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Proteinase inhibition using small Bowman-Birk-type structures

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ABSTRACT. Bowman-Birk inhibitors (BBIs) are cysteine-rich and highly cross-linked small proteins that function as specific pseudosubstrates for digestive proteinases. They typically display a "double-headed" structure containing an independent proteinase-binding loop that can bind and inhibit trypsin, chymotrypsin and elastase. In the present study, we used computational biology to study the structural characteristics and dynamics of the inhibition mechanism of the small BBI loop expressing a 35-amino acid polypeptide (ChyTB2 inhibitor) which

has coding region for the mutated chymotrypsin-inhibitory site of the soybean BBI. We found that in the BBI-trypsin inhibition complex, the most important interactions are salt bridges and hydrogen bonds, whereas in the BBI-chymotrypsin inhibition complex, the most important interactions are hydrophobic. At the same time, ChyTB2 mutant structure maintained the individual functional domain structure and excellent binding/inhibiting capacities for trypsin and chymotrypsin at the same time. These results were confirmed by enzyme-linked immunosorbend assay experiments. The results showed that modeling combined with molecular dynamics is an efficient method to describe, predict and then obtain new proteinase inhibitors. For such study, however, it is necessary to start from the sequence and structure of the mutant interacting relatively strongly with both trypsin and chymotrypsin for designing the small BBI-type inhibitor against proteinases.

Key words: Bowman-Birk inhibitor, Molecular modeling, Enzyme-linked immunosorbent assay, Enzyme specificity

INTRODUCTION

Plant defense mechanisms against herbivorous insects include the production of serine proteinase inhibitors that affect insect development (Pompermayer et al., 2001). One group of these inhibitors, called Bowman-Birk inhibitors (BBIs), function as specific pseudosubstrates for the digestive proteinases, forming a stable complex in which proteolysis is limited and extremely slow (Tiffin and Gaut, 2001). As a consequence, an amino acid deficiency occurs in insects feeding on such diet and which consequently affects the insect growth, development and fecundity (Pompermayer et al., 2001).

BBIs are cysteine-rich and highly cross-linked small proteins that typically display a "double-headed" structure (Rahbe et al., 2003). Each "head" contains an independent proteinase-binding loop that can bind and inhibit trypsin, chymotrypsin and elastase (McBride et al., 2002; Singh and Appu Rao, 2002) either independently or simultaneously. The resulting non-covalent complex renders the proteinase inactive. The realization that one BBI molecule could form a 1:1:1 complex with two enzymes led early workers to dissect this activity. This inhibition mechanism is common for the majority of serine proteinase inhibitor proteins, and many analogous examples are known.

A particular feature of the BBI protein is that the interacting loop is a particularly well-defined disulfide-linked short beta-sheet region. Moreover, small synthetic peptides based on this region keep the same structure as the corresponding part of the full-sized protein and also retain inhibitory activity (Brauer et al., 2002). It has been possible to isolate the antiproteinase activity as small (approximately 11 residues), cyclic, synthetic peptides, which display most of the functional aspects of the protein (Brauer et al., 2002).

Based on these characteristics, some serine proteinase inhibitors were over expressed in plants aiming to increase their resistance to insects. In some cases, this expected effect was

satisfactorily observed, but in others, a variable level of resistance was achieved (De Leo et al., 2001; Falco and Silva-Filho, 2003).

In recent experiments with BBI-type inhibitors described by Mello (2002), a mutated sequence was obtained (for the region 31-58) for the BBI proteinase inhibitor extracted from *Glycine max* (P01055, 1D6R:I structure). The mutant has the inhibition loop formed by the residues CTRSIPPQC and was called ChyTB2. In the present study, we used computational biology to study the structural characteristics of ChyTB2 loop, which was described as a good inhibitor for bovine trypsin and chymotrypsin. Modeling and unrestrained molecular dynamics was used to allow the final accommodation of the modeled enzyme-inhibitor complexes. The stability, hydrogen bonds, and electrostatic and hydrophobic interactions of each complex were compared to already described BBI interactions with trypsin and chymotrypsin. In our opinion, the inhibitor studied here may be used as a lead for the development of small and effective proteinase inhibitors.

MATERIAL AND METHODS

Cloning of BBI derivatives

The soybean BBI cDNA (GenBank access P01055) sequence was silent mutated at positions Ser^{38} (TCG→TCA) and Cys^{39} (TGT→TGC) to insert the unique restriction site *Nsi*I. The restriction sites *Pst*I and *Kpn*I were inserted at the ends of the sequence to be cloned into the pCTB vector. The construction was confirmed by sequencing.

