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Studies on simultaneous inhibition of trypsin and chymotrypsin by horsegram Bowman-Birk inhibitor

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Abstract. Bowman-Birk inhibitors (BBI) isolated from plant seeds are small proteins active against trypsin and/or chymotrypsin. These inhibitors have been extensively studied in terms of their structure, interactions, function and evolution. Examination of the known three-dimensional structures of BBIs revealed similarities and subtle differences. The hydrophobic core, deduced from surface accessibility and hydrophobicity plots, corresponding to the two tandem structural domains of the double headed BBI are related by an almost exact two-fold, in contrast to the reactive site loops which depart appreciably from the two-fold symmetry. Also, the orientations of inhibitory loops in soybean and peanut inhibitors were different with respect to the rigid core. Based on the structure of Adzuki bean BBI-trypsin complex, models of trypsin and chymotryspin bound to the monomeric soybean BBI (SBI) were constructed. There were minor short contacts between the two enzymes bound to the inhibitor suggesting near independence of binding. Binding studies revealed that the inhibition of one enzyme in the presence of the other is associated with a minor negative cooperativity. In order to assess the functional significance of the reported oligomeric forms of BBI, binding of proteases to the crystallographic and non-crystallographic dimers as found in the crystal structure of peanut inhibitor were examined. It was found that all the active sites in these oligomers cannot simultaneously participate in inhibition.

Keywords. Plant seed inhibitors; Bowman-Birk inhibitor; simultaneous inhibition; computer modelling.

1. Introduction

A large number of proteinase inhibitors are found in plant seeds. They appear to provide resistance against a large number of proteases of animal, fungal and bacterial origin. One of the well studied classes of these inhibitors is the Bowman-Birk family (BBI). These are small proteins made up of 60 to 80 amino acid residues. These inhibitors are known to have several isoforms, some differing in the length at the N-terminus,. and some showing significant sequence variations· They exhibit a tendency to form homo- or hetero-dimers in solution complicating the estimation of the number of isoforms. Also, elusive is the tendency of BBIs to migrate as higher molecular weight species, not only in size exclusion chromatographic studies but also in SDS polyacrylamide gels in the presence of the reducing agent like β-mercaptoethnol. Such reports are frequent in literature (Haynes and Feeny 1967; Gennis and Cantor 1976; Whitley and Bowman 1975). This had led to systematic over estimation of their molecular weights (Wu and Whitekar 1991)· Thus, often other methods like SDS PAGE after

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s-carboxymethylation of the protein or mass spectrometry have been used to obtain the correct molecular weights (Bergeron and Nielsen 1993; Prakash *et al* 1996).

BBIs are rich in disulphide bridges, with few hydrophobic residues other than prolines. Norioka and Ikenaka (1983), classified the legume double headed BBIs into four groups. Recently we have grouped the BBIs into two major classes, those from monocotyledonous seeds and dicotyledonous seeds (Prakash *et al* 1996). The further classification of dicot inhibitors is consistent with that of Norioka and Ikenaka (1983). The inhibitors from monocots are of two sizes, 8 K and 16 K. The 8 K inhibitor is single headed while the 16 K inhibitor is double headed with two 8 K like domains. On the contrary, dicot inhibitors are double headed. Crystallographic studies (Chen *et al* 1992; Suzuki *et al* 1993) have shown that the three-dimensional structure of this protein is mainly stabilized by disulphide bridges, in addition to a small hydrophobic core. The molecule consists of two domains, made of tandem repeat of homologous amino acid sequences, each consisting of three peptide chain rings made by disulphide bridges. Each domain has a reactive site against proteases, usually trypsin and chymotrypsin. The reactive site usually consists of nine residues. The amino acid residues towards the N-terminus of the scissile bond P1- P1', are denoted for convenience as P3 - P2 - P1 while those towards C-terminus as - P1'- P2'- P3' .... It is also known that a variation in the P1 residue alters the specificity of these inhibitors. Prakash and Murthy (1997) have shown that a classification on the basis of only a twelve residue stretch at the active site displays many features of phylogeny obtained for entire sequences.

