Chapter 6
Protease inhibitor-assisted religation of proteolyzed molecules

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6.1 Introduction

Since protein molecules mediate most of the myriad cellular processes that drive biological systems, a detailed understanding of such moieties and their *modus operandi* gains considerable significance. Furthermore, given the importance of insights gained from such investigations, the ability to facilitate synthesis of rationally designed molecules bearing specific functions is therefore highly desirable. In addition to the usage of several genetic engineering techniques towards synthesis of molecules, the repertoire of rationally designed molecules can be augmented by employing generic methods to incorporate unnatural amino acids or other structures that would allow much greater diversity and precision in protein design. Protein engineering by structural manipulations at the level of the protein and not the gene has been extensively reviewed (Wallace, 1995). The inherent complexities involved in fabrication of protein molecules using semi-synthetic techniques have led to an extensive employment of proteases in fragment-condensation reactions. Enzymes have been known to offer high catalytic efficiency with an equally high degree of stereospecificity, thereby circumventing the intractable synthesis problems (Bordusa, 2002). Furthermore, since proteases are known to interact with peptide bonds thereby leading to their hydrolysis, these biocatalysts have been implicated in the reverse-catalytic condensation of carboxylic and amino termini of fragments, owing largely to the microscopic reversibility of reactions proposed by van't Hoff as early as 1898.

Protease-mediated reformation of the peptide bond has been shown to occur in aqueous organic milieu with the non-covalent complexes of rabbit muscle TIM and lysozyme (Vogel and Chmielewski, 1994, Vogel *et al.*, 1999). Peptide bond resynthesis in such cases has been attributed to an increase in the pKa of the carboxyl group of the carboxylic terminus thereby leading to a shift in the equilibrium towards synthesis (Homandberg *et al.*, 1978). Additionally, crowding agents such as PEG and dextran have been used to show resynthesis of peptide bond in rabbit muscle TIM (Somalinga and Roy, 2002).

Given the ubiquity of proteases in biological pathways as well as in industrial applications and the fact that these enzymes also bring about undesirable degradation of proteins during their isolation and characterization, attempts are therefore made to inhibit
the activity of these biocatalysts using molecules that act as substrate analogues. A wide variety of molecules have been found to act as serine protease inhibitors. However they can be broadly classified into two groups: the ones that facilitate inhibition by tightly binding onto the active site of proteases through non-covalent interactions, such as aprotinin, leupeptin or Chymotrypsin Inhibitor 2 (CI 2); whereas the other class of inhibitors includes those groups that bind covalently to the active site of serine proteases thereby affecting irreversible modification of the active site and consequently rendering them useless. Phenyl Methyl Sulfonyl Fluoride (PMSF) and 4-(2-Aminoethyl) Benzene Sulfonyle Fluoride (AEBSF) are examples of the second class of protease inhibitors that bind covalently onto the active-site residue by sulfonylation, thereby affecting inhibition. Previous studies pertaining to the generation of crystals of proteolyzed lysozyme as well as investigations of the structural features of proteolyzed GST employed Subtilisin Carlsberg as the protease. Therefore, the serine protease inhibitor, PMSF was used extensively for the inhibition of the enzyme in our studies. Interestingly, addition of the inhibitor was seen to facilitate religation of the nicked substrates. Further studies were therefore conducted to understand the mode by which religation could have been possibly accomplished by the protease inhibitor.

