Chapter 4

STABILITY STUDIES ON THIOL PROTEASES
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INTRODUCTION

Stability of the protein is a function of external variables such as pH, temperature, ionic strength and solvent composition. To understand the structural and functional properties of an enzyme under different solvent conditions, is fundamentally important for both theoretical and applicative reasons; as these studies may provide insight into the molecular basis of the stability of the enzyme. Designing protocols or a protein with special properties for biotechnological applications require such results. A simple method for such studies involves monitoring of conformational changes due to perturbation of a protein molecule by various agents such as GdnHCl, urea, temperature and cosolvents [87]. One such cosolvent is poly(ethylene glycol) (PEG)- a nonpolar polymer of ethylene oxide. PEGs have different physical properties (ex- they may be liquid or low-melting solid) depending on their molecular weights, however their chemical properties are nearly identical. PEGs have the general structure: HO-(CH₂-CH₂-O)ₙ-H. The numbers that are often included in the names of PEGs indicate their average molecular weights, e.g. a PEG with n=80 would have an average molecular weight of approximately 3500 Daltons and would be labeled PEG 3500. PEG is a hydrophillic nonionic nontoxic polymer used in many biochemical and industrial applications such as cosmetics, food and pharmaceutical products. The success of this polymer in biotechnological applications depends on its mild action on the biological activity of cell components. Apart from that, PEG is also used for liquid-liquid partitioning and precipitation of biomacromolecules [88-90] for protein crystallography. Due to the extensive practical uses of PEGs, it is of fundamental importance to understand the conformational changes occurring in the protein conformation and activity in PEG-water solution.
Thiol proteases (ex- Bromelain, papain, chymopapain) are industrially very significant proteins. These belong to α+β class of proteins and have highly similar amino-acid sequence [91-93]. Thermal denaturation profile of these proteins has been found to be completely irreversible [94-95]. In this work, we have monitored the activity and conformational changes in these enzymes induced by PEG solutions of different molecular weights, i.e. 20000, 6000 and 400. Activity measurements (at different temperatures) showed that PEGs lead to destabilization of these three proteins. Far UV-CD measurements showed that PEGs lead to reduction in CD signals of papain. Fluorescence measurements indicated that papain unfolds in presence of increasing concentrations of PEGs. This observation of destabilization of thiol proteases by PEGs of different molecular weights may have important implications in industrial processes where PEG is used.

**RESULTS**

**4.1. Activity measurements**

To understand the function of thiol proteases in PEG-water solution, we performed the activity assay of these three enzymes using synthetic substrate (Z-L-Lys-ONp hydrochloride). Figure 4.1 shows the effect of 10% (w/v) PEG (20000, 6000 and 400) on activity of different thiol proteases, as a function of temperature. As can be seen from figure 4.1A, papain showed maximum activity around 50°C at pH 4-5 and activity decreased on either side of this temperature (curve 1). The figure also shows that all the PEGs used decrease the activity of papain. In the temperature range 30-70°C, papain was maximally destabilized by PEG 400 (curve 2), and to lesser extent by PEG 6000 (curve 3) while PEG 20000 was found to be the least destabilizer (curve 4), according to activity measurement data. Thus the order of activity of papain in different solutions was found to be:

Papain (at pH 4.5) > Papain + PEG 20000 > Papain + PEG 6000 > Papain + PEG 400
Figure 4.1B shows activity changes of bromelain at pH 4.5 (curve 1), in presence of 10% (w/v) PEG 400 (curve 2), PEG 6000 (curve 3) and PEG 20000 (curve 4). The results depicted that in the temperature range 30-70°C the order of activity of bromelain in different solutions was found to be:

Bromelain (at pH 4.5) > Bromelain + PEG 400 > Bromelain + PEG 6000 > Bromelain + PEG 20000.

Figure 4.1C shows temperature profile of Chymopapain activity at pH 4.5 (curve 1), in presence of PEG-400 (curve 2), PEG-6000 (curve 3) and PEG-20000 (curve 4). As can be seen here, addition of PEGs had insignificant effect on the activity of chymopapain. However, it can be seen from the curve that PEG 20000 maximally destabilized chymopapain.

