Asian Journal of Chemistry Vol. 22, No. 6 (2010), 4335-4346

Conformational Study of Intermediate States of Papain in the Presence of Different Polarities of Alcohols

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Our studies extend on the presence of intermediate state of papain at two different pH values in the presence of different kind of alcohols. A systematic investigation of the effect of aqueous alcohols on the structure of partially unfolded state at pH 3.2 and the native-like state at pH 5.0 was made using intrinsic tryptophan fluorescence and UV-visible spectroscopy. Tryptophan fluorescence studies indicate that the change in the environment of the tryptophan residue on the addition of alcohols would result in a decrease in the fluorescence intensity at pH 3.2 and 5.0. These decreases in the fluorescence intensity of papain at pH 3.2 and 5.0 can be attributed to the conformational changes in the surface-exposed tryptophan, presumably due to internal changes in a more hydrophobic environment and formation of a more compact structure. This suggest that alcohols which steer the conformational changes in the direction of the formation of native-like state and unfolded state at the two pH values of 3.2 and 5.0, respectively, lead to the formation of intermediate states along the two aforementioned pathways. UV measurement studies that indicate at pH 5.0, the addition of alcohol leads to an increase in the absorbance and demonstrate the formation of I_B and I_C states on the transition of the native-like state to the unfolded state. The native and partially unfolded states have different intermediates. In the present work, comparison of the results of fluorescence experiments shows more positive m-values as a cooperative parameter for intermediate state at pH 3.2. These effects of alcohols can be explained to some extent, by the decreased polarity of the solvent. In solvents of low polarity, hydrophobic interactions stabilizing native structures or protein aggregates are weakened and simultaneously the local hydrogen bonds are strengthened, resulting in denaturation or dissolution and stabilization of the extended α-helical structures.

Key Words: Papain, Thermodynamic studies, Intermediate states, Polarity.

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INTRODUCTION

Denaturants (*n*-alkyl sulfates, GuHCl and temperature) are most frequently employed to probe the structural aspects of a protein in solution. Thus, equilibrium unfolding studies and the presence of intermediates/molten globule state have most often been reported at low pH values or in the presence of previously mentioned denaturants[1]. Alcohol, often used as a protein precipitant or preservative has other effects on proteins and polypeptides and hence can give valuable information on the structure of proteins in solution. Alcohol disrupts the rigid tertiary structures, stabilizes and induces helicity in protein and dissolves peptide aggregation[2]. Destabilization of the tertiary structure of a protein by alcohols[3] and stabilization of the secondary structure[4] has often led to the induction of partially folded intermediates[5] in proteins, sometimes referred to as the molten globule state. Thus alcohol is now commonly used to induce partially folded states in proteins[6]. However, the behaviour of plant cyctein proteases of the papain super-family and their folding aspect in alcohol have not been reported extensively and need further consideration. Papain, a powerful proteolytic enzyme, belonging to the cystein protease family is a plant end-protease. It is isolated from the latex of the papaya fruit[7,8]. The papain molecule is a single polypeptide chain with 212 amino acid residues and has a molecular weight of 23,000 Da and the polypeptide chain is folded into two domains of roughly equal size, but of totally different conformations[9]. An interesting feature of papain molecular structure is that it is divided in the form of two distinct domains that are separated by a deep cleft. The L domain, which is mainly α-helical, is compromised of residues 10-111 and 208-212. The R domain contains residues 1-9 and 112-207 and the key feature of the R domain is its antiparallel β-sheet structure. These two domains have been shown to unfold independently. In the cleft formed between the two domains are the active site residues *viz.*, Cys-25, His-159 and Asp-178[9-12]. The structural and functional aspects of papain in response to acid and chemical denaturants such as *n*-alkyl sulfates and GuHCl were studied. Papain under acid conditions (pH 2) exists in molten globule state as characterized by spectroscopic methods. At lower concentrations of guanidine hydrochloride, at pH 2, papain tends to aggregate and the extent of aggregation is dependent on the concentration of GuHCl. Also the fluorescence spectra of the papain at different pH values with varying concentrations of sodium *n*-alkyl sulfates was also recorded to demonstrate the effect of the sodium *n*-alkyl sulfates[13,14]. Further Sathish *et al.*[9] have shown the formation of a molten globule state of papain with low denaturants concentrations and under acidic conditions. Sathish *et al.*[9] have also shown that at low concentrations of denaturants, the preferential interaction parameter (ξ3) is a negative value indicating preferential hydration. Because of the availability of a vast amount of data on the structure, papain can be used also as a model protein to study the structure-function relationship[9]. In the present work, extensive studies on papain were carried out in the presence of different organic solvents and solvent compositions. Different spectroscopic methods (*e.g.*, fluorescence and absorbance) were employed to detect and characterize the organic solvent induced states.

