Studies on Stability and Folding of Bacterial Alpha Amylases

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CERTIFICATE

This is to certify that present work entitled **"Studies on Stability and Folding of Bacterial Alpha Amylases"** submitted to the Jawaharlal Nehru University, New Delhi in partial fulfilment of the requirement for the award of the degree of Doctor of philosophy, embodies original work carried out in the School of Biotechnology, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part, or full, for the any other degree or diploma of any University.

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SYMBOLS AND ABBREVIATIONS

%	Percentage
×	Times
÷	divide
±	Plus-minus
≤	Greater than or equal to
<	Less than or equal to
°C	Degree celcius
µg	Microgram/s
µl	Microliter/s
µm	Micrometer
µM	Micromolar
Å	Angstrom
ACS	Acetyl-CoA synthetase
AHA	Alteromonas haloplanktis
AMP	Adenosine monophospahte
APS	Ammonium persulphate
AFM	Atomic force microscopy
Asn	Asparagine
Asp	Aspartate
Arg	Arginine
$BaCl_2$	Barium chloride
BAA	Bacillus amyloliquefaciens
BLA	Bacillus licheniformis
BSTA	Bacillus stearothermophilus
$Ca(OAc)_2$	Calcium acetate
CD	Circular Dichroism
cm	Centimeter
$CuSO_4$	Copper sulphate
Cys	Cysteine
$CaCl_2$	Calcium chloride
CTAB	cetyltrimethylammonium bromide
CS	Citrate Synthase
DNS	3,5-Dinitosalicyclic acid
DTAB	dodecyl trimethylammonium bromide
EDTA	Ethylenediaminetetraacetic acid
EG	Ethylene glycol
Glu	Glutamate
Gsn	Glutamine
gm	Gram
GdmCl	Guanidinium hydrochloride
GFP	Green fluorescent protein

GH	Glycosyl hydrolase
Gly	Glycine
H	Hour
HCl	Hydrochloric acid
HEWL	Hen egg white lysozyme
His	Histidine
H bond	Hydrogen bond
IDP	Intrinsically disordered protein
Ile	Isoleucine
K^+	Potassium ion
Kcal	Kilocalorie
kDa	Kilodalton
KH_2PO_4	Monopotassium phosphate
kJ	Kilojoule
Kv	kilovolt
LD	Lethal dose
Lys	Lysine
Leu	Leucine
Met	Methionine
M	Molar
mg	Milligrams
min	Minute(s)
ml	Milliliter(s)
mm	millimeter
mM	Millimoles
MQ	milliQ water
$MgSO_4$	Magnesium sulphate
$Mg\ (OAc)_2$	Magnesium acetate
$MgCl_2$	Magnesium chloride
MSG	Malate synthase G
N	Normal
NaCl	Sodium chloride
NaH_2PO_4	Monosodium phosphate
NaOH	Sodium hydroxide
$(NH_4)_2CO_3$	Ammonium carbonate
nm	Nanometer
OD	Optical density
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
pKa	-log[Ka]
pH	-log[H+]
pI	Isoelectric point

PPI	Peptidyl-prolyl isomerase
PDI	Protein disulfide isomerase
PDB	Protein data base
PPA	Pig pancrease amylase
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Room temperature
SDS-PAGE	Sodium dodecylsulphate-polyacrylamide gel electrophoresis
Ser	Serine
S	Second (s)
S.D.	Standard deviation
T_m	Mid-point of thermal denaturation
Tyr	Tyrosine
Thr	Threonine
Trp	Tryptophan
Tris	Tris(hydroxymethyl) Aminomethane
TMAO	Trimethylamine N-oxide
TTAB	tetradecyl trimethylammonium bromide
TAKA	Amylase from Aspergillus oryzae
UV	Ultraviolet
Val	Valine
w/v	Weight/volume
w/w	Weight/weight
α	Alpha
β	Beta
γ	Gamma
λ	Lambda
ε	epsilon

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Dedicated to my beloved family for their love, endless support and encouragement

CHAPTER 1

INTRODUCTION

1.1 Proteins

Proteins are one of the most important biomolecules which are found in all living organism. They are polymer of amino acids constituting the major building blocks of living beings and control the most vital processes in organisms. However, there is huge diversity exist in their size, structure, amino acid sequence, composition and function. In living system, proteins perform various functions like catalysis of metabolic reactions (enzymes), provide structural support to the body (microfilaments), act as a transporter, involved in signalling (hormones), defense (immunoglobins) and storage of the metabolic food etc. Besides this, protein also forms various transmembrane channels to regulate movement of the molecules across the cell membranes. Large repertoire of proteins in organisms interact with other macromolecules, membranes and water thus regulate various functions including DNA synthesis, DNA repair, cell cycle control, cell movement etc. (Alberts et al., 2014). The hierarchical structural organization of protein consists of primary, secondary, tertiary and quaternary structure (Linderstrøm-Lang and Schellman, 1959). Unique amino acid sequence is the primary structure of proteins which is encoded by the genetic code. Even single amino acid change in the primary structure may results in the polypeptide with entirely different properties. The local arrangement of amino acid residues gives different hydrogen bonding pattern in secondary structure, α-helix and β-sheet. α-helix consists of 'H' bonds formed between the carboxyl group of first amino acid and the amino group of fourth amino acid, which are perpendicular to its axis. Although α-helix can adopt both right-handed and left-handed topology but right-handed α-helix is more favoured due to its higher stability. There are 3.6 amino acids per turn in α-helix with a pitch of 5.4 Å. β-sheet structure forms between two or more polypeptide strands. Hydrogen-bonding pattern between polypeptides in β-sheets can be parallel or antiparallel, depending upon their orientation with respect to each other (Fersht, 1999). α-helix and β-sheets are linked by loops and turns. In recent times several studies have established the fact that in several proteins either full or part of the structure is disordered and known as intrinsically disordered proteins (IDPs) (Uversky, 2015; Toto et al., 2020). There are some structures consisting of combination of α-helix and β-sheets known as super-secondary structures for example, α-α hairpin, α-β-α structure, β-β hairpins etc. (Söding and Lupas, 2003). Such structures help to adopt specialized conformation of proteins as per the structural

and functional requirements. Domain is the independent folding unit of protein, which may represent the whole (single domain) or part of protein (multidomain). Multidomain proteins arise from the single domain proteins during the course of selective evolutionary pressure as per the adaptive requirements (Han et al., 2007). During evolution, individual domain in protein undergoes series of duplication and subsequent shuffling which gives rise to different domains in the multidomain proteins (Han et al., 2007; Bornberg-Bauer et al., 2010; Projecto-Garcia et al., 2015). Individual domain of multi domain proteins interact via both covalent and non-covalent interactions like disulfide bonds, H-bonds, hydrophobic, salt bridges, van der Waal etc. Thus, often multidomain proteins are more stable than the single domain proteins, both structurally and functionally. Domain-domain interactions play a defining role in the stability of multidomain proteins with shared interdomain surfaces (Osváth et al., 2005; Schlicker et al., 2007, He et al., 2007). Nature of the domain surfaces in addition to their stability, also affect the unfolding and folding of multidomain proteins (Osváth et al., 2005; Viswanath et al., 2018; Arviv and levy, 2012). Unlike single domain proteins, which have normally two state unfolding and folding pathways, most of the multidomain proteins have complex folding/unfolding pathways with the presence of partially folded intermediates (Batey and Clarke, 2006; Inanami et al., 2014). The final conformation which protein adopts due to the interaction between side chains of the amino acid residues and polypeptide backbone with properly folded secondary structure is referred as tertiary structure. Proteins with two or more polypeptide chains interact covalently or non-covalently to form to form quaternary structure (Sund and Weber 1966).

On the basis of their adaptations to various physiological conditions, proteins have been grouped into different categories like temperature adaptation (Thermophilic, Hypertheromophilic, Psychrophilic and mesophilic), pH adaptation (Acidophilic and Alkaliphilic) salt adaptation (Halophilic) and pressure adaptation (Barophilic) (Reed et al., 2013). Thermophilic adaptation has been considered as the most fascinating characteristics of the proteins due to their applications in different industries performing catalysis at higher temperature (Unsworth et al., 2007). Proteins from organisms growing optimally between 50 to 70˚C are classified as thermophilic proteins and the organisms are known as thermophiles. The organisms which normally grow above 80^{o}C are called hyperthermophiles. Most of the thermophilic

and hyperthermophilic organisms belong to either archaea or bacteria (Huber and Stetter, 1998). These organisms have huge repertoire of structural and functional proteins adapted to higher temperature but enzymes are the most widely studied from both, academic and industrial point of view. Enzymes obtained from thermophilic or hyperthermophilic microorgansims are also called thermozymes. There is a long list of such enzymes like cellulases, amylases, xylanase, protease, endoglucanase (Dumorné, et al., 2017) etc. which have been studied for their adaptive strategy and their applications in various industries. These proteins employ battery of structural and functional adaptations in order to maintain the stability of native conformation at extremely high temperature, which include increased hydrophobicity of protein core, salt bridges, disulfide bonds, 'H' bonding etc. As there is no general mechanism of thermal stabilization, thus contribution of the factors controlling the thermal stability may vary. In most of the cases, more than one factor synergistically enhances the thermal stability of proteins. For example, AMP (Adenosine monophosphate) forming acetyl-CoA synthetase (ACS) from *Pyrobaculum aerophilum* reported to has octameric state contrary to monomeric state observed in its mesophilic counterparts, although, oligomerization is not the universal mechanism of thermal stabilization. In addition, this protein also has very low content of thermolabile amino acids like Asn, Cys and Gln which generally undergoes chemical modifications at higher temperature (Bräsen et al., 2005). α-amylase from *Pyrococcus woesi* lacks oligomerization but still retains its structural integrity at higher temperature, which comes from multiple metal ion binding sites and disulfide bridges which are formed between Cys^{388} and Cys^{432} and another between Cys^{153} and Cys^{154} (Linden et al., 2003). Increased hydrophobicity of the protein core and the presence of three calcium binding sites in the crystal structure of α-amylase from *Bacillus licheniformis* (BLA), are the major determinant of its higher thermal stability (Machius et al., 1995; Alikhajeh et al., 2010; Fitter and Haber, 2004). Electrostatic interactions between the opposite charged groups and the salt bridges also affect the thermal stability of thermophilic or hyperthermophilic proteins. Mutational study on the ribosomal protein L30e from the thermophilic bacteria *Thermococcus coler* has shown that the salt bridges decrease the heat capacity of unfolding thus improve the thermal stability of the native state compare to the unfolded state (Chan et al., 2011). Another adaptive strategy is the presence of disulfide bonds either in protein core or between two subunits of multimeric proteins to increase the rigidity or oligomerization to enhance their

thermal stability (Cacciapuoti et al., 2012). Surface of the thermophilic proteins most often consist of increased content of charged residues as compared to the mesophilic proteins (Fukuchi and Nishikawa, 2001).

Higher kinetic and thermodynamic stability of thermophilic enzymes as compared to their mesophilic counterparts make them first choice for biotechnological and industrial applications (Unsworth et al., 2007). As the thermophilic enzymes catalyze the reactions at higher temperature, which reduces the probability of bacterial and fungal contaminations and also helps in providing the high product yield through increased kinetics of the reaction. One of the first thermostable enzyme, Taq polymerase was isolated from *Thermus aquaticus*, which catalyzes the chain reaction during *in vitro* DNA synthesis and emerged as revolutionary tool in biotechnology and molecular biology (De Champdore et al., 2007). Thermostable α-amylases from *Bacillus lichenifromis* (BLA), *Baillus stearothermophilus* (BSTA) and *Pyrococcus furiosus* etc. have been widely used in the starch-based industries like food, paper, textile and detergents (Mehta and Satyanarayana, 2016). Similar to α-amylases, other starch hydrolyzing enzymes like amylopullulanases have also been used in food, detergent and starch-based industries (Nisha and Satyanaryana, 2013). Thermostable pullulanases, glucoamylases and β-amylases are also utilized for starch liquification to help the manufacturing of glucose and fructose syrup.

1.2 Protein stability

Protein stability refers to the synergistic effect of various stabilizing interactions to keep the protein conformation in the native folded state under a given set of environmental conditions. The stability of a protein is prerequisite to work in diverse physiological conditions. The stabilizing forces of proteins can be covalent or non-covalent in nature.

1.2.1 Covalent bonding

Amino acids in a protein are linked by peptide bond. This bond keeps the entire polypeptide chain in a rigid conformation due to restricted motion along with the peptide bond which has partially double bond character. The peptide bond is highly stable and about 21 kJ/mol free energy is required to break a single peptide bond

(Branden and Tooze, 1991). The covalent bond which provides stability to proteins is disulfide bond, formed by the oxidation of two cysteine residues. It can be either intrachain or interchain depending on the location of cysteine residues involved. Although, this bond plays a defining role in the stability of extracellular proteins, but due to the reducing environment of cytosol, intracellular proteins often lack disulfide bond. The disulfide bond provides significant contribution to the protein stability by reducing the conformational freedom of unfolded state thus making the native state more stable and this mode of stabilization is known as entropic stabilization (Matsumura et al., 1989). The extent of stabilization through disulfide bond varies from protein to protein for example, intact disulfide bonds in phytase from *Aspergillus niger* are essential for its structural and functional stability (Wang et al., 2004). In addition, they also regulate the proper folding in various proteins (Branden and Tooze, 1991; Fink et al., 1999). Presence of the multiple disulfide bonds in a protein result in the high rigidity of its protein core that increases the thermal stability. Introduction of three disulfide bonds by using protein engineering in bacteriophage T4 lysozyme, results in increase of 23˚C melting temperature as compared to the wild type lacking disulfide bonds (Matsumura et al., 1989). The net effect of individual disulfide bonds on protein stability depends on structural strains imposed by it on the protein conformation. Because of this reason, disulfide bond varies in their dihedral angles and so their net effect on protein stability (Katz and Kossiakoff, 1986). Sometimes introduction of non-native disulfide bonds in proteins to enhance their thermal stabilities may also result in the loss of optimum activity due to simultaneous increase in conformational rigidity like in alkaline phosphatase (Asgeirsson et al., 2007).

1.2.2 Non-covalent interactions

The non-covalent interactions which impart substantial contribution to protein stability are hydrophobic interactions, H-bonding, electrostatic interactions and van der Waals interactions (Pace et al., 2015).

Hydrophobic interaction: Among all the non-covalent interactions, hydrophobic interaction plays a dominant role in the protein stability (Kauzmann, 1959). Protein consists of large number of non-polar amino acid residues, and their unfavourable interactions with solvent prevents protein from acquiring a stable native

conformation. Hydrophobic interactions help to reduce such unfavourable interactions up to a large extent by burying non-polar residues in the protein core (Tanford, 1962). This effect is called the hydrophobic effect. Nozaki and Tanford have purposed a hydrophobicity scale on the basis of free energy of transfer of amino acid from polar to non-polar environment (Nozaki and Tanford, 1971). Hydrophobic effect has been considered as the most prominent force in protein folding (Dill, 1990). Most of the globular proteins have more than 50% non-polar residues of their total amino acid (Schwartz et al., 2001). Generally, thermophilic and hyperthermophilic proteins have strong hydrophobic protein core than their mesophilic counterparts (Machius et al., 1995). By using various mutants of villin head piece subdomain, Pace et al., (2011) have proposed that the hydrophobic interactions contribute almost 60% of its thermodynamic stability. Extent of stabilization through hydrophobic effect vary among proteins depending on their amino acid composition, size and burial of non-polar residues in the folded state. In proteins, close packing of its interior is quite important in order to bury the both, polar and non-polar residues. It has been observed that van der Waal interactions between side chains of both polar and non-polar residues regulate the packing density of the protein core (Ratnaparkhi and Varadarajan, 2000; Chen and Stites, 2001; Pace et al., 2015).

Hydrogen bonding: The cost of burying polar side chains during protein folding is paid through intermolecular H-bonding. It has been estimated that 1.1 H-bonds per residue formed during folding (Myers and Pace, 1996; Stickle et al., 1992) and the average contribution to the protein stability is about 1 to 2 kcal/mol (Myers and Pace, 1996). The strength of H-bond depends on its geometry, average length and polarity of the solvent. In protein, average length of an H-bond is 3.04 Å, which is longer than its optimal length 2.89Å and the average strength of H-bond in α-helix is 4.9 kcal/mol (Mitchell and Price, 1990; Stickle, 1992). As the H-bonding is local in nature thus, donor and acceptor atoms should be in the proximity. In majority of proteins, most of the secondary elements involve H-bonds between polar groups of peptide backbone. The intramolecular H-bonds are the inevitable component of major secondary structure elements like α-helix and β-sheet (Stickle et al., 1992). The net contribution of a particular H-bond to the protein conformational stability depends on its position and environment in a given protein structure (Mills and Dean, 1996; Gao et al., 2009; Pace, 2009).

