Shivani Jain et al. / Journal of Pharmacy Research 2015,9(8),500-508

Review Article
ISSN: 0974-6943

Available online through
http://jprsolutions.info

Effects of Osmolytes on The Structural Stability of Bovine Trypsin: A Brief Review

Shivani Jain[1], Sarvesh Seechurn[1], Parth Gupta[2], Gaurika Garg[2], Bhavuk Dhamija[2], N. Latha[2], and Kameshwar Sharma YVR[2*]
[1]Biological Sciences, Sri Venkateswara College, University of Delhi, Delhi-110021
[2]Department of Biochemistry, Sri Venkateswara College, University of Delhi, Delhi-110021

Received on:12-01-2015; Revised on: 11-04-2015; Accepted on: 19-08-2015

ABSTRACT

Proteases represent a formidable class of industrial enzymes, accounting for about half of the total sale of the enzymes in the world. Trypsin, a protease that has strong specificity, acts as a digestive enzyme and plays a pivotal role in digestive physiology. Proteins have evolved to function within the cellular milieu, where macromolecule and small molecule solutes are present at high concentrations. It has long been known that osmolytes and other solutes transmute protein stability in vitro while certain osmolytes stabilize proteins in vivo to thermal and chemical denaturation arising in stress conditions. The role of various osmolytes and denaturants in the conformational properties and stability of the enzyme, trypsin has been scarcely investigated. In this study an attempt has been made to probe the effects of osmolytes, Glucose, Glycine, Proline, Sucrose, Trehalose and denaturants Guanidine Hydrochloride and Urea along with their concentration effects on the activity and stability of bovine trypsin. Detailed circular dichroism, steady state and temperature dependent fluorescence spectra, UV-Vis spectra and enzyme assay have been studied to scrutinise osmolyte induced stability and denaturant-induced unfolding properties of trypsin. The specific activity peak was found to be highest in sucrose and lowest in urea and GnHCl. The UV-Vis spectroscopic data showed highest absorbance peak for Proline depicting it as the strongest stabilizer, supported by low absorbance peak in fluorescence spectra. On the other hand, Urea was found to be strong denaturant with its lowest absorbance peak and highest intensity peak in UV-Vis and fluorescence spectra respectively. High osmolyte concentration demonstrated an increase in stability in case of Proline and significant rise in denaturing capacity of urea. CD analysis depicted highest positive peak in Proline, concluding it as the strongest stabilizer followed by Sucrose and Trehalose. Alpha helix and beta sheet content was estimated by K2D2 software which supported the trend in stability concordant with the rest of the data.

KEYWORDS: Trypsin, protease, osmolytes, protein folding, denaturants, spectroscopy, enzyme kinetics

INTRODUCTION

Proteins are polymers formed of smaller units known as amino acid residues held together by peptide bonds between the carboxylic and amide groups. In order to perform their biological functions, these proteins fold into specific spatial conformations, propelled by a number of non-covalent interactions such as hydrogen bonding, ionic interactions, Van der Waals forces, and hydrophobic packing[1]. These three dimensional structures discern their function in the cellular milieu to a great extent. They have evolved here to work concomitantly with other macromolecules and small molecule solutes present at high concentrations. Most cells in vivo are majorly dominated by the presence of water, electrolytes, metabolites and osmolytes as medium[2]. A protein may undergo reversible changes in its structure in order to perform its biological function. The stability studies of these transition states increasingly form a major focus of research.

***Corresponding author.**
Dr. Kameshwar Sharma YVR
Assistant Professor
Department of Biochemistry
Sri Venkateswara College
University of Delhi
Delhi-110021,India.

A protein structure is highly complicated comprising of different structural units like domains, motifs and folds. The repeating sequence of atoms along the core of the polypeptide chain is referred to as the polypeptide backbone and attached to these chains are side chains which confer upon amino acids their unique properties, setting them apart. Some of these side chains are hydrophobic or non-polar, others are charged positively or negatively[1]. The hydrophobic side chains of amino acids such as Phenylalanine, Leucine, Valine, and Tryptophan tend to cluster in the interior of the molecule enabling them to avoid contact with water surrounding them. On the other hand, the polar side chains like those of Arginine, Glutamine, and Histidine have a tendency to arrange themselves outside the molecule where

they can form hydrogen bonds with water and other polar molecules. These interactions of the side chain residues with the outside aqueous environment are known as the hydrophobic effect.

All proteins in the cell assume a structure which is thermodynamically stable under physiological conditions, known as the native state. The difference in the free energies of the folded and unfolded states quantitatively determines the net stability of the protein structure. However, these conformations get disturbed on contact with the surrounding molecules and in extreme conditions such as temperature change, high pressure and excessive salt conditions. Such perturbations may lead to unfolding of the protein and loss of its functional activity. Thus, there is a need for a mechanism to cope with these conditions and maintain the desired conformation[3]. One such mechanism is accumulation of solutes known as osmolytes which help the cell to fight unfavourable conditions and simultaneously maintain thermodynamic equilibrium between native and unfolded state.

MOLECULAR INTERACTIONS WHICH PLAY A ROLE IN CONFORMATIONAL CHANGES

Osmolytes, a class of small intracellular, neutral organic molecules get accumulated in the cells, preventing their scavenging from osmosis and other stress conditions. They raise the free energy of the unfolded state, favoring the folded state conformation. All known osmolytes are amino acids and their derivatives like Leucine, Glycine, Valine; polyols and sugars like Glucose, Sucrose, Trehalose; methylamines like Trimethylamine oxides (TMAO) and denaturants like Guanidine Hydrochloride and Urea. Unlike salt ions, these are mostly compatible with all the proteins. This ability of osmolyte to enhance the driving force for protein folding is due to a phenomenon called solvophobic effect acting on the peptide backbone, which gets exposed in the unfolded state. This implies that in the presence of solute, the change in free energy for interaction of water with the protein surface groups is more favourable than the change in free energy for solute interaction with the protein[3, 4].