In a double headed inhibitor, each reactive site might be active against two independent enzymes. In such a case, the enzymes will compete for the same sites. The two sites of the inhibitor could also be specific for two different enzymes. In this case, the binding could either be independent or competitive due to interactions between bound enzymes (Laskowski and Kato 1980).

Several crystallographic investigations have been made on these inhibitors and their complexes with the enzymes. The number of structures determined are far less than those crystallized. The quality of diffraction for many crystals has been poor. In the context of these studies, we examine the structural properties of BBIs and present studies on the simultaneous inhibition using computer modeling and competitive binding experiments.

2. Materials and methods

2·1 *Three-dimensional structures*

The coordinates of peanut BBI (PBI, Suzuki *et al* 1993), soybean BBI (SBI, Chen *et al* 1992), Adzuki bean inhibitor (ABI)-trypsin complex (Tsunagae *et al* 1986) and chymotrypsin (Cohen *et al* 1981) were obtained from the Protein Data Bank (PDB), Jan, 1996 release, from the Bioinformatics, Indian Institute of Science, Bangalore.

2.2 *Superposition of structures*

The Cα atoms of PBI were superposed on those of SBI using the program HOMOMGR (Rossmann and Argos 1975). The program determines the transformation required for the superposition of two structures and lists the residual distance between superposed Cα atoms.

2.3 *Hydropathy profiles*

The HOMOLOGY module of InsightII version 2·3 of the Biosym technologies, was used to make the hydrophobicity plots based on Kyte and Doolittle's (1982) hydrophobicity scale. A window of 3 was used for smoothening. InsightII was also used for visual examination of the superpositions that were performed, to check for short contacts between molecules and to measure bond angles and distances·

2.4 *Solvent accessible surface area calculations*

Solvent accessible surface area was calculated with the Lee and Richards (1971) algorithm. The probe radius was 1 4 A. Per cent surface accessibility (%SA) was defined as %SA = (SA/MA)* 100, where SA is the computed surface accessibility and MA is the maximum accessibility for that residue in a fully extended conformation when it occurs in a Gly-X-Gly tripeptide (Miller *et al* 1987).

2.5 *Competition experiments*

Horsegram BBI was purified by anion exchange chromatography and gel filtration. The four isoinhibitors were resolved on a DEAE-Sephacel column as described earlier (Prakash *et al* 1996). Trypsin and chymotripsin were purchased from Sigma Chemical Co. (USA). Amidolytic activity of trypsin and its inhibition by HGI was assayed using N-benzoylarginine p-nitroanilide (BAPNA) as the substrate according to the method of Kakade *et al* (1969). Chymotrypsin inhibition was assayed using benzyl tyrosine ethyl ester (BTEE) as the substrate (Hummel 1959).

Competitive binding studies were performed with the purified HGI - I II. First HGI was incubated with various concentrations of trypsin for 10 min at 37°C and inhibition of chymotrypsin was measured using BTEE as substrate. Similarly, after incubating the inhibitor with various concentrations of chymotrypsin for 10 min at 37°C, the effect on the inhibition of trypsin was measured using BAPNA as substate.