Ionic interactions: In most of the globular proteins, majority of the charged residues are located on their surface in order to avoid the electrostatic repulsion (Spassov et al., 1994). The net charge on charged residues is defined by their pKa values, which in turn depend on the environment of their side chains and pH of the protein solution (Thurlkill et al., 2006). Proteins are most stable and least soluble at their pI, as there is no net charge on protein at this pH (Pace et al., 2009). The maximum contribution of charge-charge or columbic interactions to the protein stability is approximately 10 kcal/mol, which may vary with the net charge on protein (Pace et al., 1990). There is growing interest in increasing the overall stability of protein through modulating the native interactions like salt bridges, hydrophobic etc., however increasing the stability by enhancing charge-charge interactions is relatively easier and effective (Schweiker et al., 2007). Proteins lacking native structure at neutral pH, referred as natively unfolded proteins. Presence of high content of charged residues and low hydrophobicity are the two main characteristics of this class of protein (Uversky et al., 2000). The electrostatic attractions between two charged residues separated by a distance of less than 5 Å is referred as salt bridge or ion pair. The probability of the formation of the salt bridge between two residues close to each other in primary structure is greater than distantly located amino acid residues. Although, it is the environment of salt bridge which decides its fate, whether a salt bridge is involved in protein stabilization or destabilization or it has no effect. The analysis of crystal structures of 36 monomeric proteins involving 222 salt bridges reveals that stabilizing effect of individual salt bridge on protein stability depends on factors like distance between residues forming salt bridge, geometry of salt bridge and its microenvironment (Kumar and Nussinov, 1999; Hendsch and Tidor, 1994). Kumar and Nussinov (1999) have also reported that exposed salt bridges have more stabilizing effect than the buried as later requires larger desolvation energy than the exposed salt bridges. For example buried salt bridge between Asp70 and His31 in T4 lysozyme contribute 3-5 kcal/mol to its stability (Anderson et al., 1990). Thermophilic proteins have higher number of salt bridges than their mesophilic counterparts. Thermostable protein CutA1 from hyperthermophilic bacterium *Pyrococcus horikhoshi* possess extreme thermal stability with T_m of 150˚C, much higher than the same protein from *Oryza sativa*. This difference in their stability is attributed to the presence of large number of salt bridges in CutA1 from *Pyrococcus horikoshi* (Sawano et al., 2008). Enhanced conformational stability of α-amylase from *Bacillus*

licheniformis (BLA) is due to the presence of higher number of salt bridges than its homologous α-amylase from *Bacillus amyloliquefaciens* (BAA) (Machius et al., 1995). Several computational and mutational studies have reported the significant increment on protein thermal stability upon introduction of the salt bridges (Lee et al., 2014; Pace et al., 2000).

van der Waal interaction: van der Waal's interactions are weak intermolecular interactions between two neutral molecules separated by a distance lesser than their own radii. They may be attractive or repulsive in nature. The strength of these forces varies inversely with the sixth power of distance between interacting atoms. For van der Waal's attraction or repulsion, molecules have to come close. Generally, neutral molecules having electronegative atoms withdraw electron cloud towards itself and acquire partial negative charge and simultaneously induces slightly positive charge to donor atom within the same or in the different molecules. van der Waal interaction plays a dominant role in the close packing of hydrophobic core of proteins and increases their thermodynamic stability, loss of such interactions may result in disruption of native structure of proteins (Ratnaparakhi and Varadarajan, 2000; Chen and Stites, 2001). Besides this, van der Waal interactions are also involved in the intermolecular interactions among proteins and proteins with their solvent and other ligands like metal ions (Barratt et al., 2005).

1.2.3 Thermodynamic and Kinetic stability

The intrinsic stability of protein can be expressed as thermodynamic and kinetic stability. The thermodynamic stability refers to the net free energy difference between the folded and the unfolded states. This energy can be estimated for proteins following two state model using equation 1 & 2 as given below:

$$\Delta G = G(U) - G(N) \qquad (1)$$

$$\Delta G = -RTlnK \qquad (2)$$

Where K is the equilibrium constant, ΔG is change in free energy of unfolding, U and N denote unfolded state and native state respectively. While, R and T represent gas constant and temperature respectively.

After fitting experimental data to the above equation, we get ΔG as a function of different variables like pH, temperature, denaturants etc. The actual free energy of unfolding obtained after extrapolating to physiological conditions like zero denaturant concentration, neutral pH and 37˚C. A positive value of ΔG is required for a protein to work in the given environmental conditions (Lumry and Eyring, 1954, Greene et al., 1974; Becklet and Schellman, 1987; Makhatadze and Privalov, 1995). The stability of a protein is delicate balance between various stabilizing and destabilizing interactions. Thus, the thermodynamic stability alone is not sufficient to ensure prolonged stability in harsh environmental conditions like extreme temperature, extreme pH and high salt concentrations etc. In such a circumstance, kinetic stability comes into play to provide optimal stability for sufficient time to perform important tasks. It creates obstacle to unfolding through separating folded state from unfolded state by high energy barriers like partially unfolded intermediates or molten globule states (Costas et al., 2009). In most of the cases, role of the kinetic stability becomes important under two conditions. First, when protein is thermodynamically stable but susceptible to undergo irreversible conversion to the non-functional state or the aggregated state. Second, native state is not the thermodynamically stable relative to the unfolded state (Sanchez, 2010). There are several instances where stability and folding of protein is under kinetic control like folding of α-lytic protease which synthesized as an inactive proenzyme. Although enzyme is thermodynamically stable but proper folding and full enzymatic catalytic activity requires it's binding to pro-region. The pro-region might induce some conformational changes which then results in its proper folding either through decreasing the free energy barriers or by inhibiting side reaction like aggregation (Baker and Agard, 1999). Another interesting example of kinetic stabilization is of HEWL. It is the electrostatic interactions between surface charged residues which stabilizes the folded state thus providing high energy barrier to unfolding process and thus, help to retain native conformation under harsh environmental conditions (Halskau et al., 2008; Sanchez, 2010). Many protein misfolding diseases like amyotrophic lateral sclerosis (ALS), phenylketonuria and transthyretin amyloidosis are result of the kinetic destabilization of proteins (Cleveland and Rothstein, 2001; Martinez et al., 2008; Sekijima et al., 2008). Besides *in vivo* implications, protein kinetic stabilization has also been used for *in vitro* purposes like improving the kinetic stability of biotechnologically relevant enzymes

which may guarantee their stability in adverse conditions encountered at industrial level (Rodriguez-larrea et al., 2006).

1.2.4 Factors affecting protein stability

Protein performs diverse functions in organisms inhabiting under different environmental conditions like varying temperature, pH, pressure and salt. In addition, proteins also get exposed to different inhibitors, denaturants, solvents and detergents during industrial applications (Sarmiento et al., 2015).

Proteins from thermophilic and hyperthermophilic organisms get exposed to temperature from 60^{o}C to more than 100^{o}C (Jaenicke, 1981; 1991). Each protein has optimal temperature at which it can work properly without undergoing any conformational perturbations. Therefore, abrupt increase in temperature beyond its optimum range may cause undesirable conformational changes that may lead to reversible or irreversible protein denaturation (Bull and Breese, 1973; Feller et al., 1994; Fitter and Haber, 2004). Thermal stability is the net result of numerous covalent and non-covalent interactions like 'H' bonding, hydrophobic interactions, electrostatic interactions, van der Waal interactions, disulfide bonds etc. These interactions may break as the protein gets exposed to high temperature for a longer period of time (Fitch et al., 2006). Amino acid composition of proteins also plays the significant role in its thermal stability. The amino acids share huge variations in their thermal stabilities which may be in the order of (Val, Leu)>Ile>Tyr>Lys>His>Met>Thr>Ser>Trp> (Asp, Glu, Arg, Cys) (Jaenicke and Böhm 1998). Thermolabile amino acids like asparagine and glutamine undergoes deamidation at high temperature. It is the most common chemical changes that occur during thermal denaturation (Pace et al., 2013; Bhanuramanand et al., 2014). In most of the cases upon deamidation, Asn and Glu acquire negative charges which results in disruption of the existing electrostatic interactions and thus leading to the protein aggregation (Robinson and Robinson, 2001; Flaugh et al., 2006). In addition to the high temperature, protein also loses their folded conformation at low temperature and the process is known as cold denaturation. At low temperature, structure of water changes, which results in breaking of the hydrophobic interactions and thus converting folded state into unfolded state (Feller, 2010; Karan et al., 2012; Privalov, 1990).

Electrostatic interaction between charged residues plays a key role in the protein stability, which in turn depends on the net charge on the ionizable groups of the side chains. The overall charge on a protein is the function of pH of medium in which it is dissolved. Linderstörm-Lang (1924), has reported that the pH is deciding factor in ionization state of a protein. Later, Tanford (1963) stated that each amino acid has different pKa value, which means in addition to pH of medium, amino acid composition also defines the net charge on a protein molecule. Most of the protein work in a narrow pH range, with maximum stability and activity at particular pH is called pH optimum. Any abrupt change in the pH, which might be higher or lower than the optimum value, may result in protein denaturation. The main cause of pH denaturation is the electrostatic repulsion between charged groups at both, highly acidic and basic pH, conditions (Chan and Dill, 1991; Goto and Fink, 1990; Anderson et al., 1990). Although proteins form acidophilic and alkaliphilic organisms can survive at acidic and basic pH respectively up to a certain range, beyond that they also lose their native structure (Horikoshi, 1999; Setembre et al., 2004; Parashar and Satyanarayana, 2018). pH induced denaturation of protein can be reversible or irreversible depending on the nature of proteins. In majority of the cases, multidomain proteins show irreversible and single domain proteins show reversible pH denaturation profile (Strucksberg et al., 2007; EI Kadi et al., 2006; Anderson et al., 1990).

Most of the denaturants exert their destabilizing effect on proteins through binding to the peptide backbone by forming H-bonds, thus disrupting the existing network of H-bonds. Most frequently used chemical denaturants are urea, GdmCl, GdmSCN, Sodium dodecyl sulphate (SDS) etc. (Timasheff, 1993; Lim et al., 2009; Bennion and Daggett, 2003). There is large variation among proteins with respect to their susceptibility towards chemical denaturants. Urea and GdmCl are the most commonly used chemical denaturants to determine the conformational stability of proteins and also for understanding the thermodynamics and the kinetics of protein folding (Möglich et al., 2005). Both, urea and GdmCl unfold the proteins through direct and indirect mechanisms. In the direct mechanism, they interact with proteins through ‘H’ bonds, as unfolded state forms more H bonds than native state, thus the unfolded state favoured over the native state in the aqueous solution of urea and GdmCl. While during indirect mechanism, both denaturants change the solvent properties like

viscosity, H bonding of water structure etc. which ultimately results in protein unfolding. Structural changes in water affects the hydrophobic interactions thus disrupt the protein stability (Makhatazdze and Privalov, 1992). At lower concentrations, both urea and GdmCl, show stabilizing effect on protein through weak binding (Mayr and Schmid, 1993). SDS an anionic detergent, generally used for protein purification and characterization purposes is also a potent protein denaturant (Gelamo et al., 2002; Nicholas et al., 1995).

Salts are important solvent ingredient which may have both positive and negative impact on protein stability. Their net effect depends on several factors like ionic strength of medium, physico-chemical properties of both the salt and the protein (Arakawa and Timasheff, 1982; Arakawa and Timasheff, 1984; Kohn et al., 1997). Usually salts which show preferential binding on the protein surface, exert destabilizing effect and classified as salting out agents, for example $MgSO_4$, Mg $(OAc)_2$ and Ca $(OAc)_2$ (Cohn and Edsall, 1946, Arakawa and Timasheff, 1984). On the other hand, salts with preferential hydration property show stabilizing effect on proteins and classified as salting in agents like $CaCl_2$, $MgCl_2$ and $BaCl_2$ (Von Hippel and Sehleich, 1969). As the net charge on protein molecules depends on the pH of its medium, the pH also controls the net effect of salt on proteins structural and functional stability (Goto and Fink, 1990).

1.3 Protein folding and unfolding

Acquisition of the native state from newly synthesized polypeptide is referred as protein folding. It is the primary structure of protein which guides the folding process (Anfinsen, 1973). In most of the cases, protein folding is highly cooperative and spontaneous despite large number of possible conformations (Levinthal, 1968, Malhotra and Udaonagar, 2016). The knowledge of protein folding mechanism helps to understand the molecular basis of protein stability and provides the idea that up to what extent amino acid sequence can control the stability of final structure of proteins (Ferina and Daggett, 2019, Dobson, 2000). This knowledge may also help to modulate the stability of proteins with diverse biotechnological and industrial applications (Khoury et al., 2014). The process of protein folding proceeds with two fundamental goals, one is to achieve lowest free energy state and second is to occur

with maximum speed. The first and second aspects of protein folding has been referred as thermodynamic and kinetic aspect of protein folding respectively (Chan and Dill, 1991). As per the thermodynamic control, final folded conformation must have lowest free energy state irrespective of nature of unfolded state. While on the other hand, kinetics of folding depends on the nature of both unfolded and folded state (Baker and Agard, 1994).

In order to delineate the puzzling mechanisms of protein folding, different models have been proposed. Earliest model called framework or hierarchic model was purposed by Ptitsyn (Ptitsyn, 1973). According to this model, process of protein folding proceeds in a hierarchical manner where secondary structural elements forms as initial structure and local interactions between them help to establish a framework for the formation of stable native conformation. Karplus and Weaver purposed the diffusion collision model, which shares similarity with framework model (Karplus and Weaver, 1976). This model assumes that protein consist of small structural entities having secondary structural elements and referred as microdomains. These microdomains undergoes chain of diffusion and collision which culminate with the formation of final folded state. There is a progressive increment in the stability of structural elements at each step with respect to the previous step. In hydrophobic collapse model, removal of hydrophobic chain from polar to non-polar environment of protein interior is the first step of protein folding (Agashe et al., 1995). Although there are some ambiguities exist such as whether the formation of secondary structural elements and hydrophobic collapse occur simultaneously or at different time scale. For example, folding of barnase and cytochrome c proceed through the formation of hydrophobic nucleus which precedes the formation of secondary structure and tertiary structure (Nath and Udgaonkar, 1997; Akiyama et al., 2002). According to nucleation condensation model, which suggests that the folding of polypeptide chain starts with the formation of small nucleus through hydrophobic collapse, which further increase the stability and establish all local and long-range interactions by diffusion and condensation mechanisms. Thus, formation of fully stable nucleus is the rate limiting step in protein folding (Fersht, 1997).

Modern view of protein folding is described by energy landscape and folding funnel. According to this, protein follows the route down in folding funnel and stops at

conformation with lowest free energy. Every conformational state during folding is considered as a point on energy landscape where Y axis represents the energy of all the interaction and X axis shows the entropy of each conformation. In addition, it also suggests the existence of several parallel folding pathways which collectively leads to a common folding state with minimum free energy and maximum stability. Different proteins may have different shape of folding funnel depending on their amino acid sequences (Giri Rao and Gosavi, 2016; Dill and Chan, 1997).

1.3.1 Folding pathways of single and multidomain proteins

Single domain proteins fold predominantly through simple two sate folding mechanism with the population of only native (N) and unfolded state (U) in their folding pathway (Jackson, 1998; Sosnick and Barrick, 2011). The folding pathway of majority of the small proteins devoid of any detectable folding intermediates (Schindler et al., 1995; Privalov, 1996; Krantz et al., 2002). But, in some cases, folding of small proteins do have various discrete intermediates (Baldwin, 1995). Though, it is difficult to detect them, but high resolution techniques like multidimensional nuclear magnetic resonance (NMR) spectroscopy helps in their detection. For example, folding kinetics of Rd-apocytochrome b_{562} (Rd-apocyt b_{562}) through hydrogen-deuterium exchange (HX) followed by multidimensional NMR has reported the existence of several partially folded intermediates (Feng et al., 2005). Similarly, folding pathway of another well studied, small protein cytochrome C has also reported intermediate species (Bai et al., 1995). Proteins lacking transient intermediates during their folding pathway have smooth folding funnel as per the energy landscape theory (Onuchic et al., 1995). Structural analysis of folding intermediates provides the substantial information about the folding mechanism. Although, these intermediates may have different topological features in different proteins but molten globule is the most frequently common populated intermediate species (Kuwajima, 1989; Ptitsyn et al., 1990; Ptitsyn, 1995). It is the amino acid sequence which guides the folding pathway of proteins. Therefore, proteins with different amino acid sequences fold via different folding pathways (Schellman and Gassner, 1996; Loh et al., 1995; Radford and Dobson, 1995). Temperature, pH and solvent of the folding medium also affect the folding pathway. Apomyoglobin forms

different folding intermediates in different medium having variation in pH and solvent composition (Fink, 1998; Loh et al., 1995; Goto and Fink, 1990).