Two different PCR amplifications of phagemid pCTB-BBI were used in the division of the *bbi* gene. The T1 (5'-ccgggctgcagaattcgagctcggt-3') and T2 (5'-cctctgcagaatgcatgatttacaagc-3') (both *Pst*I tailed) primers were used to amplify the trypsin (TryBBI) portion, whereas the chymotrypsin (ChyBBI) fragment was amplified using C1 (5'-cgcggtaccctgcaggttttccttg-3') and C2 (5'-cggtaccaaatcatgcatttgcgc-3') primers (Operon, California, USA), both *Kpn*I tailed. The PCR products were digested with the appropriate restriction enzyme and ligated to the pCTB vector, thus allowing for the recombinant phagemid pCTB-TryBBI and pCTB-ChyBBI. Both constructions of BBI derivatives were confirmed by sequencing and expressed on the tips of phages.

The division of the former *bbi* sequence generated two fragments: one expressing a 42-amino acid polypeptide (hereafter referred to as TryBBI) and containing the coding region for the trypsin inhibitory site, and the second one expressing a 35-amino acid polypeptide (hereafter referred to as ChyBBI) and containing the coding region for the chymotrypsin inhibitory site.

Construction of TryBBI and ChyBBI mutant libraries

The phage-display libraries were constructed varying amino acids at 4 positions in TryBBI and ChyBBI. Two degenerated oligonucleotides T-NNB (5'-gatcaatgcgcatgcNNBNN BNNBNNBcctcctcaatgccgctgttcagatatgagactgaattcgtgccattcagcttgtaaatcatgcatttgcgcac-3') and C-NNB (5'-ggtaccaaatcatgcatttgcNNBNNBtcgNNBcctNNBcagtgtttttgtgtcgacataaccgatttc-3') were synthesized containing the nucleotide sequence NNB (B = C/G/T, N = A/C/G/T) which codes for a restricted pool of amino acids at the positions P_2, P_1, P_1', P_2' of TryBBI and P_2, P_1, P_2', P_4' of ChyBBI. A PCR amplification of these two degenerated oligonucleotides using their backward primers T-NNBback (5'-gtgcgcaaatgcatgattta-3') and C-NNBback (5'-gaaatcggttat

gtcgacac3') was performed as described by Tanaka et al. (1999). The resulting double-stranded material was cleaved with *Sph*I-*Nsi*I for the trypsin inhibitors, and *Nsi*I-*Sal*I for the chymotrypsin inhibitors. After restriction, fragments were ligated into dephosphorylated phagemid vectors pCTB-TryBBI and pCTB-ChyBBI, respectively. A mix of ligation products was used to transform *Escherichia coli* TG1 cells (K12(lac-pro), supE, thi, hsdD5/F', traD36, proAB, lacIq, lacZΔM15), creating the trypsin and chymotrypsin inhibitor libraries.

Selection of the phage-display libraries

Library amplification, rescue of recombinant phage-display libraries and selection were performed as described by Mello (2002). Selection was carried out with bovine trypsin (0.2 mg/mL in 50 mM $NaHCO_3$, pH 9.6) immobilized on immunotubes (Maxi Sorb 4070319, Nunc). After five rounds of selection, bound fusion phages were eluted and used for transfection of *E. coli* cells. One hundred colonies of each selection were sequenced and evaluated for further analysis. Enzyme-linked immunosorbent assay (ELISA) of phages was performed using the Detection Module-Recombinant Phage Antibody System (Pharmacia) as described in the instruction manual. Microtiter plates (Costar) were coated with bovine trypsin and chymotrypsin (Sigma), papain (Sigma, negative control) and sugar cane borer trypsin (1 µg/mL purified protein).

Molecular modeling

The structure of the cancer chemopreventive BBI complexed with bovine trypsin (pdb 1D6R) (Koepke et al., 2000) has been used to predict the 3-D structure of the TryBBI (1-36 residues of the I chain) and ChyBBI loops (31-58 residues of the I chain) of modeled inhibitors. The atomic coordinates of the 1D6R pdb complex, solved at 2.4 Å resolution, were used as a template in comparative modeling by satisfaction of spatial restraints (Fiser et al., 2000) implemented in the program Modeller 6v2 (Sali and Blundell, 1993).

The structure of the bovine gamma chymotrypsin (pdb 1GMC) (Yennawar et al., 1994) was used in the modeling of the bovine chymotrypsin-ChyBBI complex. Final interaction model for the complex was obtained by satisfaction of spatial restrains (Fiser et al., 2000) in MODELLER 6v2, implementing CHARMM energy terms for five cycles of simulated annealing (Marti-Renom et al., 2000). For each complex, 25 different models were generated using MODELLER script, and the quality of the predicted folds was evaluated using the internal score of the variable target function (Marti-Renom et al., 2000). The stereochemical quality of the 3 best scoring models was assessed by Procheck program (Laskowski et al., 1993) at the same resolution as the 1D6R structure. The final model was selected based on the overall stereochemical quality for further energy minimization using Gromacs (Lindahl et al., 2001).