EXPERIMENTAL

Fresh latex from green fruits of *Carica papaya* was collected and immediately transported in ice bath to the laboratory, papain was isolated by anionic exchange chromatography on a column of diethylaminoethyl sepharos 6B purity and the homogeneity of the preparation was checked by SDS-polyacrylamide gel electrophoresis[15-20] (Fig. 1). To avoid any hindrance due to the autolytic nature of papain, the cystein was blocked by carboxymethylation. Such blocked protein is similar to the active form in all its physical properties. Therefore, for all the studies presented here, such inactive protein (1RCM-papain) was used. An extinction coefficient of $\varepsilon^{1\%} = 25$ was used for the determination of concentration of papain solutions[14]. Methanol, ethanol, isopropanol were purchased from the Sigma Chemical.

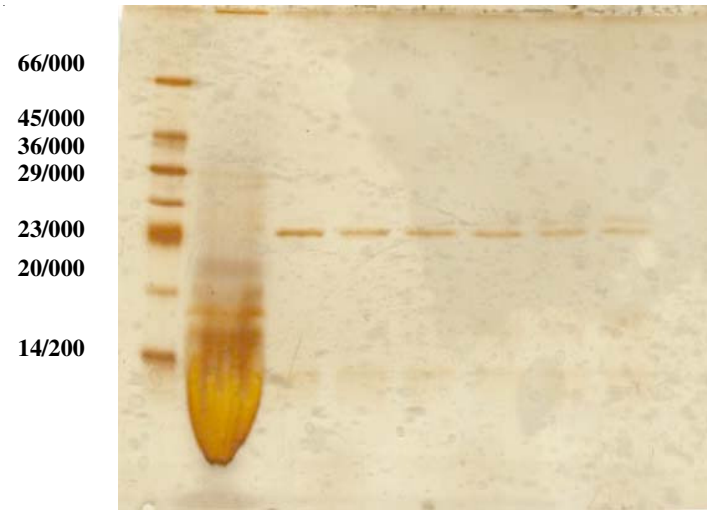


Fig. 1. Polyacrylamyde gel electrophoresis of papain. The samples in the different lanes represent: standard (line 1), crude latex extract (line 2) and different fractions of papain after purification by anionic exchange chromatography (line 3-8)

Solution preparation: The protein solution was dialyzed against buffer (50 mM glycine buffer pH 3.2 and 50 mM phosphate buffer, pH 5). The extinction coefficients were used to calculate the concentration of the native protein at different pH values. If the initial concentration and volume of protein solution are $[P]_0$ and V_0, respectively and the stock ligand concentration is $[L]_0$, then the total concentration of protein ($[P]_t$) and ligand ($[L]_t$) can be obtained by accounting for the total volume of the aliquot (V_c) added during the titration experiment[14,21]:

$$[P]_t = [P]_0V_0/(V_0 + V),\ [L]_t = [L]_0V_0/(V_0 + V_c).$$

The aliquots of alcohols were injected into the papain solution at 5 min intervals to allow for equilibration. Each experiment was repeated three times[14].