The difference in the involvement of osmolytes in the presence and absence of proteins gives an insight into a parameter known as preferential interaction which forms the basis of structural stability during folding and unfolding process. If water is enriched near the protein surface relative to its composition in the bulk solution, a preferential interaction exists between water and the protein surface, resulting in preferential hydration with respect to water or preferential exclusion from that of osmolyte. In a different scenario, if the osmolyte is enriched near the protein surface, it is said to preferentially bind to the protein surface by exclusion or replacement of water. Thus, if there is preferential binding of one component, water or osmolyte with the protein, there will necessarily be a preferential exclusion of the other component. Preferential interaction provides an impetus for folding with preferential exclusion of protecting osmolytes while similar interaction with non-protecting organic molecules known as denaturants cause protein unfolding or destabilization[5, 6, 7]. Also, the presence of denaturant increasingly shifts the protein conformational entropy towards the unfolded state, more than to the folded state and this leads to net destabilization. Urea and Guanidine Hydrochloride are two of the strongest denaturants observed so far.

The small size of osmolytes enables them to reach intracellular and extracellular molar concentrations range in the extremities and are hence indispensable for the survival of most of the organisms and their biological functions[8]. The discovery of these protecting osmolytes has given an opportunity to understand the way in which organic osmolytes stabilize proteins, a crucial subject in protein chemistry[9].

Osmolytes, therefore, promote requisite and unhindered protein folding which aids in enhancing its conformational stability efficiently.

APPLICATIONS OF OSMOLYTE INDUCED PROTEIN STABILITY IN INDUSTRY

It is an established notion that osmolytes stabilize and induce folding of aberrant proteins and hence, they can act as pharmacological chaperones for a large number of protein misfolding disorders. Polyols like Trehalose have been found to inhibit aggregation of denatured proteins like that of bovine glutamic dehydrogenase, following heat shock. It has even been exhibited to prevent fibrillation and cytotoxicity of AB-peptide and to combat prion-infected cells from induced oxidative damage[10, 11]. TMAO has been shown to prevent misfolding of prion proteins[12]. Since organic osmolytes are naturally occurring molecules, they possess potential curative applications without fear of major toxic side effects. They are commonly used to stabilize protein-based biopharmaceuticals such as recombinant human interferon, various healing antibodies and protein-based vaccines such as those for hepatitis A and B[13-17]. Cytosolic cystathionine ß-synthase (CBS) deficient mutant protein effect was found to get reversed by regaining the lost functional ability in the presence of chaperonic osmolytes like TMAO, Glycerol, Sorbitol and DMSO in yeast model[18].

Some light has been shed on the way an oral administration of an osmolyte can appreciably inhibit polyglutamine mediated protein accumulation in the transgenic mouse model of Huntington disease with further increased life span[19]. This protein impairment process constitutes a hallmark of neurodegenerative pathologies, including

Alzheimer's and Huntington's, which involve extracellular protein accumulation and fibril formation while intracellular or lewy-body formation in Parkinson's disease. So, if osmolytes can provide a unifying mechanism of action, they may have far reaching consequences in developing efficient therapeutic tools for tackling such diseases[20]. Such effects of osmolytes on protein folding pathways have become significant to study. Among other osmolytes, Trimethylamine-*N*-oxide (TMAO) has received special interest lately since it has demonstrated an extraordinary capability to support the folding of denatured to native-like species, which show significant functional activity. Recently, it has been shown that these osmolytes can be used *in vivo* also. Example being that of Glycerol and Trimethylamine N-oxide (TMAO) which can correct the temperature-sensitive folding defect of the human cystic fibrosis transmembrane conductance regulator (CFTR) mutant protein, F508 CFTR (having a deletion of a phenylalanine residue at position 508). Glycerol and TMAO have also been shown to correct the folding defect of the tumour suppressor protein, p53, mutant A125V, in cells. This mutation results in a temperature-sensitive folding defect, which is alleviated by the osmolytes and their effects are reversible[21, 22]. There is ample evidence that methylamines are better suited for the perturbation of proteins by urea, while polyols are better used as cryoprotectants and thermal stabilizers.

Osmolytes have also paved their way into biotechnological applications such as stabilization of laboratory and pharmaceutical reagents[23]. Betaine helps increase transcription and translation along with polymerase chain reaction and monoclonal antibody production by hybridoma cells[24-26]. Osmolyte Sorbitol builds up in hyperglycemia condition in diabetes mellitus which has clinical implications in its treatment[27]. Similarly, Betaine plays an important role in urinary tract infections and drug designing[28]. Recombinant DNA technology is being increasingly used to formulate novice strategies in order to incorporate osmolytes at the time of protein expression in the bacterial growth media to suppress and refold inclusion body formation[29]. Further on osmolytes are proving to be highly useful in the other fields too, such as agriculture, especially in alleviating high stress conditions and mammalian biological functions[30].

PROTEASES

Trypsin, chymotrypsin, and elastase are all digestive enzymes that are produced in the pancreas and catalyze the hydrolysis of peptide bonds. Each of these enzymes has different specificities with regards to the side chains next to the peptide bond. Chymotrypsin prefers a hydrophobic residue, trypsin is specific for a positively charged residue, and elastase prefers a small neutral residue. Chymotrypsin, trypsin and elastase are all proteins that contain a specific catalytic mechanism and hydrolyze peptides using the serine protease mechanism. Chymotrypsin and elastase are both homologs of trypsin since they are 40% alike in structure and composition. These proteases are ubiquitous and have great importance due to a wide range of industrial applications[1]. Thus, an attempt has been made to carry out a pilot study on protease, trypsin which has not been profoundly investigated till now.

Catalysis is a crucial phenomenon significant in everyday life as well as scientific research. Enzymes or biocatalysts can be both proteinaceous and non proteinaceous which in active state enhance the rate of a reaction to many folds.

