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ORIGINAL PAPER

Comparative effects of alcohols (methanol, glycerol) and polyethylene glycol (PEG-300) on acid denatured state of goat liver cystatin

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Abstract This report summarizes the effect of methanol, glycerol and polyol (PEG) on the acid induced state of goat liver cystatin by various spectroscopic techniques. Native goat liver cystatin (LC) has a fluorescence maximum at 340 nm, whereas the acid induced state shows a red shift of 15 nm with enhanced fluorescence intensity. Addition of 80% (*V*/*V*) methanol and glycerol both were found to stabilise the acid induced state of goat liver cystatin. However, glycerol was found to be a better stabilising agent than methanol. The unfolded state of liver cystatin obtained at pH 2 underwent a series of conformational changes when exposed to PEG-300 at varying concentrations. Tertiary structure was stabilized only at low concentrations of PEG-300 but higher concentrations resulted in the loss of tertiary structure.

Keywords Liver cystatin · Methanol · Glycerol · Polyethylene glycol PEG-300

Introduction

The extent of unfolding of denatured states of proteins under different conditions has been of great interest to biologist because of the possible relevance of their conformations to the protein folding pathways. It has been demonstrated that residual structural preferences, ranging from local clusters of side chains to highly ordered sub-domains persist in denatured states of proteins [1, 2, 3, 4, 5, 6].

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pH is known to influence the stability of a protein by altering the net charge on the protein. Many proteins denature at extreme pH because of the presence of destabilizing repulsive interactions between charges in the native protein [7, 8]. The exact behaviour of a given protein at low or high pH is a complex interplay between a variety of stabilizing and destabilizing forces, some of which are sensitive to the environment.

Since proteins carry out the most considerable tasks in living organisms so stabilizing the protein structure is an important issue in biological systems. Proteins are stabilized generally by a combination of hydrogen bonding, electrostatic interactions and hydrophobic interactions. Various studies have been made in improving the protein stability and it has been established that protein stability is the balancing between intramolecular interaction of protein functional groups and their interaction with solvent environment [9–11].

An extensively studied co-solvent that modifies the protein conformation is alcohol. Studies on alcohol effects provide insights into biologically important events because the alcohol solution mimics the environment of biomembrane [12] and modifies folding pathways of proteins [13, 14].

Proteins physical and chemical properties can also be altered by polymer interaction [15]. The PEG polymers are a series of non-ionic polyhydroxyl compounds that are miscible with water and display colligative properties in solution. Different molecular weight PEG's exhibit different solubilities, surface tension, viscosities, freezing points and melting points.

Cystatins are proteins that tightly bind and inhibit the harmful effect of cysteine proteinases [16]. These proteins are all related by structure and function to an inhibitor of cysteine proteinases which was first described in egg white and called as chicken egg white cystatins. Cystatins have

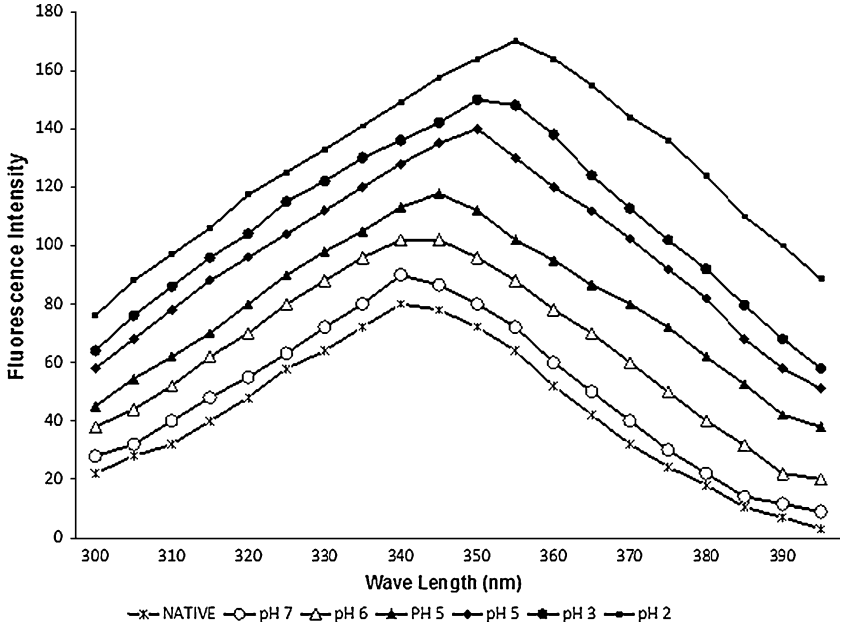

Fig. 1 Intrinsic fluorescence analysis of goat liver cystatin in the pH 7.0 to 2.0

