J. Biochem. **142,** 65–72 (2007)
doi:10.1093/jb/mvm108

Effect of Polyethylene Glycols on the Function and Structure of Thiol Proteases

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Received December 19, 2006; accepted April 21, 2007 published online May 15, 2007

Thiol proteases are industrially significant proteins with catalytic efficiency. The effect of low, medium and high molecular-weight poly (ethylene glycol) (PEG- 400, 6000 and 20000) on the stability of thiol proteases (papain, bromelain and chymopapain) has been studied by activity measurements using synthetic substrate. Structural studies performed on papain by far UV circular dichroism spectroscopic measurements indicate that there is loss in secondary structure of the protein in presence of increasing concentration of PEGs. Intrinsic fluorescence measurements lead us to conclude that tryptophan residues of protein encounter non-polar microenvironment in presence of PEG solvent while acrylamide quenching shows greater accessibility of tryptophan residues of papain in presence of PEGs. Extrinsic fluorescence measurements lead us to conclude that PEGs bind to the hydrophobic sites on the protein and thus destabilize it. Thermal denaturation studies show that melting temperature of papain is decreased in presence of PEGs. Possible mechanism of destabilization is discussed next. The results imply that caution must be exercised in the use of PEGs with thiol proteases or hydrophobic proteins in general, for different industrial applications, even at room temperature.

Key words: Poly(ethylene glycol), protein stability, synthetic substrate, thiol proteases.

Abbreviations: ANS, 8-anilino-1-naphthalene-sulphonic acid; DMSO, dimethylsulphoxide; EDTA, ethylene diamine tetra-acetic acid; GdnHCl, guanidine hydrochloride; MRE, mean residue ellipticity; NATA, N-acetyl tryptophanamide; PEG, poly(ethylene glycol); Tm, mid point of transition; w/v, weight/volume ratio.

INTRODUCTION

Stability of the protein is a function of external variables such as pH, temperature, ionic strength and solvent composition. Understanding 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 the monitoring of conformational changes due to perturbation of a protein molecule by various agents such as guanidine hydrochloride (GdnHCl), urea, temperature and cosolvents (*1*). One such cosolvent is poly(ethylene glycol) (PEG)—a nonpolar polymer of ethylene oxide. PEGs have different physical properties (*e.g*. 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: $HO–(CH_2–CH_2–O)_n–H$. 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 3,500 Daltons and would be labeled PEG 3500.

PEG is a hydrophillic, non-ionic, non-toxic 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 precipitataion of biomacromolecules (*2–4*) for protein crystallography. Due to the extensive practical uses of PEGs, it is of fundamental importance to understand the conformational changes ocurring in the protein conformation and activity in PEG-water solution.

Thiol proteases (*e.g*. Bromelain, papain, chymopapain) are industrially very significant proteins. These belong to $\alpha + \beta$ class of proteins and have highly similar amino-acid sequence (*5–7*). Thermal denaturation profile of these proteins has been found to be completely irreversible (*8*, *9*). 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) show that PEGs lead to destabilization of these three proteins, although extent of destabilization by a particular PEG is different for different protein. Far UV-CD measurements show that PEGs lead to

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reduction in circular dichroism (CD) signals of papain. Fluorescence measurements indicate 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. These observations also suggest that care should be exercised when PEG is used for protein salting out or crystallization of proteins.

MATERIALS AND METHODS

Enzymes (Papain, bromelain, chymopapain) were obtained from Sigma Chemical Co. USA. PEG 20000, 6000 and 400 were purchased from Sisco Research Laboratories, India. All the other reagents were of analytical grade.

Assay for Protease Activity—Enzyme assays were done at room temperature in 10 mM Sodium acetate buffer (pH 4.5), containing 1 mM ethylene diamine tetra-acetic acid (EDTA) and 5 mM Cysteine-HCl, using Z-L-Lys-ONp hydrochloride as the substrate. A freshly made stock solution (5 mM) of the substrate in dimethyl sulphoxide (DMSO) was added to 3 ml buffer so that final concentration of the substrate was 80 μM in the reaction mix. Protein concentration of 30 μg/ml was used for the activity assay. Protein concentration was determined spectrophotometrically using $E^{1\%}_{1\,cm}$ of 25 for papain, 20.1 for bromelain and 18.2 for chymopapain.