3. Results

3·1 *Comparison of BBI structures*

Three-dimensional structures are available for SBI (Chen *et al* 1992), PBI (Suzuki *et al* 1993), the binary complex of trypsin and ABI (Tsunagae *et al* 1986) and the ternary complex of two trypsins bound to mung bean inhibitor (Lin *et al* 1993). The coordi nates for the last of these was not available in the Jan'96 release of PDB. Each BBI has 14 cysteines or 7 disulphide bridges that are conserved in the family. In order to compare the structures of PBI and SBI, initially the two structures were superposed, using the program H OM O M G R. The information for initiating the overlap were the conserved cysteine residues. In the initial superposition, all the cysteine residues did not superpose and the overall RMS deviation between the two structures was large. A core conserved structure was obtained after removing the C-terminal stretches that includedthe last two cysteine residues. This immediatelyhinted that differences exist in

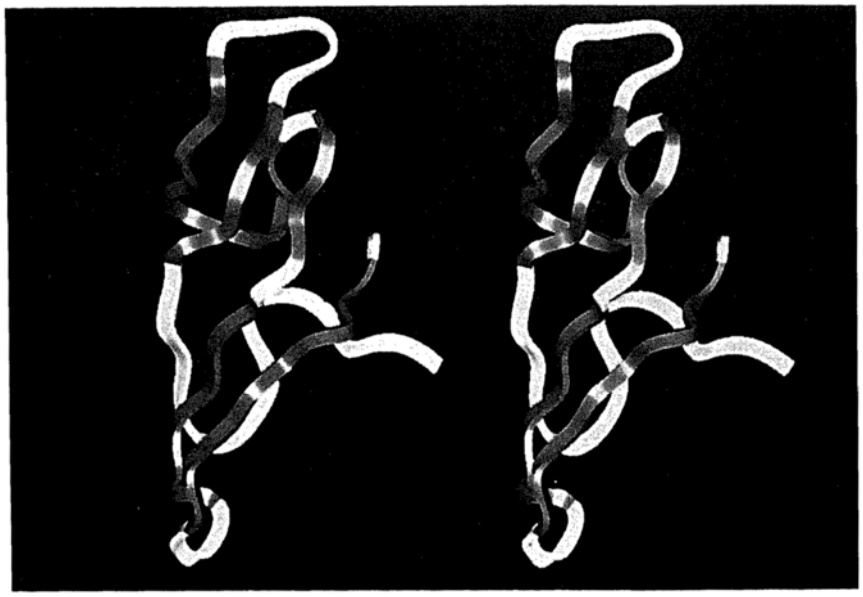


Figure 1. Ribbon diagram illustrating the residual distances when a SBI monomer is superposed on PBI monomer. The lighter regions have residual distances greater than 2·0 Å.

the environments around these last two cysteine residues. The overall RMS deviation was 1·9 A for this superposition.

Figure 1 shows the RMS deviations at different gray levels. The shaded regions in the figure correspond to residues that deviate by less than 2·0 A. It is evident that there are significant differences at the C-terminal ends·

3·2 *Hydrophobic core of BBIs*

The 8 K protein has two similar 4 K domains which probably resulted from gene duplication. The three-dimensional structure of these proteins are stabilized mostly by the disulphide bridges, apart from a small hydrophobic core· The hydrophobicity and surface accessibility plots are shown in figure 2· These plots display the lower accessibility and larger hydrophobicity of the core residues· The most accessible regions in these structures are the inhibitory loops.

3·3 *Superposition of domains*

A superposition of the two 4 K domains that constitute the 8 K BBI was achieved using HOMOM GR. The firstdomainof PBI was made upofresidues 11-29 and 63-68. The second is made up of residues 36-55. The superposition of these domains involved a rotation of 175·90°, thus suggesting that these two domains had an approximate two-fold symmetry. When the residues deviating by more than 2·0 A were omitted from superposition, the resulting rotation for superposition was 179·0° for both SBI and PBI· The superposed residues belonged to the core conserved structure.