been evolutionary related forming the "Cystatin Superfamily". The members of a protein superfamily were grouped into three families on the basis of their, carbohydrate content sequence homology and disulfide bonds [17, 18]. Members of Family 1, the stefins are found primarily intracellularly, contain about 100 amino acid residues (~11 K Da) and lack disulphide bonds. Members of family 2, the cystatins are found in body fluids and in tissues also. They contain about 120 amino acid residues (~14 K Da) and two intrachain disulphide bonds. Family 3 comprises the plasma kininogens and may therefore also be called the kininogen family with molecular weight of ~70 to 120KD. They contain additional disulphide bonds and are also glycosylated [17]. These inhibitors might protect the cells from unwanted proteolysis which may otherwise cause a number of pathologies [19], like rheumatoid arthritis [20], osteoporosis [21], Alzheimer's disease [22], metastasizing cancer [23] and microbial invasion [24].

The goat liver cystatin used in the present study was purified in our laboratory using alkaline treatment, ammonium sulphate fractionation, acetone fractionation and gel filtration chromatography on a Sephacryl S-100 column.

The aim of the present investigation was to study the effects of alcohols (methanol and glycerol) and polyethylene glycol PEG-300 on acid-induced unfolded cystatin isolated from goat liver.

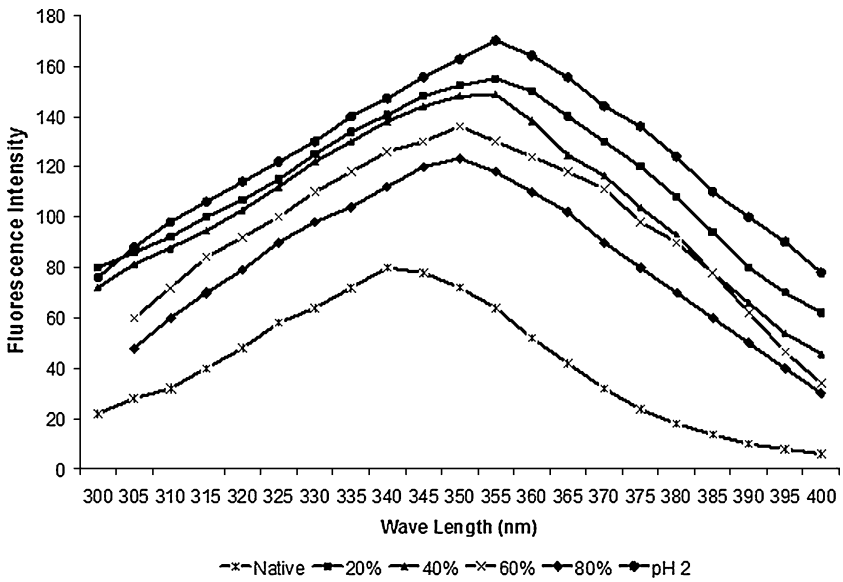

Fig. 2 Effect of varying concentration of methanol on the intrinsic fluorescence measurements of the acid-induced state of goat liver cystatin obtained at pH 2.0