Upon hydrolysis by proteases, Z-L-Lys-ONp hydrochloride is converted to yellow coloured compound that can be read at 326 nm in UV-visible spectrophotometer. Background hydrolyses of the substrate *i.e.* in the absence of enzymes, were measured and used for correcting the hydrolysis rates obtained with enzymes. Enzyme solutions (enzyme + 10% w/v PEG—20000, 6000 and 400) were incubated at different temperatures for 5 min before activity measurements were done. Protease activity was measured as rate of change in absorbance of the reaction mix at 326 nm.

Incubation of Papain in PEGs—To study the effect of PEGs on the conformation of papain at pH 4.5, different volumes of concentrated PEGs (% w/v) were added to the protein solution taken in different volumes of the buffer (in 10 mM Sodium acetate buffer, pH 4.5), so as to get the desired concentration of the PEGs. The final volume in each tube was 1.0 ml. All the spectroscopic measurements were made after half an hour of incubation at room temperature.

Circular Dichroism (CD) Measurements—Far UV-CD measurements were carried out with a Jasco spectropolarimeter, model J–720 equipped with a microcomputer. The instrument was calibrated with (+) 10-camphorsulphonic acid. Enzymes were incubated in different concentrations of PEG (20000, 6000, 400) for half an hour. All the CD measurements were made at 25°C with a thermostatically controlled cell holder attached to Neslab's RTE-110 water bath with an accuracy of ±0.1°C. Spectra were collected with scan speed of 20 nm/min and response time of 1 s. Each spectrum was the average of four scans. Far UV-CD spectra were taken at protein concentrations of 0.5 mg/ml, with 1 mm path length cell. The results were expressed as MRE (mean residue ellipticity) in deg cm^2 $dmol^{-1}$ which is defined as

$$\mathrm{MRE} = \frac{\theta_{\mathrm{obs}}}{(10 \times n \times l \times C\mathrm{p})},$$

where θ_{obs} is the CD in milli-degree, n is the number of amino acid residues, l is the path length of the cell and Cp is molar concentration.

For thermal-transition studies, a water-jacketed 1 mm pathlength cell was used for far-UV CD attached to the RTE-110 waterbath interfaced with a microcomputer. A protein concentration of 0.5 mg/ml was used. The protein solution was incubated for 3 min at desired temperature before CD measurements.

Fluorescence Measurements—Intrinsic Fluorescence

Fluorescence measurements were performed on Shimadzu spectrofluorimeter, model RF-540 equipped with a data recorder DR-3. The fluorescence spectra were measured at 25 ± 0.1°C with a 1 cm path length cell. The excitation and emission slits were set at 3 nm each. Intrinsic fluorescence was measured by exciting the protein solution at 280 nm and emission spectra were recorded in the range of 300–400 nm. Protein concentration was 0.1 mg/ml.

Acrylamide Quenching Experiments

Aliquots of 5 M acrylamide stock solution were added to the protein solution (0.1 mg/ml) to achieve the desired range of quencher concentration (0.1–0.5 M). To excite the tryptophan residues only, excitation wavelength was set at 295 nm and the emission spectra were recorded in the range of 300–400 nm. Quenching experiment on NATA, tryptophan analogue, was also done. Its concentration was determined using an extinction coefficient of $\varepsilon_M = 5690\ M^{-1}\,cm^{-1}$ at 280 nm.

The data was analysed according to Stern–Volmer equation (*10*):

$$\frac{F_o}{F} = 1 + K_{sv}[Q]$$

where F_o and F are the fluorescence intensities at 340 nm in the absence and presence of quencher, respectively; K_{sv} is the Stern–Volmer constant and [Q] is the concentration of the quencher.

ANS Binding Experiment

Binding of 8-anilino-1-naphthalene-sulphonic acid (ANS) was studied by following the changes in fluorescence intensity in 400–600 nm range after excitation at 380 nm, in the absence and presence of PEGs. Concentration of ANS was determined using an extinction coefficient of $\varepsilon_M = 5{,}000\ M^{-1}\,cm^{-1}$ at 350 nm. The molar ratio of protein to ANS was kept at 1:5 and incubation was for 5 min. Being non-polar polymer, PEGs showed significant amount of ANS binding. The respective blanks of PEGs were prepared and fluorescence emission

corrections for their possible contribution were made, in order to rule out the contributions of PEGs.

