein showing the organization of the seven disulfide bonds. The two nine-residue reactive site loops are shaded and the positions of the P_1 residues are indicated (Odani & Ikenaka).

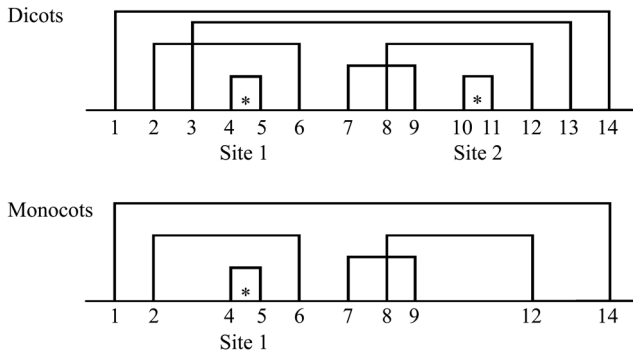

Fig. 2 Disulfide connectivity in dicot BBIs and 8 kDa monocot BBIs

* indicates the reactive site in the loop of C_4-C_5 and C_{10}-C_{11}, respectively (Prakash *et al.*).

II (SOYBN2) and the kidney bean inhibitor (PHAVU) where the P_1 position is occupied by Ala instead of Lys/Arg and hence the inhibition is specific to elastase. The P_1 residue of the second site is more variable, being Arg, Phe, Tyr, Leu or Ala. Variants of BBI proteins generated by semi-synthesis suggest that Phe is the optimal P_1 residue for chymotrypsin and that inhibition follows the series Phe>Trp>Leu>Met>Val>Ala>*D*-Trp>Gly>desLeu [26]. Deimination of Arg to citrulline destroys trypsin activity in peanut B-III, but alters chymotrypsin activity only marginally [27]. The P_1' residue in the first and the second site is a conserved Ser. The inhibition of chymotrypsin by soybean BBI was found to follow the series Ser>Ala>Thr>Val>Leu>Gly at the P_1' position [28].

In monocot BBIs with a size of approximately 8 kDa, the reactive site situated only at the N-terminal region aligns well with the first reactive site of dicot BBIs [19]. Nearly all monocot BBIs inhibit trypsin, but seem to have lost their second reactive site. The P_1 residue of the second site is not a potential target for trypsin, chymotrypsin or elastase. In addition, these inhibitors have lost the C_{10}-C_{11} bridge, which also forms a canonical loop that is indispensable for inhibitory activity in dicot BBIs (**Fig. 2**). The loss of this crucial disulfide bridge and the lack of the appropriate P_1 residue are probably the main factors for the loss of the second reactive site in monocots.

In addition to well-studied serine proteases, the inhibitory activity of BBIs against an increasing number of trypsin-like and chymotrypsin-like proteases has been described [29]. This includes activity against Janus-faced proteases such as cathepsin G and duodenase.

Although dual-headed BBI proteins are able to inhibit two molecules of protease bound to the separate reactive site, relative affinity will be altered when one site is already occupied. The soybean BBI inhibits duodenase with a K_i of 4 nM, but when precomplexed with trypsin in the other site the K_i is 400 nM [30]. Peanut inhibitor was found to be devoid of antichymotryptic activity when precomplexed with trypsin, and vice versa (that is, it would be devoid of antitrypsin activity if when precomplexed with chymotrypsin) [31]. These results suggest that though the two reactive sites are approximately 40 Å apart [32]; it is possible that in some instances there is steric hindrance when two independent proteases attempt to bind the sites.

Molecular Evolution

Mello *et al.* [33] recently constructed phylogenetic trees for the BBI family and revealed that BBIs from monocots and dicots could be clearly separated into different groups. The overall topology of the BBI tree suggests a different pattern of evolution for each group. BBIs from dicots were well conserved, showing only slight differences during their evolution while those from monocots were highly variable, indicating an interesting process of evolution based on internal gene duplications and mutation events. This confirms the theory that gene duplication events play a major role in molecular evolution [19]. Even though the amino acid composition of different BBIs has been changed during evolution, their cysteine residues are highly conserved.