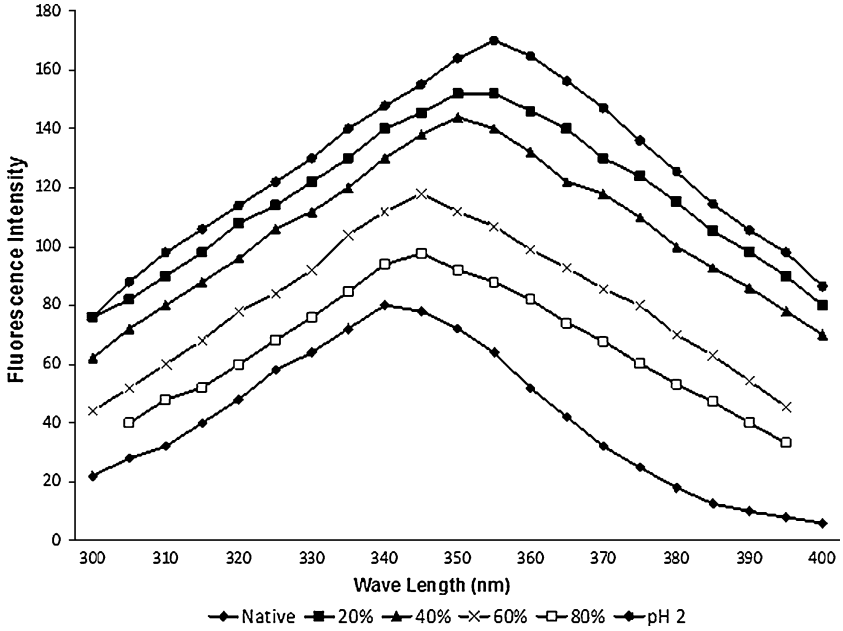

Fig. 3 Effect of varying concentration of glycerol on the intrinsic fluorescence measurements of the acid-induced state of goat liver cystatin obtained at pH 2.0

Materials and Methods

Materials

Papain, Sephacryl-S100HR, casein, acrylamide, ethylene di-amine tetra acetic acid (EDTA), acetone, commassie brilliant blue R-250, Cysteine were from Sigma Chemical company St. Louis USA. Medium range molecular weight markers were from genei. Methanol, glycerol and PEG 300 were purchased from Sigma Chemical company.

Sample Preparation

Methods

Purification of Liver Cystatin

Cystatin was isolated and purified from goat liver with a yield of 370 by using a three step procedure. Fresh liver tissue (100) g was homogenized in 200 ml extraction buffer (0.05 M sodium phosphate buffer, pH 7.5, 1.0% NaCl,

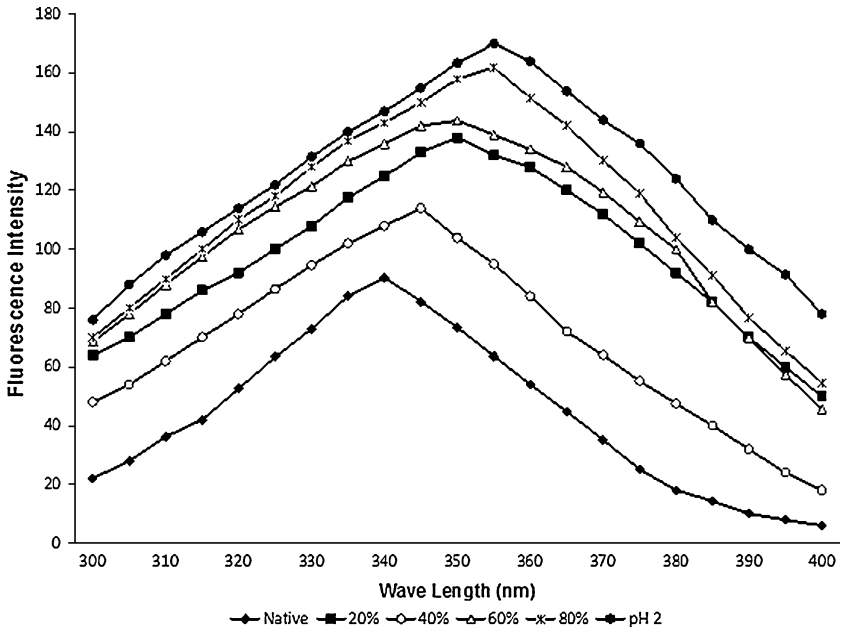

Fig. 4 Effect of varying concentration of polyethylene glycol (PEG-300) on the intrinsic fluorescence measurements of the acid-induced state of goat liver cystatin obtained at pH 2.0

Table 1 Fluorescence properties of the acid-induced state of goat liver cystatin under the influence of alcohols (methanol & glycerol) and PEG-300

Conditions	Intensity	Emission (λmax)
Acid induced state	170	355 nm
Native	90	340 nm
Methanol (80%)	123	350 nm
Glycerol (80%)	100	345 nm
PEG-300 (40%)	114	345 nm

3 mM EDTA, 2.0% n-butanol). The homogenized tissue was subjected to alkaline treatment. The supernatant obtained after homogenization was then subjected to 40% ammonium sulphate. The supernatant obtained was then treated with acetone in the ratio of 1:1 and was later subjected to 60% ammonium sulphate saturation. The precipitated protein after extensive dialysis was applied on Sephacryl S-100 column, pre-equilibrated with 0.05 M sodium phosphate buffer (pH 7.5). Fractions eluted were analysed for protein concentration and inhibitory activity. Purified liver cystatin (LC) gave a single band on PAGE. The molecular weight of the inhibitor was found to be 38.8KDa as calculated by its subunit structure on SDS-PAGE.