Thus the results presented in figure 4.1 lead us to conclude that different PEGs have different degree of destabilization of the three proteins belonging to same structural class. However the general conclusion driven from these experiments was that all the three PEGs used, of low and high molecular weights, lead to destabilization of the thiol proteases. Since the maximum difference in activity of protein in absence and presence of different PEG solutions was found to be in the case of papain, we performed further studies on papain. Structural perturbations in papain were monitored by spectroscopic methods, i.e. far UV-CD for changes in secondary structure and fluorescence measurements as a probe for tertiary structure.
Figure 4.1: (A) Temperature profile of Papain activity at pH 4.5 (curve 1), in presence of PEG-400 (curve 2), PEG-6000 (curve 3) and PEG-20000 (curve 4). (B) Temperature profile of Bromelain activity at pH 4.5 (curve 1), in presence of PEG-400 (curve 2), PEG-6000 (curve 3) and PEG-20000 (curve 4). (C) Temperature profile of Chymopapain activity at pH 4.5 (curve 1), in presence of PEG-400 (curve 2), PEG-6000 (curve 3) and PEG-20000 (curve 4).
4.2. CD measurements

Figure 4.2 shows the effect of different molecular weight polyethylene glycols concentrations (PEG-400, 6000 and 20000) on papain at pH 4.5 as monitored by the measurements of MRE at 222 nm. Alteration in the ellipticity at this wavelength is a useful probe for monitoring varying secondary structure contents. The figure shows that with increasing concentrations of PEGs significant decrease in the $MRE_{222}$ was observed, indicating that there was disruption of secondary structure content of the protein.

![Plot of MRE at 222nm of Papain in presence of PEG 20000 (■), 6000 (∆) and 400 (●).](image)

To ascertain the extent of disruption of protein structure by PEGs, we compared far UV-CD spectra of papain at pH 4.5 (curve 1), papain in presence of 10% PEG w/v 20000 (curve 2), 10% w/v PEG 6000 (curve 3) and 10% w/v PEG 400 (curve 4) in Figure 4.3A. Spectrum of papain at pH 4.5 reveals two negative peaks at 222 and 208 nm that are characteristic of helical content of the protein [96]. Maximum decrease in far UV-CD spectra was observed in case of PEG-400 and least with
PEG 20000 with PEG 6000 showing intermediate values, as was also supported by activity measurements (figure 4.3A). These results suggested that addition of PEGs, lead to loss of secondary structure content of papain. Figure 4.3B shows far UV-CD spectra of papain at pH 4-5 (curve 1); papain in presence of 45% w/v PEG 20000 (curve 2), 45% w/v PEG 6000 (curve 3), 90% w/v PEG 400 (curve 4), indicating that at very high concentration of PEGs, far UV-CD spectra had very similar ellipticity values. At low concentrations of PEGs (i.e. 10% w/v) extent of disruption of secondary structure was different while at high concentration where maximum disruption of structure was observed, loss of structure upto similar extent was observed for all the three PEGs studied.

Figure 4.3: (A) Far UV-CD spectra of papain at pH 4-5 (curve 1); papain in presence of 10% w/v PEG 20000 (curve 2), 10% w/v PEG 6000 (curve 3) and 10% w/v PEG 400 (curve 4)
(B) Far UV-CD spectra of papain at pH 4-5 (curve 1); papain in presence of 45% w/v PEG 20000 (curve 2), 45% w/v PEG 6000 (curve 3) and 90% w/v PEG 400 (curve 4).
4.3. Fluorescence measurements

4.3.1. Intrinsic Fluorescence: As the intrinsic fluorophore tryptophan is highly sensitive to the polarity of its surrounding environment, PEGs induced changes in conformation of papain were monitored using fluorescence spectroscopy. Papain contains 5 tryptophan residues out of which three are buried in hydrophobic core while two are located near the surface of the molecule [97]. Figure 4.4A shows changes in fluorescence intensity of papain at 340 nm with increasing concentration of PEGs, the changes in wavelength maxima are depicted in figure 4.4B and the spectra of papain in presence of PEGs can be seen from figure 4.4C. As can be seen from the figures, with increasing concentration of PEGs fluorescence intensity at 340 nm increased with blue shift in wavelength maxima indicating that structural changes occurred in the vicinity of tryptophan residues. Since PEGs are non-polar polymers, interaction of protein with increasing concentration of PEGs may lead to the observed blue shift. This indicated that tryptophan residues moved to more hydrophobic environment in the presence of PEGs.
Figure 4.4: (A) Changes in Fluorescence Intensity at 340 nm of Papain in presence of increasing concentration of PEG 20000 (■), 6000 (△) and 400 (●).
(B) Changes in wavelength maxima of Papain in presence of increasing concentration of PEG 20000 (■), 6000 (△) and 400 (●).
(C) Intrinsic fluorescence spectra of Papain at pH 4.5 (curve 1); papain in presence of 40% w/v PEG 20000 (curve 2), 40% w/v PEG 6000 (curve 3), 80% w/v PEG 400 (curve 4).