Energy minimization and molecular dynamics of modeled complexes

To refine the models of the enzyme-BBI complexes and to allow best accommodation of the contacting residues in the enzyme-inhibitor interface, additional energy minimization and equilibrating molecular dynamics simulations were carried out with the program Gromacs 3.2 (Lindahl et al., 2001) on a dual-CPU Linux work station. Complexes were solvated in explicit single point charge solvent model, using octahedral water box subjected to periodic boundary

conditions and ionizable amino acids were protonated according to pH 7.0. The simulated system (~39,000 atoms) was fully solvated with 12,000 water molecules. To neutralize the system, one Na^+ and 5 Cl^- ions were added in the box. Initial complex models were submitted to a steepest-decent energy minimization (5000 steps), to remove bad van der Waals contacts. For further relaxation, the minimized structure of the complexes was used in unrestrained molecular dynamics for 500 ps with berendsen-type temperature (312 K) and pressure (1 atm.) coupling in an 8 x 12 x 8-nm simulation cell, implementing the smooth particle mesh Ewald method of electrostatic treatment (Essman et al., 1995). Production dynamics were carried out under the same conditions for 2-3 ns. Cluster analysis of the trajectory obtained was performed using *g_cluster* program of Gromacs 3.2 (Lindahl et al., 2001) with 2.5 Å cut-off to obtain the average structures of the complex during production simulation. The most stable complex in the MD run was selected for unrestrained multiple step conjugate-gradient minimization process (0.1 kJ mol^{-1} nm^{-1}) to obtain the final minimized structure of the complex for further analysis.

Evaluation of protein-inhibitor interactions in enzyme-BBI complexes

For evaluation of the trypsin-inhibitor and chymotrypsin-inhibitor interactions, the Java Protein Dossier (JPD, Neshich et al., 2004) program of the Diamond Sting Suite (Neshich et al., 2005) was used. The cut-off distances for hydrogen bonds, salt bridges, and aromatic interactions were 2-3.2, 2-6 and 4.0 Å, respectively. To evaluate the stability of the secondary structure elements at the contact surface of the enzyme-inhibitor complex, the program *do_dssp* in Gromacs package (Lindahl et al., 2001) was used.

RESULTS

Structure of the TryBBI and ChyBBI models

In order to study the TryBBI and ChyBBI interactions, the sequence of soybean BBI was used to model and analyze the inhibition mechanism for each complex. The 1-36 residues in the sequence/structure of soybean BBI (pdb 1D6R:I) (Lindahl et al., 2001) were used to model our TryBBI, and 31-58 residues of the same sequence/structure were used to model the ChyBBI. After the modeling procedure and validation of the model using Procheck, molecular dynamics simulation was used to allow the proper accommodation of new "*in silico*" constructed complexes (Figure 1A and B).

A 15-residue finger-like structure, but with different physicochemical characteristics composes each BBI loop. The TryBBI-interacting loop (Figure 1A) is composed mainly of charged and polar residues. At the same time, the ChyBBI-interacting loop (Figure 1B) is composed of hydrophobic residues.

Contact surface differences in bovine TryBBI- and ChyBBI-modeled complexes

In BBI-trypsin complex (1D6R pdb), the important BBI-contacting residues are Lys_{16} and Asn_{18} at the "left" side, Gln_{21} at the "right" side and Arg_{23} at the "upper" side of the contacting surface (Figure 1A). The most important residue in the contact interface is Lys_{16}, able to make a salt bridge with Asp_{189}, and hydrogen bonds with Gly_{193} and Ser_{214}. The Cys_{14}-Gly_{216}