6.2 Results

6.2.1 Proteolysis and quenching of lysozyme-SC reaction

As in the earlier cases, lysozyme was subjected to proteolysis with SC at a molar ratio of 100:1 in the presence of 50mM Tris pH 8.0 and 2mM DTT at 37 °C. Aliquots were taken at regular intervals to monitor the progress of the reaction and the reaction mixture was finally quenched by the immediate addition of 2 mM PMSF dissolved in DiMethyl Sulfoxide (DMSO). Figure 6.1(A) depicts the Coomassie-stained 17% SDS-PAGE showing that SC facilitates the complete proteolysis of lysozyme within 10 min. from the initiation of the reaction. However, upon addition of the protease inhibitor, the proteolytic fragments were no longer detected. Instead a band showing mobility similar to that of the native molecule could be clearly seen on the gel, therefore possibly indicating
Figure 6.1: SDS-PAGE profiles of lysozyme and hydantoinase subjected to SC treatment. (A) Proteolysis of lysozyme and subsequent religation of the nicked molecules in the presence of PMSF (Lane 1: control lysozyme, lane 2: reaction after ~30 sec, lane 3: after 10 min., lane 4: after 30 min., lane 5: after 60 min., lane 6: after addition of PMSF. (B) Proteolysis of hydantoinase and subsequent religation of the nicked molecule in the presence of PMSF (Lane 1: marker, lane 2: control hydantoinase, lane 3: reaction after 5 min., lane 4: after 30 min., lane 5: after 60 min., lane 6: after 180 min., lane 7: after 210 min., lane 8: after addition of PMSF)
that the nicked termini of the proteolyzed lysozyme had religated to generate the native form upon addition of PMSF.

6.2.2 Proteolysis and inhibition of hydantoinase-SC reaction

In an attempt to observe the effect of PMSF quenching on the proteolysis reaction of a different substrate, D-hydantoinase, a 54 kDa, monomeric protein from the Bacillus sp. AR9 was expressed and purified to homogeneity using immobilized metal affinity chromatography (as described in the materials and methods section). Purified hydantoinase was subjected to proteolysis with SC at several w/w ratios of protein to the protease. However, complete proteolysis of the molecule was not observed. Experiments were performed with a 100:1 w/w ratio of hydantoinase: SC in the presence of 50mM Tris pH 8.0 at 37°C and the progress of the reaction was monitored by collecting aliquots at intermediate time points. Quenching of the proteolytic reaction was once again facilitated by the addition of 3 mM PMSF. Figure 6.1 (B) illustrates that the partially proteolyzed hydantoinase no longer exhibits fragmentation after addition of the inhibitor. Interestingly, all the cleaved peptide bonds of the substrate appeared to have religated upon treatment with PMSF, similar to the observations with lysozyme.

6.2.3 Proteolysis and inhibition of GST-SC reaction

Furthermore, to assess the ubiquity of PMSF-assisted religation of peptide bonds, GST was subjected to proteolysis with SC at 100:1 w/w ratio under conditions used for earlier studies pertaining to characterization of the non-covalent complex of the molecule. Upon treatment with 3mM PMSF after 10 min. from the initiation of the reaction, no bands corresponding to the proteolyzed fragments could be detected. However, a band similar to that of the native molecule could be clearly discerned from the SDS-PAGE (Figure 6.2(A)), thus suggesting that peptide bond reformation had taken place as a consequence of addition of the protease inhibitor.

Taken together the investigations performed, demonstrated the remarkable efficacy of PMSF in bringing about religation of the nicked termini of proteolyzed substrates.
Fig 6.2: SDS-PAGE proteolysis profiles of GST and RNase A subjected to treatment with SC. (A) Proteolysis of GST and subsequent religation upon addition of PMSF (Lane 1: control GST, lane 2: reaction mixture after ~20 sec., lane 3: reaction mixture after 5 min., lane 4: reaction mixture after 10 min., lane 5: after treatment with PMSF). (B) Proteolysis of RNase A and subsequent quenching of the reaction mixture with 3 mM PMSF (Lane 1: marker, lane 2: control RNase A, lane 3: after ~30 sec. from start of reaction, lane 4: after 10 min., lane 5: after 30 min., lane 6: after 60 min., lane 7: after 90 min., lane 8: after addition of PMSF, lane 9: 10 min. after addition of PMSF).
6.2.4 Proteolysis and inhibition of RNase-SC reaction