An evolutionary scheme of BBIs, especially from monocots, is shown in **Fig. 3** [33]. It appears that BBIs of less than 100 residues from monocots have lost two or four cysteine residues. When two cysteines are absent, it is most probable that these are residues C_{10}-C_{11} of the second reactive loop, as in the case of sugarcane and wheat BBIs (**Fig. 3**, MI-I). The elimination of four cysteines implies that residues C_3-C_{13} and C_{10}-C_{11} (**Fig. 3**, MI-II), or C_4-C_5 and C_{10}-C_{11} are missing (**Fig. 3**, MI-III). It appears that BBIs lacking four cysteines have probably evolved from the MI-I group, and further separated into two groups: the first being the MI-II group from wheat, rice and foxtail millet, and the second being the MI-III group from sugarcane, rice and maize. The MI-III group has a striking feature in that there is a sequence of 15 hydrophobic amino acids with a potential N-glycosylation site, indicating that BBIs in this group could be secretory glycoproteins without inhibitory activity. The BBIs of groups MI-IV and MI-V (approximately 180 residues) from monocots were probably generated by internal gene duplications in MI-II BBIs (**Fig. 3**). The MI-IV pattern

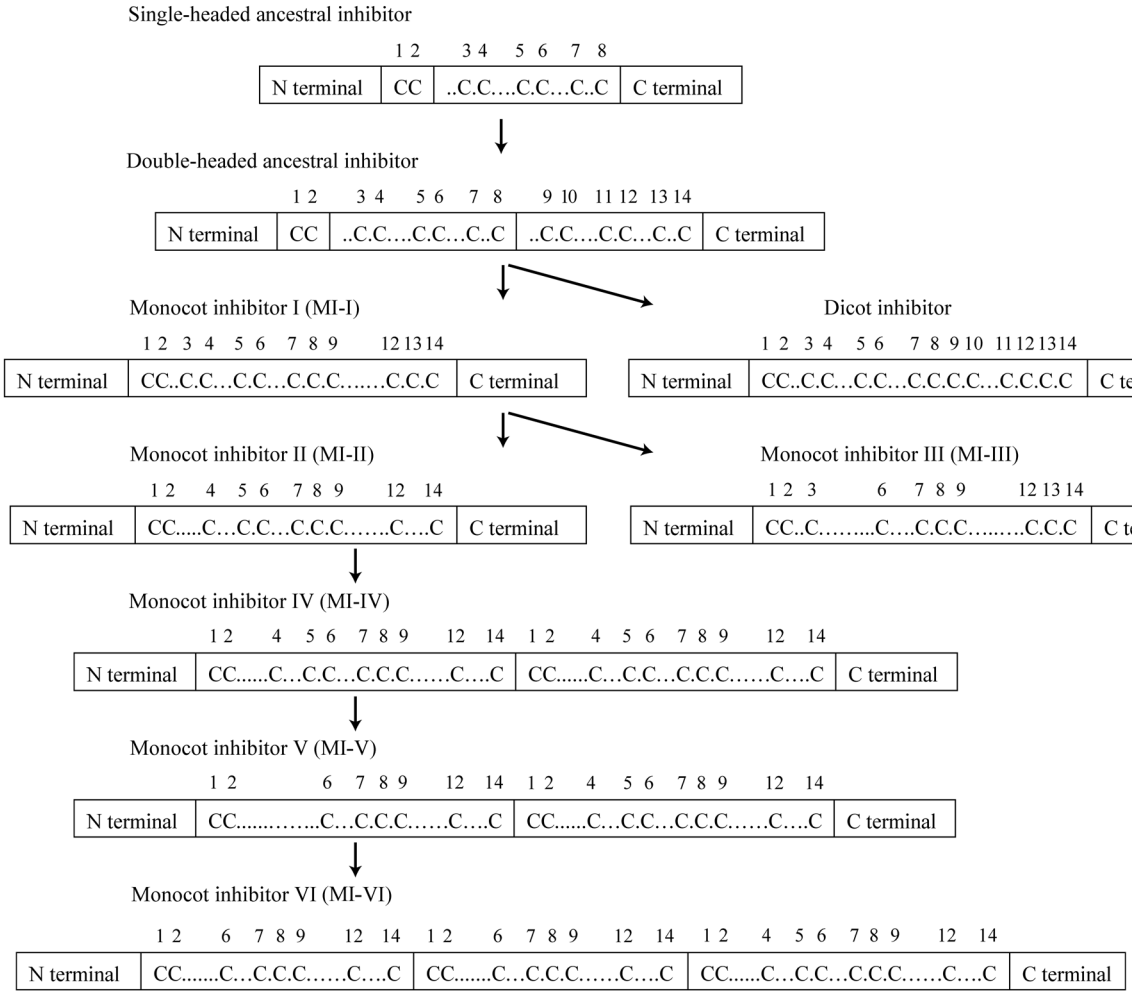

Fig. 3 A proposed evolutionary scheme for the Bowman-Birk-type proteinase inhibitor

Cysteines are numbered in order of appearance along the double-headed ancestral inhibitor (Mello *et al.*).

was only observed in a single barley expressed sequence tag (EST) consensus, whereas MI-V was found in sugarcane, rice and maize. It appears that MI-IV has lost four cysteines in both duplicated regions (C_3-C_{13} and C_{10}-C_{11}, another reactive site in loop C_4-C_5 still remains). The C-terminal region of MI-V is identical to that of MI-IV; however, the N-terminal region has further lost another two cysteines C_4-C_5, then two reactive sites were lost. The BBIs of the MI-VI group (approximately 250 amino acid residues in size) found in rice have probably evolved from the MI-V group by an additional duplication of the first region, that is, the MI-VI group is characterized by three duplications in tandem. The reactive sites of BBIs from monocots are extremely variable, indicating that duplication can be followed by deletion, insertion, and/or mutation.

Canonical Loop Conformation

The BBI family of protease inhibitors is unique in comprising a disulfide-linked nine-residue loop [34] that adopts the characteristic canonical conformation formed of a short type VIb β-turn [35] from P_1' to P_4' centered on a *cis*-Pro in P_3' reversing an antiparallel β-strand (an 11-residue loop is found at one reactive site of the peanut inhibitors A-II, B-II and B-III [6,36]). The nine-residue loop responsible for protease inhibition, also called the protease-binding loop, exists in all known BBI structures [34]. Through the exposed convex protease-binding loop, which is complementary to the concave active site of the target protease, BBIs bind to and then inhibit the cognate enzyme. This loop, which is constrained by the presence