Proteases, also known as peptidases or proteinases are systematic enzymes whose catalytic function is to perform proteolysis, i.e., hydrolysis of peptide bonds that link amino acids together in the polypeptide chain which make up proteins. These can be obtained from varied sources like plants, animals and microbes, where aquatic sources have been relatively less explored. They are amongst the most eminent enzymes used in the world today for various industrial purposes. Proteolytic enzymes are very important in digestion as they breakdown the peptide bond in protein rich foods to liberate the amino acids needed by the body. They have been used for a long time in various forms of therapy; their use in medicine and clinical studies is indispensable for further studies in enzyme therapy. They have multiple industrial applications such as in detergents, food, and pharmaceuticals etc.[31].

All proteases have an active regulatory mechanism where their inactive state or zymogen is cleaved by other protein molecules, sometimes of the same type to get activated. Some of them like serine proteases are more active post autolysis. The serine proteases are a family of enzymes that cut certain peptide bonds in other proteins. This activity depends on a set of amino acid residues in the active site of the enzymes, one of which is always serine. In mammals, serine proteases perform many functions especially in digestion and blood clotting. Trypsinogen is the precursor form or zymogen of trypsin and is secreted in the lysosome of the cell. The zymogen precursor is necessary in order to prevent the destruction of cellular proteins and to allow the enzyme to be in its active state only when in appropriate conditions.

The use of hydrolytic enzymes such as protease, lipase and amylase in the industrial enzyme market is growing steadily due to their excellent specificity, being atom economic, mild reaction conditions, energy saving process and simplicity[32, 33]. Proteases alone account for about 50% of the total sale of the enzymes in the world. Trypsin is one such protease, structural stability of which in presence of osmolytes we have tried to elucidate.

TRYPSIN AND ITS IMPORTANCE

Trypsin [EC 3.4.21.4], an autocatalytic enzyme, belongs to a class of serine proteases, found in the digestive system of many vertebrates, where it cleaves peptide chains mainly at the carboxyl side of the amino acids lysine or arginine, except when either is followed by proline[34]. This specificity is driven by the residue which lies at the base of the enzyme's S1 pocket, generally a negatively charged Aspartic acid or Glutamic acid. It is a medium sized globular protein of molecular weight 24 kDa, consisting of 223 amino acid residues and has high stability and catalytic activity. Hence, it plays a pivotal role in the process of digestion. It is composed of 13 beta-strands, six of which form a beta-barrel structure. There are four regions of alpha-helix, and six disulfide bridges. In the duodenum, it catalyzes the hydrolysis of peptide bonds, which is a necessary step in protein absorption as proteins are generally too large to be absorbed through the lining of small intestine. Trypsin is synthesized in the pyloric caecum as an inactive precursor trypsinogen, which is secreted into the intestinal lumen and activated by enteroproteases[35]. It has an optimal pH of 7.5-8.5 and an optimal temperature of 37°C.

Trypsin breaks down peptides by catalytic mechanism common to all serine proteases. The active site where this mechanism occurs in trypsin is composed of three amino acids and is known as a catalytic triad. The three catalytic residues are Serine 195, Histidine 57, and Aspartate 102. In this mechanism, serine is bonded to the imidazole ring of histidine. When histidine accepts a proton from serine an alkoxide nucleophile is formed. This nucleophile attacks the substrate when present. The role of the Aspartate residue is to hold Histidine in proper position to make it a good proton acceptor. The three residues function to hold each other in the proper positions in the pocket formed for nucleophilic attack. The steps of the mechanism involve two tetrahedral intermediates and an acyl-enzyme intermediate. A motif is formed in this reaction known as an oxyanion hole. This oxyanion hole is specifically formed between the amide hydrogen atoms of Serine 195 and Glycine 193. This oxyanion hole stabilizes the tetrahedral intermediate through the distribution of negative charge to the cleaved amide[1].

Trypsin is the most discriminating of all proteolytic enzymes in terms of restricted number of chemical bonds that it attacks. Good use of this fact has been made by chemists to determine the amino acid sequence of proteins. The isolation and purification of trypsin has been done for various species, including fishes and carps. Fish viscera in particular, contain a rich source of digestive proteases which accounts for 5% of the total mass[36].

Partially purified preparations of trypsin are used in meat tenderizing, leather industry, cell culture industry and in proteinaceous preparation of immunoglobin fragments[37]. Due to its powerful proteolytic function and specificity, trypsin has a wide range of applications as in the manufacturing of insulin, production of recombinant proteins for clinical use, in wound care market and as an oral treatment for inflammatory edema, haematoma etc.[38]. Other applications include its use in pre-digestion of baby food, fingerprinting and sequencing work, and environmental monitoring.

Trypsin has been isolated from a number of animal sources including bovine, porcine, ovine, goat, mouse, whale, elephant, seal, African lung fish, cat, turkey, fish and human[38]. Among all trypsins, fish trypsins are of great interest as they exhibit high catalytic activity at low temperatures, making them very suitable for food processing and biotechnological applications[39].

INFLUENCE OF OSMOLYTES AND DENATURANTS ON TRYPSIN BY USING ENZYME KINETICS AND SPECTROSCOPIC METHODS

Protein folding is an extremely complex process and extensive research is being conducted for elucidation of new methods to study this mechanism at molecular and atomic levels. Experimentally, the degree of folding is generally measured by tracking some spectroscopic proxy, such as intrinsic fluorescence or ellipticity as measured by circular dichroism. Thus, our objective is to assess parameters like specific activity, UV-Vis & fluorescence spectroscopy and CD analysis in both osmolytes and denaturants for complete understanding of protein folding/unfolding and investigation of its structure and function.