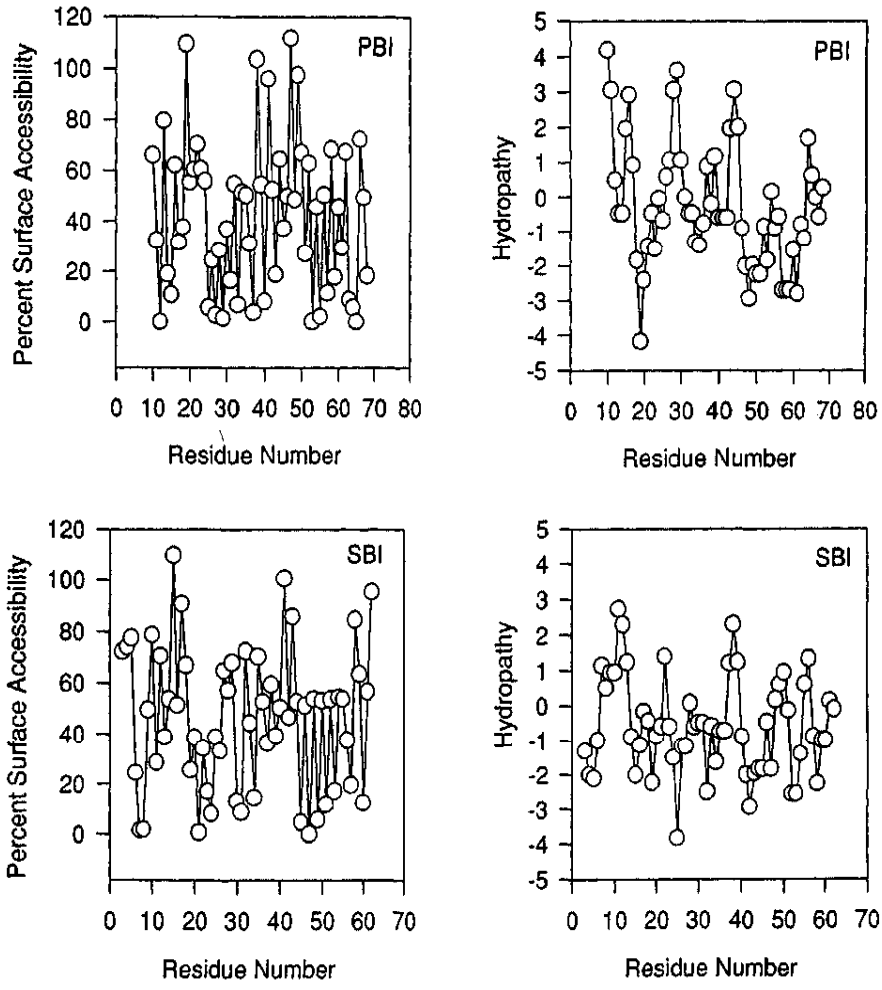
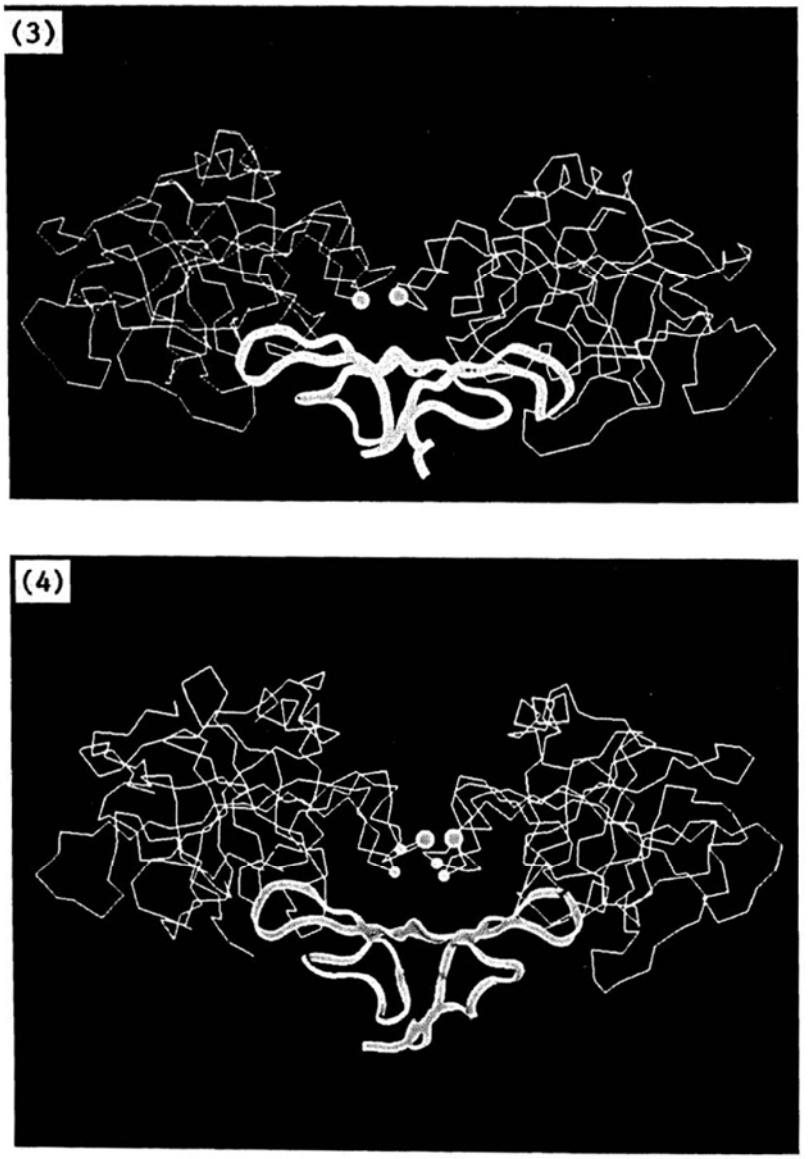