of disulfide bridges and an extensive hydrogen-bonding network, is thought to have the same conformation as the productively bound peptide substrate [2,34]. BBIs interact with protease in a substrate-like manner and the protease-binding loop (substrate-like loop) is kept in a well ordered conformation. There is a consensus sequence (P_3-P_6') for most of the reactive site loops of BBIs, especially in the first reactive site loops: $CTP_1SXPPQC$ (P_1 indicates the residue that determines the specificity of inhibition; X indicates any of the 20 residues) [19].

In addition to the cysteine residues, the nine-residue loop also has several highly conserved residues: proline (which incorporates a *cis* peptide bond) at P_3' and serine at P_1' [32]. Tsunogae *et al*. [37] described this inhibitory loop as a two-stranded antiparallel β-sheet turned by the *cis*-Pro at P_3'. The "back side" β-sheet acts principally to restrain the reactive-site in an inhibitory conformation, not directly to interact with the target protease/enzyme. All contacts with the protease are via the "front side" β-sheet of the loop (P_3-P_2'). Of these interacting residues, the P_1' serine residue is well conserved, possibly due to its involvement in intra-molecular hydrogen bonding within the inhibitor loop [20], and the P_3 cysteine residue is essential for disulfide formation.

BBI-derived/synthesized protein fragments that encapsulate this loop retain the structure and inhibitory activity of the parent protein [38]. The most common BBI loop sequence has a proline-proline element with a *cis-trans* geometry at P_3'-P_4'. Brauer *et al*. [39] have examined this element by analysis of the inhibitory activity and structure for a series of synthetic fragments where one of two proline residues has been systematically replaced with alanine. The results show that only when a *cis* proline is present at the P_3' position could a potent inhibition be observed. Although a P_4' proline is not essential for activity, it effectively stabilizes this *cis* conformation by suppressing alternative conformations. The most evidence for this is from the Pro-Ala variant, which comprises a 1:1 mixture of slowly exchanging and structurally different *cis* and *trans* isomers. Monitoring the action of trypsin on this mixture by NMR spectroscopy shows that this protease interacts selectively with the *cis* P_3' structure, providing direct evidence for the link between activity and the native-like structure of the *cis* isomer. This is the first example where *cis* isomer selectivity can be demonstrated for a protease.