In comparison to the small single domain proteins, multidomain proteins have complex folding pathways. This complexity may arise due to the cis-trans isomerization of proline, disulfide bond formation and protein aggregation (Feng et al., 1995). Beside these factors, domain-domain interactions also play a significant role in the folding of multidomain proteins. It has been observed that proteins sharing domain interfaces show cooperative folding transition with two state mechanism. But sometimes, this cooperativity may lose on changing the folding conditions. As in the case of chicken brain α-spectrin, R16 and R17 domains show cooperative folding at 25˚C and low ionic strength but upon changing the temperature and ionic strength, they completely lose the cooperativity and fold independently with intermediate species (Batey et al., 2005). In the absence of domain-domain interactions, individual domains of multidomain proteins may show independent unfolding as observed in the case of titin and fibronectin (Head et al., 2001; Steward et al., 2002). In the case of malate synthase G (MSG), which is a large protein (82 kDa), the inter-domain interactions are weak and due to this, the folding pathway of MSG contains functional intermediates with differentially folded domains (Dahiya and Chaudhary, 2014). Multidomain proteins also have complex folding kinetics as compared to the single domain proteins (Batey et al., 2006).

1.3.2 Protein unfolding

Stability of the protein is the result of various covalent and non-covalent interactions like 'H' bonding, hydrophobic interactions, ionic interactions, van der Waal's forces and disulfide bridges. But, their individual contribution to protein stability may vary among the proteins. Thus, the quantitative measurement of all the stabilizing interactions through protein unfolding experiments are extremely helpful to understand the origin of protein stability. Additionally, protein unfolding also provides the substantial insight in to the protein folding mechanism (Greene and Pace, 1974; Fersht, 1997). A protein can unfold via exposing to the extreme conditions of temperature, pH, pressure and chemical denaturants. Although, thermodynamic properties of protein unfolding like enthalpy, entropy etc. are independent of the mode

of denaturation, but the extent of perturbation of secondary and tertiary structure depends on the type of denaturants used in protein unfolding (Pfeil and Privalov, 1976; Makhatadze and Privalov, 1992). Hence, for realistic information of forces stabilizing the proteins, it is essential to use different denaturation methods. Irrespective of denaturants used, thermodynamic stability can only be estimated for proteins which undergo reversible unfolding with two state mechanism (Tanford, 1968; Becklet and Schellman, 1987). For proteins with two state folding mechanisms, classical unfolding studies may provide accurate measurement of free energy, entropy and enthalpy of folded conformation. Thermal unfolding is widely used method for determining the protein stability. Quantitative measurement of protein stability during thermal transition is possible by putting experimental data to the following equation:

$$\Delta G = \Delta H - T\Delta S + \Delta Cp(T - To - Tln(\frac{T}{To}) \qquad (3)$$

Where ΔG, ΔH and TΔS represent the change in free energy, enthalpy and entropy of thermal unfolding respectively, ΔCp is the change in heat capacity upon unfolding as function of temperature. *T* represents any given temperature during thermal transition, while T_0 indicates reference temperature (Schellman, 1987). Values of these parameters determine the stability of a protein in a given set of environmental conditions. Heat capacity is the most important thermodynamic property, as it gives the idea about the extent of opening of protein core and exposure of the non-polar residues to the polar environment. More positive value of heat capacity indicates larger contribution of hydrophobic interactions to the protein stability (Tanford, 1968; Schellman, 1987; Pace et al., 1996,). The graphical representation of change in thermodynamic parameters like ΔH, TΔS, ΔG and ΔCp as a function of temperature is called protein stability curve. Although, the thermal transition is an effective method for stability measurement, but the relative contribution of various non-covalent interactions in protein stability can be estimated through equilibrium unfolding experiments in different denaturants.

Thermal Unfolding: During thermal transition, melting temperature (T_m) represents the temperature at which protein loses half of its native structure. T_m is the characteristic property of a protein, but its value may change upon changing the environmental conditions. In addition to providing the estimate of thermal stability,

T_m can also give idea about the number of domains present in multidomain proteins, if domains unfold independently (Ingham et al, 1995). Reversibility of thermal unfolding transition depends on the type of protein, solvent and other unfolding conditions like pH, ionic strength and heating rate etc. For example, extent of reversibility of thermal unfolding transition of lysozyme varies with pH and presence and absence of cosolvent like trehalose, maltose etc. (Blumlein and McManus, 2013). Most often, single domain proteins show reversible thermal unfolding and follows two state model. Thermal unfolding of bovine alpha-lactalbumin exhibits reversible two state transition (Hiraoka and Sugai, 1984). Similarly, chymotrypsin inhibitor-2 also shows reversible thermal unfolding transition with 7.18 kcal/mol free energy of folding (Jackson and Fersht, 1991). While most of the multidomain proteins show irreversible thermal unfolding transition, which might be due to the onset of aggregation upon unfolding at high temperature (Yan et al., 2004, Fitter and Haber, 2004). In the case of bacterial α-amylase like BAA, BLA, BSTA, thermal unfolding transitions are irreversible (Brosnan et al., 1992; Fitter and Haber, 2004). Thus, it is not possible to determine the thermodynamic parameters of thermal unfolding which are irreversible in nature. Some multidomain proteins also show reversible thermal unfolding like amylase from *Alteromonas haloplanktis* (AHA) at low temperature (Feller, 1994). Multidomain proteins like β-glucosidase I and II from *Pichia etchellsii*, although, show fully irreversible thermal unfolding, but their unfolding in pH and chemical denaturants exhibit substantial reversibility (Shah et al., 2018).

***Chemical unfolding*:** GdmCl and urea are the two commonly used chemical denaturants for protein unfolding studies (Wong and Tanford, 1973; Pace et al., 1986). The simulation studies on protein L and α/β proteins like chymotrypsin inhibitor 2 indicate that the β-sheets are generally unfolds first during urea denaturation than the α-helix, while opposite result was observed during GdmCl unfolding. During GdmCl induced unfolding, α-helix unfolds first followed by unfolding of β-sheets (Camilloni et al., 2008; Parui and Jana, 2019). Both urea and GdmCl denature the protein either through direct or indirect mechanism. During direct mechanism, urea and GdmCl form intermolecular 'H' bonds with peptide group and thus disrupt the exiting intramolecular 'H' bonding in protein. Urea has more potential of forming such bonds than the GdmCl (Pace, 1986; Bennion and Daggette, 2003; Shirley, 1995). Urea and GdmCl have different ionic character, urea is neutral

molecule, while GdmCl is charged chaotropic agent. Because of this, GdmCl in addition to hydrophobic interactions, also breaks ionic interaction thus considered to be more potent chemical denaturant than the urea (Greene and Pace, 1974; Monera et al., 1994). Susceptibility of proteins towards urea and GdmCl unfolding depends upon the extent of their relative stabilization by hydrophobic and ionic interactions (Dempsey et al., 2005). Difference in destabilizing potential of GdmCl and urea is more pronounced in proteins having large contribution of ionic interaction in their structural stabilization (Smith and Scholtz, 1996). It has been observed that the chemically unfolded polypeptide is more expanded and consist of less structural elements than the thermally unfolded (Tamura and Gekko, 1995; Wilson et al., 1996; Gast et al., 1997). The unfolding transitions by GdmCl and urea might be reversible or irreversible depending upon the several factors like structural properties of protein, pH, temperature, and solvent properties etc. The unfolding of small single domain proteins mostly shows two state folding mechanism. For example, three heme proteins, leghemoglobin, myoglobin and cytochrome C follow two state unfolding in both, urea and GdmCl (Basak et al., 2015). But there are some multidomain proteins which also unfolds reversibly with two state mechanism like two skeleton proteins, erythroid and non-erythroid spectrin exhibit reversible two state unfolding transition during chemical unfolding (Patra et al., 2015). Thermostable protein, tyrosine phosphatase also unfolds reversibly with monophasic unfolding pathway in urea and GdmCl (Wang et al., 2014). Majority of the multidomain proteins do not obey two state model and unfolds irreversibly in urea and GdmCl. Thermostable α-amylases, BLA and BAA, also unfold irreversibly in urea and GdmCl (Strucksberg et al., 2007; Fitter and Haber, 2004). In some cases, intermediate species with different structural properties like partially unfolded state or molten globule might populate during chemical denaturant induced unfolding and characterization of such species may help to understand the stability and structure- function relationship in several multidomain proteins (Kuwajima et al., 1976; Khurana and Udgonkar, 1994; Gokhale et al., 1999; Hornby et al., 2000). The equilibrium unfolding in urea and GdmCl has long been used as a method for measuring conformational stability of proteins (Pace et al., 1990; Shirley, 1995). Such studies also provide the knowledge about the relative contribution of various interactions in conformational stability of proteins. Thermostable alcohol dehydrogenase from *Thermoanaerobacter brockii* (TBADH) shows unusual stability towards urea unfolding at pH 7.0, which might be due to the

presence of increased electrostatic interactions (Mishra et al., 2008). Kinetics of unfolding in urea and GdmCl may provide the substantial information about the stability of individual domains in multidomain proteins (Tripathi et al., 2008; Singh et al., 2015).

pH unfolding: As protein's net stability is the result of fine balance between stabilizing and destabilizing interactions, this balance is highly dependent on the net charge of protein. The net charge of a protein can be modulated by changing the pH of the medium. Any abrupt change in the ionic strength or the pH, changes the net charge on protein which may results in disruption of stabilizing interactions like 'H' bonding, salt bridges, charge -charge interactions and finally leads to the unfolding of the protein (Anderson et al., 1990; Fitch et al., 2006). Maximum stability of a protein is observed at their isoelectric point (pI), because at this pH, protein has no net charge and therefore no electrostatic repulsion exist (Mathew and Gurd, 1986). In majority of the cases, proteins follow bell shaped unfolding transition during pH induced unfolding, with decreased stability at both extremes of pH. These unfolding transition of proteins might be reversible or irreversible depending on the amino acid composition and other environmental factors (Anderson et al., 1990; Fitch et al., 2006). Generally, pH induced unfolding is less effective than the chemical and thermal unfolding (Tanford et al., 1968; Luke et al., 1967). Acid induced unfolding of *Staphylococcal* nuclease shows that the unfolded state binds more protons than the native state (Fitch et al., 2006). pH titration curve of several proteins has indicated that the proteins like chymotrypsin have reversible transition over wide range of pH, while others like pepsin show reversible unfolding transition only within a narrow pH range. Thus, reversibility of the pH induced unfolding transition depends on the physicochemical properties of proteins (Tanford, 1963).

1.4 Protein refolding and aggregation

Protein unfolding leads to the formation of the denatured state of protein in which non-covalent interactions are broken. Proteins containing disulfide bonds are completely unfolded by breaking the disufide bonds by reducing agents. Upon removal of denaturant, proteins do not always refold back completely. The efficiency of *in vitro* refolding of proteins is severely affected by unproductive side reaction like

aggregation. Aggregation also reduces the efficiency of *in vivo* folding of proteins, for example, tailspike protein from bacteriophage P22 shows extensive aggregation during in *vivo* folding (Betts and King, 1999). Although, refolding yield is determined by kinetic competition between refolding and aggregation (Kiefhaber et al., 1991), but higher rate of folding under *in vivo* is ensured by accessory proteins like peptide-prolyl isomerases, protein disulfide isomerases and molecular chaperones (Balchin et al., 2016). These accessory proteins increase the refolding yield either by stabilizing the folding intermediates thus reduces the extent of aggregation or through increasing the rate of folding. Two most common rate limiting steps of protein folding are cis-trans isomerization of Xaa-pro bond and correct disulfide bond formation which are catalyzed by peptidyl-prolyl isomerase (PPI) and protein disulfide isomerase (PDI) respectively (Balchin et al., 2016). While molecular chaperones like GroEL and GroES bind to the hydrophobic surfaces of unfolded polypeptide in a sequential manner, prevent non-specific association thus prevent aggregation (Fink, 1999; Agashe and Hartl, 2000). Because accessory proteins are absent during *in vitro* refolding, therefore the chances of misfolding and aggregation increases. This compromises the refolding yields up to a large extent. The role of folding enhancer has been investigated in several studies during *in vitro* refolding for example, refolding of mitochondrial enzyme, rhodanese is assisted by molecular chaperones from *E. coli*, GroEL and GroES in combination with MgATP and K^+ ion (Mendoza et al., 1991). Similarly, folding of green fluorescent protein is facilitated by GroES, independent of ATP (Bandyopadhyay et al., 2019). Another example includes the refolding of malate synthase G (MSG) with the help of GroEL/GroES system. During refolding of MSG, folding rate is greatly enhanced by the sequential binding of GroES and GroEL to the folding intermediates (Dahiya and Chaudhuri, 2014). Small heat shock proteins have molecular chaperone like activity and prevent misfolding and aggregation during both *in vivo* and *in vitro* refolding (Mogk and Bukau, 2017; Haslbeck et al., 2019). As most of the small, single domain proteins have simple folding pathway devoid of partially folded intermediates, therefore, the probability of aggregation is quite low during refolding (Jackson, 1998; Sosnick and Barrick, 2011). On the other hand, multidomain and oligomeric proteins have complex folding pathways with comparatively higher propensity of aggregation and misfolding reactions. These complexities are due to the exposure of hydrophobic surfaces and their nonspecific interactions during folding resulting into the misfolding and

aggregation (Dobson et al., 1998). Other important reason behind inefficient refolding of multidomain protein is non-native interactions between individual domains (Fitter, 2009; Batey, 2006), however, individual domains of multidomain proteins with less shared topology, fold independently without aggregation (Han et al., 2007). As the interdomain interactions increase, misfolding and aggregation become the dominant side reactions during refolding of multidomain proteins (Batey, 2006; Fitter, 2009; Borgia et al., 2011; 2015, Tian and Best, 2016). For example, refolding pathway of malate synthase G (MSG) is sequential in nature with two kinds of intermediate species. First, the burst phase intermediate (I_M), populated during early phase of refolding having less structural topology which gets converted into native like intermediate. Finally, slow conversion of native like intermediate into native state, completes the refolding of MSG. This slow step of MSG refolding corresponds to the correct pairing of domains (Kumar and Chaudhury, 2018). The degree of reversibility of unfolding-refolding transitions in multidomain proteins varies depending upon the nature of protein, solvent and type of denaturants being used for unfolding. Like, unfolding transition of three different multidomain proteins phosphoglycerate kinase (PGK), α-amylase from *Aspergillus oryzae* (TAKA) and α-amylase from *Bacillus licheniformis* (BLA) exhibit different degree of reversibility during pH, chemical and thermal unfolding. The unfolding transition of PGK shows some degree of reversibility in all the three modes, while some amount of reversibility is also observed for TAKA during pH and GdmCl induced unfolding. Contrary, BLA shows fully irreversible unfolding transitions during pH, chemical and thermal unfolding. This differential behaviour of unfolding transition might be due to the differences in structural properties like domain architecture, hydrophobicity of protein core, number of domains etc. (Strucksberg et al., 2007). Efficient refolding of multidomain proteins remain a serious challenge from both academic and industrial point of view.

Inclusion bodies formation is the bottleneck during recombinant protein production. The inclusion bodies are insoluble protein aggregates which are formed during the over-expression of proteins in the host organism like *E.coli* during recombinant protein production (Clark, 2001; Tsumoto et al., 2003). The process of obtaining refolded protein from inclusion bodies consist of four separate steps, isolation of inclusion body from host cell, solubilization through chemical denaturants, refolding and purification (Alibolandi and Mirzahoseini, 2011). To get optimally active protein,

efficient refolding is the most critical step and thus requires extra care, as the aggregation is the most common side reaction during refolding. Aggregation is the leading cause of low refolding yield of recombinant protein during their overexpression in *E. coli* (Clark, 1998). Although use of the cosolvent additives during refolding is the most convenient and effective method for enhancing the refolding yield of recombinant proteins (Arakawa, 2018). But these cosolvents have diverse effect on protein aggregation which depends on the physico-chemical properties of osmolyte and the nature of proteins. In addition, protein aggregation also hampers *in vivo* refolding of several proteins which results into pathological conditions like Huntington's, Parkinson's and Alzheimer's disease etc. (Chiti and Dobson, 2017). Therefore, there is a need of cosolvents which are effective against aggregation during *in vivo* and *in vitro* refolding (Singer and Lindquist, 1998; Ignatova et al., 2006).

1.5 Cosolvent assisted protein refolding

Cosolvents like polyols, sugars and amino acids etc. are well known to enhance the stability of the native proteins (Arakawa and Timasheff, 1982; kaushik and Bhat, 1998). They have been also explored for enhancing the refolding yield of proteins as well, although, there is no direct correlation between the two roles of cosolvents as protein stabilizer and refolding enhancer, but attempts have been made in this direction (Mishra et. al., 2005). Polyols and sugars are well known as protein stabilizers, while urea and GdmCl are the protein denaturants. Beside this, there are some cosolvents which do not belong to either of the two classes discussed above, like arginine, cationic salts, polyamine etc. Refolding additives like denaturants and detergents need to be removed after completion of refolding in a separate step as they might be harmful for the prolong stability of proteins. This separate step may enhance the cost of recombinant protein production. Thus, there is a need of cosolvent which does not need to be separated after the refolding.