Absorption measurements: The absorption spectra of papain were obtained with a spectrophotometer, Model Shimadzu PC 1650, with cells of 1 cm widths. The protein concentration was determined at 0.04 %. Samples containing different concentration of alcohols were equilibrated at room temperature for 5 min before recording for absorbance measurements.

Fluorescence measurements: Fluorescence spectra were recorded with a JASCO FP-2600 spectrofluorometer. Samples containing different concentrations of alcohols were equilibrated at room temperature for 5 min before recording for tryptophan fluorescence measurements. The excitation wavelength was at 278 nm and the emission was recorded from 300 to 400 nm.

RESULTS AND DISCUSSION

The 15 mL fresh milky latex extracted from samples of *C. papaya* contained 121.6 mg/mL papain and showed 3648000 units of protease activity/mL. As papain is a protease of broad specify and no specific synthetic substrate is available, casein was used as a substrate to determine the total protease activity present in the latex while the purity of papain was determined by electrophoresis and chromatography. On anionic gel electrophoresis, the protein in the latex separated as seven bands (Fig. 1). One of this protease was identified as papain according to its mobility that was equal to that of the standard papain and its *in situ* proteolytic activity on polyacrylamide gel[22-27].

Absorbance measurements: Absorbance spectroscopic technique in UV region can be used to study the binding and folding-unfolding of a protein, initiated by alcohols. Addition of various concentrations of alcohols to the papain at pH 5 enhances the absorption intensities (Fig. 2C).The marked change in the absorbance value of the mixture at various concentrations of alcohols corresponds to the change of the conformation of the protein, *i.e.* the protein folding. The results show (Fig. 2C) that in presence of alcohol, maximum hydrophobic residues are exposed and it is clear that the protein has started to denature. Hence in the presence of alcohols, the exposure of hydrophobic groups falls between those of the native-like state and the denaturated state. Thus it can be concluded that alcohols are responsible for making hydrogen bonds within the polypeptide chains and weakening the hydrophobic interactions, which in turn are responsible for tertiary structures resulting in the retention of secondary structures with partial tertiary structures[28].

Fluorescence measurement: The intrinsic fluorescence maximum (λ_{max}) is an excellent parameter to monitor the polarity of tryptophan environment in the protein and is sensitive to the protein conformation[13]. Figs. 2A and 2B summarizes the results of fluorescence studies on protein at pH 3.2 and 5.0 in the presence of alcohol, respectively. According to Figs. 2A and 2B, the addition of various concentrations of alcohols to the partially unfolded state at pH 3.2 and native-like state at pH 5 of papain causes a decrease in the fluorescence intensity.