Protein Estimation

The protein content was quantitated by Folins phenol reagent by the method of Lowry et al. [25].

Acid Denaturation of Goat Liver Cystatin and the Assay of Inhibitory Activity of LC

Acid unfolding of liver cystatin was carried out using 50 mM sodium phosphate buffer. 2 μM of goat liver cystatin was incubated with buffers of pH 2.0 (glycine-HCl buffer), sodium acetate (pH 3.0–5.0) and sodium phosphate (pH 6.0–7.0) for 2 h at 37 °C. The inhibitory activity of all the incubated samples was assessed by its ability to inhibit the casienolytic activity of papain by the method of Kunitz (1941) [26]. Intrinsic fluoresence measurements were carried out on a Shimadzu spectrofluorimeter model RF-540. The fluoresence was recorded in the wavelength range 300 nm–400 nm after exciting the protein solution at 280 nm for total protein fluoresence.

Addition of Alcohols (Methanol, Glycerol) and Polyethylene Glycol on Acid-denatured State of Cystatin

Co-solvent containing solutions were freshly prepared for each study with a volumetric adjustments of concentrations. Methanol, glycerol and PEG-300 were added to the acid denatured state in concentration range upto 80% (*V*/*V*).

UV-measurements

UV-measurements of the co-solvent containing solutions was done on a UV spectrophotometer in the wavelength range of 220 nm–400 nm using a cell holder of 1 cm path length.

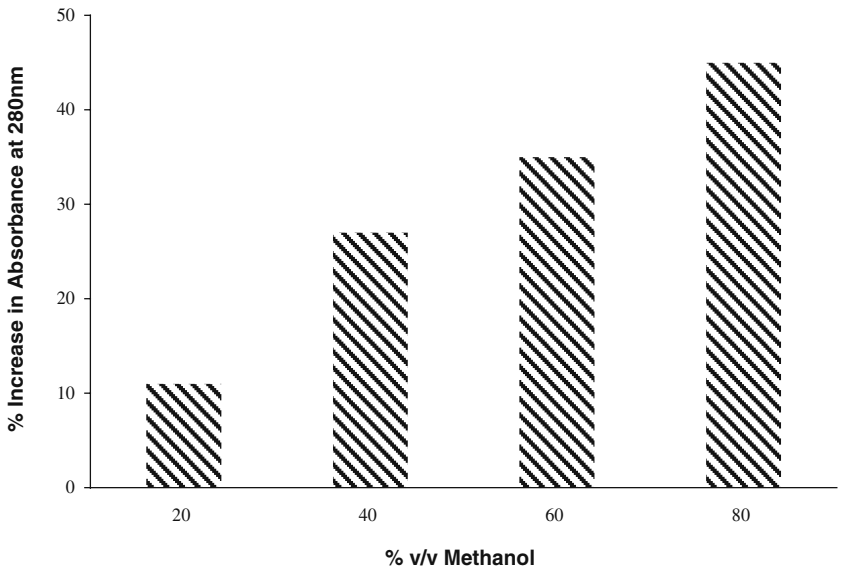

Fig. 5 Percentage increase in absorbance at 280 nm in the acid-induced state of goat liver cystatin obtained at pH 2 upon addition of varying concentration of methanol