1.5.1 Polyols

Polyhydric alcohols like, ethylene glycol (EG), glycerol, erythritol, xylitol, sorbitol etc. and sugars like trehalose, sucrose have long been used as cosolvent for enhancing the stability of proteins (Gekko and Timasheff, 1981; Kaushik and Bhat, 1998, 2003;

Back et al., 1979). The stabilization of proteins in the aqueous solution of polyols and sugars achieved through their preferential hydration mechansims (Gekko and Timasheff, 1981; Gekko and Morikawa, 1981 a). Upon transfer of polypeptide from water to solutions of stabilising and destabilising osmolytes, osmolyes shows preferential exclusion and preferential binding, respectively, based on free energy calculations (Street et al., 2006). The polypeptide backbone makes more favourable interactions with the polar groups of cosolvents than the non-polar groups (Street et al., 2006). In addition to their effect on the stability of native state of proteins, polyols also show positive effect on the refolding of aggregation prone proteins (Mishra et al., 2007; 2005). Idea behind using polyols and sugars for refolding of proteins comes from the compaction of protein structure in the presence of these cosolvents, and the extent of stabilization varies with physico-chemical properties of polyols and proteins (Gekko and Timasheff, 1981; Feng and Yan, 2008). In the case of large cosolvents like sugars, relative stabilization of proteins is governed by excluded volume effect (Schellman, 2003). While in the case of small size polyols like glycerol, increase in the excluded volume effect might be originated from the electrostatic orientation of glycerol molecules with respect to protein surface (Vagenende et al., 2009). There are number of studies where series of polyols with different lengths of hydrocarbon chains and number of hydroxyl groups were used for enhancing the refolding of proteins. In the case of arginine kinase, glycerol, sorbitol, glucose and sucrose have been used for improving the refolding, but only glycerol, sorbitol and sucrose were found to be effective. Though, all cosolvents including glucose were found to prevent the aggregation during refolding (Yu and Li, 2003). In a systematic study, the role of polyols on refolding of aggregation prone citrate synthase have been studied. Polyols like ethylene glycol, glycerol, sorbitol, xylitol and erythritol were employed for increasing the refolding of chemically denatured citrate synthase at neutral pH and room temperature. Maximum refolding of CS was observed in 7M glycerol up to 83%. Aggregation kinetics have shown that aggregation suppression effect of polyols depends upon the number of hydroxyl group (Mishra et al., 2005). Polyethylene glycols (PEG) have also been used for enhancing the refolding yield of proteins. Polyethylene glycols with molecular weight between 1000 and 8000 were found to be effective for improving the refolding yield of carbonic anhydrase by forming complex with folding intermediates just like molecular chaperones and prevent the aggregation during refolding (Cleland et al., 1992; Cleland and Wang, 1990). Different polyols

have different effect on solvent properties like viscosity, surface tension, hydrogen bonding network etc. Glycerol usually decreases the surface tension but at the same time, it increases the viscosity of the solution and exerts its stabilization effect on protein conformation by preferential hydration of proteins (Gekko and Timasheff, 1981). The Preferential hydration of proteins in the presence of glycerol is due to the unfavourable interaction of protein with solvent which is known as solvophobic effect. Unlike glycerol, most of the polyols increases the surface tension of water, which might play a dominant role in the protein stabilization through preferential hydration. Thus, extent of the stabilization of protein structure is directly related to the degree by which they increase the surface tension of water (Kaushik and Bhat, 1998; 2003). The order of preferential hydration of BSA by polyols is inositol>sorbitol>mannitol>xylitol which is in accordance with the order of increase in surface tension of water (Gekko and Morikawa, 1981 a). Similarly, polyhydroxy sugars like glucose, sucrose, lactose and trehalose also increase the surface tension of water to different degree depending on the physico-chemical properties and stabilize the protein through preferential hydration (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982). Although, trehalose shows preferential exclusion at normal temperature, but in the case of RNase, both trehalose and sorbitol have been reported to show weak binding to native state of ribonuclease at high temperature and this binding is greater for trehalose than sorbitol (Xie and Timasheff, 1997). Thus, at high temperature trehalose exert additional stabilization effect on native conformation of protein and also helps to retain its biological activity at high temperature (Kaushik and Bhat, 2003). Like glycerol, both sorbitol and xylitol increase the viscosity of the solution. But they have opposite effect on surface tension, unlike glycerol, both sorbitol and xylitol are known to increase the surface tension of aqueous solution, but this effect is more for sorbitol. Thus, sorbitol is a strong stabilizer than the xylitol (Kaushik and Bhat, 2003). As the stabilizing efficiency of polyols depends on their hydrophilic character, EG may induce protein denaturation and promote formation of β-sheet structure due to less hydrophilic nature. Hence EG, unlike other polyols has some destabilizing effect on protein structure, but this effect is more at high temperature (Gekko, 1981; Naidu et al., 2020). However, refolding of citrate synthase (CS) in EG, increases up to 52 % in the presence of 5 M, but further increase in EG concentration results in down fall in the refolding yield (Mishra et al., 2005). On the other hands, glycerol at its higher concentration decelerates the refolding kinetics of

proteins due to the high viscosity of the solvent. Glycerol also affects the refolding pathway of proteins as it was observed in the case of lysozyme. The refolding pathway of lysozyme shows folding intermediates with the hydrophobic core, which is not present without glycerol (Wu et al., 2008). Besides this, polyols have also been found to affect the amyloid fibril formation, through stabilizing the aggregation prone species (Roy and Bhat, 2018; Verma et al., 2020).

1.5.2 Amino acids and their derivatives

In addition to polyols and sugars, certain amino acids like arginine, proline, glycine and their derivatives are also known to show positive effect on protein folding (Singer and Lindquist, 1998, Diamant et al., 2003, Ignatova et al., 2006). Arginine is one of the most widely used amino acids for suppressing aggregation during protein refolding (Arora and Khanna, 1996). Anti-aggregation property of arginine was first reported by Rudolph and Fischer (1990), since then, it has been continuously being used for enhancing the refolding yield of aggregation prone proteins. Arginine increases the refolding yield of various proteins like lysozyme (Hevehan and Clark, 1997), immunotoxin (Brinkmann et al., 1992), Casine kinase (Lin and Traugh, 1993) and Fab antibody fragments (Tsumoto et al., 1998) etc. The comparative effect of arginine on refolding of proteins with or without disulfide bonds like, recombinant human granulocyte colony stimulating hormone (rhG-CSF), recombinant green fluorescent protein (GFP) and BSA was observed that arginine is less effective for protein containing disulfide bonds might be due to the fact that arginine cannot prevent formation of incorrect disulfide bonds in proteins. Although, cause of aggregation can be intermolecular association of protein either through hydrophobic interaction or via covalent linkage like disulfide bonds (Chen et al., 2008). But the effect of arginine on suppression of aggregation is specific for hydrophobic interaction mediated aggregation. Thus, arginine mediated effect on refolding of proteins also depends on their amino acids' compositions and nature of intermediate populated during protein refolding. Arginine has also been reported to shows negative effect on refolding of certain proteins but exact mechanism behind this effect is still not clear (Lange and Rudolph, 2009; Chen et al., 2015). Arginine may act as a protein destabilizer for certain proteins, depending on their amino acid compositions. For example, structural and functional stability of phosphoenol pyruvate is inhibited due

to the presence of arginine in solvent medium (Yancey et al., 1982). This denaturation effect of arginine is possibly due to its weak binding to the protein surface. Arakawa and his group (2007) have reported that although like GdmCl, arginine also shows favourable interactions with side chains of amino acids like tryptophan, tyrosine etc., but it behaves differently than GdmCl at protein level and exhibit limited binding at protein surface. In the presence of arginine, various proteins like ribonuclease, BSA and lysozyme, show preferential hydration, which is greater than GdmCl but less than other stabilizing amino acids, like alanine and betaine (Arakawa et al., 2007). As the side chain of arginine has guanidinium group which may undergo additional interactions like hydrogen bonding with side chains of other amino acids and л interactions with aromatic amino acids (Flocco and Mowbray, 1994; Mitchell and Price, 1990). Thus, effect of arginine on protein aggregation is the result of its favourable interactions with amino acid side chains thus reducing the intermolecular interactions between protein molecules and hence preventing the aggregation. But in few cases, interactions between arginine and amino acid side chains may have negative effect on the protein stability and refolding, as unfolded polypeptide has more exposed side chains for arginine binding, thus it would prefer more unfolded state over the native state (Arakawa and Tsumoto, 2003; Baynes et al., 2005; Arakawa et al., 2007).

Proline is the second most commonly used amino acid for improving the refolding yield of aggregation prone proteins. Kumar et al (1998), reported the chaperone like activity of proline during refolding of carbonic anhydrase. They have shown that the proline forms supramolecular assembly through hydrophobic interaction between its side chain and exposed hydrophobic region of folding intermediates. In addition, proline also undergoes intermolecular self-association through hydrophobic stacking (Greenstein and Wintiz, 1961; Schobert and Tschesche, 1978). Proline has also been effective in improving the refolding yield of proteins like lysozyme, arginine kinase, creatine kinase, amino-cyclase etc. (Samuel et al., 2000; Meng et al., 2001; Kim et al., 2006; Xia et al., 2007).

Glycine and its methyl derivatives which are generally referred as methylamine also show preferential exclusion and thus positive effect on protein stability. Although their capability of increasing the protein stability decreases with increasing number of

methyl group in the order of Gly>N-methylglycine (sarcosine)>TMAO~ Dimethylglycine>Trimethylglycine (Betaine) (Bruździak et al., 2016). They also have been found to be effective in improving the refolding of proteins. For example, in the case of lactate dehydrogenase, methylamine increases the extent renaturation of acid denatured state in the order of TMAO>betaine>Sarcosine>β alanine which is in contrast to their effect on protein stability (Yancey and Somero, 1979). Mitchell et al (2001), have also shown that glycine and methylamine like sarcosine, betaine and TMAO protect serpin α-antitrypsin inhibitor from thermal deactivation, while at the same time sarcosine was found to be ineffective for enhancing the refolding yield of the same protein (Chow et al., 2001). Although individual osmolytes like betaine, sarcosine and sorbitol, show positive effect on the refolding yield of RNase A, but mixture of these osmolytes exhibit negative effect on the refolding yield of RNase A (Warepam and Singh, 2015). It has also been observed that betaine and sarcosine decrease the formation of insoluble protein aggregates which are generally observed during pathological conditions of neurogenerative diseases like Parkinson, Alzheimer etc. (Natalello et al., 2009; Venkatraman et al., 2020).

1.5.3 Denaturants

In majority of the cases chemical denaturation of the proteins are carried out with the help of urea and GdmCl, as both are highly effective chaotropic agent. Although, exact denaturation concentration of urea and GdmCl may vary from protein to protein but most of the proteins denatured at higher concentration of both chaotrops. At their lower concentrations, both urea and GdmCl, show weak binding to proteins and thus behave as aggregation suppressing molecule (Orsini and Goldberg, 1978). Inclusion body formation is the bottleneck in the recombinant protein production in *E. Coli*. However native protein can be recovered from the inclusion bodies by denaturing and refolding the inclusion bodies (Mirazhoseini et al., 2003; Mirazhoseini and Alibolandi, 2009). In order to recover the native protein, inclusion bodies are subjected to two-step process. During the first step, inclusion bodies are solubilized with the help of chemical denaturants like urea and GdmCl. In the second step, denatured protein obtained in the first step undergoes refolding in the presence of additives. At low concentrations urea and GdmCl have been utilized as cosolvent additive during refolding of inclusion bodies (Alibolandi and Mirzahoseini, 2011;

Arora and Khanna, 1996). Lysozyme was successfully recovered from solubilized inclusion bodies after renaturation with the help of low concentrations of GdmCl and arginine (Hevehan and Clarke, 1997). The positive effect of GdmCl during refolding might be arising due to the favourable interactions of guanidinium group with peptide backbone and side chains of charged residues through 'H' bonding. In addition, GdmCl also interacts with aromatic residues through stacking interactions (Orsini and Goldberg, 1978; Arakawa and Timasheff, 1984).

1.5.4 Detergents

Detergents are amphipathic molecules having both polar and nonpolar groups. Their non-polar end consists of hydrophobic tail, which can bind to the exposed hydrophobic patches of folding intermediates thus preventing non-specific interactions between them and aggregation. Both, ionic and non-ionic, detergents being used as efficient refolding additives for enhancing the refolding yield of proteins. Refolding of GdmCl induced unfolded rhodanese has been achieved with the help of non-ionic detergents, dodecyl-β-D-maltose or lauryl maltoside. Here, lauryl maltoside stabilizes the folding intermediates and reduces the aggregation during refolding (Tandon and Horowitz, 1987). Rozema and Gellman developed a two-step method called artificial chaperone assisted refolding (Rozema and Gellman, 1995; 1996 a, b). This method is based on the sequential binding of GroES and GroEL during *in vivo* refolding (Harlt, 1996). In the first step, detergent binds to the hydrophobic surface of folding intermediates to capture them thus prevents aggregation. In the next step, detergent molecules are removed with the help of striping agents like cyclodextrins to allow the refolding of proteins. This approach has been used for improving the refolding yield of various proteins. The refolding yield of chemically denatured α-amylases was improved by using both cationic (CTAB, DTTAB and TTAB) and anionic (Tween 80 and Triton X-100) detergents as capturing agents while α-β cyclodextrins were used as striping agents (Khodagholi and Yazdanparast, 2005). Similarly, refolding of other proteins like hen egg white lysozyme, carbonic anhydrase and citrate synthase has also been enhanced by using artificial chaperone assisted method, with different combination of detergents as capturing and cyclodextrins as striping agents (Kurganov and Topchieva, 1998; Machida et al., 2000; Daugherty et al., 1998).

1.6 Alpha amylase

The enzyme which catalyzes the hydrolysis of O-glycosidic bonds of starch and other polysaccharides is referred as amylase. The amylases which produce end product with α- anomeric configuration are called α-amylases. They are endo-acting glycosyl hydrolases, which break the internal α,1-4 glycosidic bonds (Suganuma et al., 1978; MacGregor, 1993). Beside endo-acting amylases, there are various exo-acting amylases like maltogenic α-amylases, maltotriohydrolase, maltotetrahydrolase etc. are also found. They act on non-reducing end of a polysaccharide chain and produce oligosaccharides of same size. Unlike exo-acting amylases, endo-acting amylases produce multiple size products. In addition, endo-acting amylases have higher reaction yield than the exo-acting amylases. These differences could be due to the presence of a greater number of subsites in their active center and thus have ability to act on more than one bond at a time. α–amylases are ubiquitous in nature and are found from bacteria to mammals. The mammalian porcine pancreatic α-amylase, attacks on six glycosidic bonds in a single encounter with substrate and enzyme has four subsites in its active site (Robyt and French, 1967). Unlike mammalian α-amylase, other α-amylases like α-amylase from *Aspergillus oryzae*, *Bacillus licheniformis*, barley etc. give comparatively large size products due to the extended geometry of their active site (Machius et al 1995, Matsuura et al., 1984; Kadziola et al., 1994; Qian et al., 1993).

Due to their highly efficient catalytic properties, α-amylases occupied the important place among industrially important enzymes and provide the significant contribution to the world enzyme market (Rao and Satyanarayana, 2007; Rajgopalan and Krishanan, 2008). Although, they are highly ubiquitous in nature and present in almost all kingdom of life from bacteria to human, but industrially relevant α-amylases mostly obtained from bacteria and fungi. Due to their simple life cycle, bacteria and fungi are easy to handle and grow. Thus, large scale production of bacterial and fungal α-amylases are quite economical in comparison to the other sources (Gupta et al., 2003; Soni et al., 2003). In addition, they can be easily manipulated for the increased thermal stability and enhanced catalytic efficiency by protein engineering approaches (Machius et al., 2003; Declerck et al., 2000). Most industrially important bacterial α-amylases are from *Bacillus licheniformis*, *Bacillus*

amyloliquifaciens, *Bacillus subtilis*, *Bacillus stearothermophilius* etc. (Pandey, 2000; Prakash and Jaiswal, 2010). In comparison to the bacterial α-amylases, fungal α-amylases have limited source for industrially important enzyme. Most common sources of industrially important fungal α-amylases are from *Aspergillus sp*, *Penicillium sp*, *Thermomyces longuginosus* etc. (Jin et al., 1998, Jensen et al., 2002; Kathiresan and Manivannan, 2006, Hernández et al., 2006). α-amylase exhibit huge variation in their physico-chemical properties and optimum values like pH, temperature, salt etc. Their high industrial importance and environmental diversity make them a potential model enzyme from both academic and industrial point of view. Thermal stability is the most desirable property of industrially important enzymes including α-amylases, because this enables enzyme to work at higher operational temperature during various industrial applications (Mehta and Satyanaryana, 2016; Gupta et al., 2003).

1.6.1 Classification of alpha amylases

Starch hydrolyzing enzymes like α-amylase, α-glucosidase, dextran glucosidase, isoamylase, pullulanase, amylopullulanase, neopullulanase etc. with similarity in their amino acid sequences and structural properties have been classified in α-amylase family. Enzymes of this group have some characteristic features, which are hallmark of α-amylase family. They can break α-glycosidic bonds of various polysaccharides to produce low molecular weight products with α-anomeric configuration and their catalytic site consists of three conserved residues Asp, Glu and Asp. Most of the α-amylase family enzymes have TIM barrel like structure which also includes catalytic site residues (Kuriki and Imanaka, 1999; MacGregor et al., 2001). Main representative enzymes of this family include α-amylase, isoamylase, pullulanase, CGTase, α-glucosidase, amylopullulanase, oligo-1,6 glucosidase, cyclodextrinase and glycogen debranching enzymes etc. (Takata et al., 1992; Svensson et al., 1994; Kuriki Imanaka, 1999). Further, on the basis of their catalytic activity, they have been divided in to four categories, namely (1) endoamyalses (2) exoamyalses (3) transferases (4) debranching enzymes.