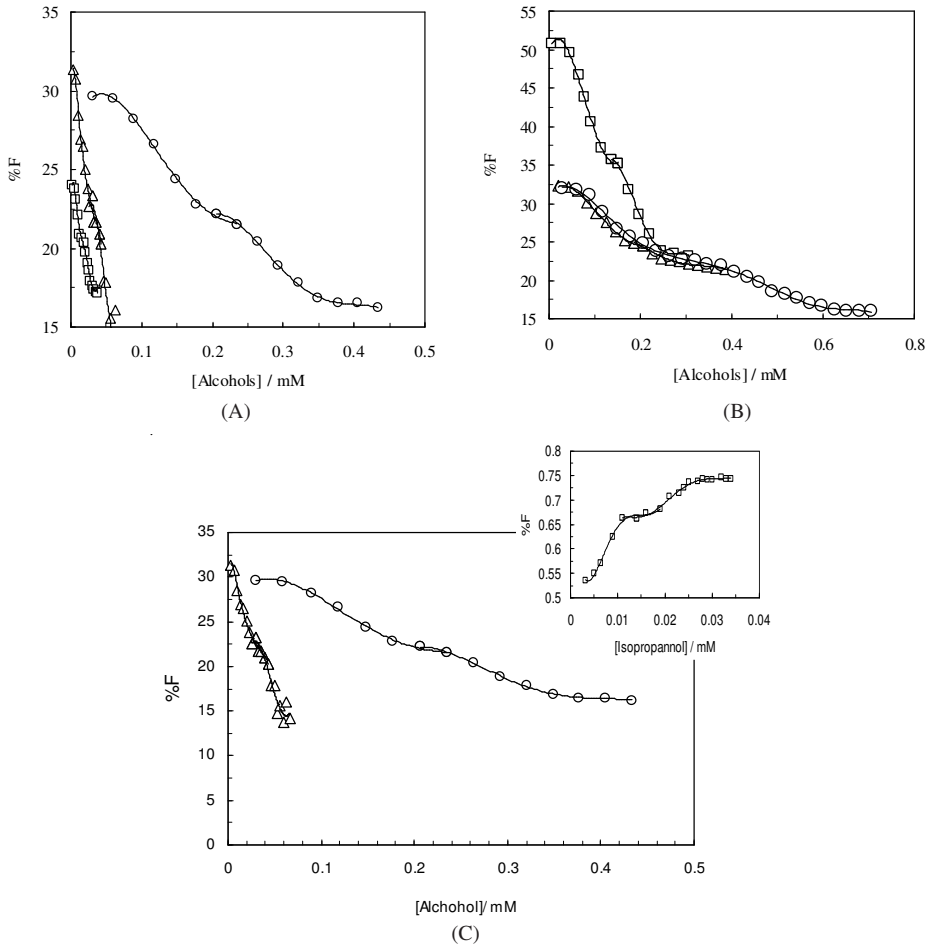
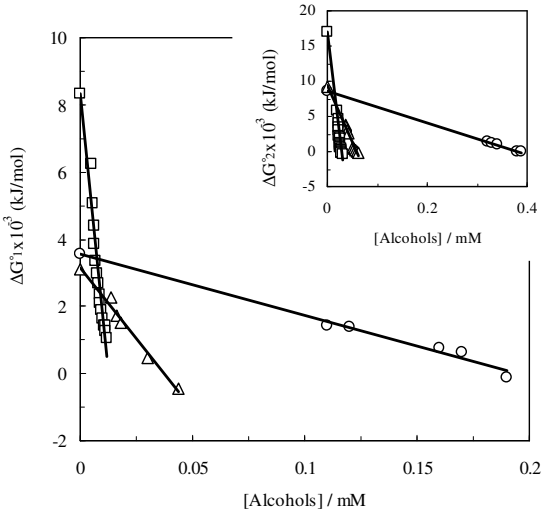


Fig. 2: (A) Plot of intensity fluorescence *versus* concentrations of alcohols at pH 3.2, methanol (○), 12 mM; ethanol (△), 3.4 mM; isopropanol (□) 2.6 mM; (B) plot of intensity fluorescence *versus* concentrations of alcohols at pH 5, methanol (○), 12 mM; ethanol (△), 8.5 mM; isopropanol (□) 2.6 mM. (C) Plot of absorbance spectra *versus* concentrations of alcohols at pH 5. methanol (○), 12 mM; ethanol (△), 8.5 mM; isopropanol (□) 6 mM