Since protease-catalyzed religation had been earlier demonstrated with RNase A in the presence of organic co-solvents, albeit with yields up to 50% after time periods of about two weeks (Homandberg et al., 1979), we attempted to assess the efficacy of PMSF in bringing about the religation reaction of this molecule. RNase A was subjected to proteolysis with SC at 100:1 w/w ratios in the presence of 50 mM Tris pH 8.0 and aliquots of the reaction mixture were collected for visualization of the proteolytic profile at different time points from the start of the reaction. Finally, quenching of the reaction was induced by the addition of PMSF to the proteolysis reaction mixture. Surprisingly, no band with mobility similar to that of native RNase A could be visualized on the SDS-PAGE (Figure 6.2(B)). Literature suggested that proteolysis of RNase A using SC results in the cleavage of peptide bond between Ala 20 and Ser 21 residues that mapped to a flexible loop region connecting the RNase S (21-124) with the helical S-peptide (1-20) (Richards and Vithayathil, 1959; Kim et al., 1992). Comparison of the results obtained from these studies with those gained from studies of lysozyme and GST suggested that the inherent conformational flexibility of the loop in RNase A augmented the entropic barrier towards peptide bond formation.

6.2.5 Proteolysis and inhibition of GST-Eps8SH3 fusion protein-SC reaction

To further analyze the effect of conformational flexibility on PMSF mediated religation, GST-Eps8 SH3 fusion protein was purified to homogeneity and proteolysis was conducted with 100:1 w/w ratio of the fusion protein with SC in the presence of 50 mM Tris pH 8.0 at 37 °C. Figure 6.3 illustrating the proteolytic profile of the fusion protein indicates that addition of PMSF resulted in the generation of a religated molecule with mobility similar to that of intact GST. However, the religation reaction did not result in the generation of the fusion protein, thereby indicating that the flexible linker region between GST and the fusion partner SH3 obviated the generation of the fusion protein.
Figure 6.3: **Proteolysis profile of GST-Eps8 SH3 upon treatment with SC.** SDS-PAGE profile showing the proteolysis of GST-Eps8SH3 fusion protein and subsequent effect of quenching by PMSF. (Lane 1: marker, lane 2: control GST-Eps8SH3, lane 3: reaction at 5 min., lane 4: at 10 min., lane 5: at 30 min., lane 6: at 60 min., lane 7: after addition of PMSF). Profile depicts the change in mobility between the control molecule and the religated product.
6.2.6 Role of DMSO in PMSF mediated religation

DMSO has been shown to enhance the religation reaction in case of lysozyme (Vogel et al., 1996), albeit at high concentrations. Since PMSF used for facilitating inhibition in our proteolysis reactions was dissolved in DMSO, the ability of this molecule to facilitate religation was studied using lysozyme and GST as model systems. Since the final concentration of DMSO consequent upon addition of PMSF to the reaction mixture was 1%; similar, five-fold and ten-fold higher concentrations of DMSO were added to the lysozyme reaction mixture. Results showed that no religation seemed to have occurred at such concentrations of DMSO therefore precluding the possibility of this reagent in bringing about religation (Figure 6.4(A)). Similar experiments performed with proteolyzed GST reiterated the inability of DMSO in religating the nicked GST molecule (Figure 6.4(B)). Furthermore, 3 mM of a water-soluble analog of PMSF, 4-(2-Amino)ethyl benzene sulfonyl fluoride (AEBSF) was used to bring about inhibition of the proteolytic reaction with GST as the substrate. Figure 6.4(C) depicting the effect of AEBSF on GST shows that near-complete religation of nicked GST occurred in the presence of AEBSF, therefore suggesting that DMSO used at low concentrations in the earlier proteolysis reaction mixtures had little role to play in aiding religation. Additionally, results asserted the role of sulfonyl fluoride inhibitors in assisting the religation reaction.