Endoamylases: Enzyme which acts only on internal glycosidic bonds comes under this category of glycosyl hydrolases. α-amylases from different organisms are the ideal representative of this group. They generally act on α-glycosidic bonds in

polysaccharides and produces oligosaccharides of different chain lengths with retention of α-configuration. This group of enzymes are present from lower to higher organisms and involved in several steps of carbohydrate metabolisms which are essential for the storage and assimilation of energy (Pandey et al., 2000; van der Maarel et al., 2002).

Exoamylases: They act on end unit of glycosidic chain and produce single size products like glucose or maltose etc. Some of the members like β-amylase catalyzes the hydrolysis of external α,1-4 glycosidic bonds, while others like amylopullulanase or glucoamylase, α-glucosidase possess dual property of hydrolyzing both α,1-4 and α,1-6 glucosidic linkages. Members of this group also differs in term of their substrate specificity, like α-glucosidase acts on small size maltooligosaccharides and forms glucose as the end product, while other members like α-glucoamylase exclusively acts on polysaccharides with long hydrocarbon chain (Pandey et al., 2000). Beside this, there are some other exoamylases like maltotriohydrolases, maltogenic α-amylase, maltotetrahydrolase etc. which share the sequence similarity with endoamylases except, maltogenic α-amylase (Robyt and Ackerman, 1971; Diderichsen and Christionsen, 1988; MacGregor et al., 2001).

Transferases: These enzymes have both, hydrolytic and transferase activities in a single active site. They catalyze the hydrolysis of α,1-4 glycosidic linkage in one polysaccharide (donor) and simultaneously transfer it to another polysaccharide which acts as an acceptor molecule. Enzymes like amylomaltases and cyclodextrin glycosyltransferase are the example of this class. Although they exhibit higher similarity in catalytic process, but produces different products. Amylomaltase forms linear products, while cyclodextrin glucosyltransferase forms cyclic oligosaccharides (Takaha and Smith, 1999). All the enzymes with α-amylase like activity belong to mainly two glycosyl hydrolase (GH) family, which are GH13 and GH57. There are 19 members in GH13 family including CGTase, maltotriose forming α-amylase and α-glucosidase. While there are only 13 members in GH57 family (Henrissat, 1991).

Debranching enzyme: This group of enzymes exclusively hydrolyze α,1-6 glycosidic bonds in starch and other branched polysaccharides. Two main representatives of this class are isoamylase and pullulanase, which differ in their catalytic activities. Isoamylases act on α,1-6 glycosidic linkages in amylopectin thus used in debranching

of starch. On the other hand, pullulanase has unusual property of catalyzing α,1-6 glycosidic bonds in two unrelated substrates, amylopectin and pullulan (Israilide et al., 1999; Kelkar and Deshpande, 1993). Unlike this, there are enzymes which are capable of hydrolyzing both α,1-4 and α,1-6 glycosidic bonds, like amylopullulanase. In most of the cases, both catalytic activity of this enzyme resides in the same active site. Another member of this group is neopullulanase, having dual function of both hydrolyzing and synthesizing α,1-4 and α,1-6 glycosidic bonds with the help of a single active site (Kuriki et al., 1991; Hatada et al., 1996; MacGregor et al., 2001).

1.6.2 Catalytic mechanism of alpha amylases

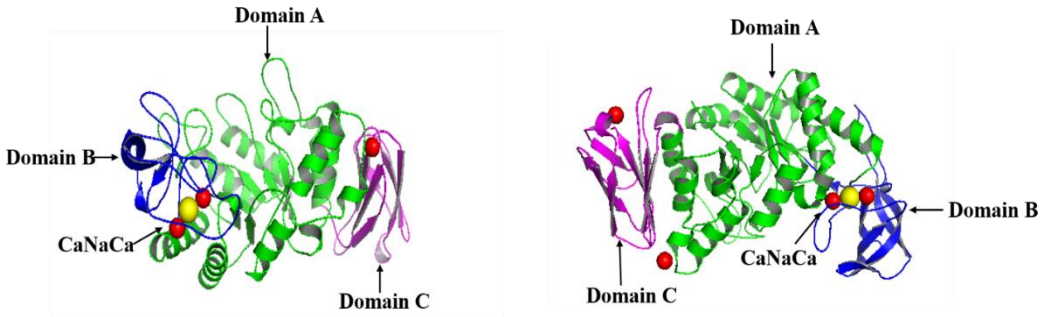
The catalytic residues in α-amylases are highly conserved with Asp, Glu and Asp forming catalytic triad (Svensson et al., 1994). Although, these enzymes catalyze the hydrolysis of α,1-4 linkage in variety of polysaccharides, but starch is the most commonly used substrate. In most of the cases, raw starch is obtained from maize, wheat, potato, tapioca etc. (Agrawal et al., 2005; Goyal et al., 2005). Starch is a highly branched polymer of glucose which consists of two components, one is amylose where glucose units are joined by α,1-4 glycosidic bonds in a linear chain, while other is amylopectin, relatively shorter than amylose with linear chain consist of 10-16 units and branching occurs after 10–15-unit interval through α,1-6 glycosidic linkage. Before its conversion to useful products, starch undergoes series of modifications through different enzymatic process like gelatinization, during which starch dissolved into smaller molecules and forms viscous suspension. Next steps are liquification and saccharification which involve hydrolysis and production of syrup through hydrolysis (Gupta et al., 2003; Prakash and Jaiswal, 2010). The catalytic mechanism of α-amylase is based on the double displacement reaction with the retention of α-configuration. First displacement reaction involves the protonation of glycosidic oxygen to break the glycosidic bond, which results in the formation of oxocarbenium intermediates. β-glycosyl intermediate formed via nucleophilic attack of protein group on sugar anomeric center. In the second displacement reaction, activated water molecules attack the anomeric center and transition state which get hydrolyzed to form product (Davies et al., 1998, Uitdehaag et al., 1999). Three universally conserved amino acid residues (Asp, Glu, Asp) in α-amylases were first reported in α-amylases from *Aspergillus oryzae* (TAKA) (Matsuura et al., 1984). The

mutational studies have shown that the presence of these conserved catalytic residues in the active site is the prerequisite criteria for enzymes belonging to the α-amylases family (Svensson et al., 1994; Janecek, 1997). All three catalytic residues perform specific role in the enzymatic activity of α-amylases. Glutamate acts as proton donor and first aspartate as proton acceptor while the second aspartate is supposed to be involved in the stabilization of oxocarbenium intermediate formed during catalytic process (Uitdehaag et al., 1999). There are several subsites in the active site of α-amylases which interact with the glucose of starch and other polysaccharides, although their number may vary among different α-amylases (MacGregor et al., 2001).

1.6.3 Structural features of alpha amylases

Alpha Amylase consists of a single polypeptide chain, which folds into three distinct domains, namely domain A, B and C. The domain A is the largest and forms central part of the enzyme (Figure 1 a and b). It consists of $(\beta/\alpha)_8$ topology where eight β-strands wrapped around eight α-helixes. Presence of this topology in domain A is the characteristic feature of the enzymes of α-amylase family. However, it is also present in other enzymes as well (Svensson and Søgaard, 1992). Number and sequence of amino acids in domain A are highly conserved among different member of α-amylase family. One of the notable feature of this domain is the presence of catalytic site which have conserved sequence. The substrate binding subsite of active center is located in $(\beta/\alpha)_8$ barrel structure. Calcium and Sodium binding sites are also situated in this domain. Although number of such sites may vary among α-amylases depending upon their origin, but in most of the cases, metal binding sites play an important role in the structural and functional stability of α-amylases (van der Marrel et al., 2002; De Souza et al., 2010). In some α-amylases from *Bacillus sp.* KR8104 and *Bacillus sp*. DR90 which are alkaliphilic and aciodophilic respectively, calcium has functional role but no role in the stability of the protein (Sajedi et al., 2005; Asoodeh et al., 2013). In majority of the cases, domain A is located towards the N-terminal end of enzyme. Some α-amylases have additional N-terminal domain called N' domain which also includes some residues of the active site. For example, α-amylases from *Pyrococcus furiosus* has four domain architecture with N, N', B and C (Park et al., 2013).

A loop like structure formed by combination of third β-strands and the helix of TIM barrel of domain A, referred as domain B. It is the most flexible part of the enzyme structure and exhibits huge variation in terms of both, sequence and length, among different α-amylases (Janecek, 1997; MacGregor et al., 2001). The domain B consists of β-sheet structure and it is the least stable part of the enzyme. As it has been observed that during unfolding of the psychrophilic α-amylase from *Alteromonas haloplanktis* (AHA), unfolding initiated from domain B (Feller, 1994; D' Amico et al., 2006). C-terminal part of the enzyme consists of another domain called C domain which is mainly made up of β-strands. Although there is no specific role has been assigned to this domain but it is speculated that it might be involved in the stabilization of domain A and also helps in the substrate binding in some members of α-amylases family (Lawson et al., 1994; Dauter et al., 1999). This domain consists of Greek key motif in some α-amylases like BAA and BLA (Machius et al., 1995, Alikhajeh et al., 2010). Some other members of α-amylase family like amylomaltases lack this domain (Przylas et al., 2000). Although, true α-amylase has three domain topology but, some other member of α-amylase family like cyclodextrin glucotransferase and maltogenic α-amylases from *Bacillus sp.* have more than three domains. For example maltogenic α-amylase from *Bacillus stearothermophilus* possess two extra domains, domain D and E. However, the role of these domains are not clear (Dauter et al., 1999; Hofman et al., 1989; Harata et al., 1996; Matsuura et al., 1995). In addition to Calcium and Sodium, Chloride binding site has also been reported in some α-amylases. Most of the mammalian α-amylases and few bacterial α-amylases like AHA have chloride binding sites (Brayer et al., 1995; Ramasubbu et al., 1996; Aghajari et al., 2010).



(a) **(b)**

Figure 1: Crystal structure of bacterial α-amylases, BLA (a), PDB IBL1 (Machius et al 1995) and BAA (b) PDB BH4 (Alikhajeh et al 2010). Domain A, B and C have been shown in green, blue and magenta colour respectively and calcium binding sites are illustrated by red and sodium binding site by yellow.

1.6.4 Industrial applications of alpha amylase

The hydrolyzing property of α-amylase makes it a valuable candidate for starch-based industries like food, textile, paper, beverage, fuel, pharmaceutical and detergent industry.

Food industry***:*** α-amylases have successfully been used for variety of purposes in the food industry. Like preparation of glucose and fructose syrups, production of good quality digestive aids, baking, brewing etc. The baking industry most extensively uses α-amylases, mostly from thermophilic sources like *B. stearothermophilus* or *B. licheniformis* (van der Marrel et al., 2002). In this industry, α-amylases have been used for increasing the fermentation rate and for improving the quality of products like texture, taste, crust colour etc. Addition of α-amylases to dough also enhances the half-life of the product (Gupta et al., 2003). In beverage industry, α-amylases used for removing unwanted starchy substances thus uplift the quality of beers and fruit juices. As the animal feed is highly fibrous in nature which may create digestive problems, thus α-amylases are used for softening the animal feed to make it easily digestible (van der Marrel et al., 2002; Ghorai et al., 2009).

Fuel industry: Biofuel is one of the main alternative for petroleum where ethanol is widely used. Starch often acts as a cheapest substrate for the same due its low price and easy availability. The process of conversion of starch into ethanol consists of three steps, namely liquification, saccharification and fermentation. During liquification, starch is hydrolyzed into low molecular weight substances like dextrin. The saccharification of dextrin and other low molecular weight substrates takes place with the help of other enzymes. These sugars finally convert into ethanol through fermentation with the help of *Saccharomyces cerevisiae* (Moraes et al., 1999). Thermophilic α-amylases from different bacteria like *Bacillus stearothermophilus, Pyrococcus woesi* etc. are the best choice for hydrolyzing the starch in industry (Sanchez and Cardona, 2008).

Detergent industry: Hydrolytic enzymes including α-amylases have been widely used in the detergents industry as additives. Presence of α-amylases in the detergent formulation enhances their potential to remove dark stain from cloths. In addition, α-amylases help to clear spots containing starch food like chocolate, gravy etc. (Gupta et al., 2003; Mitidieri et al., 2006; Hmidet et al., 2009). α-amylases possess huge variation in terms of their adaptation to environmental variables like temperature, pH etc. In the detergent industry, α-amylases having low temperature and high pH optima are the most suitable candidates. α-amylases from *Bacillus* or *Aspergillus* species are frequently used in the detergent industry. However enzymes used in the detergent industry must have adequate oxidative stability due to the oxidizing environment (Kirk et al., 2002; Chi et al., 2009).

Textile industry: Textile industry uses α-amylase at large scale for improving the quality of fabric wherein, the starch is used as sizing agent as it is cost effective and widely available. α-Amylases are used during desizing purpose to remove the starch from the surface of fiber. During sizing process, starch has been applied to thread which provides protective covering. But after completion of weaving, it has to be removed and this is achieved through applying α-amylases, which selectively remove starch from the surface of thread (Feitkenhauer, 2003; Gupta et al., 2003; Ahlawat et al., 2009).

Paper industry*:* Like textile industry, starch has also been used as sizing agent for pulp and paper industry. This process enhances appearance like finishing, erasibility,

strength and stiffness of paper. But for proper sizing, viscosity of starch should not be too high, thus α-amylases are used to control the viscosity of starch. Alpha amylase partially breaks the starch through batch or continuous process and produces low viscosity starch. This less viscous starch perfectly improves the smoothness of paper without affecting its quality that might be the case with highly viscous starch.

1.6. 5 Homologous bacterial alpha amylases

In this study, two homologous α-amylases, one from *Bacillus licheniformis* (BLA) and other from *Bacillus amyloliquefaciens* (BAA) have been chosen to study the stability and folding. Both share 81% sequence identity and 88% sequence similarity but possess significant differences in their melting temperature (T_m) (Machius et al., 1995; Alikhajeh et al., 2010). There is no cysteine residue in both the enzymes and thus lacking disulfide bond. Although, both bacteria are mesophilic in origin, but BLA behaves like thermophilic while BAA as mesophilic counterpart (Fitter and Heberle, 2000; Declerck et al., 2000). X-ray crystallographic studies have revealed that tertiary structures are conserved in both the enzymes and consist of three domains A, B and C (Machius et al., 1995; Alikhajeh et al., 2010). Despite of this much similarity, BLA shows greater structural flexibility than BAA at higher temperature as shown by Neutron scattering (Fitter and Heberle, 2000) and has higher energy of activation for thermal unfolding in comparison to BAA (Duy and Fitter, 2005). The factors which govern this differential stability of BLA and BAA are still not clear. It has been observed that the unfolded state of BLA is more compact than BAA during thermal and GdmCl induced unfolding, points towards the entropic stabilization of BLA (Fitter and Haber, 2004). By using site directed mutagenesis, residues affecting thermal stability has been identified, for example, few lysine residues in BLA form additional salt bridges which are absent in its counterpart BAA that might be responsible for its higher thermal stability (Tomazic and Klibanov, 1988). X-ray crystallographic structures of both proteins provide some hints for higher thermal stability of BLA relative to BAA(Machius et al., 1995; Alikhajeh et al., 2010) but it is still not possible to understand completely the stability differences by comparing the amino acid sequence and crystal structure of both proteins. There are several factors which imparts thermal adaptation to BLA relative to its mesophilic counterpart BAA. The structural comparison and mutational studies showed that BLA has few

critical intra domain (Asp121-Arg127 in B domain and Glu250-Lys251 in A domain) and inter-domain salt bridges (between Asp60-Arg146 and Asp204-Lys237 connect domain A and B) (Machius et al., 1995; Declerck et al., 2000). Mutational analysis through replacement of Asp190 by Phy190 shows the importance of aromatic-aromatic interactions in the stabilization of BLA (Machius et al., 2003). One of most significant factors is the presence of large number of histidine residues in BLA relative to BAA, which might be decreasing the entropy of unfolded state and stabilizes the native state (Alikhajeh et al., 2010). It is one of the few examples where comparative study from structural and folding point of view has been carried out in some detail. Thermal unfolding of BLA and BAA shows that both enzymes undergo irreversible unfolding with T_m of 101°C and 86°C respectively in the presence of 2mM Calcium (Fitter and Haber, 2004). The comparative unfolding of both BLA and BAA in urea and GdmCl indicates that at pH 7.0, BLA shows unusually higher stability towards urea denaturation than BAA and molten globule like intermediates were observed during GdmCl unfolding of BLA (Ahmad and Mishra, 2020). As per the structure and the sequence comparison of BLA and BAA, shortening of loop region due to the deletion of Glu178-Gly179 in the domain B and the orientation of Ala298 which helps to interact with Lys234 and Glu189 through ionic interactions in domain B, found to be key structural determinant of the thermal stability of BLA (Wu et al., 2018).