RESULTS

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. Figure 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 Fig. 1A, papain shows maximum activity around 50°C at pH 4.5 and activity decreases 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 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 depict 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 1C depicts temperature profile of Chymopapain activity at pH 4.5 (curve 1), in presence of PEG 400

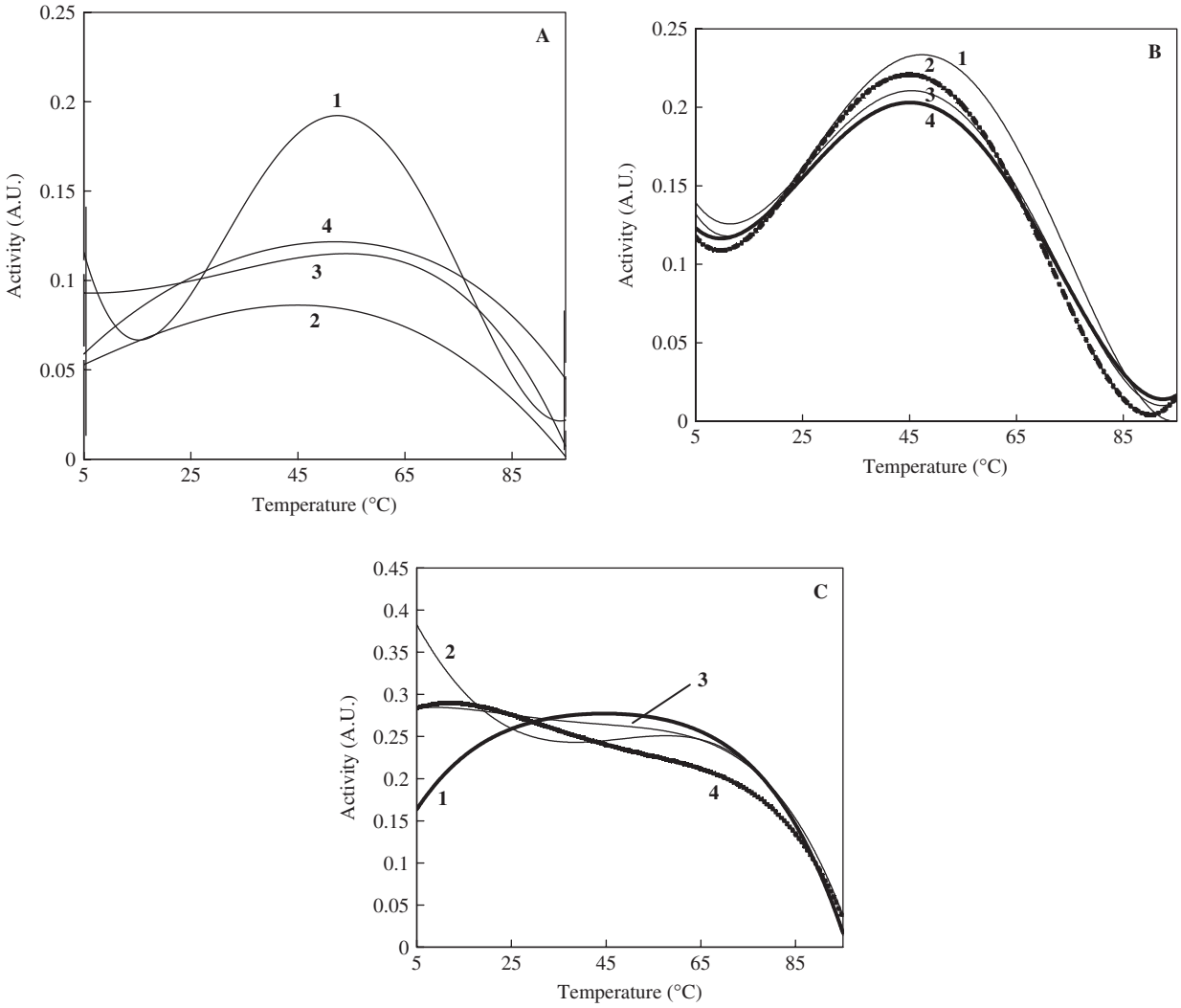

Fig. 1. **(A) Temperature profile of papain activity at pH 4.5 (curve 1), in presence of PEG-400 (curve 2), PEG-6000 (curve 3) and PEG-20000 (curve 4).** (B) Temperature profile of bromelain activity at pH 4.5(curve 1), in presence of PEG-400 (curve 2), PEG-6000 (curve 3) and PEG-20000 (curve 4). (C) 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). Protein concentration of 30 μg/ml was used.