Figure 2. Plots illustrating the variation in surface accessibility and hydrophobicity along the polypeptide chain in PBI and SBI. The least surface accessible and most hydrophobic residues of this plot belong to the segments structurally conserved between PBI and SBI (figure 1).

3.4 *Simultaneous binding of trypsin and chymotrypsin*

Attempts were made to understand the mode of binding of trypsin and chymotrypsin to BBI using SBI and PBI as models· The structure of Adzuki bean BBI-trypsin complex was used as the basis for initiating modeling studies· The program HOMOMGR, was used to superpose the inhibitory loop of ABI onto the first inhibitory loop of SBI· The resultant matrix was applied to the trypsin coordinates (of the ABI-trypsin complex). Chymotrypsin coordinates, obtained from the PDB, were rotated such that they superposed onto the trypsin coordinates of the ABI-trypsin complex· The superposition of the inhibitory loop of ABI onto the second inhibitory loop of SBI yielded a transformation which was applied to the rotated chymotrypsin coordinates· These operations resulted in a model of SBI bound to trypsin and chymotrypsin at the two

sites (figrue 3)· The lack of extensive short contacts in figure 3 suggests that the two sites can be accessed simultaneously· However, there are a few short contacts involving residues 174 and 95 of trypsin and chymotrypsin respectively· These short contacts can be relieved so as to enable formation of hydrogen bonds that might stabilize the ternary complex· Such hydrogen bonds have been reported to exist in the crystal structure of



Figures 3 and 4. Binding of trypsin and chymotrypsin to PBI **(3)** and SBI **(4)** modelled as described in the text. The inhibitor is shown as a ribbon diagram while the enzymes as wire drawings. The circles mark the residues in the two enzymes which make short contacts. The reduced unfaourable interaction in **(3)** is due to the different disposition of the active site loops in SBI and PBI with respect to their conserved cores.

the complex of mung bean BBI with two trypsin molecules (Lin *et al* 1993). The inhibitory loops adopt different spatial conformations when the structures of SBI and PBI are superposed· Therefore, a similar superposition was carried out to obtain a ternary complex of trypsin, chymotrypsin and PBI· In this model the number of short contacts were less (figure 3)·