6.2.7 Role of protease in religation of proteolyzed lysozyme and GST

Since the only other component, apart from DMSO and PMSF in the reaction mixture was SC, attempts were made to understand the role of subtilisin in aiding religation. To that effect, protease VIII A cross-linked to 4 % beaded-agarose was used to affect proteolysis of lysozyme as well as GST under conditions used earlier. Calculations were performed to ensure that the substrate: protease ratio remained unchanged from the previous experiments. Proteolysis of lysozyme was carried out for 30 min. and the supernatant was then collected by centrifuging the reaction mixture at 8000 g for 1 min. Furthermore, the supernatant was applied to ~100 μl of p-aminobenzamide beads pre-equilibrated with 50 mM Tris pH 8.0. Following incubation for 20 minutes with intermittent mixing of the beads, the supernatant was recovered by centrifugation of the
Figure 6.4: SDS-PAGE profiles of lysozyme and GST depicting the effect of DMSO and AEBSF.
(A) Proteolysis of lysozyme with SC and subsequent addition of DMSO (Lane 1: marker, lane 2: control lysozyme, lane 3: reaction mixture at 30 min., lane 4: after addition of 1% DMSO, lane 5: after addition of 5% DMSO, lane 6: after addition of 10% DMSO). (B) Proteolysis of GST and subsequent addition of DMSO (Lane 1: marker, lane 2: control GST, lane 3: reaction mixture at 5 min., lane 4: after addition of 1% DMSO, lane 5: after addition of 10% DMSO). (C) Gel profile depicting the effect of AEBSF on proteolyzed GST (Lane 1: marker, lane 2: control GST, lane 3: reaction mixture after 5 min., lane 4: after 10 min., lane 5: after addition of AEBSF).
sample at 8000 g for 1 min. In order to remove most of the protease, the step involving p-
aminobenzamidine bead incubation was performed at least five times prior to the final
recovery of the supernatant. 3 mM PMSF was then applied onto the supernatant and the
sample was subsequently prepared for visualization on a 17 % SDS-PAGE. Results
showed that a band corresponding to the native lysozyme was found to occur upon
addition of PMSF to the protease-deficient supernatant (Figure 6.5(A)). In a similar
experiment conducted with proteolyzed GST, application of PMSF to the supernatant
resulted in the generation of intact GST molecule (Figure 6.5(B)), therefore hinting
towards protease-inhibitor assisted religation being a protease-independent phenomenon.
Additionally, determination of the amount of protease present in the supernatant using the
standard FITC-casein assay indicated that the amount of SC in the supernatant was as low
as 25 ng. Therefore suggesting that protease removal resulted in much lower amounts of
protease than that detected for reactions carried out in the presence of SC. Since the rate
of appearance of the religated molecule (GST or lysozyme) is quite similar for proteolysis
reactions conducted in the absence and in the presence of protease, the insignificant role
played by the protease is further evidenced.