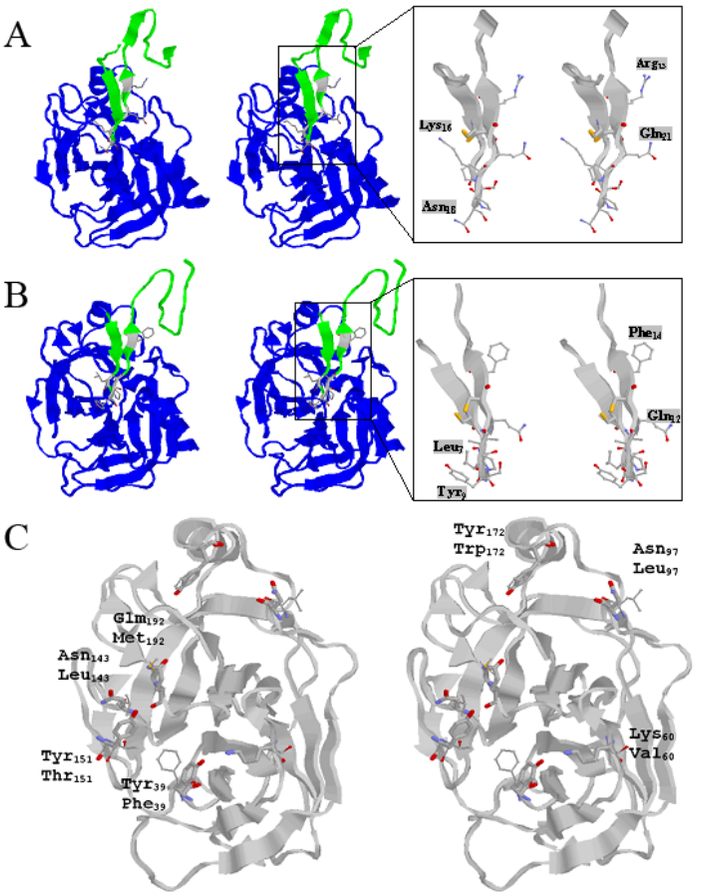

Figure 1. Analysis of the contacting residues in TryBBI-bovine trypsin and ChyBBI-bovine chymotrypsin complexes. **A.** A stereo view of the model for the TryBBI-trypsin interaction. The polar/charged residues (Lys_{16}, Asn_{18}, and Gln_{21}, Arg_{23}) inducing electrostatic interactions and weak hydrophobic contacts were predominant in TryBBI-trypsin complex. *Inset*, The stereo view of the loop with above described residues involved in interface interactions. **B.** A stereo view of the model of the ChyBBI-chymotrypsin interaction. Close hydrophobic contacts across the interface (Leu_7, Tyr_9, and Phe_{14}) were characteristic in ChyBBI-chymotrypsin complex. *Inset*, The stereo view of the loop with above described residues involved in interface interactions. **C.** Stereo view of the structural alignment of bovine trypsin (the upper of the labeled residues) and chymotrypsin (the lower of the labeled residues) and important differences in the residues of the contacting inhibitor surface for each enzyme. Trypsin residues are represented in thicker wire frame lines and chymotrypsin residues in thinner wire frame lines. For clarity, only secondary structure elements of the chymotrypsin were represented.

hydrogen bond at the center of the β-barrel structure of the BBI loop, and Arg_{23}-Asn_{97} and Cys_{12}-Ser_{217} hydrogen bonds stabilize the position of the BBI at the upper and right sides of the contact interface.

In our modeled BBI-chymotrypsin complex, the most important BBI-contacting residues are Leu_7 and Tyr_9 at the "left" side, Gln_{12} at the "right" side and Phe_{14} at the "upper" side of the contacting surface (Figure 1B). The most important residue in the contact interface is Leu_7, able to make hydrogen bonds with Gly_{174} and Ser_{195}, and hydrophobic contact with Leu_{180} and

Ala_{194} ring. The hydrophobic pocket formed by Trp_{153}, Trp_{196} and Ile_{82} residues of chymotrypsin allows the accommodation of the Ile_4 and Phe_{14} BBI residues at the upper side of the contact surface through hydrophobic interactions. The position of the Tyr_9 is coordinated by aromatic stacking with Phe_{22} and a hydrogen bond with Phe_{24} enzyme residues. The Cys_5-Gly_{197} and Ser_8-His_{40} hydrogen bonds also maintain the overall secondary structure of the ChyBBI loop-chymotrypsin complex.

After structural alignment in JPD, bovine trypsin (pdb 1D6R) (Koepke et al., 2000) and chymotrypsin (pdb 1GMC) (Yennawar et al., 1994) were fitted at less than 1.5 Å RMSD and residues located on the inhibitor-contacting surface were compared. The important residues in the inhibitor-contacting surface were positioned in a ring form, comprising the 97 and 172 positions at the "upper" side, 151, 143 and 192 positions at the "left" side, and 39 and 60 positions at the "right" side of the surface (Figure 1C). The Asn-Leu change at 97 and 143 positions, and the Tyr-Phe_{39}, Lys-Val_{60}, Asn-Leu_{143}, Tyr-Trp_{172}, and Gln-Met_{192} substitutions were mapped in the trypsin/chymotrypsin structures, and are shown in Figure 1C. The physicochemical properties in these 7 different residues are responsible for the general change of polar to hydrophobic contacting at interface in the trypsin and chymotrypsin enzymes, respectively.