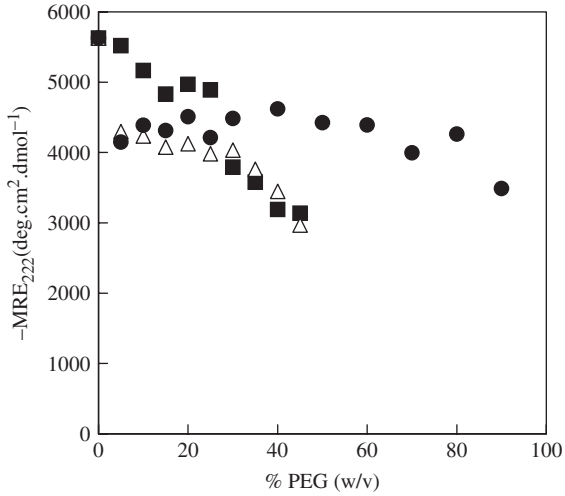

Fig. 2. **Plot of MRE at 222 nm of Papain in presence of PEG 20000 (filled square), 6000 (open triangle), and 400 (filled circle).** Protein concentration used was 0.5 mg/ml.

(curve 2), PEG 6000 (curve 3), and PEG 20000 (curve 4). As can be seen here, addition of PEGs has insignificant effect on the activity of chymopapain. However, it can be seen from the curve that PEG 20000 maximally destabilizes chymopapain.

Thus the results presented in Fig. 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 this experiments was that all the three PEGs used, of low and high molecular weights, lead to destabilization of the thiol proteases. Since the maximum differences 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.

CD Measurements—Figure 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. 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 Fig. 3A. Spectrum of papain at pH 4.5 reveals two negative peaks at 222 nm and 208 nm that are characteristic of helical content of the protein (*11*). Maximum decrease in far UV-CD spectra was observed in case of PEG-400 and least with PEG 20000 with PEG 6000 showing intermediate

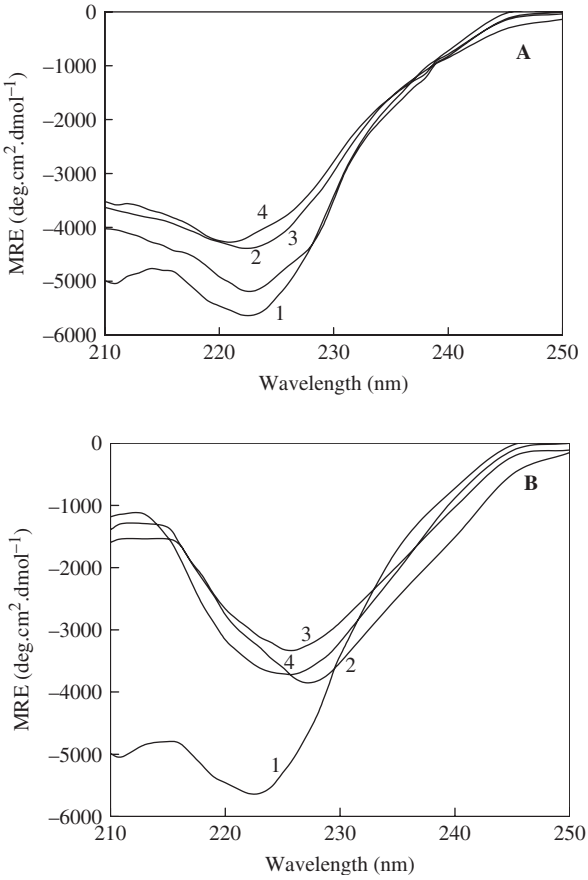

Fig. 3. **(A) Far UV-CD spectra of papain at pH 4.5 (curve 1); papain in presence of 10% PEG 20000 (curve 2), 10% PEG 6000 (curve 3) and 10% PEG 400 (curve 4)** (B) Far UV-CD spectra of papain at pH 4.5 (curve 1); papain in presence of 45% PEG 20000 (curve 2), 45% PEG 6000 (curve 3) and 90% PEG 400 (curve 4). Protein concentration was kept at 0.5 mg/ml.

values, as was also supported by activity measurements (Fig. 1A). These results suggest that addition of PEGs lead to loss of secondary structure content of papain. Figure 3B shows far UV-CD 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 have 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.