Awanish *et al*. analysed the stability of alpha chymotrypsin, protease similar to trypsin in different solvents using the above stated spectroscopic methods[40]. Raj Kumar and co-workers also performed these methods for a comparative study between trypsin and chymotrypsin[41-43].

Stability studies of trypsin have not been explored to its potential and there is a paucity of information available on this subject. Therefore, we have tried to probe the same through our studies using the following parameters.

A) Enzyme Kinetics

The initial velocity of trypsin activity in the presence of selected osmolyte solutions was determined from the slope of the first order reaction velocity in the linear range of the hyperbolic curve of the reaction. The change in absorbance was assessed on Varian Cary UV–Vis spectrophotometer under kinetic mode for 3 minutes at 410

nm by measuring the production of *p*-nitroanilide using L-BAPNA (N-Benzoyl-DL-arginine-4-nitroanilide hydrochloride) of Sigma-Aldrich Corporation, St. Louis, MO, USA as the substrate[44]. Further, we also calculated the specific activity of the respective osmolyte solutions which determines the amount of product formed by an enzyme in a given amount of time under given conditions, per milligram of total protein and gives a measurement of the enzyme purity[33].

Pankaj Attri along with co-workers measured the activity of CT (Chymotrypsin) by the method of Erlanger *et al*. using SAPNA in 50 mM Tris-HCl, pH 8.2 having 20 mM $CaCl_2$ as a substrate solution[44, 45]. Chymotrypsin activity units were expressed as the change in absorbance per minute per milligram of protein. With reference to the data collected by them, it was quite evident that Urea and GnHCl had zero enzyme activity as CT was in the unfolded state. Their previous results show that, among all the osmolytes, TMAO is a powerful stabilizer and the most compatible of osmolytes, whereas the activity results reveal that Sucrose has eminent activity among the rest of the osmolytes. This phenomenon explains that chymotrypsin activity is not significantly enhanced by any osmolytes. The lack of change in CT activity under similar conditions indicates that osmolyte-induced folding is not specific. Results reveal that Trehalose acted as the best enhancer for CT stability among the set of polyols, with varying efficacies and efficiencies. Urea and GnHCl show very low specific activity as the protein is in unfolded state as observed by Awanish *et al*.[40]. S. Sahu and P. Das demonstrated that inclusion of osmolytes in assay medium significantly enhanced the activity. The stimulation was registered to be an osmolyte concentration dependent phenomenon. Maximum stimulation was visualized in the range of 300-500 mM with sugar alcohols and between 200-300 mM with sucrose. Excesses of sucrose beyond 300 mM induced a decline in activity. While a comparable stimulation in activity was discernible with sugar alcohols like Sorbitol and Mannitol, a significant higher stimulation was achieved by inclusion of sucrose in the medium[46].

While in trypsin, the rate of substrate digestion is significantly increased in the presence of TMAO in the presence of 3M glycerol or in buffer alone[41].

Our results are consistent with the experimental results of TMAO-induced enzyme activity. Glycine, Sucrose and Proline showed high activity while GnHCl and Urea showed little activity. The higher the specific activity, more stable is folded state and vice versa. If specific activity is more, then conformational states are not disturbed and its active sites are not blocked as in case of Sucrose. Although Sucrose is a weak stabilizer, it shows highest enzyme activity with respect to others. This shows that trypsin activity is not significantly enhanced by rest of the osmolytes.

B) UV Spectroscopy

Absorption emission spectra of commercially available bovine trypsin in the presence of various osmolyte solutions were recorded using UV Spectrophotometer (UV-1800 Shimadzu) within a range of 300-400nm.

In case of CT, unfolding in presence of denaturants leads to exposure of Tyr residues and thus, tyrosyl ionization as observed by Awanish *et al*.[40].

Absorbance in the visible light spectrum correlates to the stability of proteins as it depends on the environment of the protein. Through our pilot studies, we concluded that higher the absorbance maxima value, higher is the stability of protein at the specific wavelength at which a peak is recorded and vice versa. This observation is opposite to that observed in case of fluorimetry where, the higher the recorded intensity, lower is the stability and vice versa. Highest UV-Vis absorbance was recorded for Proline at 250 nm at lower concentration of 0.25M, suggesting it was the most stabilizing amongst all osmolytes. Trehalose and GnHCl showed the least absorbance, showing their effects as denaturants.

C) Fluorescence Spectroscopy

Fluorescence emission spectroscopy, a biophysical method is extensively used in research for unravelling the structural conformation and dynamics of macromolecules like proteins. This information is gained by studies of phenomenon that affect the excited state such as the local environment, quenching process and energy transfer[47]. The fluorescence intensity of a molecule depends on its quantum yield, which is obtained by the ratio of emitted photons to that of excited ones. Tryptophan and Tyrosine both act as fluorophores and get excited at a wavelength of 280 nm, while Tryptophan alone has a peak at 295 nm. Their fluorescence properties are sensitive to the environment which changes when a protein folds or unfolds.

In native or folded protein, Tyr and Trp reside inside the protein core where hydrophobic effect becomes prevalent, giving high quantum yield. While unfolded proteins are exposed to solvents and give rise to hydrophilic environment. One example of denaturant is GnHCl, where all Trp residues get hydrated as a consequence of loss of stability, increasing the fluorescence intensity by a large factor resulting in a stokes shift. There are 5 Trp and 12 Tyr residues in trypsin, which are found throughout the molecule, and several of them are located in the vicinity of active sites, indicating that the

environmental changes in the Trp and Tyr residues may involve some of the active sites. In case of chymotrypsin, there are eight Trp and four Tyr residues, which are also spread throughout the molecule[41].