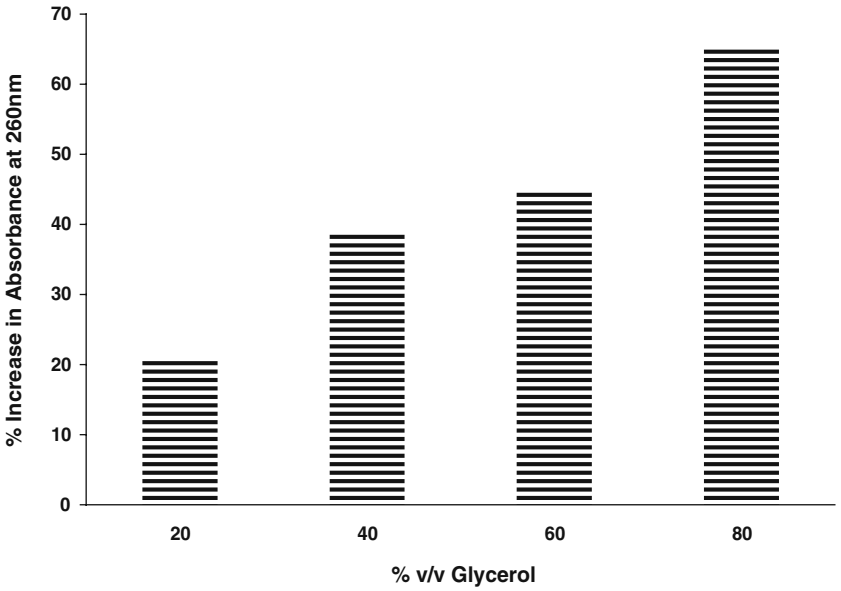

Fig. 6 Percentage increase in absorbance at 260 nm in the acid-induced state of goat liver cystatin obtained at pH 2 upon addition of varying concentration of glycerol

Fluorescence Measurements

The fluorescence spectra were recorded on a Shimadzu RF 540-samples containing different concentrations of PEG-300, methanol and glycerol were incubated at room temperature for 2 h before recording fluorescence. The excitation wavelength was 280 nm and the emission was recorded from 300 to 400 nm. The final protein concentration was taken into consideration.

Results and Discussion

Acid Induced Denaturation of Goat Liver Cystatin

The acid denaturation of goat liver cystatin was studied over a pH range of 2.0–7.0 in terms of intrinsic fluorescence. The spectral parameters of tryptophan fluorescence, such as position, shape and intensity are dependent on the dynamic and electronic properties of the chromophore

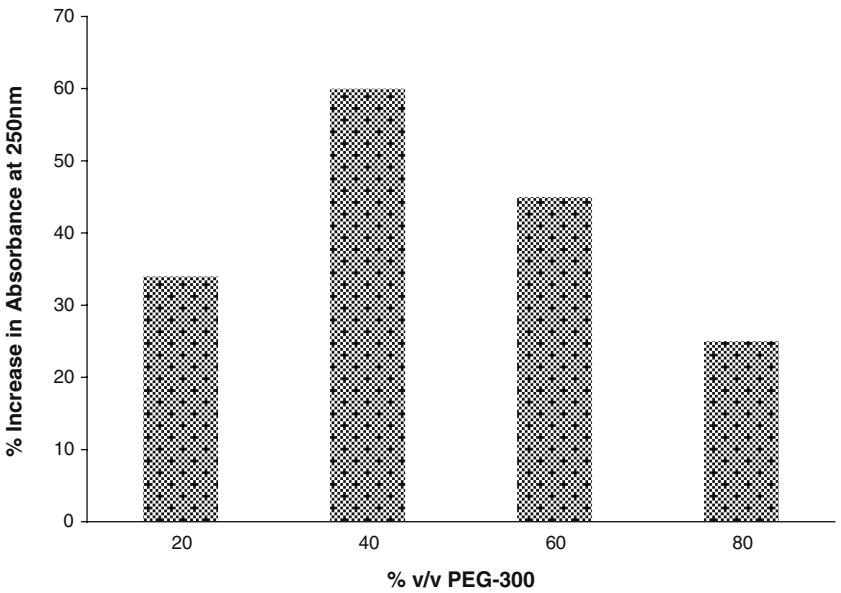

Fig. 7 Percentage increase in absorbance at 250 nm in the acid-induced state of goat liver cystatin obtained at pH 2 upon addition of varying concentration of polyethylene glycol PEG-300

environment, hence steady state fluorescence has been extensively used to obtain information on the structural and dynamic properties of proteins [27].

Figure 1 shows the intrinsic fluorescence emission spectra of goat liver cystatin (LC) under different conditions. In the native state at pH 7.5, GLC is characterized by a peak at 340 nm. As the pH was decreased from 7.0 to 2.0 the fluorescence intensity was found to gradually increase with a red shift. At pH 2.0 goat liver cystatin is substantially unfolded at low ionic strength as evidenced by the considerable increase in fluorescence intensity and a red shift of 15 nm. The acid induced state has a λ max at 355 nm. This red shift of 15 nm and the increase in fluorescence intensity indicate that the protein conformation is being altered and that the microenvironment of the aromatic amino acids is being changed.