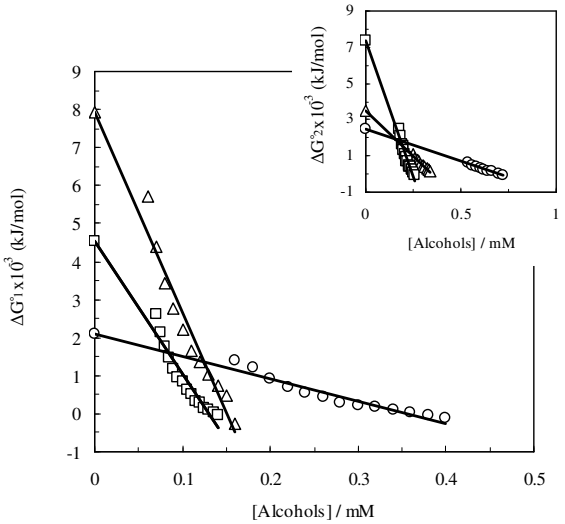
Thermodynamic analysis of intermediate state formation: Figs. 2A-C show the sigmoidal curves (drawn by a numerical analysis method, called qubic-spline in the MATLAB program, version 6.1) for the transition of the partially unfolded state to the native-like state (P→N') at pH 3.2 and for the native-like state to unfolded state of papain (N'→U) at pH 5 upon the addition of alcohols. Papain is a small protein with a single subunit. Therefore, a two-state analysis based on the pace theory was performed[29]. It is now possible to obtain the equilibrium constant (K) for the P→I and I→N' pathways and to calculate the corresponding Gibb's free energy changes, ΔG^{o}, as follows:

$$\Delta G^{o} = -RT \ln (A_{obs} - A_P)/(A_I - A_{obs})$$

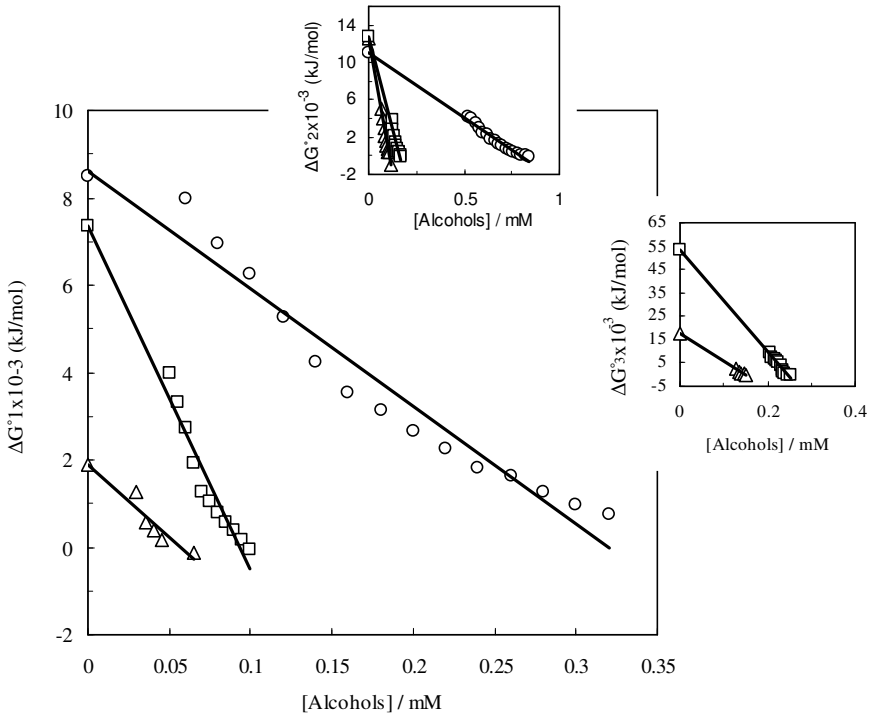
where R is the gas constant, T is the absolute temperature, A_P, A_I and A_{obs} are the physical parameters of extinction coefficient and percentage of fluorescence of P, I and any observed states, respectively. Figs. 3A-C shown the plot of ΔG^{o} against total alcohol concentrations ($[alcohol]_{total} = [alcohol]_{free} + [alcohol]_{bound}$). The free energies of intermediate formation in the absence of alcohols, $\Delta G^{o}(H_2O)$, were calculated by the least-squares method from the following equation[14,29-31].