Structural model for the ChyTB2 inhibition mechanism

Obtained in phage-display experiments, the ChyTB2 mutant (CTRSIPPQC loop) showed high affinity for bovine trypsin and chymotrypsin enzymes in ELISA experiments (Table 1). These characteristics are ideal for developing a good inhibitor for both enzymes. ChyTB2 inhibitor in complex with bovine trypsin (ChyTB2-Try) and bovine chymotrypsin (ChyTB2-Chy) were modeled and submitted to molecular dynamics simulation in order to study the dynamics of inhibition and the important residues to both these complexes. The molecular dynamics trajectories obtained were submitted to cluster analysis (Figure 2), and the representative structure of the most represented cluster was used as the complex structure for interaction analysis. For ChyTB2-Try complex, a stable cluster was obtained in 3.8-4.2 ns of

Table 1. Results of the analyses of inhibitor-enzyme contacts in modeled complexes, using JPD and Table of Contacts (Neshich et al., 2004).

	Trypsin		Chymotrypsin	
	TryBBI	ChyTB2	ChyBBI	ChyTB2
Loop sequence	*CTKSNPPQC*	*CQRSRPGQC*	*CALSYPAQC*	*CQRSRPGQC*
H-bonds	14	8	7	9
Attractive charge	1	1	0	1
Repulsive charge	0	0	0	0
Hydrophobic	8	5	8	11
Aromatic stacking	0	0	1	1
IFR energy[1]	67.2	67.7	23.9	70.0
ELISA[2]	1.621 ± 0.0947	1.771 ± 0.0545	1.484 ± 0.377	1.765 ± 0.382

[1]The Table of Contacts (Neshich et al., 2004) calculates the accumulative energy of contacts in the protein-inhibitor interface, using 0.6 kcal/mol for hydrophobic interactions, 1.5 kcal/mol for aromatic stacking, 2.6 kcal/mol for H-bonds, and 10.0 kcal/mol for salt-bridges (attractive-repulsive charge). [2]$OD_{410\ nm}$ ± standard deviation (n ≥ 4).

dynamics (Figure 2A), and the representative structure for further analysis was tacked for 3800 ps. For ChyTB2-Chy complex, stable complex was obtained after 700 ps of dynamics (Figure 2B), and representative structure was tacked for 1800 ps.

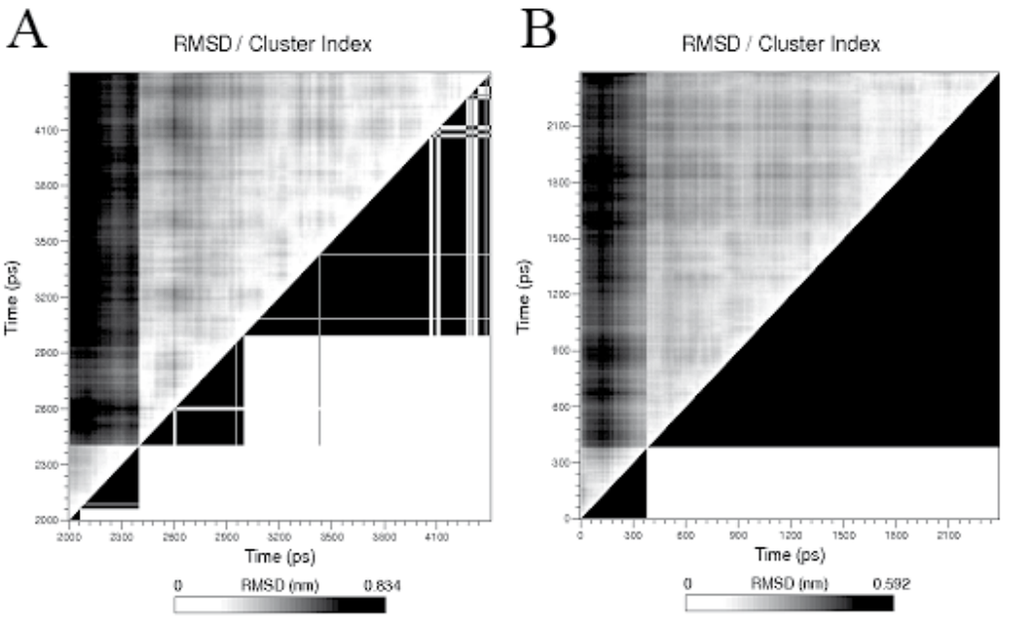

Figure 2. Cluster analysis results for trajectories obtained in molecular dynamics experiments for ChyTB2-trypsin (A) and ChyTB2-chymotrypsin (B). Graphical representation for clustering matrices were obtained using the *g_cluster* program of the GROMACS 3.3.1 package (Lindahl et al., 2001). RMSD = root mean square deviation.

Although the ChyTB2 mutant maintains the hydrophobic characteristics of the ChyBBI type loop, an Arg present at position P1 allows for the formation of salt bridges and hydrogen bonds with residues of the bovine trypsin (Figures 3 and 4).