As observed by Raj Kumar *et al*., the enzyme activity of trypsin is significantly enhanced in TMAO compared to glycerol. A quantum yield and blue shift in wavelength maxima was observed, suggesting stable state in the presence of TMAO[41]. Pankaj Attri and co-workers performed experiments on trypsin which show that TMAO is the strongest stabilizer being the most effective compatible osmolyte, whereas Sucrose is a weak stabilizer and the least protective osmolyte[44]. The stabilizing effects of the four polyols on chymotrypsin in the decreasing order were seen to be as, Trehalose, Sucrose, Glycerol and Sorbitol as reported by Awanish *et al*.[40]. GnHCl was found to be a stronger denaturant than Urea for the same. According to Venkatesu *et al.*, the efficiency of stabilizing effects in decreasing order follows the trend TMAO, Proline, Betaine, Sarcosine and Sucrose[48]. Polyols like Glucose, Trehalose, and Glycine produced a significant blue shift indicating increased stability as reported by Pankaj Attri[44]. N. Gheibi and co-workers with their studies on mushroom tyrosinase (MT) from *Agaricusbisporus* showed that in the case of Trehalose, there is a pronounced reduction in maximum emission of intrinsic fluorescence leading to higher stability[49]. Edward P. O'Brien *et al*. performed their experiments on protein L and cold shock protein and found that the efficiency of denaturation follows the trend in decreasing order GnHCl, Urea and Betaine[50].

Fluorescence emission spectra of commercially available Bovine Trypsin were recorded in this study in Tris buffer, 10 mM, pH 8.0 on Cary Eclipse Spectrofluorimeter at an excitation wavelength of 295 nm in the presence of the osmolytes Glucose, Glycine, Sucrose, Proline and Trehalose; denaturants, GnHCl and Urea at three concentrations 0.25 M, 0.5 M and 1 M. The experiments were carried out at temperatures, 25^{0}C, 45^{0}C and 65^{0}C respectively. The results of our experiments for enzyme trypsin were found to be in support of the data mentioned above. Glucose, Trehalose and Proline were found to be good stabilizers as compared to Glycine and Sucrose while GnHCl was found to be a strong denaturant.

D) Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy measures the difference in the absorbance of right- and left-circularly polarized light by a substance and thus**,** is a well-known method for understanding the structure and interaction of proteins in a solution. This phenomenon is highly sensitive to secondary structure of proteins and can analyze the content of alpha helix, parallel & anti- parallel beta sheets and random coils. It spans the range from 180-250 nm wavelengths and is a widely used technique. Changes in far UV reflect major changes in backbone of protein. Here, absorbing group is mainly peptide bond or amide chromophore. The tertiary conformation can also be obtained from studying the CD spectrum in the near UV range of 250-350 nm. It can be mainly attributed to the presence of aromatic amino acids, Tyrosine (Tyr), Tryptophan (Trp) and Phenylalanine (Phe) and disulphide bonds between Cysteine residues. The major bands of Trp are located in the 290-305 nm region while that of Tyr in the 275-282 nm region. The bands for Phe lies in the range of 255-270 nm but are generally so weak that they go unnoticed as trypsin belongs to family of globular proteins having a low content of aromatic residues. The near UV absorbance of disulphide bond occurs near 260 nm and is generally quite weak. However, the intensity depends on a number of factors like dihedral angle of disulphide bond[51].

There is a paucity of data available for the review of this technique for enzyme trypsin. So, in this study, we have tried to correlate the data available for other similar enzymes with that of trypsin.

Studies carried out by Mohammed Suleiman Zaroog and co-workers on the effect of various polyols on the native and thermal denatured state of Glucoamylase (GA) revealed that the far UV CD spectrum of the thermally denaturated state of GA showed ~48% loss in the mean residue ellipticity at 222 nm value compared to the native GA, along with a shift in the CD spectral signals, suggesting denaturation of GA at high temperature as observed with other proteins. Addition of Glucose or Trehalose to the Thermal Denatured state of GA markedly increased the $MRE_{222\ nm}$ value by ~110% and ~91%, respectively, showing a significant reversal in the CD spectral characteristics close to the native GA. Glycine was able to induce ~22% regain in this value[52]. Quantitatively, various polyols showed the increase in order of effectiveness directing GA to adopt a more stable conformation.

As observed by Awanish Kumar *et al.*, in their studies on chymotrypsin, polyols are found to be excellent protein stabilizers. These may include Glycerol, Erythritol, Xylitol, Sorbitol, Sucrose, and Trehalose. The efficiency of stabilizing effects follows the trend, TMAO, Proline, Betaine, Sarcosine and Sucrose in increasing order as per their analysis[40]. Pankaj Attri *et al*. showed that the far CD spectrum of Chymotrypsin (CT) in buffer has no positive band, whereas TMAO and Sarcosine showed a positive band. Proline and Betaine have also very high positive absorption. TMAO, Betaine, Sarcosine, and Sucrose show clear improvement in the ß-structure estimated using K2D2 software. They further concluded that apart from far UV, near UV also depicted some changes though not significant. The near-UV CD spectra of CT shows small differences in intensity obtained for Trehalose, Sorbitol, Glycerol, and Sucrose in between 280 and 300 nm

which could again be attributed to a compact conformation. On the contrary, the drop-off ß-structure in denaturants vividly demonstrated the ability of Urea and GnHCl to ruin the compact and flexible native conformation of CT[44].

Pincock and co-workers proposed that a major factor involved in the denaturation of protein is the "concentration effect." It is evident that with an increase in the concentration of Urea, the denaturation extent of the molecule is gradually increased. On adding denaturants such as GnHCl to the solution of the enzyme, the band obtained at 230 nm becomes less negative[53]. Studies show that GnHCl is a stronger denaturant than Urea[40]. The interaction of Proline with Bovine liver catalase increased the percentage of α-helices the most, followed by Xylitol and Valine, whereas the noncompatible Histidine destabilized the macromolecular structure which was eminent from the decrease in the α-helical content, as a registered benchmark for the destabilization[54].