4.3.2. Acrylamide Quenching Experiments:
The topological properties of proteins can be obtained using fluorescence properties of tryptophan residues. To confirm the extent of exposure of tryptophan residues in papain in different PEG solvent systems, we conducted fluorescence quenching experiment using uncharged molecules of acrylamide. Figure 4.5 shows Stern Volmer plot of papain at pH 4.5 (curve 1), papain in presence of 40% w/v PEG 20000 (curve 2), 40% w/v PEG 6000 (curve 3) and 80% w/v PEG 400 (curve 4); while the Stern-Volmer constants ($K_{sv}$) are compared in Table 4.1. Results for the tryptophan analogue NATA (curve 5) are also included as a standard for complete accessibility to quencher.
As given in table 4.1, $K_{sv}$ was found to be maximum for papain in presence of 80% w/v PEG 400 followed by 40% w/v PEG 6000 and then for protein in 40% w/v PEG 20000 and was minimum for papain in buffer. This implies that tryptophan residues were maximally exposed in presence of PEG 400, relatively less exposed in PEG 6000, even lesser in case of PEG 20000 and were least accessible to quencher in buffer, indicating that addition of PEGs to the buffer lead to exposure and greater solvent accessibility of these residues. These results corroborate with activity measurement data pertaining to destabilization of protein in presence of PEGs.

Figure 4.5: Stern Volmer plot of Papain at pH 4.5 (◊, curve 1), papain in presence of 40% w/v PEG 20000 (■, curve 2), 40% w/v PEG 6000 (△, curve 3) and 80% w/v PEG 400 (●, curve 4). Curve for NATA has been included for comparison (×, curve 5)
ABLE 4.1: Acrylamide quenching data of papain in different PEGs

<table>
<thead>
<tr>
<th>Particulars</th>
<th>$K_{sv} \text{ (M}^{-1}\text{)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain at pH 4.5</td>
<td>4.4</td>
</tr>
<tr>
<td>Papain + 40% PEG 20000</td>
<td>6.3</td>
</tr>
<tr>
<td>Papain + 40% PEG 6000</td>
<td>7.2</td>
</tr>
<tr>
<td>Papain + 80% PEG 400</td>
<td>8.5</td>
</tr>
<tr>
<td>NATA</td>
<td>21.5</td>
</tr>
</tbody>
</table>

4.3.3. **ANS Binding Experiment:**

PEGs are non-polar solvents and hence the possibility that interaction of PEG with papain involves hydrophobic sites can be tested using ANS that has widely been used as fluorescence probe for hydrophobic patches. ANS acquires fluorescence emission when bound to protein while emission of free ANS is negligible. Therefore, if ANS is displaced from its binding sites on papain by competition with PEG, a decrease of its fluorescence would be induced.

Figure 4.6 compares extrinsic fluorescence spectra of ANS bound to papain (curve 1) with that of ANS bound to papain in presence of 50% w/v PEG 400 (curve 2). It can be seen that ANS binds to papain with $\lambda_{\text{max}}$ at 470 nm. When papain was incubated in PEG-400 before addition of ANS, fluorescence spectrum showed decrease in intensity together with red-shift to 490 nm, a characteristic of free ANS. Similar results were also obtained with PEG 20000 and 6000 (data not shown). This shows that interaction of papain with PEG lead to displacement of ANS, probably due to competition for hydrophobic sites.
4.4. Thermal denaturation studies

The structural stability of the papain in presence of 10% (w/v) PEGs was determined by temperature induced unfolding, followed by MRE measurements at 222 nm. Figure 4.7 shows the normalized transition curves for thermal unfolding of papain (pH 4.5) and in presence of 10% (w/v) PEG- 20000, 6000 and 400. As can be seen from the figure, papain at pH 4.5 showed cooperative transition, while in presence of PEGs transition became non-cooperative one, with PEGs leading to destabilization of papain. Temperature at mid-point of transition (Tm) (Table 4.2) has been used as a measure of stability as free energy change, $\Delta G$ could not be determined because of the irreversibility of the unfolding process. Comparison of Tm at 10% (w/v) of different PEGs indicated that PEG-400 maximally destabilized papain, followed by PEG 6000 and least by PEG 20000; as also supported by previous experiments. These results, together with decrease in
activity measurements and in far UV-CD spectra with increasing concentration of PEGs, indicated that the protein was denatured in PEG-water solution.