6.2.8 Effect of PMSF on crystals of nicked lysozyme

Since our results showed a stark difference from the earlier studies pertaining to
protease-catalyzed religation, further elucidation of the insignificance of proteases in
PMSF-mediated religation was deemed necessary. Amongst the several techniques
employed to purify proteins from other associated impurities, crystallization has been one
of the most primitive. Although little data is available pertaining to the feasibilty of
protein crystallization as a tool for protein purification, recent research has shown
potential for this age-old technique as a means for purifying proteins. Studies performed
to elucidate the purity of lysozyme crystals as a consequence of accompanying
macromolecular impurities such as avidin, ovalbumin and conalbumin at concentrations
as high as 50 % have shown that high crystal purity (> 99.99 %) was observed for
lysozyme (Judge et al., 2000). Since crystals of proteolyzed lysozyme obtained after
removal of the protease were readily available, the effect of PMSF on the highly
homogeneous population of nicked lysozyme in the crystals was studied. Crystals of
nicked lysozyme were washed thoroughly with 50 mM Tris pH 8.0 buffer and the
Figure 6.5: SDS-PAGE profiles depicting proteolysis of lysozyme and GST attached to agarose beads and the effect of addition of PMSF to the protease-deficient supernatant (A) Proteolysis of lysozyme and subsequent effect of PMSF addition (Lane 1: marker, lane 2: control lysozyme, lane 3: reaction after 10 min., lane 4: after 30 min., lane 5: after 60 min., lane 6: supernatant of reaction mixture collected after centrifugation, lane 7: addition of PMSF to supernatant). (B) Proteolysis of GST and subsequent effect of PMSF addition (Lane 1: marker, lane 2: control GST, lane 3: reaction mixture after 5 min., lane 4: reaction mixture supernatant obtained after centrifugation, lane 5: effect of PMSF on supernatant).
surrounding milieu along with the crystal was repeatedly dried with the help of fine bore capillaries or paper wicks. Following several rounds of washing and drying, a population of the crystals to which PMSF had been added was prepared for SDS-PAGE analysis along with another set wherein no such addition was performed. Figure 6.6 illustrates that addition of PMSF to the highly purified population of lysozyme in the crystals resulted in efficient religation of the substrate molecule, therefore implying that PMSF alone could facilitate religation of the nicked species of lysozyme.

6.2.9 Effect of variable concentrations of PMSF on the religation of GST

Since results showed that religation of GST and lysozyme was essentially dependent on the protease inhibitor, studies were taken up to assess the effect of variable concentrations of the inhibitor on the religation of GST. Variable concentrations of PMSF (1nM, 10nM, 100nM, 1μM, 10 μM, 100 μM and 1mM) were added to the reaction mixture containing 366 nM of SC, after 5 min. from the start of the reaction (Figure 6.7). Aliquots were taken at regular intervals to observe the progress of the reaction in the presence of the inhibitor. Results indicated that no considerable religation of GST was seen to occur at concentrations of PMSF upto 1.0 μM even after 4 hrs. from the addition of PMSF (Figure 6.7(D)). However, at a concentration of 10 μM PMSF, immediate religation of GST was observed with the profile remaining unchanged upto 4 hrs from the time of addition of PMSF. Furthermore, the addition of higher concentrations of the inhibitor showed a gel profile that was similar to that obtained at a concentration of 10 μM, which was in effect ~25 fold higher than the concentration of SC used. Data therefore suggests that the extent of GST religation depends largely on the concentration of PMSF used, with low concentrations of PMSF unable to aid religation. Literature reveals that, depending upon the pH of the medium and the inhibitor-protease ratio, sulfonyl fluoride compounds are known to form covalent adducts with protein molecules (PCT/EP97/03668). More importantly, tyrosine and lysine residues along with the free amino terminus have known to be linked with these compounds (www.roche-applied-applied-science.com). It was therefore surmised that PMSF would perhaps act by binding onto the groups at the nicked termini, thereby leading to their activation.
Figure 6.6: SDS-PAGE profile illustrating the effect of PMSF on crystals of nicked lysozyme. (Lane 1: marker, lane 2: control lysozyme, lane 3: crystal of nicked lysozyme, lane 4: crystal of nicked lysozyme after PMSF addition)
Figure 6.7: **SDS-PAGE profiles illustrating the effect of variable concentrations of PMSF on proteolyzed GST.** Each gel profile (A-F) shows a control GST (lane 1), reaction mixture after 5 min. (lane 2), immediately after addition of PMSF (lane 3), 30 min. after PMSF addition (lane 4), 1 hr. after addition of PMSF (lane 5), 2 hrs. after addition of PMSF (lane 6), 3 hrs. after addition of PMSF (lane 7), 4 hrs. after addition of PMSF. (A) reaction mixture containing 1.0 nM PMSF, (B) reaction mixture containing 10 nM PMSF, (C) reaction mixture containing 100 nM PMSF, (D) reaction mixture containing 1 μM PMSF, (E) reaction mixture containing 10 μM PMSF, (F) reaction mixture containing 100 μM PMSF, (G) reaction mixture containing 1 mM PMSF (lane 1: reaction mix. after 5 min., lane 2: control GST, lane 3: immediately after PMSF addition, lane 4: 30 min. after PMSF addition, lane 5: 1 hr. after PMSF addition, lane 6: 2 hrs. after PMSF addition, lane 7: 3 hrs. after PMSF addition).
6.2.10 Effect of p-toluene sulfonyl chloride (tosyl chloride) on proteolysis of lysozyme