(A)



(B)



(C)

Fig. 3: (A) Free energy values $\Delta G°_1(H_2O)$ of fluorescence spectra *versus* concentrations of alcohols at pH 3.2. Inset: Free energy values $\Delta G°_2(H_2O)$ of fluorescence spectra *versus* concentrations of alcohols at pH 3.2. (B) Free energy values $\Delta G°_1(H_2O)$ of fluorescence spectra *versus* concentrations of alcohols at pH 5. Inset: Free energy values $\Delta G°_2(H_2O)$ of fluorescence spectra *versus* concentrations of alcohols at pH 5. (C) Free energy values $\Delta G°_1(H_2O)$ of absorbance spectra *versus* concentrations of alcohols at pH 5. Inset: Free energy values $\Delta G°_2(H_2O)$ of absorbance spectra *versus* concentrations of alcohols at pH 5. Inset: Free energy values $\Delta G°_3(H_2O)$, absorbance spectra *versus* concentrations of alcohols at pH 5

$$\Delta G° = \Delta G°(H_2O) - m[\text{alcohol}]$$

when m is the slope of a linear curve reflecting the cooprativity and also hydrophobicity of the transition state. The m-value correlates strongly with the amount of protein surface, exposed to the solvent upon unfolding[32]. Thus, for proteins that undergo a simple two-state unfolding mechanism, the amount of the surface, exposed to the solvent upon unfolding is a main structure to determined m-values and hydrophobicity. The $\Delta G°(H_2O)$ and m-values at pH 3.2 and 5.0 are tabulated in Tables 1-3. It is apparent from Tables 1-3 that the values of $\Delta G°(H_2O)$ and m-values of the conformational transitions induced by methanol, ethanol and isopropanol increase with proportionately the chain lengths of *n*-alkyl sulfates.

TABLE-1
$\Delta G^o(H_2O)$, m-VALUES OF FLUORESCENCE SPECTRA FOR THE INTERMEDIATE STATES OF PAPAIN AT pH 3.2 UPON INTERACTION WITH ALCOHOLS

	$\Delta G^o_1(H_2O)$ (kJ mol^{-1})	$\Delta G^o_2(H_2O)$ (kJ mol^{-1})	$\Delta G^o_{1+2}(H_2O)$ (kJ mol^{-1})	m_1 (kJ mol^{-1} M^{-1})	m_2 (kJ mol^{-1} M^{-1})
Methanol	3.574	8.839	12.114	18.381	18.381
Ethanol	3.139	9.271	12.511	83.827	83.827
Isopropanol	8.334	17.097	25.431	653.640	653.640

TABLE-2
$\Delta G^o(H_2O)$, m-VALUES OF FLUORESCENCE SPECTRA FOR INTERMEDIATE STATES OF PAPAIN AT pH 5 UPON INTERACTION WITH ALCOHOLS

	$\Delta G^o_1(H_2O)$ (kJ mol^{-1})	$\Delta G^o_2(H_2O)$ (kJ mol^{-1})	$\Delta G^o_{1+2}(H_2O)$ (kJ mol^{-1})	m_1 (kJ mol^{-1} M^{-1})	m_2 (kJ mol^{-1} M^{-1})
Methanol	2.111	2.492	4.603	5.933	3.593
Ethanol	7.922	3.510	11.433	52.612	10.006
Isopropanol	4.534	7.351	11.886	34.931	29.642

TABLE-3
$\Delta G^o(H_2O)$, m-VALUES OF ABSORBANCE SPECTRA FOR INTERMEDIATE STATES OF PAPAIN AT pH 5 UPON INTERACTION WITH ALCOHOLS