The present CD spectroscopic studies conducted on a JASCO J-815 spectrophotometer with osmolytes prepared in Tris buffer, 10 mM, and pH 8.0 at concentrations, 0.25 M, 0.5 M and 1 M. Trypsin concentration was taken as 1 mg/ml. Samples were scanned at room temperature in the far UV range (180-250 nm) using a 0.1 cm path length cuvette. The alpha and beta secondary structures were calculated using K2D2 software. The results obtained from our data were found to be consistent with the trends observed in other similar proteases as stated above. Trehalose shows a dramatic enhancement in tertiary structure at 1 M concentration, while in the rest of the polyols, the update of the tertiary structure was not too high. However a slight increase in structural stability was observed in the case of Glycerol at 1M concentration.

CONCLUDING REMARKS

From our pilot study, we conclude that the presence of co solvents in the form of osmolytes and denaturants play a pivotal role in determining the stability status of a protein molecule like trypsin present with the myriad other components in a cell. Osmolytes like polyols, trimethylamines and amino acids act as stabilizers preventing the cell from unfavourable conditions like desiccation, stress, high temperature, extreme pressure and presence of other chaotropic agents. Denaturants like GnHCl and Urea act as strong destabilisers. Proteins are surrounded by an aqueous medium and the interplay of varied molecular interactions like hydrophobicity, solvophobic effect, preferential hydration and preferential exclusion with solvents influence their conformational dynamics profoundly. Concentration of co-solvents plays a critical role in determining the extent of folding and unfolding of a protein molecule. Spectroscopic techniques such as UV-Vis spectroscopy, fluorescence emission spectroscopy and circular dichroism spectroscopy act as great proxy for probing the structural changes in a macromolecule like protein. To conclude, trypsin is an important protein from the perspective of great industrial applications and hence more emphasis on studies to purify it and to maintain its structural stability in vitro is required.

ACKNOWLEDGEMENT

Authors are thankful to Department of Biotechnology (DBT), Govt. of India for funding the project under DBT-STAR College Scheme "BT/HRD/11/07/2011". Sincere thanks to the Principal, Sri Venkateswara College for providing lab space & kind support and Dr. P. Venkatesu, Department of Chemistry, University of Delhi for necessary help.

REFERENCES

1. D. Voet, J. G. Voet, and C. W. Pratt, "Principles Of Biochemistry" *John Wiley And Sons*, Ed. 3, 2008.
2. P. H. Yancey, M. E. Clark, S. C. Hand, R. D. Bowlus, And G. N. Somero, "Living With Water Stress: Evolution Of Osmolyte Systems." *Science*, Vol. 217, No. 4566, Pp. 1212–1222, 1982.
3. R. Kumar, "Role of Naturally Occurring Osmolytes In Protein Stability and Stability." *Archives of Biochemistry and Biophysics*, Vol. 491, No. 1-2, Pp. 1–6, 2009.
4. D. W. Bolen, "Effects Of Naturally Occurring Osmolytes On Protein Stability And Solubility: Issues Important In Protein Crystallization." *Methods*, Vol. 34, No. 3, Pp. 312–22, 2004.
5. D. J. Felitsky and M. T. Record, "Application of the Local-Bulk Partitioning and Competitive Binding Models To Interpret Preferential Interactions of Glycine Betaine and Urea with Protein Surface." *Biochemistry*, Vol. 43, No. 28, Pp. 9276–9288, 2004.
6. D. J. Felitsky, J. G. Cannon, M. W. Capp, J. Hong, A. W. V. Wynsberghe, C. F. Anderson, And M. T. Record, "The Exclusion Of Glycine Betaine From Anionic Biopolymer Surface: Why Glycine Betaine Is An Effective Osmoprotectant But Also A Compatible Solute." *Biochemistry*, Vol. 43, No. 46, Pp. 14732–14743, 2004.
7. S. N. Timasheff, "Stabilization of Protein Structure by Solvent Additives." *In Stability Of Protein Pharmaceuticals, Part B: In Vivo Pathways Of Degradation And Strategies For Protein Stabilization*, Pp. 265–285, 1992.
8. J. Rosgen, B. M. Pettitt, And D. W. Bolen, "Protein Folding, Stability, And Solvation Structure In Osmolyte Solutions." *Biophysical Journal*, Vol. 89, No. 5, Pp. 2988-2997, 2005.
9. M. Auton and D. W. Bolen, "Predicting The Energetics of Osmolyte-Induced Protein Folding/Unfolding." *Proceedings*

Of The National Academy Of Sciences Of The United States Of America, Vol. 102, No. 42, 15065–15068, 2005.