Fluorescence Measurements—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 (*12*). Papain contains five tryptophan residues out of which three are buried in hydrophobic core, while two are located near the surface of the molecule (*13*). Figure 4A shows

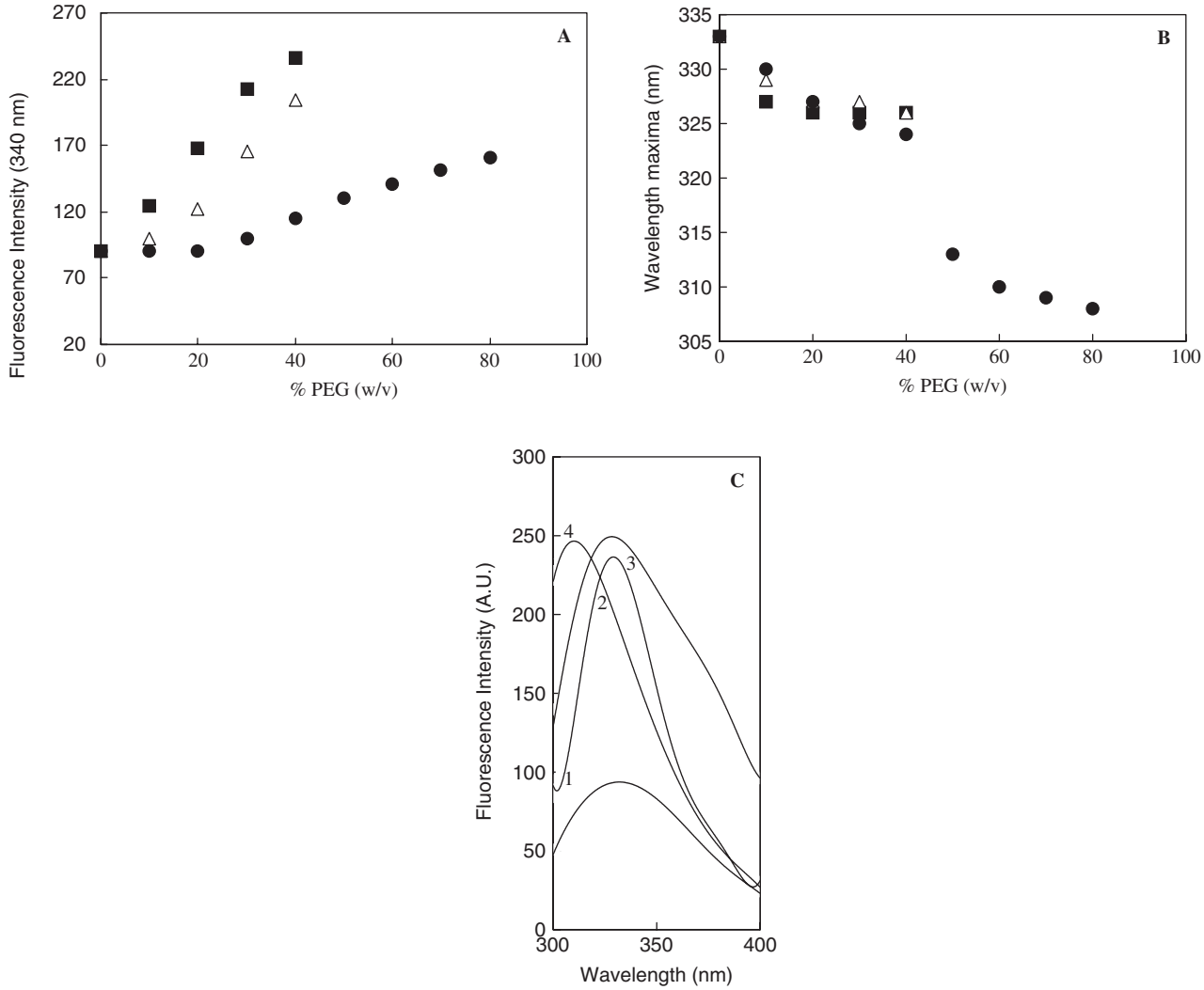

Fig. 4. **(A). Changes in fluorescence intensity at 340nm of papain in presence of increasing concentration of PEG 20000 (filled square), 6000 (open triangle) and 400 (filled circle).** (B) Changes in wavelength maxima of papain in presence of increasing concentration of PEG 20000 (filled square), 6000 (open triangle), and 400 (filled circle). (C) Intrinsic fluorescence spectra of papain at pH 4.5 (curve 1); papain in presence of 40% PEG 20000 (curve 2), 40% PEG 6000 (curve 3), 80% PEG 400 (curve 4). Concentration of protein was 0.1 mg/ml.

changes in fluorescence intensity of papain at 340 nm with increasing concentration of PEGs, the changes in wavelength maxima are depicted in Fig. 4B and the spectra of papain in presence of PEGs can be seen from Fig. 4C. As can be seen from the figures, with increasing concentration of PEGs fluorescence intensity at 340 nm increases with blue shift in wavelength maxima indicating that structural changes occur 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 indicates that tryptophan residues moved to more hydrophobic environment in the presence of PEGs.

Acrylamide Quenching Experiments

The topological properties of properties 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 5 shows Stern–Volmer plot of papain at pH 4.5 (curve 1), papain in presence of 40% PEG 20000 (curve 2), 40% PEG 6000 (curve 3), and 80% PEG 400 (curve 4); while the