The BBI active loop also possesses a highly conserved Thr at the P_2 position. The importance of this residue has been confirmed by the synthetic BBI reactive site loops. It was found that Thr was the optimal residue at this position when inhibiting chymotrypsin. The Thr side chain has a dual role: intra-loop hydrogen bonding (via the -OH group) and formation of hydrophobic interactions with the enzyme (via the $-CH_3$ group) [32,40].

Crystal Structure

Several three-dimensional conformations of BBIs have been solved either by X-ray crystallography or by NMR spectroscopy. These include the crystal structures of PI-II from tracy soybean [21], A-II from peanut [41], BBI from soybean [10,42], and PsTI-IVb from pea seeds [43]. The structure of BBI from barley seeds at 1.9 Å resolution remains the highest refinement [32]. The crystal structures of BBIs in complex with trypsin from adzuki bean [37], mung bean [20] (**Fig. 4**), soybean [44], barley seed [45] and wheat germ [46], and that of mung bean BBI in complex with Dengue virus NS3-proteinase [12] are also available. A three-dimensional model of the black-eyed pea BBI-chymotrypsin complex has been constructed based on the homology of BBIs [47]. The crystal structures of the complexes of the Lys active domain and its partial fragment from the mung bean inhibitor with trypsin have also been elucidated [48–50]. The crystal structure of SFTI-1 in complex with trypsin has exhibited both sequence and conformational similarity to the trypsin-reactive site loop of BBIs [51]. Several solution structures of SFTI-1 alone or in complex with enzyme have also been solved, including structures of SFTI-1 [9], and MSTI from snail medic seeds [52].

Sunflower Trypsin Inhibitor-1

Among BBIs, sunflower trypsin inhibitor-1 [SFTI-1, **Fig. 5(A)**] is the most potent known naturally occurring Bowman-Birk inhibitor, and has recently attracted much attention due to its small size and cyclized-backbone. SFTI-1 also encompasses a disulfide-linked loop of nine amino acids, which is homologous in sequence to the first reactive loop of BBIs. There is only one residue difference in the loop between SFTI-1 and mung bean, with the residue Ile at position 10 being replaced by Gln [**Fig. 5(B)**] [9,51]. The crystal structure of SFTI-1 showed that the Ile side chain in this position is close to the surface and makes no contacts with the enzyme. Hence this change does not appear to affect the interaction of the inhibitor and the enzyme [51]. The other five SFTI-1 residues form a second backbone-cyclized loop instead of a disulfide bridge in BBIs. As expected, SFTI-1 in complex with bovine

β-trypsin also adopts the same conformation that has been previously found in the crystal structure of the complex of the Lys domain fragment of mung bean inhibitor with trypsin [53], that is, a double-stranded anti-parallel β-sheet conformation stabilized by a single disulfide bond bridging the β-sheet region [51]. Their active disulfide-linked loops are superimposed very well [**Fig. 5(D)**]. An extended reactive loop directly associated with protease is located at one end of the β-sheet with a hairpin turn between Gly^{1} and Asp^{14} completing the circle at the other end. The solution structure of SFTI-1 is similar to its crystal structure in complex with trypsin [9].