Experiments were therefore performed wherein attempts were made to prevent PMSF-assisted religation by protecting the amino terminus using tosyl chloride, a compound not known to be used as a protease inhibitor. Furthermore, owing to the similarity of the sulfonyl groups present in AEBSF, PMSF with tosyl chloride, this molecule proved to be a suitable candidate for experiments done with a perspective of preventing religation. 3mM concentration of tosyl chloride was applied to proteolyzed lysozyme obtained as a result of the protease-on-beads reaction. Following incubation of proteolyzed lysozyme with tosyl chloride for 10 min., an aliquot was prepared subsequently for visualization on the SDS-PAGE (Figure 6.8). Interestingly, treatment of the nicked molecule with tosyl chloride showed religation of the nicked form, therefore hinting towards a lowering of the enthalpic barrier of the termini and subsequent peptide bond synthesis.

6.2.11 Biophysical characterization of religated GST

In order to assess the properties of GST obtained as a result of protease-inhibitor religation, spectroscopic and chromatographic examinations were performed with the religated molecules and subsequently comparisons were made with the native counterpart. SDS-PAGE profiles shown for the religation reactions indicated that the religated molecule bore mobility similar to that of the native molecule, therefore suggesting that all the cleaved peptide bonds had been religated upon addition of PMSF. Size exclusion chromatography showed that the religated molecule obtained as a consequence of PMSF addition exhibited an elution profile similar to that of the native GST, thereby suggesting that the quaternary, dimeric structure of religated molecule was same as that of the native counterpart (Figure 6.9(A, B)). Furthermore, fluorimetric investigations of religated GST showed that the tertiary structure was similar to that of the native molecule (Figure 6.9(C)). Activity studies performed with religated GST indicated that the molecule showed nearly 98 % activity in comparison to the native GST. Finally, secondary structural estimation of the religated GST indicated that the overall secondary structural content of the religated molecule was unchanged (Figure 6.9(D)).
Figure 6.8: SDS-PAGE profile depicting the effect of tosyl chloride on protease-deficient supernatant of lysozyme. Gel shows proteolysis profile of lysozyme in the presence of SC on beads and the subsequent effect of tosyl chloride addition. (Lane 1: marker, lane 2: control lysozyme, lane 3: reaction mixture after 2 min., lane 4: after 30 min., lane 5: supernatant obtained after removal of protease beads, lane 6: effect of tosyl chloride on supernatant)
Figure 6.9: Biophysical characterization of PMSF-religated GST. (A) Gel filtration chromatogram of native GST (elution volume indicated) (B) Gel filtration chromatogram of religated GST (elution volume indicated) (C) Comparative fluorescence emission spectra of religated and native GST (D) Comparative CD spectra of religated and native GST
together, the biophysical and biochemical characterization of religated GST indicates little differences with the native counterpart.