3.5 *Competitive binding*

The competitive binding experiments were carried as described in § 2· In order to check the simultaneous binding of trypsin and chymotrypsin by HGI, two experiments were carried out· In the first experiment, the trypsin inhibitory activity was measured in the presence of varying amounts of chymotrypsin. There was a blank and two controls· The first control had trypsin (2 μM), while the second contained inhibitor and trypsin in 1:2 ratio· Since HGI-III binds trypsin in 1:1 ratio (Sreerama *et al* 1997), the second control would show half the activity when compared to the first control· The other tubes had

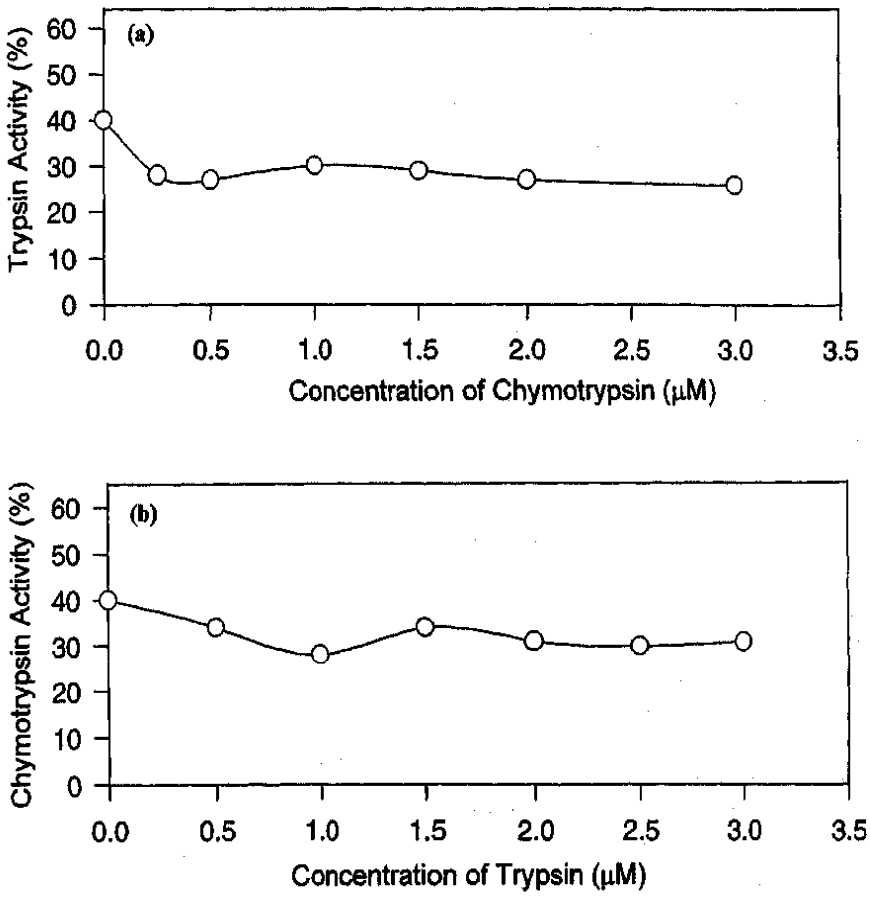


Figure 5. (a) Tryptic inhibition by HGI in the presence of varying concentrations of chymotrypsin· **(b)** Inhibiton of chymotrypsin by H G I in the presence of varying concentrations of trypsin. In bith cases, a marginal reduction in activity is observed· The inhibitor to enzyme ratio was 1:2.

varying amounts of chymotrypsin apart from the inhibitor and trypsin in the ratio 1:2. In these, the activity of trypsin was measured in the presence of chymotrypsin. Similarly, in the second experiment chymotrypsin inhibitory activity was measured in the presence of varying amounts of trypsin.