As we described above, in the TryBBI-trypsin complex the most important interactions are salt bridges and hydrogen bonds, and the central residue in the interface is the Lys_{16} at the P1 loop position. In our "*in silico*" experiments, the Lys_{16} residue at P1 loop position is able to make a salt bridge with Asp_{189}, and hydrogen bonds with Gly_{193} and Ser_{214} enzyme residues (Figure 3A). In the ChyTB2 mutant, the Lys residue is replaced by Arg at the P1 loop position, allowing the formation of a salt bridge with Asp_{189} and hydrogen bond with Gly_{193} and Ser_{214} (Figure 3A-C). In the direct comparison, although the trypsin contacting residues in TryBBI and ChyTB2 inhibitors are different (Figure 3B,E), the Lys_{16}-contacting residues in the TryBBI-trypsin complex, Arg_{16}-contacting residues in ChyTB2-trypsin complexes, and the type of contacts in both complexes are almost the same (Figure 3C,F).

At the same time, our "*in silico*" experiments showed that in the ChyBBI-chymotrypsin complex, the most important interactions are hydrophobic. The Leu_7 residue (P1 loop position) is anchored through hydrophobic interactions and makes hydrogen bonds with Gly_{174}

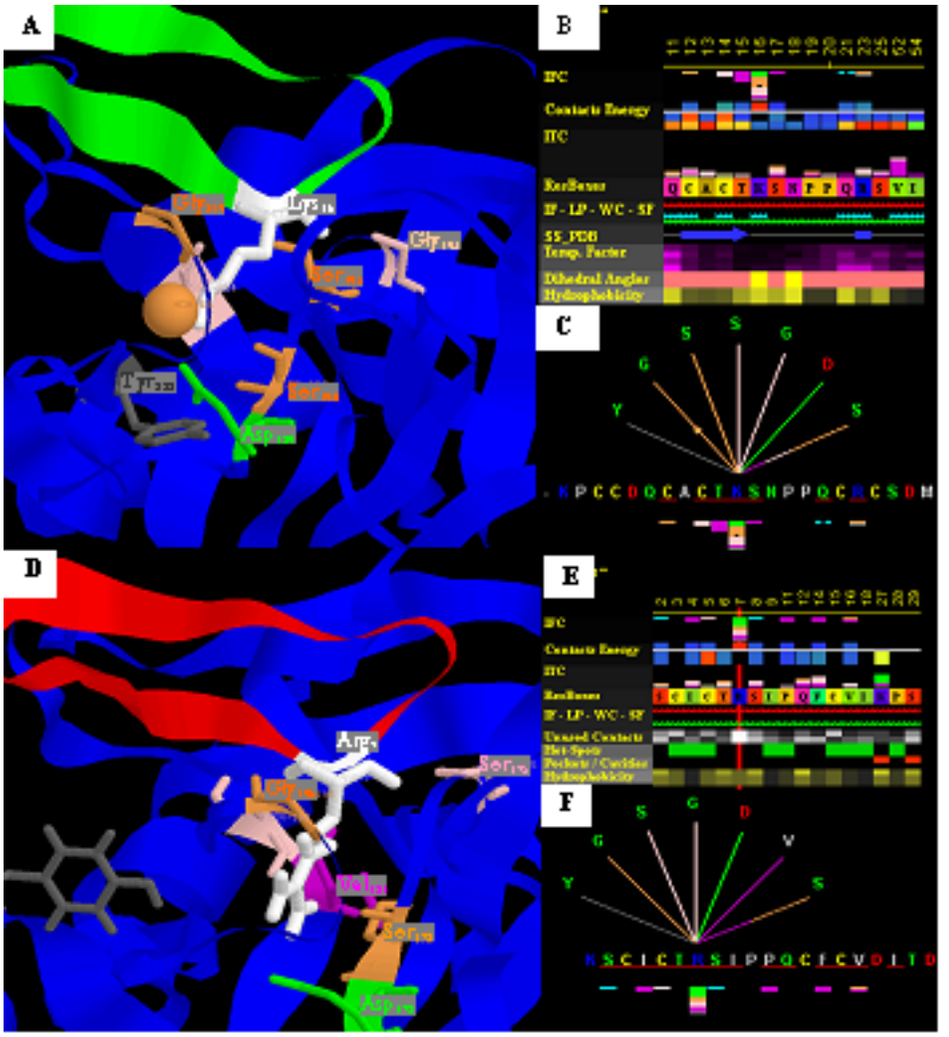

Figure 3. Analysis of the interface-forming residues in the TryBBI-trypsin and ChyTB2-trypsin complexes using the JPD program (http://sms.cbi.cnptia.embrapa.br/SMS/index_m.html). In JPD representations, the contacting residues at the interface, energetic levels, types of contacts, secondary structure, temperature factor, hydrophobicity, and other physicochemical parameters (total of 306 of them) are mapped in colored boxes. Contacts are represented in color code, according to the contact type. Aromatic stacking contacts are represented in dark grey, hydrophobic interactions in magenta, hydrogen bonds are represented in light grey, and electrostatic interactions are represented in green. **A.** Structure of the TryBBI-trypsin complex. TryBBI loop was represented in green color and trypsin in blue ribbon presentation. The central residue in white color is Lys_{16}, the most important contacting TryBBI residue at the interface. Contacting trypsin residues are represented in color code, according to the contact type. Aromatic stacking contacts are represented in dark grey (Tyr_{228}), hydrogen bonds are represented in dark yellow (Ser_{214}, Gly_{193}) and electrostatic interactions are represented in green (Asp_{189}). **B.** JPD representation of the interface-forming residues in TryBBI-trypsin complex. In colored boxes are mapped the residues contacting at the interface, energetic levels and types of contacts, secondary structure, temperature factor, hydrophobicity, and other physicochemical parameters. **C.** Trypsin residues contacting the Lys_{16} of the TryBBI loop. The contact type was mapped in color code as described above. **D**, **E** and **F.** The JPD representation of the ChyTB2-trypsin complex and type of contacts established with emphasis on Arg_{7}. Contacting residues and type of contacts are mapped in color code as described above.