6.3 Discussion

Protease-catalyzed semi-synthesis of protein molecules has been in vogue for quite some time now. Application of organic co-solvent milieu to the proteolysis reaction mixture at concentrations as high as 60-90% have been used to reform the peptide bond of cleaved substrates (Vogel et al., 1996). In addition, studies with rabbit muscle TIM utilizing crowding agents such as PEG and dextran have shown that for efficient religation to occur, the product molecule must exclude a volume that is lesser than that excluded by the reactant molecule. The concept of protease-catalyzed peptide bond synthesis had been propounded on the basis of the theory of microscopic reversibility of reactions furthered by van't Hoff in 1898. Since proteases are known to be reactive towards the peptide bond, the reverse-catalysis phenomenon seen in the proteolysis reaction mixtures was easily attributed to them. Covalent bond formation in any system has been understood to utilize energy in order to overcome the entropic and the enthalpic barriers towards the synthesis of bonds. Additionally, it has also been realized that the greater the energy barrier (entropic and enthalpic) towards bond formation, the lesser is the probability of its generation. Researchers have demonstrated that the ribosome acts as an "entropy trap" whilst facilitating peptide bond formation (Sievers et al., 2004). Furthermore, substrate-assisted catalysis as facilitated by the A76 2' OH of the P-site tRNA has also been suggested to aid peptide bond synthesis in the ribosome (Weinger et al., 2004). Based on these observations, it has been indicated that peptide bond synthesis by the ribosome does not follow the general acid-base catalysis mechanism. On the contrary, reduction in entropy of the reacting termini and their activation by the A76 2' OH moiety of tRNA is sufficient to catalyze bond formation. Enzymes have been widely used to lower the energy barrier towards the synthesis of desired products by providing a microenvironment wherein the enthalpic barrier is lowered owing to activation of the reacting termini due to interactions with atoms in the active site. Furthermore, the active site serves to augment the proximity of the reacting termini as a consequence of non-
covalent interactions between the catalyst and the substrate. Understandably, activation of the reactive termini and their proximity would augment the formation of product.

Our results pertaining to religation showed that reformation of the peptide bond in nicked species occurred in the absence of protease. Thus suggesting a remarkable departure from the hitherto accepted concept of peptide bond reformation in the presence of proteases being essentially catalyzed by the enzyme itself. PMSF, the serine protease inhibitor generally employed for quenching proteolysis reactions has been shown to bind stoichiometrically to the active site of SC. Sulfonylation of the $\text{O}\gamma$ atom of the serine residue in the catalytic site leads to an irreversible inhibition of the catalytic activity of SC. Structural studies performed with the inhibitor complexed with SC have shown that access to the other catalytic site residues is completely obliterated as a consequence of the binding of PMSF to the active site serine. Since the active site of SC is blocked due to the presence of the inhibitor, we proposed that the protease is rendered useless in catalyzing either proteolysis or religation reactions. Therefore leading to the important question pertaining to the religation of peptide bonds in absence of the protease. Furthermore, to explore the extent of universality of PMSF in facilitating religation, we sought to use diverse systems. Experiments performed with hydantoinase and GST suggested that the religatory potential of the protease inhibitor could be extended to several systems. However, the absence of religated product in the RNase A reaction raised further questions about the generality of PMSF as a religation-facilitating agent. Literature suggested that studies performed with proteolyzed RNase A in the presence of glycerol led to the formation of 50% religated product only after a time period as long as about two weeks in contrast to other systems wherein efficient religation was observed as early as 10 min. from the addition of the organic co-solvent (Vogel et al., 1996). Additionally, crowding agents have been found not to assist in reformation of the cleaved peptide bond in RNase A (Somalinga and Roy, 2002). Fragment complementation studies performed on RNase A showed that subtilitic cleavage occurred at the peptide bond between the Ala 20 and Ser 21 residues that mapped to a flexible region in the RNase A molecule. Furthermore, structural studies of the nicked species of RNase A by Kim et al. (1992) showed that crystallographic densities were absent for residue 16-23 in the nicked form of RNase A, therefore suggesting an inherent flexibility of these residues as a consequence of proteolysis. It was therefore thought that religation on the presence of PMSF was not facilitated largely due to the inherent flexibility of the nicked termini of the molecule. To
further attest the importance of lowering of entropy in the phenomenon of peptide bond reformation, we subjected the fusion protein GST-Eps8SH3 to proteolysis with SC. GST-Eps8SH3 fusion protein was a suitable choice for such studies, since the fusion molecule comprises the GST module known to show religation in the presence of PMSF, in addition to a flexible linker region known to connect the Eps8SH3 domain to the GST molecule. Results showed that only the religated GST molecule was obtained as a result of PMSF addition. Absence of the religated fusion protein consequent upon addition of PMSF, suggested that the high entropic barrier posed by the linker region prevented facile religation of the SH3 domain with the GST module, thus highlighting the importance of entropic compensation in PMSF-assisted religation reactions.