	$\Delta G^o_1(H_2O)$ (kJ mol^{-1})	$\Delta G^o_2(H_2O)$ (kJ mol^{-1})	$\Delta G^o_{1+2}(H_2O)$ (kJ mol^{-1})	$\Delta G^o_{1+2+3}(H_2O)$ (kJ mol^{-1})	m_1 (kJ mol^{-1} M^{-1})	m_2 (kJ mol^{-1} M^{-1})	m_3 (kJ mol^{-1} M^{-1})
Methanol	8.592	10.989	–	19.581	26.938	13.829	–
Ethanol	1.878	12.652	17.970	32.500	33.235	118.39	121.96
Isopropanol	7.356	12.788	53.109	73.262	78.689	78.178	215.70

The native conformation of a protein is a characteristic property of the macromolecule itself, governed by the number, arrangement and covalent/non-covalent interactions of its amino acids. Structural studies of proteins in different solvent systems can provide information about the structure of a protein molecule and of the roles of various stabilizing and destabilizing forces[33]. Therefore, the effects of various non-fluorinated organic solvents on the structure of papain were studied to obtain further insight in to enzyme folding and unfolding. Organic solvents can denature proteins. Moreover, the resulting products are not completely unfolded but possess a measure of conformation. In addition, the structure of a protein can be made highly ordered or disordered relative to the native state by changing the solvent system employed. Generally, partially unfolded states arise due to the different contributions of hydrogen bonds, hydrophobic interactions and electrostatic interactions of the protein molecule and the solvent system. A wide range of organic solvents, capable of denaturing proteins are known and alcohols are most widely used as solvent due to their high miscibility with water. Moreover, the usage of alcohols provides unique opportunities to dissect the contributions made by alkyl

groups to protein conformation and to examine the changes induced by altering chain length. Alcohols in general have three distinct effects upon protein and polypeptides: (i) the destruction of the rigid native protein structure, (ii) the induction or enhancement of helixes, (iii) and the dissolution of peptide aggregates. Their role in stabilizing helical structure[4], which destabilizes tertiary structure[34], is well documented. The ability of an alcohol to disrupt the internal folding of a protein increases with its chain length and hydrocarbon content, and follow the order: isopropanol > ethanol > methanol[35]. Thus, the destabilization of a tertiary structure and the stabilization of a secondary structure may induce partial protein folding which is often referred to as the molten globule[36]. Under conditions of moderately low pH, transition to a molten globule-like state can be induced by various means, one being the inclusion of alcohol in the solvent composition[37]. The molten globule state is a commonly evoked term for partially folded protein structures with considerable secondary structure, but with few, if any, fixed tertiary structural contacts. Addition of various concentrations of alcohols including methanol, ethanol and isopropanol (non-fluorinated, aliphatic, monohydric alcohol) to the papain at pH 3.2 and 5.0 decrease the fluorescence intensities. The marked change of fluorescence intensity value of the mixture at various concentrations of alcohols corresponds to the change in the conformation of the protein, *i.e.*, the protein folding[14]. This result shows that in the presence of methanol, ethanol and isopropanol a sudden switch to the conformation of the protein occurred along with a decrease in fluorescence intensity at pH 5.0. Papain assumes a native conformation at pH 8.7, while the conformation at pH 3.2 is a partially unfolded state. Moreover it could be hypothesized that at pH 3.2 in presence of alcohols, papain folds from a partially unfold state to a native-like structure followed by an decrease in fluorescence intensity. At lower pH the protein molecule is under stress because of the electrostatic repulsions that are due to the increased positive charge. Thus the addition of different kinds of alcohols tends to cause varying effects by opening up the molecule, depending on the nature of the alcohol which drives the molecule to a new conformation (intermediate state). Also the UV measurement studies indicate an increase in the UV absorbance on the addition of alcohols to native-like state. At pH 5.0, addition of alcohols leads to an increase in the UV absorbance which demonstrates the formation of two intermediate states, I_1 and I_2 and this effect is enhanced with increasing concentration and chain length of alcohols.