Outline of the present research problem

The marginal stability of proteins under extreme temperature remains an open question in the field of protein science because thermal stability of proteins is of great significance for both academic as well as industrial point of view. Although there are several studies where thermophilic and mesophilic proteins have been compared to find the origin of thermal stability but most of the studies have been focused on amino acid sequence and structural comparisons between these two classes of proteins. By comparing amino acid sequence and structure of homologous proteins, it may be possible to predict the possible reasons behind the differential thermal stability. Several factors like differences in proline content, number of charged residues, salt bridges, hydrophobicity etc. have been pointed out for the higher stability of thermophilic proteins. However for better understanding of mechanism of thermal stability, it is important to understand mechanism of unfolding and refolding and comparing with its homologous counterpart. Protein folding is hierarchic and progresses through discrete pathways consisting of one or more intermediates. Most often kinetic intermediates escape the detection and non-amenable to structural characterization due to their transient nature and accumulation to an insufficient level. The Kinetic unfolding intermediates are best described if their equilibrium accumulation is demonstrated. Presence of one or more intermediates in both equilibrium and kinetic process would, therefore strengthen the pathway concept of folding.

First part of the present study is based on identification and characterization of different intermediates populated during equilibrium and kinetic unfolding of α-amylases (BLA and BAA) by chemical denaturants (urea and GdmCl). Here emphasis has been given to compare the two homologous α-amylases in terms of their unfolding pathway, in order to understand the possible origin of protein stability. Our results suggest that BLA and BAA have different unfolding pathways during urea and GdmCl induced unfolding transitions. The unfolding route of BLA in GdmCl consists of partially unfolded intermediates or molten globule like intermediates, while BAA lack such detectable intermediates. Another important outcome of our study is the unusual stability of BLA during urea unfolding. BLA retains its native, biologically active conformation even after incubating in 8M urea at pH 7.0 for 30 days. In

contrast to BLA, BAA shows cooperative unfolding transition and its unfolding completes after incubating in urea for four days.

As α-amylase is a highly aggregation prone protein and undergoes irreversible thermal and chemical unfolding. So, in second and third part of this study, efforts have been made to enhance the refolding yield and prevent the aggregation of BLA and BAA. Polyols and sugars are known to stabilize the native state of proteins. However, stability and folding do not always correlate. Here objective is to find out the effect of polyols and sugars on the refolding of thermophilic and mesophilic proteins BLA and BAA. The idea behind this study is to see if there is differential effect of cosolvents during refolding of thermophilic like (BLA) and mesophilic protein (BAA). Among polyols and sugars, glycerol, sorbitol and trehalose were found to be the most effective during refolding of BAA, although much less effect was observed for BLA.

In the third and last part of the work, amino acids like arginine, proline, glycine and its derivatives which are also known to stabilize the native state of proteins have been explored for enhancing the refolding yield. Mechanism of the stabilization of amino acids are different than the polyols and sugars. Most of the polyols and sugars stabilize the proteins by altering the solvent properties and thus causing preferential hydration of proteins, while some amino acids like arginine show weak binding to the proteins. Therefore, in this study amino acids and their derivatives have been used to enhance the refolding yield and prevent the aggregation of BLA and BAA during refolding. Higher concentrations of arginine and proline showed inhibitory effects on the refolding and also on native state stability of BLA and BAA. Similarly, glycine and sarcosine also showed inhibitory effects on both refolding and native state stability of BLA and BAA.

CHAPTER 2

MATERIAL AND METHODS

2.1 Materials

α-amylases BLA, BAA and other chemicals like Anilino-1-napthalene-8-sulfonate (ANS), maltose, sodium chloride, calcium chloride, sodium phosphate monobasic, sodium phosphate dibasic, starch, 3, 5-Dinitrosalicyclic acid (DNS), trehalose, sorbitol, xylitol, ethylene glycol, glycerol, sucrose, erythritol, arginine, proline, glycine, betaine, sarcosine, sodium potassium tartrate, acrylamide, sodium hydroxide and dialysis membrane were purchased from Sigma-Aldrich (St. Louis, U.S.A.). GdmCl and urea of highest purity grades were purchased from MP Biomedicals (IIIkirch, France). All the other chemicals were of highest purity grade.

2.2 Methods

2.2.1 Enzymatic solution preparation

Enzyme solutions were prepared by dissolving powdered α-amylase in filtered 20mM Sodium phosphate buffer, pH 7.0. Calcium was neither added nor depleted from the solutions of both the α-amylases. Stock solutions of BLA and BAA were stored at 4°C for further use. Enzymatic solution was not used for more than one week even after storing at 4°C to minimize the chances of bacterial and fungal contaminations.

2.2.2 Determination of protein concentration

The primary structures of BLA and BAA consist of 17 tryptophan (Machius et al., 1995; Alikhajeh et al., 2010). Thus, UV-absorption spectroscopy is the best method to determine the concentration of their protein solutions. Wavelength scan between 250 to 350 nm of protein solution was performed using UV-Vis spectrophotometer (Analytic Jena, Germany). Concentrations were calculated by using extinction coefficient 136410 M^{-1} cm^{-1} and 136710 M^{-1} cm^{-1} for BLA and BAA respectively at 280 nm (Strucksberg et al., 2007).

2.2.3 Preparation of urea and GdmCl solutions

Urea and GdmCl were used as a denaturing agent during equilibrium and kinetic unfolding experiments. The stock solutions of urea and GdmCl were prepared in 20 mM phosphate buffer, pH 7.0. Various concentrations were made by diluting the

stock solutions. Refractive index of urea was checked through refractometer (Reichert, AR200), and concentration was determined with the help of equation 4 Concentration of GdmCl was calculated using equation 5 given below (Pace, 1989). pH of both urea and GdmCl were adjusted to 7.0 with help of pH meter (Hach lange).

$$\text{Molarity}_{(\text{urea})} = 117.66(\Delta N) + 29.753(\Delta N)^2 + 185.56(\Delta N)^3 \quad (4)$$

$$\text{Molarity}_{(\text{GdmCl})} = 57.147(\Delta N) + 38.68(\Delta N)^2 - 91.60(\Delta N)^3 \quad (5)$$

2.2.4 Enzyme activity assay

The activity assay of α-amylase was performed according to method developed by Bernfield (Bernfield, 1955). For the measurement of enzymatic activity of α-amylase, starch was used as a substrate. Both starch (10%) and enzymatic solutions (4.2 µM, if not stated otherwise) were prepared in 20mM phosphate buffer, pH 7.0 without added Calcium. According to this method, amount of reducing sugar produced upon action of α-amylase on polysaccharides like starch is directly proportional to the intensity of colour. Here, DNS was used as colouring agent which was prepared in distilled water and consist of, sodium potassium tartrate (28.2 % w/v), sodium hydroxide (2M) and DNS (1%). The enzyme reaction started by adding 0.5 ml enzymatic solution to 0.5 ml of 1% starch and vortex for at least 40 seconds after that resultant solution incubated for 3 min at 25°C. After 3 min of incubation, reaction was stopped by adding 1 ml DNS solution. DNS reacts with the reducing sugars and form yellow colour product. The resultant solution was heated for 5 min in a water bath at 100°C. Samples were allowed to cool at room temperature and diluted tenfold before measurement of absorbance. Finally, absorbance was measured at 540 nm using UV-Vis spectrophotometer. Intensity of the colour gives the estimate of enzymatic activity. The absorbance of native protein at 540 nm, was considered as 100 % enzymatic activity, to determine the relative activity of unfolded and refolded samples.

2.2.5 Tryptophan fluorescence

All the fluorescence spectra were recorded on spectrofluorimeter, coupled with peltier (Agilent, Cary Eclipse). As the tryptophan residues are distributed throughout the protein structure in both α-amylases (Machius et al 1995, Alikhajeh et al 2010),

hence, the conformational changes during protein unfolding were monitored by tryptophan fluorescence emission spectra after excitation at 295 nm and spectra recorded from 305 to 450 nm. The scanning speed was set at 50 nm per minute and excitation and emission slit widths were set at 5 nm each. All the spectra were corrected by taking spectrum of buffer solution and subtracting it from the protein spectra. For monitoring the extent of unfolding, changes in wavelength maxima were analyzed as a function of urea and GdmCl concentrations. All the fluorescence experiments were performed with 4.2 µM of protein concentration, if not stated otherwise.

2.2.6 ANS fluorescence

Anilino-1-napthalene-8-sulfolate (ANS) is a hydrophobic fluorescent dye (Stryer, 1965) which is generally used for monitoring the extent of opening of hydrophobic core upon protein unfolding. Upon binding to the hydrophobic patches, ANS fluorescence intensity increases and blue shift in wavelength maxima is observed. Stock solution of the ANS was prepared in 20 mM phosphate buffer, pH 7.0 and the concentration was determined by using extinction coefficient 5000 M^{-1} cm^{-1} at 350 nm. For proper binding, 1:100 molar ratio of protein and ANS was used respectively. Final concentration of the protein and ANS was 4.2 and 420 µM respectively. Before measuring ANS fluorescence, protein samples were incubated with ANS for at least 30 minutes for proper binding. ANS fluorescence emission spectra were recorded after exciting at 380 nm and collecting spectra from 410 nm to 600 nm, with excitation and emission slit width at 10 and10 nm respectively. All ANS spectra of protein were corrected by subtracting the spectrum of solution containing ANS and buffer.

2.2.7 Circular dichroism (CD) spectroscopy

Conformational changes during protein unfolding and refolding was monitored by far-UV CD spectra in the range of 200 to 250 nm. All the spectra were recorded on JASCO-815 CD spectropolarimeter with attached peltier, using 1mm path length cuvette and 4.2µM of protein concentration, if not stated otherwise. In order to minimize the signal to noise ratio, spectra were collected and averaged over five scans and during spectral analysis, buffer signal was subtracted. During CD measurement it

was assured that high tension (HT) voltage should not exceed more than 800, because it can damage the optics of the instrument. Thus, extreme care has been taken during selection of protein concentration, solvent and cuvette path length, because these factors affect, both the quality of the spectra and HT voltage. As the chemical denaturants like urea and GdmCl show strong absorbance in the far-UV region (200-350 nm), thus CD spectra of such samples were recorded above 210 nm only to avoid the possibility of high HT voltage. Heat generated during the operation of instrument is neutralized by circulating water bath.

2.2.8 Equilibrium unfolding

The conformational stability of proteins as a function of various denaturants can be measured through equilibrium unfolding (Hornby et al., 2000; Singh et al., 2015; Canchi et al., 2010). During equilibrium unfolding, changes in secondary and tertiary structural properties as a function of different concentrations of denaturants were monitored through various spectroscopic techniques like CD and fluorescence. Here equilibrium unfolding of bacterial α-amylases, BLA and BAA have been carried out in urea and GdmCl. For unfolding, both BLA and BAA were incubated in different concentration range from 0 to 8 M for urea and 0 to 4 for GdmCl with 0.5 and 0.25 interval respectively. To achieve the equilibrium, unfolding reaction was allowed to occur for several days. The extent of unfolding was followed by tryptophan fluorescence. Equilibrium during unfolding reaction is supposed to be attained when there is no further shift in the wavelength maxima. All the equilibrium unfolding experiments were performed at 25°C and pH 7.0. The extent of unfolding at each concentration was measured by determining the fraction unfolded (F_U) using equation (Greenfield, 2006; Pace and Hermans, 1975). By calculating the fraction of unfolded at each concentration of denaturants using equation (6), free energy of unfolding at each particular concentration can be determined by using equation (7) and (8). Free energy of unfolding in water (ΔG_{H20}) was determined by extrapolating the plots of free energy of unfolding to 0 M denaturant concentration.

$$F_u = A_x - A_N / A_D - A_N \qquad (6)$$

$$K_D = F_u / 1-F_u \qquad (7)$$

$$\Delta G = -RT \ln K_D \qquad (8)$$

Where $A_{N,}$ A_D and $A_{x,}$ denotes the spectral signal of protein in native state, denatured state and protein at particular concentration of denaturant respectively. $K_{D,}$ F_u and $1\text{-}F_u$ denotes the equilibrium constant, fraction of unfolded and folded state respectively. Change in free energy (ΔG) was calculated using equation 8, where R denotes gas constant (0.002 kcal/mole) and T is the absolute temperature (298 K).

2.2.9 Equilibrium refolding

For equilibrium refolding, enzymes were unfolded at protein concentration of 252µM, in 6M GdmCl and 10M urea for 24 hours at 25°C. Equilibrium refolding was achieved by diluting the denatured enzymes in 20 mM phosphate buffer, pH 7.0 and containing various final concentrations of urea and GdmCl, as mentioned in equilibrium unfolding experiments. All the equilibrium refolding transitions were measured at 4.2µM protein concentration. As the equilibrium achieved quickly during refolding in comparison to unfolding, thus equilibrium refolding of BLA and BAA in urea and GdmCl were monitored only for three days, unlike for five days in unfolding. Like equilibrium unfolding, refolding transitions were also followed by tryptophan fluorescence measurement, and change in fluorescence wavelength maximum was used as a probe.

2.2.10 Unfolding kinetics

Unfolding kinetics of both enzymes in urea and GdmCl were performed through manual mixing procedure. During which, buffer containing desired concentration of denaturants and enzyme were mixed outside the instrument and dead time for this process is about 10 seconds. After that, solution was placed in the spectrofluorimeter and extent of unfolding over time was monitored by tryptophan fluorescence. Protein concentration was kept the same as during equilibrium unfolding experiments i.e. 4.2 µM. Kinetics were measured in 20 mM phosphate buffer, pH 7.0, at 25 °C. The unfolding kinetics for BAA and BLA in four different concentration of urea (7, 7.5, 8 and 8.5M) and GdmCl (1, 2, 3 and 4M) were carried out up to the point when no further changes in tryptophan fluorescence wavelength maximum was observed. During initial phase of kinetics, spectra were recorded at small period of interval, for 30 minutes, spectra were collected after every 2 minute interval. After that duration between two consecutive spectra increases to 5 minutes till 3 hours. After 3 hours,

time interval between two consecutive spectra, further increases to 30 minutes till complete unfolding. This might be due to the fact that conformational changes occur with slower rate at later stages of unfolding.

2.2.11 Cosolvent assisted refolding

Refolding of BLA and BAA were performed by dilution method in which denatured protein solution is diluted rapidly while mixing. This leads to the decrease in concentrations of protein and denaturant several fold immediately. Although low protein concentration is beneficial for refolding to avoid intermolecular association thus aggregation but sudden decrease in denaturant concentration may impose constraint during refolding, due its high diffusion rate it may induce protein aggregation (Yamaguchi and Miyazaki, 2014; Mishra et al., 2005). To avoid this, proper mixing of refolding solution was carried out through vortex. The final volume of refolding solution was 2 ml to facilitate the smooth mixing of refolding solution. The enzymes were unfolded by incubating in 6M GdmCl at 25°C for 24 hours. Then refolding was carried out by diluting the unfolded protein in refolding buffer containing various concentrations of different cosolvents. Refolding in buffer without any cosolevnt was used as control experiment. Residual concentration of GdmCl has no inhibitory effect on enzyme activity. Refolded samples were incubated for 3 hours at 25°C to complete the refolding. In order to see the effect of different temperature on refolding yield of bacterial α-amylases, spontaneous refolding was performed at 4, 15, 35 and 45°C. For this, refolding buffer was pre-equilibrated at desired temperature in the circulated water bath for at last 15 minutes. Although, refolding yield of BLA and BAA was measured through enzymatic activity measurement but the estimation of formation of secondary and tertiary structure carried out with help of CD and fluorescence spectroscopy. Appropriate controls were used to see the relative effect of different cosolvents on refolding of alpha amylase. The single shot dilution was performed in glass tubes by diluting the unfolded samples 60 folds in refolding buffer and vortex for at least 40 seconds. This step has been performed with extreme care to get enhanced and reproducible refolding yield. The final residual concentrations of protein and denaturant (GdmCl) were 0.025 mg/ml and 0.1M respectively. The effect of residual concentration of GdmCl on proteins native conformation was checked by measuring the activity of native α-amylase with 0.1M of GdmCl.

2.2.12 Static light scattering

Static light scattering was used for monitoring the formation and characterization of protein aggregates during the refolding of α-amylases. The experiments were performed on spectrofluorimeter using 10 mm path length cuvette. The kinetics were measured both in the presence and absence of cosolvents, in refolding buffer at 25 °C. The excitation and emission wavelength were set at 400 nm with 2.5 nm slit width. The continuous magnetic stirring required during aggregation kinetics measurement. The contribution of buffer in the light scattering was subtracted to get signal only from protein.