10. R. Liu, H. Barkhordarian, S. Emadi, C. B. Park, and M. R. Sierks, "Trehalose Differentially Inhibits Aggregation and Neurotoxicity of Beta-Amyloid 40 And 42." *Neurobiology of Disease*, Vol. 20, No. 1, Pp. 74-81, 2005.
11. F. Béranger, C. Crozet, A. Goldsborough, and S. Lahmann, "Trehalose Impairs Aggregation of PrP^{Sc} Molecules and Protects Prion-Infected Cells against Oxidative Damage." *Biochemical and Biophysical Research Communications*, Vol. 374, No. 1, Pp. 44-48, 2008.
12. B. J. Bennion and V. Daggett, "Counteraction of Urea-Induced Protein Denaturation by Trimethylamine N-Oxide: A Chemical Chaperone at Atomic Resolution." *Proceedings Of The National Academy Of Sciences Of The United States Of America*, Vol. 101, No. 17, Pp. 6433-6438, 2004.
13. M. Besman, E. Bjornson, F. Jameel, R. Kashi, M. Pikal, S. Tchesalov, And J. Carpenter, "Novel Albumin Free Factor VIII Formulations." Patent No. US0176791-A1, 2008.
14. J. F. Carpenter, H. Sathish, T. W. Randolph, B. Salinas, C. Allan, and S. Bishop, "Stabilized Antibody Formulations and Uses Thereof." Patent No. WO039761- A2, 2008.
15. S. D. Webb, J. L. Cleland, J. F. Carpenter, And T. W. Randolph, "Effects Of Annealing Lyophilised And Spray-Lyophilised Formulations Of Recombinant Human Interferon-Gamma." *Journal of Pharmaceutical Sciences*, Vol. 92, No. 4, Pp. 715-729, 2003.
16. E. Y. Chi, J. Weickmann, J. F. Carpenter, M. C. Manning, and T. W. Randolph, "Heterogeneous Nucleation-Controlled Particulate Formation of Recombinant Human Platelet-Activating Factor Acetylhydrolase In Pharmaceutical Formulation." *Journal of Pharmaceutical Sciences*, Vol. 94, No. 2, Pp. 256-274, 2005.
17. D. T. Brandau, L. S. Jones, C. M. Wiethoff, J. Rexroad, And C. R. Middaugh, "Thermal Stability Of Vaccines," *Journal Of Pharmaceutical Sciences*, Vol. 92, No. 2, Pp. 218-231, 2003.
18. L.R. Singh, X. Chen, V. Kozich, And W.D. Kruge, "Chemical Chaperone Rescue of Mutant Human Cystathionine ß-Synthase." *Molecular Genetics and Metabolism*, Vol. 91, No. 4, Pp. 335-342, 2007.
19. M. Tanaka, Y. Machida, S. Niu, T. Ikeda, N. R. Jana, H. Doi, M. Kurosawa, M. Nekooki, And N. Nukina, "Trehalose Alleviates Polyglutamine-Mediated Pathology In A Mouse Model Of Huntington Disease," *Nature Madicine*, Vol. 10, No. 2, Pp. 148–154, 2004.
20. R. Kumar and E. B. Thompson, "Transactivation Functions Of the N-Terminal Domains of Nuclear Hormone Receptors: Protein Folding and Coactivator Interactions." *Molecular Endocrinology*, Vol. 17, No. 1, Pp. 1-10, 2003.
21. D. Michalovitz, O. Halevy, And M. Oren, "Conditional Inhibition of Transformation and of Cell Proliferation by a Temperature Sensitive Mutant Of p53," *Cell*, Vol. 62, No. 4, Pp. 671-680, 1990.
22. J. Martinez, I. Georgoff, J. Martinez, And A. J. Levine, "Cellular Localization And Cell Cycle Regulation By A Temperature-Sensitive p53 Protein," *Genes And Development*, Vol. 5, Pp. 151-159, 1991.
23. T. Arakawa, D. Ejima, Y. Kita, and K. Tsumoto, "Small Molecules Pharmacological Chaperones: From Thermodynamic Stabilization to Pharmaceutical Drugs." *Biochimica et Biophysica Acta*, Vol. 1764, No. 11, Pp. 1677-1687, 2006.
24. W. Henke, K. Herdel, K. Jung, D. Schnorr, and S. A. Loening, "Betaine Improves the PCR Amplification of GC-Rich DNA Sequences." *Nucleic Acids Research*, Vol. 25, No. 19, Pp. 3957-3958, 1997.
25. C. Hethke, A. Bergerat, W. Hausner, P. Forterre, and M. Thomm, "Cell-Free Transcription at 95 Degrees: Thermostability of Transcriptional Components and DNA Topology Requirements of Pyrococcus Transcription." *Genetics*, Vol. 152, No. 4, Pp. 1325-1333, 1999.
26. K. Oyaas, T. E. Ellingsen, N. Dyrset, and D. W. Levine, "Utilization of Osmoprotective Compounds By Hybridoma Cells Exposed To Hyperosmotic Stress." *Biotechnology and Bioengineering*, Vol. 43, No. 1, Pp. 77-89, 1994.
27. M. B. Burg and P. F. Kador, "Sorbitol, Osmoregulation, and the Complications of Diabetes." *The Journal of Clinical Investigation*, Vol. 81, No. 3, Pp. 635-640, 1988.
28. B. A. Peddie, M. Lever, K. Randall, And S. T. Chambers, "Osmoprotective Activity, Urea Protection and Accumulation Of Hydrophilic Betaines In Escherichia coli And Staphylococcus aureus." *Antonie Van Leeuwenhoek*, Vol. 75, No. 3, Pp. 183-189, 1999.
29. K. Tsumoto, D. Ejima, I. Kumagai, and T. Arakawa, "Practical Considerations In Refolding Proteins from Inclusion Bodies." *Protein Expression and Purification*, Vol. 28, No. 1, Pp. 1-8, 2003.
30. M.C. Tarczynski, R.G. Jensen, and H.J. Bohnert, "Stress Protection of Transgenic Tobacco by Production of the Osmolyte Mannitol." *Science*, Vol. 259, No. 5094, Pp. 508-510, 1993.
31. B. K. Khangembam, K. S. YVR, and R. Chakrabarti, "Purification and Characterization of Trypsin from the Digestive System of Carp *Catla catla* (Hamilton)." *International Aquatic Research*, Vol. 4, No. 9, 2012.
32. K. S. YVR, N. Boora, And P. Tyagi, "Isolation, Purification

and Characterisation of Secondary Structure and Kinetic Study of Lipase from Indian Major Carp, *Catla catla* (Catla)." *Enzyme Engineering*, Vol. 3, No. 1, 2014.