The lack of a major conformational change upon binding to the enzyme suggests that the structure of SFTI-1 is rigid and already pre-organized for maximal binding due to minimization of entropic losses compared with a more flexible ligand [**Fig. 5(C)**]. Thus, its trypsin inhibitory activity is considerably stronger than that of other naturally occurring members of the Bowman-Birk family of inhibitors and small synthetic peptides based on the trypsin binding loop of soybean BBIs. The solution structure of a synthetic acyclic variant of SFTI-1, which introduces Gly^{1} at the N-terminal and Asp^{14} at the C-terminal, is remarkably similar to that of native SFTI-1 [**Fig. 5(C)**], confirming that the stable arrangements of cross-linking hydrogen bonds between the β-strands and a single disulfide bond greatly contribute to their structural rigidity and allow both of them to inhibit trypsin with very high potency (0.5 nM and 12.1 nM, respectively) [9]. Recently, Zabłotna *et al*. determined the trypsin inhibitory activity and hydrolysis rates of SFTI-1 and its two analogues, indicating that head-to-tail cyclization is significantly less important than the disulfide bridge for maintaining trypsin inhibitory activity [54]. SFTI-1 inhibited β-trypsin with an impressive sub-nanomolar K_i of 0.1 nM, and it inhibited cathepsin G with a comparable K_i. SFTI-1 has a considerable selectivity, for example, it proved to be 74-fold less inhibitory for chymotrypsin, and was found to be three orders of magnitude less inhibitory for elastase and thrombin. In contrast, it had no effect on Factor Xa.

It should be stressed that the obtained results are very useful as a starting point for the study of enzyme-inhibitor interactions. Because of the advantages outlined herein, SFTI-1 turned out to be a very attractive template for the design and chemical synthesis of new protease inhibitors with potential uses as therapeutic agents.

Potential Applications and Future Prospects

The BBIs have been thought to play a vital role in the arsenal of defense mechanisms that plants use to protect against insect predators and against environment hazards during germination and seedling growth. Due to their inhibitory activity, BBIs may protect cereal seeds by suppressing the digestion of food, which is necessary for the growth of pathogens and pests. After part of a seedling is bitten or wounded, the gene expression of BBIs is dramatically increased to resist an invasion of pests [55]. It has been reported that cowpea BBI can confer enhanced insect resistance to transgenic tobacco plants [56]. When both soybean BBIs and Kunitz inhibitors were introduced and expressed in sugarcane, the growth of neonate larvae of *Diatraea saccharalis* feeding on leaf tissue from transgenic plants was significantly retarded as compared to larvae feeding on leaf tissue from untransformed plants [57].

In addition to protease inhibitory activity, the anticarcinogenic activity and radioprotective activity of BBIs from legumes have been widely studied. Populations that consume relatively large amounts of legumes in their diets have relatively low incidences of prostate, colon, breast, and skin cancer [58]. It is believed that the anti-carcinogenic effect is the result of the inhibition of proteolytic activity in transformed cells [59]. The soybean extract that is enriched in BBI, called BBI concentration (BBIC), was granted investigational new drug status by the USA food and drug administration (FDA) in April 1992 (IND No. 34671; sponsor, Ann R. KENNEDY). BBIC is in clinical trials [60] to investigate its anti-carcinogenic effects (reviewed by Kennedy [13]). Phase I and Phase IIa studies of BBIC in patients with oral leukoplakia have demonstrated clinical activity without detectable side effects after oral administration [61].

Dengue fever and Dengue hemorrhagic fever caused by the Dengue virus place approximately 2.4 billion people in tropical regions at risk. There is no known therapy or effective vaccine. An intensive study of the replication of the virus in host cells, with a view to defining and characterizing chemotherapeutic targets for drug design, has identified a viral protease NS3 as a potential target that plays a critical role in viral replication. Recently, Murthy *et al*. found that NS3 could be inhibited by mung bean BBI, and then the crystal structure of a complex of the protease with the mung bean inhibitor was solved [12]. The structure provides a starting point for the design of specific inhibitors of enzymatic activity.