2.2.13 Atomic force microscopy

The morphology of protein aggregates formed during refolding of α-amylases in the absence and presence of cosolvents were examined by atomic force microscopy (AFM). Details of AFM measurements has been described elsewhere (Rahamtullah and Mishra, 2021). Briefly, a sample volume of 20 µl was spread over piece of mica sheet of highest purity grade V1 (TED PELLA Inc., USA). After drying at room temperature, samples were washed with filtered miliQ water and left for overnight drying. The dried samples were used for capturing image with help of WITec alpha 300 atomic force microscope (WITec GmbH, Germany). Data analysis has been performed using Project 5 software.

CHAPTER 3

STABILITY AND UNFOLDING OF BLA AND BAA IN CHEMICAL DENATURANTS

The work described in this chapter has been published as:

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3.1 Introduction

BLA and BAA are homologous α-amylases from *Bacillus licheniformis* and *Bacillus amyloliquefaciens* respectively. Although, both have mesophilic origin but from thermal stability point of view, BLA behaves as a thermophilic and BAA as a mesophilic counterpart (Fitter and Haber, 2004). Both, BLA and BAA share 88% and 81% amino acids sequence similarity and identity respectively, moreover they also have similar structural fold (Alikhajeh et al., 2010; Machius et al., 1995). To understand the stability differences between thermophilic and mesophilic proteins most of the work has been focussed on comparing amino acid sequence and atomic resolution structure. In general, thermophilic proteins have more number of charged residues, increased ionic interactions, higher number of disulfide bonds, additional hydrogen bonds, increased packing of hydrophobic core, higher number of proline residues than their mesophilic counterparts (Janicke and Böhm, 1998; Veteriani et al., 1998; Wantnabe et al., 1994). But, relative contribution of each of these factor is specific for a particular pair of mesophilic and thermophilic protein. Despite of differences in the thermal stability of BLA and BAA, they have a lot of similarities, like absence of disulfide bonds, similar content of charged residues and proline content. However, there is significant difference in the number of histidine residues. The number of histidine residue in BLA and BAA is 24 and 14 respectively (Machius et al., 1995; Alikhajeh et al., 2010).

This study is intended to address the origin of the stability difference between BLA and BAA. To achieve this, conformational stability of both α-amylases was compared in two chemical denaturants with different ionic character, urea and GdmCl. Therefore, this study has been focused on investigating the relative contribution of each type of interactions in the stability of these two α-amylases. For this, the equilibrium unfolding of both α-amylases in GdmCl and urea has been performed. The equilibrium unfolding has been considered a robust method, which can provide the realistic measure of conformational stability (Pace et al., 1986; 1990). In several studies, urea and GdmCl have been used for deciphering the existence of equilibrium and kinetic intermediates during the folding pathway of proteins (Kim and Baldwi, 1990; Jaenicke, 1987). Due to the different ionic nature, urea and GdmCl provide varying estimate of conformational stability of proteins (Monera et al., 1994).

Moreover, for the quantitative conformational stability measurements, chemical denaturants (GdmCl and urea) induced unfolded state of proteins are more favoured over temperature induced, as former devoid of any residual secondary structure and tertiary content (Tamura and Gekko, 1995; Gast et al., 1997).

From the current study, it becomes clear that BLA has higher conformational stability than BAA in both urea and GdmCl. Interestingly, BLA shows extreme resistance towards urea induced unfolding at pH 7.0 and retain its folded conformation even after 30 days incubation. In addition, study of the structural and functional relationship of BLA and BAA during GdmCl and urea induced unfolding by using CD, intrinsic fluorescence and enzyme activity suggest that, BLA does not obey two state model and unfolds non-cooperatively with partially unfolded or molten globule like intermediates. This observation is further confirmed by ANS binding. To clarify the kinetic origin of the stability of bacterial α-amylases, kinetic unfolding was performed in different concentrations of GdmCl and urea. Different unfolding rates of BLA and BAA in GdmCl and urea may also shed light on the stability differences between them.

3.2 Results

3.2.1 Equilibrium unfolding and Refolding of BLA and BAA in GdmCl

The quantitative measurement of conformational stability of BLA and BAA were carried out through equilibrium unfolding in GdmCl and urea. The equilibrium unfolding transitions of BLA in GdmCl exhibit sigmoidal behaviour. During unfolding transition on day one, structural perturbation initiated at 0.5M GdmCl and ends up at 2.5M. No further changes in the wavelength maxima were observed above this concentration. The apparent midpoint of denaturation (C_m) for day one was 1.6M, which shifted to 1.4M and 1.3M on second and third day respectively. No further change in apparent C_m was observed upon extending the incubation till fifth day (C_m 1.3M) as shown in Figure 2a, Table 1. Although, pre-denaturation baselines of different days were superimposable but drifting of post-denaturation baselines was observed up to three days during equilibrium unfolding BLA in GdmCl. In the case of BAA, change in wavelength maxima indicates that denaturation begins at 0.25M and

completed around 1.5M. Further no change in the tryptophan wavelength maxima was observed upon extending the denaturation up to 4 M. The tryptophan fluorescence of BAA exhibits large red shift from 340 to 358 nm upon complete unfolding in GdmCl. After one day incubation in GdmCl, the apparent C_m was observed to be 0.70 M. As the incubation period increases, apparent C_m slightly decreases to 0.65M, 0.62M, 0.6 M and 0.6M for day two, three, four and five respectively (Figure 2b and Table 1). The equilibrium was attained after four days of incubation. Even at sub-denaturing concentrations of GdmCl, BAA shows increase in the wavelength maxima due to slight destabilization of native structure (Figure 2b). The pre and post denaturation baselines of different days were not superimposable, which can be attributed to the higher susceptibility of BAA towards GdmCl. The equilibrium unfolding transition of BAA in GdmCl also shows sigmoidal pattern. These results show that BLA has higher apparent C_m in GdmCl than BAA. The quantitative measurement of conformational stability of α-amylases in GdmCl were carried out using equations given by Greenfield (Greenfield, 2006). The apparent free energy of unfolding (ΔG_{H2O}) of BLA and BAA in GdmCl was estimated to 2.94 and 2.53 kcal/mole respectively (Figure 2e). Thus, thermodynamically BLA is marginally stable in GdmCl than the BAA. Although the free energy of unfolding of BLA is less in comparison to other thermophilic and hyperthermophilic proteins (Mishra et al 2008, Ogasahara et al 1998). In addition to the equilibrium unfolding, equilibrium refolding of BLA and BAA in GdmCl has also been carried out (Figure 2c, d). The equilibrium refolding was monitored for 3 days, but it was achieved quickly. The equilibrium refolding transitions of both α-amylases in GdmCl show no pretransition base lines. In the case of BLA, midpoint of renaturation (C_m) for day 1 is 0.5M and no further changes were observed in the C_m values of day 2 and 3. Thus, it can be concluded that equilibrium was achieved within one day of refolding (Figure 2c).While for BAA, although midpoint of renaturation (C_m) occur at 0.5M on day 1, as it was observed in BLA, but shifted to 0.25M on day 2 and 3.

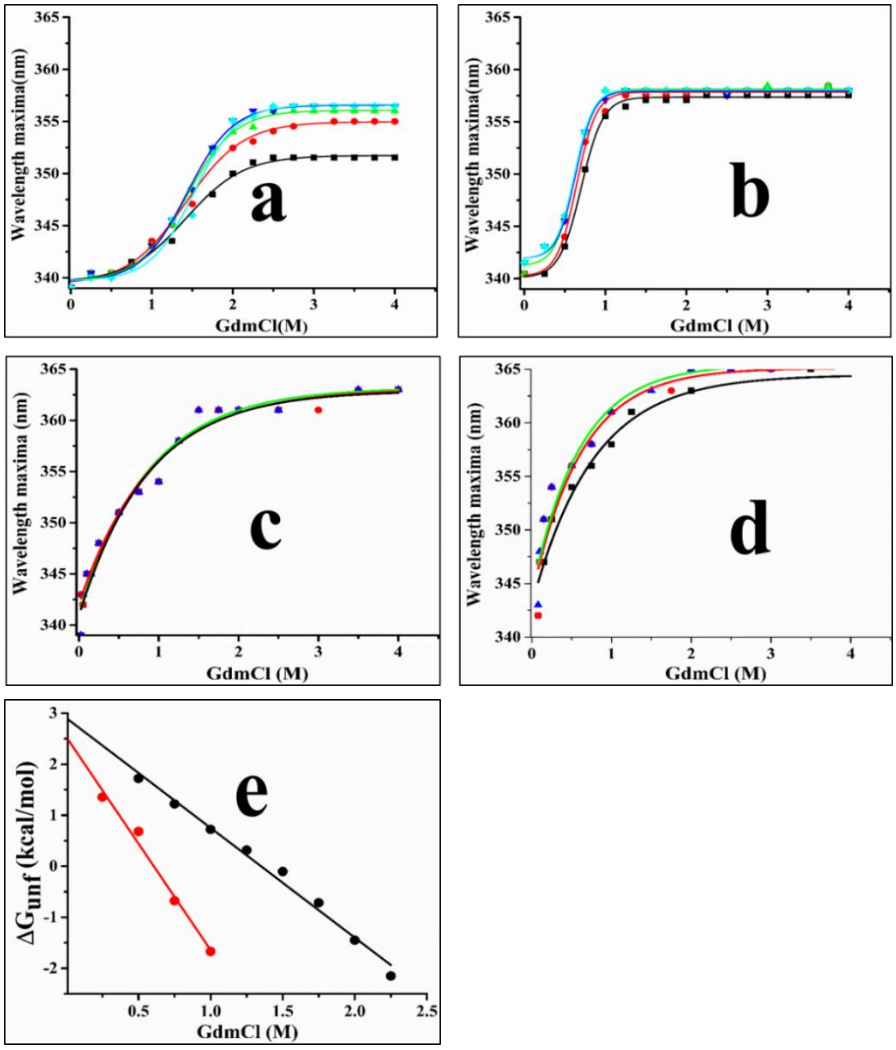

Figure 2: Apparent equilibrium unfolding and refolding transitions of BLA and BAA in GdmCl. a & b, represent equilibrium unfolding and c & d represent equilibrium refolding of BLA and BAA respectively. Both the enzymes were incubated at different concentrations of GdmCl for day 1(black), 2 (red), 3 (green), 4 (blue) and 5 (cyan) for unfolding and day 1 (black), day 2 (red) and day 3 (green) for equilibrium refolding. All the experiments were performed with 4.2µM of protein concentration, at 25°C and 20mM phosphate buffer pH 7.0. The change in free energy of unfolding of BLA and BAA was plotted against various concentrations of GdmCl (e).

Unlike BLA, where equilibrium refolding was achieved in one day, equilibrium during refolding of BAA was achieved in 2 days (Figure 2d). Shifting of C_m to the lower values might be due to the more susceptibility of refolded state of BAA towards GdmCl. The lower C_m values of BLA and BAA during equilibrium refolding transitions in GdmCl compare to equilibrium unfolding, indicate the lower stability of

their refolded state in GdmCl (Figure 2c, d). Difference in the C_m values of BLA and BAA during equilibrium unfolding and refolding hints towards the kinetic origin of conformational stability of both enzymes, but this effect is more prevalent in the case of BLA than the BAA. Equilibrium unfolding transitions of bacterial α-amylases in chemical denaturants are not fully reversible, due to dominance of aggregation at below the C_m values in GdmCl and urea (Fitter et al., 2004).

Table 1. C_m of BLA and BAA during equilibrium unfolding in GdmCl

Incubation time (days)	BLA (M)	BAA (M)
1	1.55	0.70
2	1.45	0.65
3	1.45	0.62
4	1.40	0.60
5	1.42	0.60

3.2.2 Equilibrium unfolding and refolding of BLA and BAA in urea

Urea is uncharged and weaker denaturant compare to the GdmCl (Monera et al 1994). The equilibrium unfolding of BLA and BAA (Figure 3a, b) in urea was monitored for six days. BLA exhibit no change in the wavelength maxima upon incubating in 8 M urea for six days (Figure 3a). In fact, urea unfolding of BLA was monitored over 30 days but did not included due to possible known effects of urea forming isocyanate over time resulting from the carbamylation of amines in lysine and arginine residues (Stark et al.,1960). BAA shows no appreciable change in the wavelength maxima between 0 to 2M urea, which ranges from 340 to 342 nm.

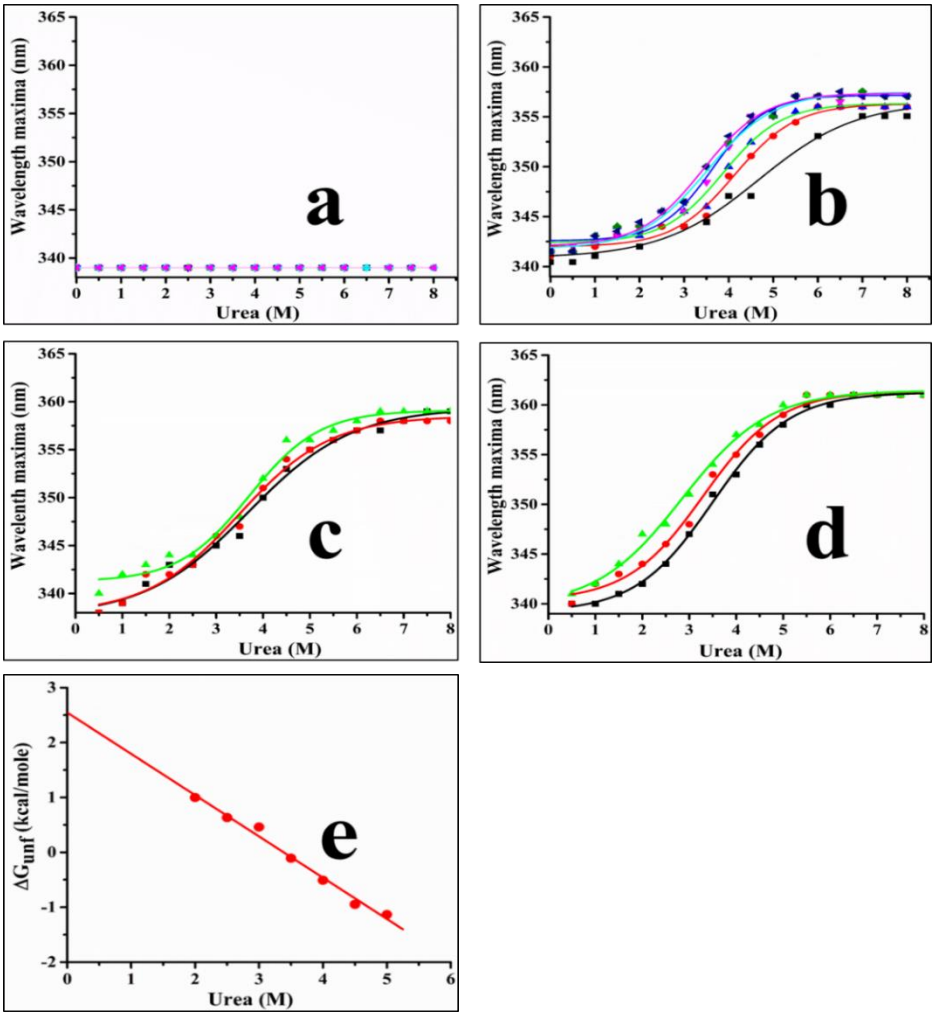

Figure 3: Apparent equilibrium unfolding and refolding transitions of BLA and BAA in urea. a & b represent equilibrium unfolding while c & d represent refolding of BLA and BAA respectively. During equilibrium unfolding both enzymes were incubated in various concentrations of urea for day 1 (black), 2 (red), 3 (green) 4 (blue), 5 (cyan) and 6 (magenta) respectively. While during equilibrium refolding incubation period was only 3 days for both enzymes, for BLA transition for day 1 (black), day 2 (red) and day 3 (green). Change in free energy of unfolding of BLA and BAA was plotted against different concentrations of urea (e). All the experiments were performed with 4.2µM of protein concentration, at 25°C and 20mM phosphate buffer pH 7.0.

Maximum changes in the wavelength maxima from 342 to 358 nm were observed upto 7 M urea. No further changes were observed at 7.5 and 8M urea. The apparent C_m after one day was calculated to be 4.4M. Similar to GdmCl induced unfolding of BAA, here also the apparent C_m shifted to lower concentration of urea upon increasing the incubation period. The apparent C_m for two, three, four, five and six days of incubation, were 4.0M, 3.8 M, 3.54 M, 3.4 and 3.4 M respectively (Figure 3d, Table 2). The unfolding transitions of BAA in urea are sigmoidal shape and cooperative in nature. The post denaturation baselines remain non-superimposable up to four days. After that post denaturation transition baselines for day five and six superimposed on each other. This indicates that equilibrium attained after 4 days of incubation. The pre transition shows drifting baselines for multiple days in urea due to susceptibility of BAA towards sub-denaturation concentrations of urea. The free energy of unfolding (ΔG_{H2O}) of BAA in urea was estimated to be 2.22 kcal/mole (Figure 3e).