Stern–Volmer constants (K_{sv}) are compared in Table 1. Results for the tryptophan analogue NATA (curve 5) are also included as a standard for complete accessibility to quencher. As given in Table 1, K_{sv} was found to be maximum for papain in presence of 80% PEG 400 followed by 40% PEG 6000 and then for protein in 40% PEG 20000 and is 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 are least accessible to quencher in buffer, indicating that addition of PEGs to

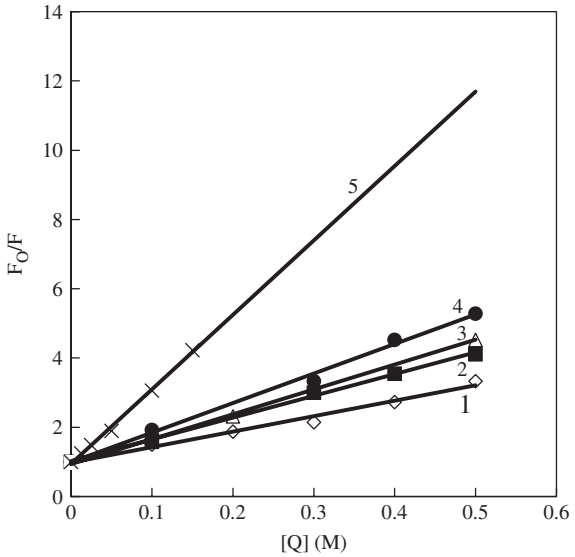

Fig. 5. **Stern Volmer plot of Papain at pH 4.5 (open diamond, curve 1), papain in presence of 40% PEG 20000 (filled square, curve 2), 40% PEG 6000 (open triangle, curve 3) and 80% PEG 400 (filled circle, curve 4).** Curve for NATA has been included for comparison (multiplication symbol, curve 5). Protein concentration used was 0.1 mg/ml.

Table 1. **Acrylamide quenching data of papain in different PEGs.**

Particulars	K_{sv} (M^{-1})
Papain at pH 4.5	4.4
Papain + 40% PEG 20000	6.3
Papain + 40% PEG 6000	7.2
Papain + 80% PEG 400	8.5
NATA (*N*-acetyl tryptophanamide)	21.5

the buffer lead to exposure and greater solvent accessibility of these residues. These results corroborate activity measurement data pertaining to destabilization of protein in presence of PEGs.

ANS Binding Experiment

PEGs are non-polar solvents and hence the possibility that interaction of PEG with papain involve 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 6 compares extrinsic fluorescence spectra of ANS bound to papain (curve 1) with that of ANS bound to papain in presence of 50% PEG 400 (curve 2). It can be seen that ANS binds to papain with λ_{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).