Human tryptase, a trypsin-like enzyme that is the main protein in most mast cells, is involved in inflammatory and allergic disorders, among them asthma, multiple

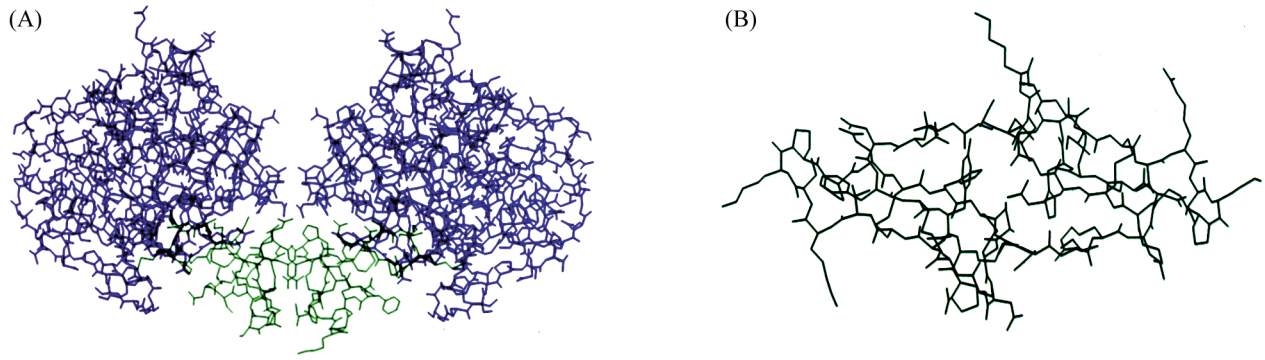

Fig. 4 Crystal structure of mung bean trypsin inhibitor

(A) The crystal structure of the complex of the mung bean inhibitor with trypsin at a molar ratio of 1:2. (B) The crystal structure of the mung bean inhibitor itself.