One of the best criteria for determining protein stability is free energy in the absence of ligand, $\Delta G^o(H_2O)$ or the m-value. The m-values for a given transition are generally interpreted as a measure of change in solvent exposure for that transition[14] and this makes them a useful estimate of the gross compactness of different states on the folding pathway relative to the two end-stations, the denatured state D and the native state N. Ligand-induced refolding is useful for understanding the mechanism of conformational stability. In particular, the m-value is important since it is a measure of the coopratívity of the folding and unfolding transitions and is proposed to be

approximately proportional to the difference in solvent-accessible surface area between the folded and unfolded states[14]. Tables 1-3 show the m-values and $\Delta G^{o}(H_2O)$ for the intermediate states of papain upon the addition of alcohols at pH 3.2 and 5.0, respectively. Tables 1-3 show the increase in $\Delta G^{o}(H_2O)$ and m-values that corresponds to the length of the hydrophobic chains. It is noted that the m-value for the intermediate states induced by alcohols at pH 3.2 are significantly higher than those at pH 5. On other hand, m-value is a sign of coopravity, therefore intermediate states, induced by alcohols at pH 3.2, is more cooperative than intermediate states at pH 5.the folding pathways at pH 3.2 and the conformational changes of the papain at pH 5, in the presence of various concentrations of alcohols revealed that intermediate states differ in m-value and $\Delta G^{o}(H_2O)$. However the effects of various alcohols on papain at pH 3.2 and 5.0 indicate that the longer the chain length, the higher are the m-values and $\Delta G^{o}(H_2O)$. Therefore, the quality of solvent with various chain lengths can determine different levels of stability and conformations on intermediate states. UV measurement studies in presence of alcohols exhibits two intermediate states for the conformational changes of the protein at pH 5. These results suggest the presence of two structural parts in the molecular structure of the protein with different stabilities. The different stabilizations of the structural parts in the intermediate state may be a reflection of the differential stabilization of local conformations of alcohols. Since papain like cytein proteinases, except cathepsin c, is a monomer, whose structure consists of two domains referred to as R and L domains[38]. The occurrence of two transition midpoints in the presence of organic solvents is consistent with the view that papain has two domains that fold sequentially suggesting that one region in the protein molecule is stabilized while the other is destabilized in the presence of alcohols. The domain interactions in papain, which are perturbed due to the presence of alcohols, might be dominated by hydrophobic stabilization. Also different domains have different relative hydrophobic stabilization and these results best witness to the hypothesis that folding of domains in papain proceeds independently. The coopravity could be due to side-chain interactions of the domains interface. This confirms the conclusion that papain consists of two domains in its molecular structure. Hydrophobic interactions play crucial roles in stabilizing the native conformations of proteins. Moreover, a marked reduction in hydrophobic interactions due to almost any non-aqueous water miscible solvent must be critically involved in the observed conformational changes. Intra- molecular hydrogen bonds are important in determining these structures, but hydrophobic interactions make the largest single contribution to stabilize the native conformations of these macromolecules in solution. In the absence of these interactions, the stability of a molecule is reduced[39]. Several hypotheses have been proposed to account for the folding/unfolding of enzymes in organic solvents[40]. (i) water molecules in the enzyme are stripped away or replaced by solvent molecules therefore causing deformation or denaturation (ii) organic molecules bind to specific enzyme sites (iii) interfacial or the surface tension of solvents destroy the tertiary structures of

enzymes in a two-phase system. However, enhanced stability in the I_{Iso}-state > I_E-state > I_{met}-state is probably due to the hydrogen bond forming ability of alcohols.

Conclusion

The results described in this paper indicate that there are different structures of intermediate states of papain induced by various concentrations of alcohols at two different pH values. In addition evidence for the stabilization of the intermediate states of papain has been presented in different solutions with varying polarities and hydrophobicity of the alcohols.

ACKNOWLEDGEMENT

The financial support of the Research Council of the Islamic Azad University-Mashhad Branch is gratefully acknowledged.

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(*Received*: 15 July 2009; *Accepted*: 8 February 2010) AJC-8412