![Figure 4.7: Thermal denaturation profile showing changes in MRE at 222nm of Papain at pH 4·5 (◇), papain in presence of 10% w/v PEG 20000 (■), 6000 (△) and 400 (○).](image)

**TABLE 4.2: Thermal transition midpoint of papain in different PEGs**
(Obtained from Temperature-induced normalized transition curves shown in figure 4.7)

<table>
<thead>
<tr>
<th>Protein state</th>
<th>T_m (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papain at pH 4·5</td>
<td>73</td>
</tr>
<tr>
<td>Papain + 10% w/v PEG 20000</td>
<td>70·5</td>
</tr>
<tr>
<td>Papain + 10% w/v PEG 6000</td>
<td>69</td>
</tr>
<tr>
<td>Papain + 10% w/v PEG 400</td>
<td>68·5</td>
</tr>
</tbody>
</table>
DISCUSSION

Preferential interaction is a thermodynamic measure of how solute and solvent redistribute around a protein in solution [98]. If water is enriched near the surface relative to its composition in bulk solution, there is preferential hydration of the protein molecule. If this is the situation, it stands to reason that if water is enriched near the surface then the solute will be in deficit, so the condition of preferential hydration is also one of preferential exclusion of solute. The condition, where the solute concentration at or near the protein surface is enriched relative to that in bulk solution, is described as solute binding or preferential interaction of solute with the protein. A strong correlation between solution stabilization and preferential interaction has been observed for many compounds. Those compounds that are strongly excluded from the protein surface stabilize proteins against various stresses imposed on the proteins in solution [99]. However PEG is an exception to this rule [100-102]. Exclusion of PEG from the protein surface is quite large, and even exceeds the level of exclusion achieved by the well-known protein stabilizers, for example- sugars, salts etc. However, PEG is a weak protein destabilizer and has been found to decrease the melting temperature of proteins [103,104].

Our results in this paper indicate that PEGs (20000, 6000 and 400) lead to destabilization of thiol proteases. The possibility to explain this effect could be based on the proposal by Lee and Lee [102] that high molecular-weight PEGs assume compact structure stabilized by intramolecular hydrophobic interactions, thereby having lower PEG solvent interaction than the fully extended ones. Accordingly the effective exclusion size of PEG should be reduced at high PEG concentration and the change in exclusion size should be lower for large PEGs; this allows penetration of hydration layer of the protein. Thus decrease in preferential exclusion of PEG (20000 and 6000) might lead to increase in interaction of PEG with the protein surface. Low molecular weight PEG (ex-400) may show preferential interaction with protein displacing the folded-unfolded
equilibrium to the unfolded form, that is manifested as significant decrease in secondary structure content and melting temperature of the protein. Thus destabilization of proteins by PEGs could be explained in two ways: due to its small molecules, low molecular weight PEG shows positive interaction with the unfolded state of protein while high molecular weight PEGs acquire a compact form which allows the interaction with protein.

Structural studies on papain suggest that protein assumes unfolded conformation in the presence of PEGs. PEG is a non-polar polymer that can assume a compact structure in aqueous solutions by intramolecular hydrophobic interactions. PEG can bind to hydrophobic sites on protein based on the fact that PEG is essentially non-polar [105]. PEGs may have stabilizing or destabilizing effect on proteins depending on their chemical nature. Thiol proteases are hydrophobic proteins rich in uncharged amino-acid residues (~82%) [91-93]. We propose that PEGs bind to the hydrophobic sites on the thiol proteases and hence lead to their destabilization. Evidence for claim can be drawn from the results obtained in ANS binding experiment (figure 4.6). This is in agreement with the findings of Pace and Marshal [106] that non-polar solvents destabilize hydrophobic proteins more than less-hydrophobic ones.

**SIGNIFICANCE OF THE STUDY**

On the basis of the results, it is possible to conclude that PEGs lead to destabilization of thiol proteases (papain, bromelain and chymopapain). However, extent of destabilization by different PEGs (20000, 6000 and 400) is different for the three proteases studied. Destabilizing effects of PEGs suggest that care should be exercised when PEG is used, even at room temperature, for protein salting out or crystallization or in industrial applications, for thiol proteases and for hydrophobic proteins in general.