Effect of Alcohols (Methanol and Glycerol) on the Acid Induced State of Goat Liver Cystatin

The intrinsic fluorescence maximum (λmax) is an excellent parameter to monitor the polarity of tryptophan environment in the protein and is sensitive to protein conformation [28]. The additive effects of two alcohols methanol and glycerol was studied on the acid-induced goat liver cystatin. As the concentration of methanol was increased from 20% (*V*/*V*) to 80% (*V*/*V*) the fluorescence intensity was found to decrease with a blue shift.

As can be seen in Fig. 2, 80% (*V*/*V*) methanol showed a λ max emission at 350 nm thus indicating that methanol stabilized the acid-denatured goat liver cystatin particularly at high concentrations.

Glycerol was also found to have stabilizing effect on acid-denatured goat liver cystatin. However, glycerol showed a better stabilizing co-solvent effect as compared to methanol. This is evident from Fig. 3 as 40% (*V*/*V*) glycerol concentration brought the same stabilizing effect as was brought by 80% methanol. At even higher concentration of glycerol that is at 60% (*V*/*V*) and 80% (*V*/*V*) glycerol further stabilized the acid-induced denatured cystatin and revealed a state that was very near to the native cystatin. This could be because glycerol has three –OH groups as a result of which it exhibited more hydrogen bonding and more polar–polar interactions, hence glycerol being a better stabilizing agent.

On addition of PEG-300 (20% *V*/*V*) to the acid induced state of goat liver cystatin, there was a decrease in fluorescence intensity with a blue shift of 5 nm (Fig. 4). The fluorescene intensity further goes on decreasing with polyol concentration upto 40% and the emission maximum at this concentration was 345 nm. Increasing the polyol concentration to 60% and 80% resulted in a sudden increase in fluorescence intensity especially at 80% PEG-300 concentration. This change in tertiary conformation might have occurred in such a way that exposed the tryptophan residues accounting for the sudden increase in tryptophan fluorescence at 60% and 80% PEG-300 concentration. Thus it could be concluded from the results that PEG-300 stabilized the acid-denatured state only at low concentrations (Table 1).

Effect of Alcohols (Methanol & Glycerol) and PEG-300 on the Absorption Patterns of Acid Induced State of Goat Liver Cystatin

The acid induced state of goat liver cystatin showed one peak at 230 nm and a smaller peak at 270 nm with only slight increase in the apparent absorbance as compared to the native state of goat liver cystatin. However, addition of alcohols (methanol & glycerol) and PEG-300 resulted in the significant increase in the absorbance.

Addition of methanol in varying concentrations of 20% (*V*/*V*) to 80% (*V*/*V*) resulted in increase of absorbance at 280 nm. Figure 5 shows that increasing the concentration of methanol resulted in linear increase of apparent absorbance. The increase in absorbance around 280 nm could be attributed to the change in the environment of tyrosine residues.

Addition of glycerol with three (–OH) groups resulted in maximum apparent absorbance at 260 nm. The increase in absorbance around 260 nm could be attributed to the change in conformation of tryptophan and tyrosine residues. As shown in Fig. 6 maximum increase in absorbance could be seen in 80% *V*/*V* glycerol followed by 60, 40% and 20%. The magnitude in absorbance was maximum for glycerol, which could be due to the three (–OH) groups present in glycerol.

Addition of PEG was also found to increase apparent absorbance at 250 nm due to the change in the microenvironment of phenylalanine residues. However, the magnitude of increase was lesser as compared to methanol and glycerol. This could be because of the more number of carbon atoms present in the n-alkyl chain of PEG (Fig. 7).

Conclusion

Thus on the basis of these studies it can be concluded that polyol induced state has a midway conformation lying in between the native and acid-induced state at pH 2.0. Since cystatins have an important role to play as thiol protease inhibitors in normal body processes owing to their cysteine proteinase inhibitory activity. Thus the refolding studies on goat liver cystatin may prove useful in understanding of the pathway by which the protein attains its native conformation and various conditions that can be employed to refold denatured protein back to its native state.