Figure 5 shows the results of competitive binding experiments. The tryptic activity is reduced by approximately 2 5 % when chymotrypsin to inhibitor ratio was 0·25:1. The activity does not reduce further even at 3:1 ratio of chymotrypsin to inhibitor· Similarly the chymotryptic activity is also reduced by almost the same amount and behaved similarly up to 3:1 ratio of trypsin to inhibitor·

3.6 *Functional significance of the oligomeric forms*

Peanut inhibitor crystal structure consists of tetramers with approximate 222 symmetry. One of the two-folds is crystallographic· In order to examine the functional significance of the crystallographic dimer (with fewer intersubunit contacts) and non-crystallographic dimers (with more extensive contacts), models of trypsin and chymotrypsin bound to one of the monomers (of the dimers), were constructed. The bound enzymes penetrated into the free monomer, suggesting that in this kind of dimeric association, steric hindrance prevents the binding of enzymes simultaneously to both sites of a given monomer· None of the sites of the crystallographic dimer can bind to any enzyme without severe short contacts· On the contrary, one of the non-crystallographic dimers can bind only to two enzymes, one on each monomer.

4. Discussion

BBIs are proteins that are relatively small in size (Mw ~ 8 kDa)· The inhibitors from dicotyledonous seeds are double headed and usually inhibit trypsin and chymotrypsin. The structural superposition of the BBIs from soybean and peanut showed that there is a considerable difference in the two structures at their C-termini and at the loops. Also, the orientation of the reactive loops with respect to the conserved cores in these two inhibitors are different. This is also reflected in the binding of trypsin and chymotrypsin to PBI and SBI (figures 3 and 4). Examination of the hydrophobicity plots and surface accessibility (figure 2) showed that the loops are the most accessible regions while the less accessible regions form a small hydrophobic core that is conserved. Between the two domains that comprise the 8 K monomer, the cores are related by an almost exact two-fold symmetry. Deviation from this nearly strict two-fold symmetry is observed when more residues are included in the superposition. The reactive site loops deviate appreciably from the two-fold symmetry relating the core (figure 1)·

Models of trypsin and chymotrypsin bound to BBI were constructed based on the known structure of ABI inhibitor-trypsin complex. Figure 3 show that there are minor short contacts concentrated mainly around the residues 174 of trypsin and 95 of chymotrypsin. The crystal structure of the mung bean inhibitor complex showed that the two trypsin molecules interact by means of hydrogen bonds between the residues 174 of one trypsin and 95 of the other (Lin *et al* 1993). In our model, most of the atoms involved in the short contacts are separated by a distance of about 2·0 A. About 10 atoms have distances less than 2·0A. This shows that the two enzymes can be

simultaneously inhibited by the inhibitor. Examination of the models of enzymes bound to the iligomeric units of the inhibitor as observed in PBI structure suggested that these forms are unlikely to be functionally significant.

The differences in the loop structures between SBI and PBI were reflected in the lesser number of short contacts between the two enzymes (figure 3) when a similar model was constructed with PBI. This shows that these contacts can be relieved by minor rearrangements in the reactive loop of the inhibitor. It is also possible that the bow shaped inhibitor undergoes a small change inorder to accommodate the two enzymes. This could also lead to a small degree of cooperative behaviour in the binding of the two enzymes to the inhibitor.

Inorder to examine this aspect, the competitive binding studies were carried out. The tryptic activity in the presence of inhibitor (2 μM of trypsin and 1 μM of HGI) was 40% of the activity in the absence of inhibitor. This reduced to 30% in the presence of chymotrypsin. Similarly the residual chymotryptic activity, measured after incubating the inhibitor in varying concentrations of trypsin, reduced by a small amount. The activity did not reduce further even at saturating concentrations of the other enzyme. This shows (figure 5) that the binding of one enzyme in the presence of the other is associated at best with a minor negative cooperativity. The short contacts between the two enzymes as seen in our model appear to be responsible for this effect.

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