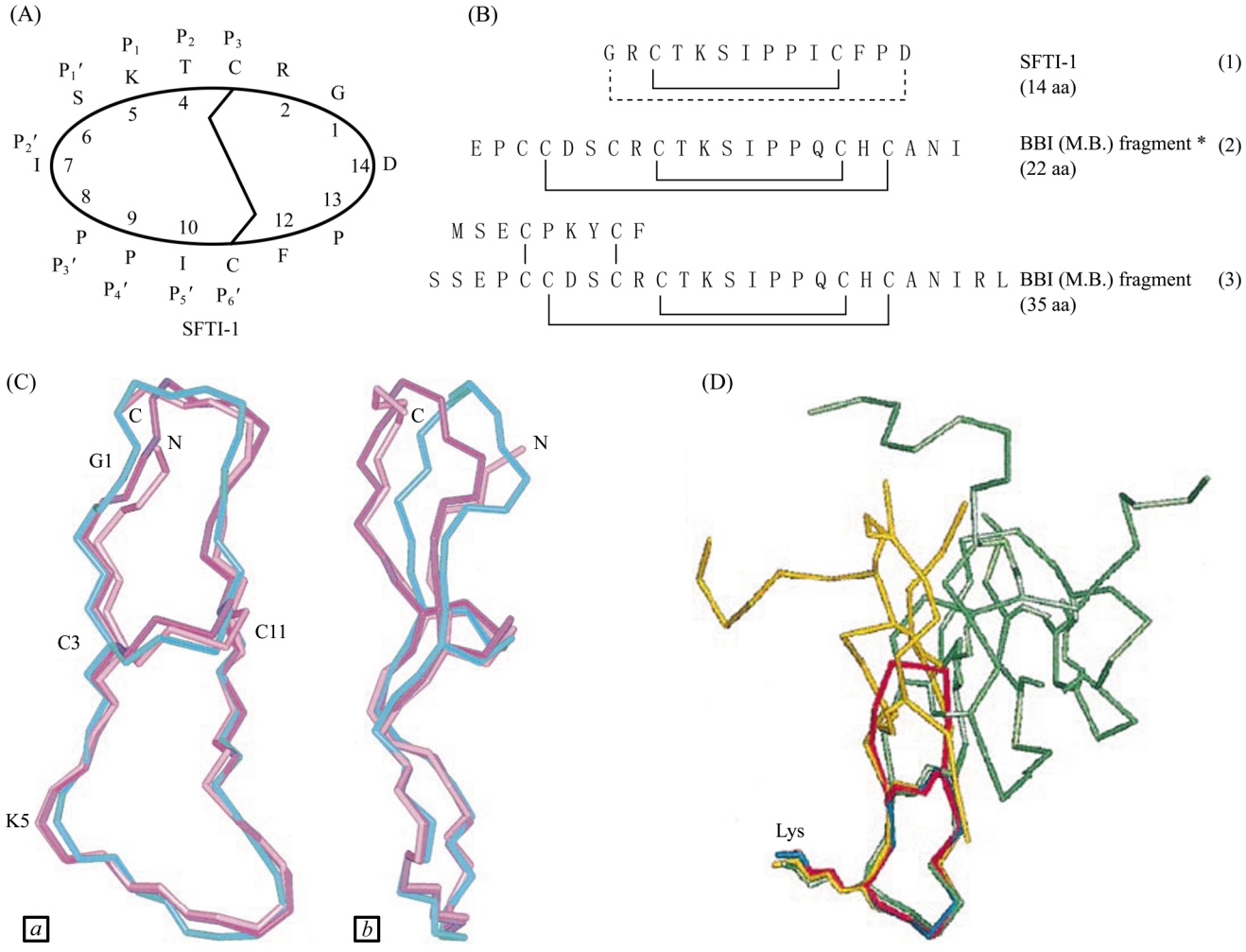

Fig. 5 Sequence and structure of the sunflower trypsin inhibitor SFTI-1 and the Lys domain fragments of mung bean inhibitor

(A) Sequences of the head-to-tail cyclic peptide SFTI-1 (Korsinczky *et al.*). (B) Sequence alignment of SFTI-1 and the Lys domain fragments of mung bean inhibitor (* synthetic material; aa, amino acid; M.B., mung bean). Solid lines show the positions of disulfide bonds; dotted lines indicate head-to-tail cyclization in SFTI-1. (C) Superimposition of the solution structures of cyclic SFTI-1 (purple), acyclic SFTI-1 (pink) and the crystal structure of bovine trypsin-complexed cyclic SFTI-1 (light blue). The N and C termini are labeled on acyclic SFTI-1. (a) and (b) views are rotated through 90° (Korsinczky *et al.*). (D) Alpha-C traces showing superimpositions of SFTI-1 (red) with the mung bean inhibitor [53] shown in blue, Adzuki bean inhibitor [37] shown in yellow, and soybean inhibitor [42] shown in green. The P1 lysine residue is indicated (Luckett *et al.*).

sclerosis, arthritis and rheumatoid arthritis. Different from trypsin, tryptase is an atypical serine protease; its active form is a tetramer that is formed of four almost identical monomers in the presence of heparin or acidic polysaccharides. Most serine protease inhibitors, including the potent bovine pancreatic trypsin inhibitor and BBIs, cannot inhibit tryptase because they are not able to access the central pore of the enzyme tetramer. Recently, Scarpi *et al*. reported that using the "canonical scaffold" of BBI (CTKSIPPQC), a series of extended peptides at both the C- and N-termini were synthesized. One of them is a potent tryptase inhibitor with a K_i value of 1.0 nM. Thus, it provides a prospect for drug design to target tryptase [14].

Long *et al*. found that SFTI is also a very potent inhibitor, with a K_i of 0.92 nM of the epithelial serine protease matriptase, which is a type II transmembrane protease involved in the development and pathogenesis of epithelial tissues [62]. Matriptase may function to degrade the extracellular matrix and to activate hepatocyte growth factor, urokinase and protease activated receptor-2 (PAR-2). Thus, matriptase blockage could potentially modulate the proliferation, motility, invasion and differentiation of cells. The design and synthesis of various analogues based on the matriptase catalytic site have been intensively studied.

One goal of inhibitor research is to reduce the size and simplify these inhibitor proteins to a minimal structure element [63]. Proteinomimetics offers the potential to combine the functional benefits both of a macromolecular and of a low molecular weight system. The canonical nine-residue loop of BBIs/STFI-1 has presented itself as a good template in protein mimicry [38]. In 1999, McBride *et al.* [64] identified a potent inhibitor of the serine protease human leukocyte elastase (HLE/HNE) from screening a combinatorial peptide library based on the disulfide-bridged protein β-sheet segment that forms the active site loop of BBIs. The inhibitor is an 11-residue peptide, NleCTASIP-PQCY (cyclized via a disulfide bridge), which inhibits HLE with a K_i value of 65 nM and also exhibits the highest degree of resistance against hydrolytic turnover by the enzyme [64]. HLE plays an important role in the tissue destruction associated with pulmonary emphysema, rheumatoid arthritis, cystic fibrosis, adult respiratory distress syndrome and chronic bronchitis [65]. Later, Brauer *et al.* determined the NMR structure of the 11-residue core peptide and superimposed its 30 lowest energy simulated annealing structures onto the X-ray structure of the reactive site loop from mung bean BBI protein, validating that the nine-residue loop within the core peptide retains not only biological activity but also the structure of the corresponding native protein [**Fig. 6(A)**] [66].