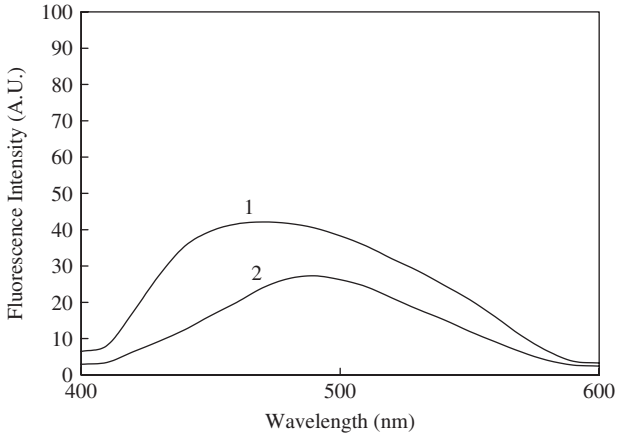

Fig. 6. **Fluorescence emission spectra of ANS bound to papain at pH 4.5 (curve 1) and to papain in presence of 50% PEG 400 (curve 2)**. Curve 2 has been obtained after subtraction of spectrum obtained for 50% PEG 400 + ANS from the emission spectrum of papain incubated with 50% PEG 400 followed by addition of ANS. Protein at the concentration of 0.1 mg/ml was used.

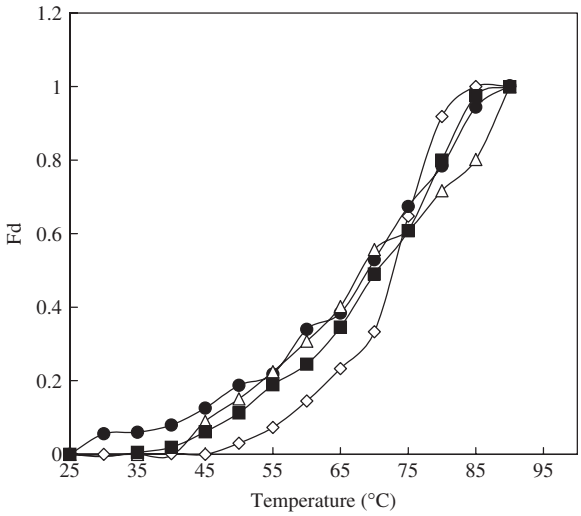

Fig. 7. **Thermal denaturation profile showing changes in MRE at 222nm of papain at pH 4.5 (open diamond), papain in presence of 10% PEG 20000 (filled square), 6000 (open triangle) and 400 (open circle).** 0.5 mg/ml protein was used.

This shows that interaction of papain with PEG lead to displacement of ANS, probably due to competition for hydrophobic sites.

Thermal Denaturation Studies—The structural stability of the papain in presence of 10% (w/v) PEGs were determined by temperature induced unfolding followed by MRE measurements at 222 nm. Figure 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 7, papain at pH 4.5 shows cooperative transition, while in presence of PEGs transition becomes

Table 2. **Thermal transition midpoint of papain in different PEGs.**

Protein state	T_m (°C)
Papain at pH 4.5	73
Papain + 10% PEG 20000	70.5
Papain + 10% PEG 6000	69
Papain + 10% PEG 400	68.5

Obtained from temperature-induced normalized transition curves shown in Fig. 7

non-cooperative one, with PEGs leading to destabilization of papain. The *T*m-values (Table 2) have been used as a measure of stability as $\triangle G_U$ could not be determined because of the irreversibility of the unfolding process. Comparison of *T*m at 10% (w/v) of different PEGs indicates that PEG-400 maximally destabilizes 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, indicate that the protein undergoes denaturation in PEG–water solution.

DISCUSSION

Preferential interaction is a thermodynamic measure of how solute and solvent redistribute around a protein in solution (*14*). 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 (*15*). However PEG is an exception to this rule (*16–18*). 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 (*19*, *20*).

Our results in this article 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 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 (*21*). PEG can bind to hydrophobic sites on protein based on the fact that PEG is essentially non-polar. 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%) (*5–7*). 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 (Fig. 6). This is in agreement with the findings of Pace and Marshal (*21*) that non-polar compounds destabilize hydrophobic proteins more than less- hydrophobic ones.

On the basis of the results discussed in this article, 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.

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