33. S. Kulshrestha, P. Tyagi, V. Sindhi, and K. S. YVR, "Invertase and Its Applications- A Brief Review." *Journal of Pharmacy Research*, Vol. 7, No. 9, Pp. 792-797, 2013.
34. W. R. Repniewski, A. Perrakis, C. E. Vorgias, and K. S. Wilson, "Evolutionary Divergence and Conservation of Trypsin." *Protein Engineering*, Vol. 7, No. 1, Pp. 57-64, 1994.
35. M. Marcuschi, T. S. Esposito, M. F. Machado, I. Y. Hirata, M. F. Machado, M. V. Silva, L. B. Carvalho, V. Oliveira, And R. S. Bezerra, "Purification, Characterization And Substrate Specificity Of A Trypsin From The Amazonian Fish Tambaqui *(Colossoma macropomum)*." *Biochemical and Biophysical Research Communications*, Vol. 396, No. 3, Pp. 667–673, 2010.
36. A.Gildberg, "Recovery of Proteinase and Protein Hydrolysates from Fish Viscera." *Bioresource Technology*, Vol. 39, No. 3, Pp. 271–276, 1992.
37. N. B. Iannucci, G. J. Albanesi, M. M. Marani, H. M. F. Lahore, O. Cascone, and S. A. Camperi, "Isolation Of Trypsin From Bovine Pancreas Using Immobilized Benzamidine And Peptide CTPR Ligands In Expanded Beds." *Separation Science and Technology*, Vol. 40, No. 16, Pp. 3277-3287, 2007.
38. S. Mukherjee, D. Roy, and P. Bhattacharya, "Comparative Performance Study Of Polyethersufone and Polysulfone Membranes for Trypsin Isolation from Goat Pancreas Using Affinity Ultrafiltration." *Separation Science and Technology*, Vol. 60, No. 3, Pp. 345-351, 2007.
39. N. F. Haard and B. K. Simpson, "Digestive Proteases from Marine Animals." *Seafood Enzymes*, Pp. 191-213, 2000.
40. A.Kumar and P. Venkatesu, "Overview of the Stability of a-Chymotrypsin In Different Solvent Media." *Chemical Reviews*, Vol. 112, No. 7, Pp. 4283-4307, 2012.
41. R. Kumar, J. M. Serrette, and E. B. Thompson, "Osmolyte-Induced Folding Enhances Tryptic Enzyme Activity." *Archives of Biochemistry and Biophysics*, Vol. 436, No. 1, Pp. 78-82, 2005.
42. R. Kumar, J. M. Serrette, S. H. Khan, A. L. Miller, and E. B. Thompson, "Effects Of Different Osmolytes on the Induced Folding of the N-Terminal Activation Domain (AF1) of the Glucocorticoid Receptor." *Archives of Biochemistry and Biophysics*, Vol. 465, No. 2, Pp. 452-460, 2007.
43. R. Kumar, J. C. Lee, D. W. Bolen, And E. B. Thompson, "The Conformation Of The Glucocorticoid Receptor AF1/tau1 Domain Induced By Osmolyte Binds Co-regulatory Proteins." *The Journal of Biological Chemistry*, Vol. 276, No. 21, Pp. 18146-18152, 2001.
44. P. Attri and P. Venkatesu, "Thermodynamic Characterization of the Biocompatible Ionic Liquid Effects on Protein Model Compounds and Their Functional Groups." *Physical Chemistry Chemical Physics*, Vol. 13, No. 14, Pp. 6566-6575, 2011.
45. B. F. Erlanger, N. Kokowsky, and W. Kohen, "The Preparation and Properties of Two New Chromogenic Substrates of Trypsin." *Archives of Biochemistry and Biophysics*, Vol. 95, Pp. 271-278, 1961.
46. S. Sahu, P. Das, M. Ray, and S. C. Sabat, "Osmolyte Modulated Enhanced Rice Leaf Catalase Activity under Salt-Stress." *Advances In Bioscience and Biotechnology*, Vol. 1, 39-46, 2010.
47. J. R. Lakovicz, "Principles of Fluorescence Spectroscopy." *Springer*, Ed. 3, 2006.
48. P. M. Reddy, M. Taha, A. Kumar, P. Venkatesu, and M. J. Lee, "Interruption Of Hydration State Of Thermoresponsive Polymer, Poly (N-Isopropylacrylamide) In Guanidinium Hydrochloride." *Polymer*, Vol. 54, No. 2, Pp. 791-797, 2011.
49. N. Gheibi, A. A. Saboury, K. Haghbeen, and A. A. Moosavi-Movahedi, "The Effect of Some Osmolytes on the Activity and Stability of Mushroom Tyrosinase." *Journal of Biosciences*, Vol. 31, No. 3, Pp. 355–362, 2006.
50. E. P. O'Brien, G. Ziv, G. Haran, B. R. Brooks, and D. Thirumalai, "Effects of Denaturants and Osmolytes on Proteins Are Accurately Predicted By the Molecular Transfer Model." *Proceedings Of The National Academy Of Sciences Of The United States Of America*, Vol. 105, Pp. 13403-13408, 2008.
51. S. M. Kelly, T. J. Jess, and N. C. Price, "How to Study Proteins by Circular Dichroism." *Biochimica et Biophysica Acta*, Vol. 1751, No. 2, Pp. 119-139, 2005.
52. M. S. Zaroog, H. A. Kadir, and S. Tayyab, "Stabilizing Effect of Various Polyols on the Native and the Denatured States of Glucoamylase." *The Scientific World Journal*, Vol. 2013, 2013.
53. R. E. Pincock and W. S. Lin, "Denaturation of a-Chymotrypsin In Frozen Aqueous Solutions." *Journal of Agricultural and Food Chemistry*, Vol. 21, No. 1, Pp. 2-5, 1973.
54. H. S. Tehrani, A. A. Moosavi-Movahedi, H. Ghourchian, F. Ahmad, A. Kiany, M. S. Atri, S. Ariaeenejad, K. Kavousi, And A. A. Saboury, "Effect Of Compatible And Noncompatible Osmolytes On The Enzymatic Activity And Thermal Stability Of Bovine Liver Catalase." *Journal of Biomolecular Structure and Dynamics*, Vol. 31, No. 12, Pp. 1440-1454, 2013.

Source of support: Department of Biotechnology (DBT), Govt. of India**, Conflict of interest:** None