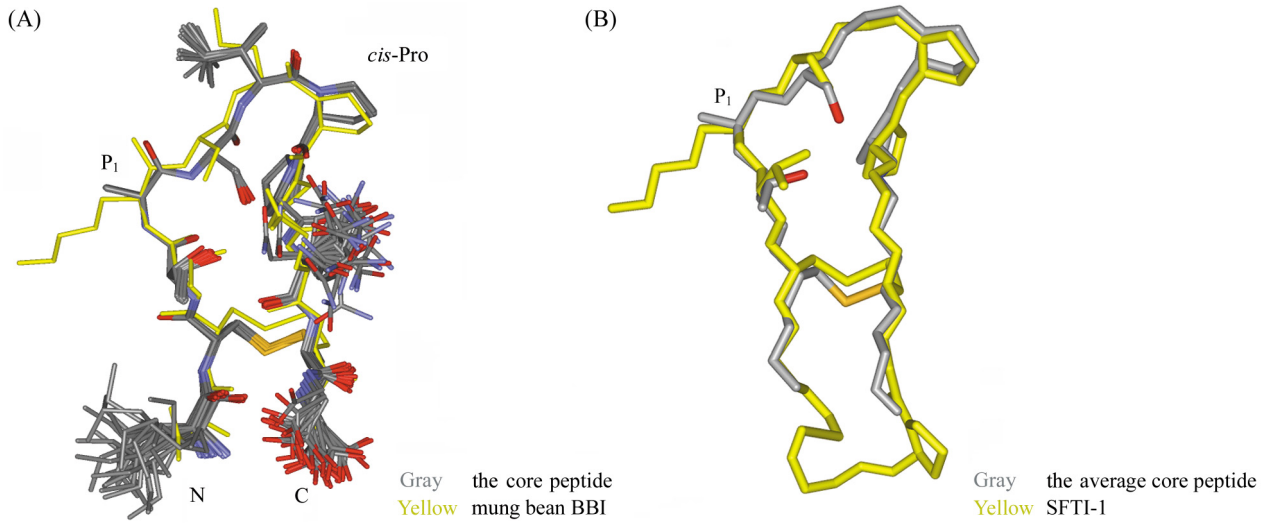

Fig. 6 Conformation comparison of the synthetic human leukocyte elastase inhibitor with the mung bean BBI fragment and SFTI-1 (Brauer *et al.*)

(A) Stereo view of the 30 best simulated annealing structures calculated from the NMR data (gray) of the synthetic peptide superimposed onto the X-ray structure of the reactive loop (yellow) from the mung bean BBI protein. The positions of the P_1 residue and the *cis*-Pro at P_3' are indicated, as are the locations of the N- and C-termini. The disordered side-chain of the C-terminal residue Tyr11 is omitted for clarity. (B) Superimposition of the averaged minimized simulated annealing structure of the entire backbone of the anti-HNE 11-residue peptide (gray) onto the corresponding atoms of the bicyclic Sunflower trypsin inhibitor 1 (SFTI-1, yellow). The RMS (root-mean-square) deviation between the two structures is 0.72 Å. Most side chains are omitted for clarity.

Brauer *et al.* also superimposed the averaged minimized simulated annealing structure of the entire peptide backbone onto the structure of native SFTI-1 [**Fig. 6(B)**].

Recently, several peptide variants synthesized in our lab based on the Lys active fragment of the mung bean trypsin inhibitor appear to strongly inhibit furin, which plays a vital role in many important physiological systems (unpublished data), further confirming that the canonical nine-residue loop of BBIs could be used as a useful scaffold for the design of pharmaceuticals for different purposes.

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