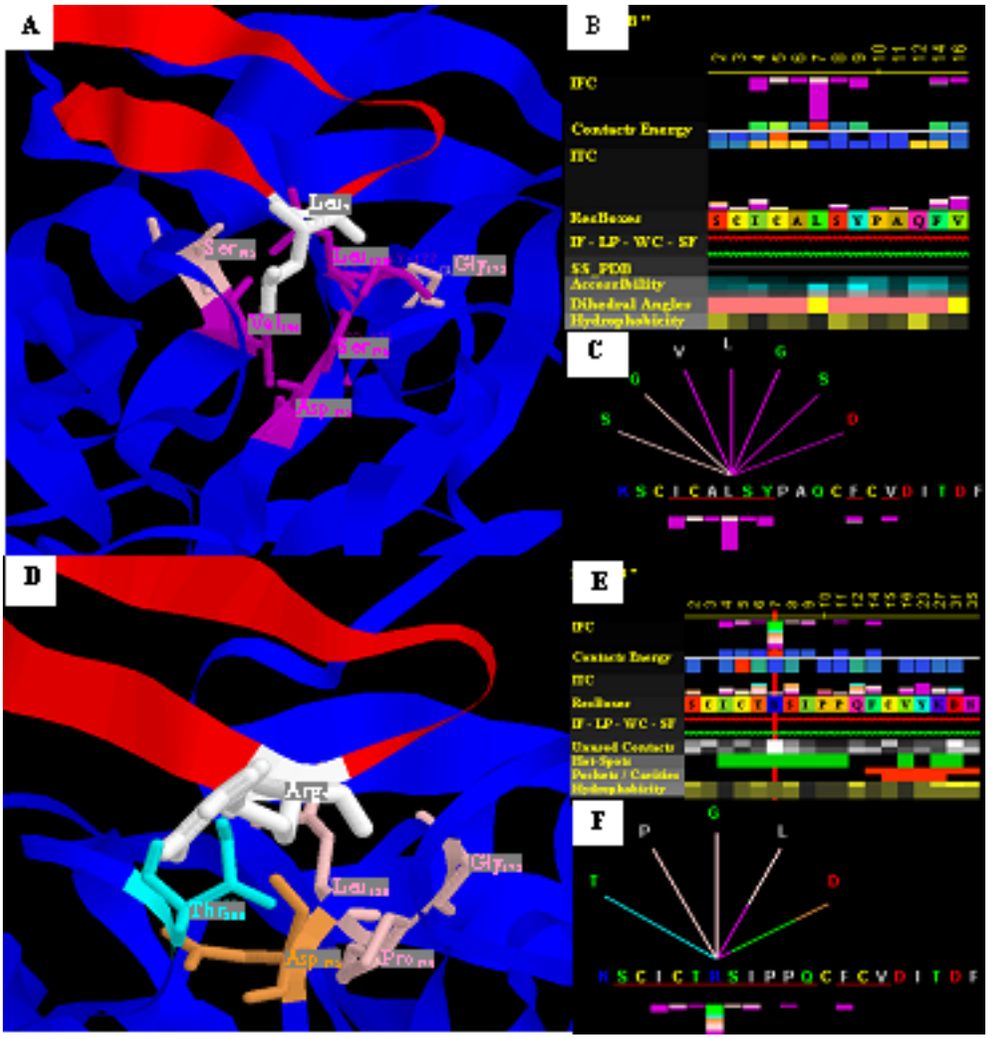

Figure 4. Analysis of the interface-forming residues and contacts in the ChyBBI-chymotrypsin compared to ChyTB2-chymotrypsin complex using the JPD program (http://sms.cbi.cnptia.embrapa.br/SMS/index_m.html). **A.** ChyBBI-chymotrypsin complex. ChyBBI loop was represented in red color and chymotrypsin in blue. The central residue in white color is Leu_7, the most important contacting ChyBBI residue at the interface. Contacting chymotrypsin residues are colored according to the contact type. The hydrophobic interactions are represented in magenta (Val_{194}, Val_{195}, Ser_{176}) and hydrogen bonds are represented in light grey (Ser_{195}, Gly_{178}). **B.** JPD representation of the interface forming residues in ChyBBI-chymotrypsin complex showing number of parameters pertinent to the sequence stretch containing Leu_7. **C.** Chymotrypsin residues contacting Leu_7 ChyBBI loop residue colored according to type of contacts. **D**, **E** and **F.** JPD representation of the ChyTB2-chymotrypsin complex and important contacts. Contacting residues and type of contacts are mapped in color code as explained in Figure 2.

and Ser_{195} in the ChyBBI-chymotrypsin complex (Figure 4A-C). The ChyTB2 mutant interacts with the bovine chymotrypsin hydrophobic surface through Ile, Thr, Ile, Pro, and Phe residues. The Arg_7 residue at the P1 loop position maintains a hydrogen bond with the Gly_{178}, making a new one with Pro_{179} and Leu_{180}, and a salt bridge with Asp_{175} (Figure 4D-F).

As the ChyTB2 mutant in ELISA experiments revealed significantly enhanced binding capacity with trypsin and chymotrypsin as compared to the native BBI (Table 1), the sequence of this mutant must be used as a starting structure for a BBI-type inhibitor for both enzymes.

DISCUSSION

The ChyTB2 (CTRSIPPQC) mutant's high affinity for both bovine trypsin and chymotrypsin in ELISA experiments (Table 1) is an ideal characteristic to maintain in the development of a strong dual enzyme inhibitor. Although this mutant maintains the hydrophobic characteristics of the ChyBBI-type loop and continues to interact with the bovine chymotrypsin hydrophobic surface through Thr, Ile and Pro residues at the P2, P2' and P4' sites, respectively (Figure 4), it also has an Arg at position P1 that allows the formation of salt bridges with Asn_{189} and hydrogen bonds with the Gly_{193} and Ser_{214} residues of bovine trypsin (Figure 3). An interesting observation is that the sequence of the inhibitory loop in this molecule