References

1. Kim PS, Baldwin RL (1990) Intermediates in the folding reactions of small proteins. Annu Rev Biochem 59:631–660
2. Dobson CM (1991) Characterization of protein folding intermediates. Curr Opin Struct Biol 1:22–27
3. Redfield C, Smith RAG, Dobson CM (1994) Structural characterization of highly ordered molten globule at low pH. Nat Struct Biol 1:23–29
4. Ptitsyn OB (1995) Molten globule and protein folding. Adv Protein Chem 47:83–229
5. Arcus VL, Vuilleumier S, Freund SMV, Bycloft M, Fresht AL (1995) A comparison of the pH, urea and temperature denatured states of barnase by heteronuclear NMR: implications for the initation of protein folding. J Mol Biol 254:305–321
6. Shortle D (1996) The denatured state (the other half of the folding equation) and its role in protein stability. FASEB J 10:27–34
7. Creighton TE (1993) Chemical properties of polypeptides, proteins:Structure and molecular properties. Freeeman, New York, pp 1–48
8. Tan YJ, Oliverberg M, Davies B, Fersht AR (1995) Perturbed pKa values in the denatured states of proteins. J Mol Biol 254:980–992
9. Yancey PH, Somero GN (1980) Methylamine osmoregulatory solutes of elasmobranch fishes counter act urea inhibition of enzymes. J Exp Zool 212:205–213
10. Timasheff SN (1993) The control of protein stability and association by weak interactions with water: how do solvents affect these processes? Annu Rev Biophys Biomol Struct 22:67–97
11. Zou Q, Bennion BJ, Dagget V, Murphy KP (2002) The molecular mechanism of stabilisation of proteins by TMAQ and its ability to counteract the effect of urea. J Am Chem Soc 124:1192–1202
12. Bychkova VE, Dujeskima AE, Klemin SI, Tiktopulo EI, Uversky VN, Ptitsyn OB (1996) Biochemistry 35:6058–6063
13. Chiti F, Taddei N, Webstal P, Hamada D, Fiaschi T, Ramponl G, Dobson CM (1999) Nat Struct Biol 6:380–387
14. Lu H, Buck M, Radford SE, Dobson CM (1997) J Mol Biol 265:112–117
15. Raqi C, Sedaghat-Herati MR, Ouameur AA, Tajmir-Reahi HA (2005) Biopolymers 78:231–236
16. Ekiel I, Abrahamson M, Fulton DB, Lindahl P, Storer AC, Levadoux W, Lafrance M, Labelle S, Pomerleau Y, Groleau D, LeSauteur L, Gehring K (1997) (NMR) Structural studies of human cystatins C dimmers and monomers. J Mol Biol 271:266–271
17. Barrett AJ, Rawlings N, Davies M, Machleidt W, Salvesen G, Turk V (1986a) Cysteine Proteinase inhibitors of the cystatin superfamily. In: Barrett A, Salvesen G (eds) Proteinase inhibitors. Elsevier, Amsterdam, pp 515–569
18. Barrett AJ, Fritz H, Grubb A, Isemura S, Järvinen M, Katunuma N, Machleidt W, Müller-Esterl W, Sasaki M, Turk V (1986) Biochem J 236:312
19. Shah A, Bano B (2009) Cystatins in health and diseases. Int J Pept Res Ther 2009(15):43–48
20. Trabant A, Gay RE, Fassbender HG, Gay RS (1991) Cathepsin b in synovial cells at the site of joint destruction in rheumatoid arthritis. Arthritis Rheum 34:1444–51
21. Delaisse JM, Ledent P, Vaes G (1991) Collagenolytic Cysteine proteinases of bone tissue. Cathepsin B, procathepsin L and a cathepsins L like 70KDa proteinases. Biochem J 279:167–174
22. Bernstein HG, Kirschke H, Wiederander B, Pollak KH, Zipress A, Rinne A (1996) Mol Chem Neuropathol 27(3):225–247
23. Köppel P, Baici A, Keist R, Matzku S, Keller R (1984) Cathepsin B-like proteinases as a marker for metastatic tumors cell variants. Exp Cell Biol 52:293–299
24. North MJ, Moltram JC, Coombs GH (1990) Parasitol Today 6:270–275
25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. Biol Chem 193:265–270
26. Kunitz M (1947) Crystalline soyabean trypsin inhibitor, general properties. J Physiol 30:291–310
27. Ghisla S, Massey V, Lostle JM, Mayhew S (1974) Fluoresence and optical characteristics of reduced flavins and flavoproteins. Biochemistry 13:589–597
28. Stryer L (1986) Science 162:526–540