In an attempt to ensure that the religation reaction in the presence of PMSF had no role of the protease, we sought removal of SC from the reaction mixture after proteolysis had been performed. Proteolysis reactions of lysozyme and GST with protease linked to agarose beads showed that the nicked molecule obtained in the supernatant after centrifugation appeared at mobility similar to that of the native counterpart, therefore suggesting that proteases might not be needed for religation when PMSF was added. Additionally, studies performed to assess the amount of protease in the supernatant indicated that much lower amounts of the protease was detected for the supernatant. Furthermore, since the rates of appearance of the religated molecules (lysozyme and GST) were similar in the presence and in the absence of the protease, the insignificance of proteases in catalyzing the religation reaction is further confirmed.

Since our investigations reveal a novel perspective of the protease-catalyzed religation reactions, further experiments showing the absence of proteases were fundamental to further this viewpoint. Since the purity of lysozyme crystals has been demonstrated to be as high as 99.99% even in the presence of high concentrations of other proteins with concentrations as high as 50%, experiments were performed with the highly purified population of nicked lysozyme in the crystals. Results indicated that the addition of PMSF to the thoroughly washed and dried crystals lead to the formation of intact lysozyme with mobility similar to that of the native molecule. Additionally, the absence of any fragments in the religated sample suggested that the entire population of nicked lysozyme comprising the crystal had religated. Results obtained with religation observed after removal of proteases from the solution and studies with the nicked lysozyme in crystals, therefore confirmed that religation was mediated primarily by PMSF.
Additionally, investigations performed to show that DMSO, the solvent used for solubilization of PMSF did not aid in religation, demonstrated that AEBSF, a water-soluble analog of PMSF also facilitated religation of nicked termini. Therefore, it can be further concluded that sulfonylating agents led to efficacious religation.

On the basis of the results obtained thus far, it is argued that the sulfonylating agents not only lead to modification of the catalytic site of serine proteases, but also bind onto the amino groups available in proteins. Furthermore, literature showed that sulfonyl compounds undergo non-specific reactions with lysine residues as well as the free-amino terminus (PCT/EP97/03668). To further understand the role played by PMSF and AEBSF in religation, we sought to block the reactive group at the nicked termini using tosyl chloride. Extensive review of literature pertaining to properties exhibited by tosyl chloride showed that it was not widely used as a protease inhibitor, however derivatives of this compound such as N-tosyl-L-lysine-chloromethyl ketone (TLCK) and tosyl phenylalanyl chloromethyl ketone (TPCK) have been used extensively as serine protease inhibitors. Additionally, reports are available wherein the compound has been used as a protecting agent for amino groups that is readily cleaved (www.chemicalland21.com). Experiments conducted with the objective of blocking the nicked termini in lysozyme in order to prevent religation by PMSF led to the usage of p-toluene sulfonyl chloride in these experiments. Interestingly, religation was observed to be facilitated in the presence of tosyl chloride. Insights gained from literature suggested that tosyl chloride behaves as a good leaving group in nucleophilic reactions owing to the distribution of negative charge over the three oxygen atoms. Furthermore, since both PMSF as well as AEBSF contain sulfonyl groups like toluene sulfonyl chloride, we therefore suggest that the attack of the sulfonyl groups of PMSF and AEBSF on the nicked termini leads to the activation of the terminal groups, thereby facilitating lowering of the enthalpic barrier of the nicked termini.

Overall, the results present a novel perspective of peptide bond religation and gains importance due to the remarkably low concentrations of religating agents used for bringing about peptide bond formation.