occurs naturally in five different BBIs from pea (CAC24566), alfalfa (S56647, P80321, P16346) and peanut (P01067) (Mello et al., 2003). Moreover, one of the alfalfa inhibitors (S56647) is a double-headed BBI that contains the same sequence in both heads. Single-headed inhibitors of serine proteinases, which can interact with both trypsin and chymotrypsin, have been reported in potato (Pearce et al., 1982; Moura and Ryan, 2001), and more effective inhibition of chymotrypsin by a trypsin inhibitor was also observed by Moura and Ryan (2001) in pepper.

The P1 position of proteinase inhibitors determines up to 40-70% of the total association energy of the complex formation (Lu et al., 1997). Positively charged Arg residues are able to make hydrogen bonds, salt bridges and even aromatic stacking type contacts (Figures 3 and 4). Another position that plays an important role is P2' since it fits into an apolar S2 enzyme pocket. P2' Ile and Met are the residues most frequently found in BBIs from dicotyledonous plants, present in 78 of 121 inhibitory loops from 61 different dicotyledonous species. Gariani and co-workers (1999) studying the residues at the P2' position, observed that large aliphatic side chains give the best inhibitors while positively charged residues are tolerated at this position and negatively charged side chains give poor inhibitors. According to Brauer and Leatherbarrow (2003), when Pro is present at the P4' position, an additional restraint on the backbone of the peptide occurs which appears to reduce the rate of tryptic hydrolysis.

The results showed that modeling combined with molecular dynamics is an efficient method to obtain new proteinase inhibitors. Therefore, the sequence of the mutant strongly interacting with both types of enzymes (trypsin and chymotrypsin) should be used as a starting structure for constructing an efficient BBI-type inhibitor for both chymotrypsin- and trypsin-type enzymes. Our contribution also emphasizes the need for having an integrated environment that can display the highest possible number of parameters, which could influence the specificity of binding. Such environment has been proven to add to both the efficiency of the work and more complete analysis of the system. The STING environment in its current Diamond version (Neshich et al., 2005) has been very helpful in order to acquire and simultaneously display and analyze the largest number of sequence/structure/function attributes relevant for the current study.

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