Table 2. C_m of BAA during equilibrium unfolding in urea

Incubation time (days)	BAA (M)
1	4.42
2	4.00
3	3.80
4	3.54
5	3.40
6	3.40

BLA shows exceptionally higher stability in urea unfolding. Equilibrium refolding of BLA and BAA has been carried out in different urea concentrations. As BLA does not unfold even in 8M urea at pH 7.0 (Figure 3a), thus for equilibrium refolding, it was unfolded in 8M urea by lowering pH to 3.0. The results indicate that equilibrium refolding transition of BLA shows sigmoidal, two state behaviour without any detectable intermediate species. The midpoint renaturation (C_m) of BLA for day one was achieved at 4M, no further changes in C_m values were observed for day two and

three. BAA exhibit lower C_m values than BLA with midpoint of renaturation for day one, two and three attained at 3.5 M, 3.2 and 3.0M respectively.

3.2.3 Effect of low pH and ionic strength on urea induced unfolding of BLA

Since BLA shows extreme resistance towards urea induced unfolding and retain folded conformation, even after incubation for 6 days in 8M urea at 25°C and pH 7.0 (Figure 3a). To understand this unusual stability of BLA in urea, the effect of low pH and ionic strength (1M NaCl) on urea induced unfolding of BLA was done as shown in Figure 4a and b respectively. The results show that unlike unfolding of BLA at pH 7.0, urea in combination with low pH (pH 3.0) is able to unfold BLA completely with apparent C_m varying from 1.92M for day one to 1.78, 1.75 and 1.75M for day two, three and four respectively (Figure 4a).

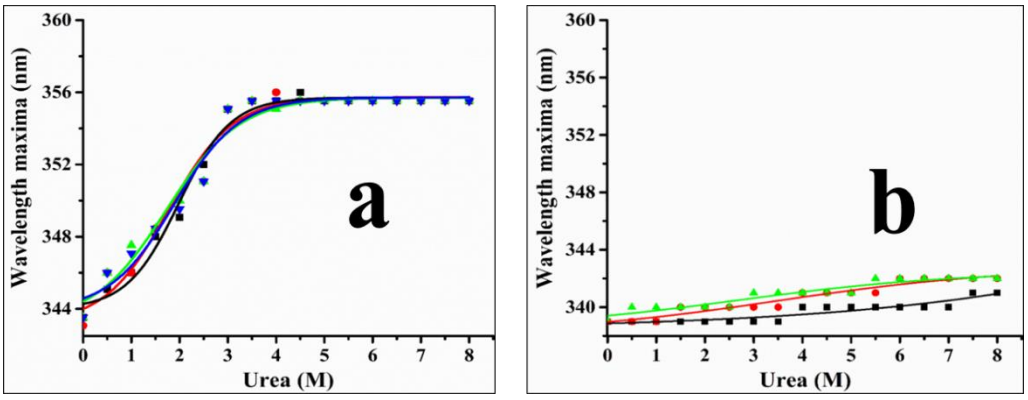

Figure 4: Effect of acidic pH (a) and ionic strength (b) on urea induced unfolding of BLA. For unfolding at pH 3.0 (a), BLA was incubated in various urea concentrations for day 1 (black), day 2 (red), day 3 (green), day 4 (blue). Unfolding in varying concentration of urea with 1M NaCl (b) was done for day 1 (black), day 2 (red) and day 3 (green). All the measurements were performed with 4.2µM of protein and at 25°C.

It seems that the equilibrium has achieved after two days and thus no further change in C_m values were observed. The equilibrium reached very quickly even faster than the equilibrium unfolding of BLA in GdmCl (Figure 2a). Which means combination of urea and pH proven to be even stronger unfolding condition than the GdmCl alone at pH 7.0. Effect of ionic strength on the urea induced unfolding to mimic the GdmCl was carried out out for three days with urea in the presence of 1M NaCl. Results indicates that net change in wavelength maxima was from 340 to 342 nm (Figure 4b) which means NaCl is not as potent in breaking electrostatic interactions as GdmCl was for BLA.

3.2.4 Structural and functional relationship during chemical induced unfolding

In order to investigate the correlation between structural and functional stability of BLA and BAA in chemical denaturants, equilibrium unfolding of BLA and BAA in GdmCl and urea were followed through different spectroscopic probes. The extent of loss of secondary and tertiary structure of both α-amylases during equilibrium unfolding in GdmCl and urea were monitored by using circular dichroism (CD), tryptophan fluorescence and enzymatic activity measurements. Changes in ellipticity at 222nm $(\theta)_{222nm}$ provide the conformational change in the secondary structure during unfolding. During BLA unfolding in GdmCl, both tryptophan fluorescence wavelength maxima and ellipticity $(\theta)_{222nm}$ remain unchanged up to 0.5M GdmCl. While the loss of enzymatic activity begins even at 0.25M. At 1M GdmCl, BLA loses all of its secondary structure contents as indicated by maximum change in the ellipticity. Although, change in tryptophan fluorescence and enzymatic activity signal indicates the retention of tertiary structure. No further changes in the fluorescence and the enzymatic activity signals were observed above 2.5 and 3.25M of GdmCl. BLA shows apparent C_m of 1.70, 1.00 and 0.75M for fluorescence, enzyme activity and CD respectively after one day of incubation (Figure 5a). During BLA equilibrium unfolding in GdmCl, loss of secondary structure content occurs prior to the loss of tertiary structure, which followed by enzymatic activity and fluorescence respectively. Thus, unfolding transitions of BLA in GdmCl through different probes are non-coincidental. Like BLA, no appreciable changes in ellipticity, fluorescence and enzyme activity of BAA were observed up to 0.5M. Above this, all three probes

exhibit drastic changes in their signal and maximum signal of CD, fluorescence and enzymatic activity of BAA were observed at 1M, 1.25 and 1.5 respectively. Unlike BLA, BAA shows close apparent C_m, 0.70, 0.70 and 0.65 M for fluorescence, enzyme activity and CD respectively (Figure 5 b). It seems that unlike BLA, unfolding transitions of BAA in GdmCl through all three different probes are coincidental. Similarly, equilibrium unfolding of BLA and BAA in urea were also monitored using CD, fluorescence and enzymatic activity measurements. As it has been shown that BLA retains its native conformation even in 8M urea at pH 7.0 (Figure 3a).

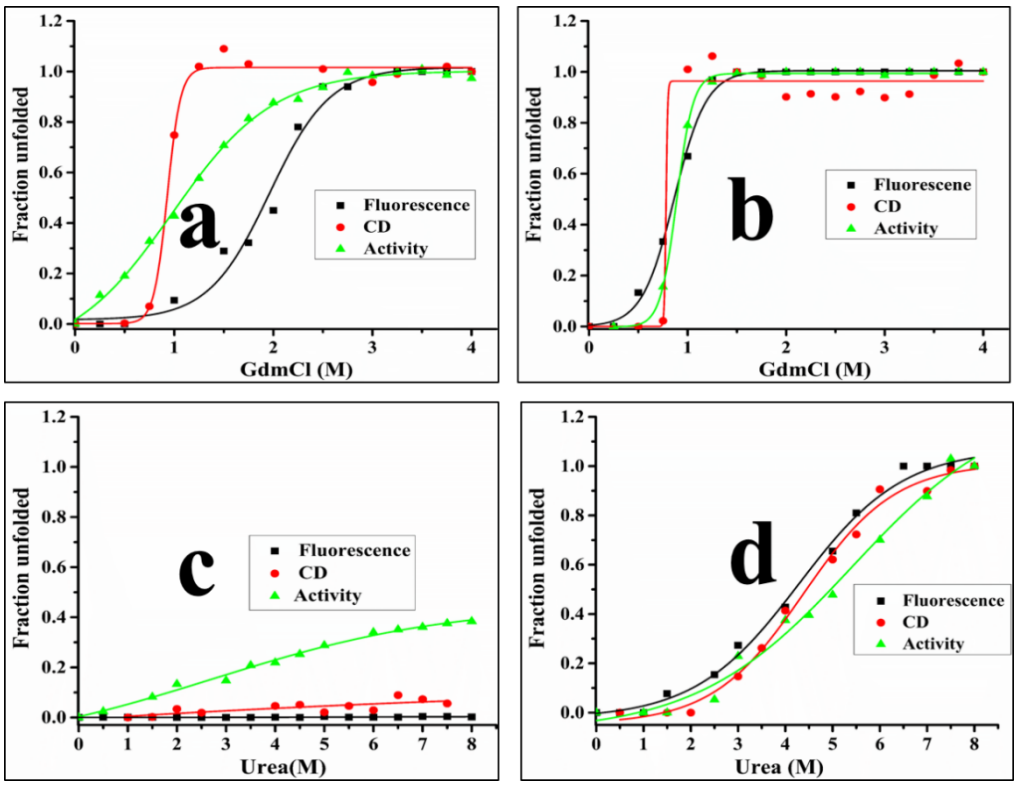

Figure 5: Structure-function relationship during GdmCl and urea unfolding of BLA and BAA. Figure a, b represent the loss of structural and functional properties during GdmCl induced unfolding of BLA and BAA respectively. Figure c and d represent the loss of structural and functional properties during urea induced unfolding of BLA and BAA respectively. Both the enzymes were incubated in various concentrations of GdmCl and urea for 24 hours and unfolding was monitored through fluorescence (black), CD (red) and enzyme activity (green).

Thus, tryptophan fluorescence signal remains unchanged during equilibrium unfolding of BLA in urea. While, change in ellipticity $(\theta)_{222nm}$ indicates the loss of some amount of secondary structure content and also about 25 % of enzymatic activity of BLA was lost at 8M urea (Figure 5c). On contrary to BLA, equilibrium unfolding of BAA in urea follows the sigmoidal path by all three probes. Change in fluorescence signal begins at 0.5 M, while no change in ellipticity and enzymatic activity was observed till 2M urea. Unexpectedly, loss of enzymatic activity, requires higher concentration of urea than the change in fluorescence and CD in transition zone. Thus, BAA shows identical apparent C_m 4.20M for CD and fluorescence, while slightly higher (4.50) M for enzyme activity (Figure 5d). Similar to GdmCl, unfolding transitions of BAA in urea were almost coincidental, which indicates that intermediates are either not populated or not detected during urea unfolding.

3.2.5 ANS binding during equilibrium unfolding in GdmCl and urea

Conformational changes during equilibrium unfolding of BLA and BAA in GdmCl and urea have also been monitored through ANS fluorescence. As ANS is a hydrophobic dye, thus it specifically binds to the exposed hydrophobic patches and exhibits marked increase in its fluorescence intensity and blue shift in emission wavelength from 510 to 480 nm (Semisotnov et al., 1991; Stryer, 1965). The ANS fluorescence spectra of native state of BLA and BAA are quite similar to ANS only, with very small increase in the ANS fluorescence intensity and no change in the wavelength maxima (510 nm). During equilibrium unfolding of BLA in GdmCl, increase in the ANS fluorescence intensity started at 0.25M, but maximum increase was observed at 0.5M, with approximately fourfold increment in ANS fluorescence relative to native protein. Along with this, blue shift in ANS fluorescence maxima was also observed from 510 to 480 nm during the unfolding in GdmCl (Figure 6a). Beyond 0.5M, ANS fluorescence intensity exhibits drastic decrease. At 4.0M GdmCl, intensity approaches close to that of the native state. The species at 0.5M GdmCl represents the partially folded intermediates with exposed hydrophobic patches having strong affinity for the ANS binding. Unlike BLA, ANS fluorescence exhibit comparatively less increase in its intensity during GdmCl unfolding of BAA. In the case of BAA, maximum intensity was observed at 1.0M GdmCl, which is almost two fold relative to the native state. Similar to BLA, blue shift in wavelength maxima

from 510 to 480 nm was also observed. BAA, which is more susceptible to GdmCl than the BLA. Even at 0.25M, protein core of BAA opens up to some extent and thus accessibility of ANS to hydrophobic patches increases (Figure 6b).

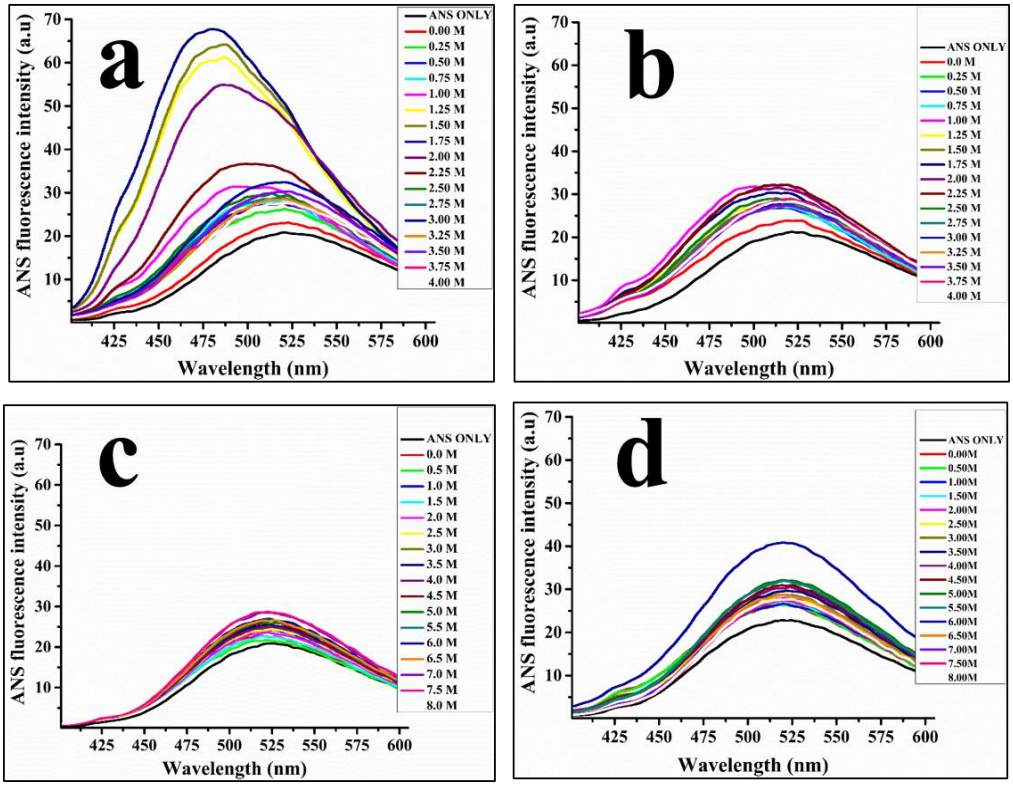

Figure 6: ANS binding during equilibrium unfolding of BLA and BAA in GdmCl and urea. Figure a, b represent ANS binding during GdmCl induced unfolding of BLA and BAA respectively. Figure c and d represent urea induced unfolding of BLA and BAA respectively. Both the enzymes were incubated for 24 hours at various concentration of GdmCl and urea in the presence of ANS. The extent of core opening was monitored by the ANS fluorescence.

Further increase in GdmCl concentration leads to the decrease in ANS fluorescence intensity at 4.0M, intensity becomes almost equal to that of the native BAA. Equilibrium unfolding of both BLA and BAA in urea was also monitored with the help of ANS. The results indicate that BLA exhibit significantly less increase in ANS fluorescence intensity during urea induced unfolding as compared to the GdmCl. At initial concentrations of urea, from 0 to 5M apparently negligible increase was observed. Above this concentration, some increase in ANS intensity was detected with maximum increase was observed at 7.5M urea which is less than two-fold,

relative to native enzyme but no blue shift in wavelength maxima was observed (Figure 6c). This result indicates the partial exposure of hydrophobic surface during urea induced unfolding, though enzyme remains folded even in 8M urea at pH 7 as shown in Figure 2c. While on other hand, during BAA unfolding in urea, increase in ANS fluorescence intensity began from 0.5M urea and intensity exhibit two fold increase at 1M urea, but no blue shift in wavelength maxima was observed. No further increase in ANS intensity was detected by increasing the urea concentration beyond 1M (Figure 6d). Thus, from the above results, it can be infer that unlike GdmCl induced unfolding, no significant ANS binding was observed during equilibrium unfolding of BLA and BAA in urea (Figure 6c, d).

3.2.6 Kinetic unfolding in GdmCl and urea

In most of the cases, thermodynamic stability of proteins does not always correlate with its kinetic stability (Kim and Baldwin, 1990; Sanchez, 2010). Thus, in order to understand this lack of correlation, it is important to study the kinetic stability of proteins as well. In this study, unfolding kinetics of both BLA and BAA were carried out in four different concentrations of urea and GdmCl. The selected concentrations of Urea and GdmCl were 7, 7.5, 8, 8.5M and 3, 4, 5 and 6M respectively. As the unfolding reaction started by manual mixing of protein with GdmCl, within duration of mixing protein undergoes some structural perturbations. These structural changes were reflected in change in fluorescence signal at 0 time point, which could be referred as burst phase signal sentence is not clear. The intensity of the burst phase signal depends upon the denaturant concentration. The results show that the unfolding kinetics of BLA and BAA in various GdmCl concentrations have different starting point due to the differences in their burst phase signals (Figure 7a, b). Clearly, initial and final values of wavelength maxima of BLA and BAA were changes with increasing GdmCl concentration during their kinetic unfolding.

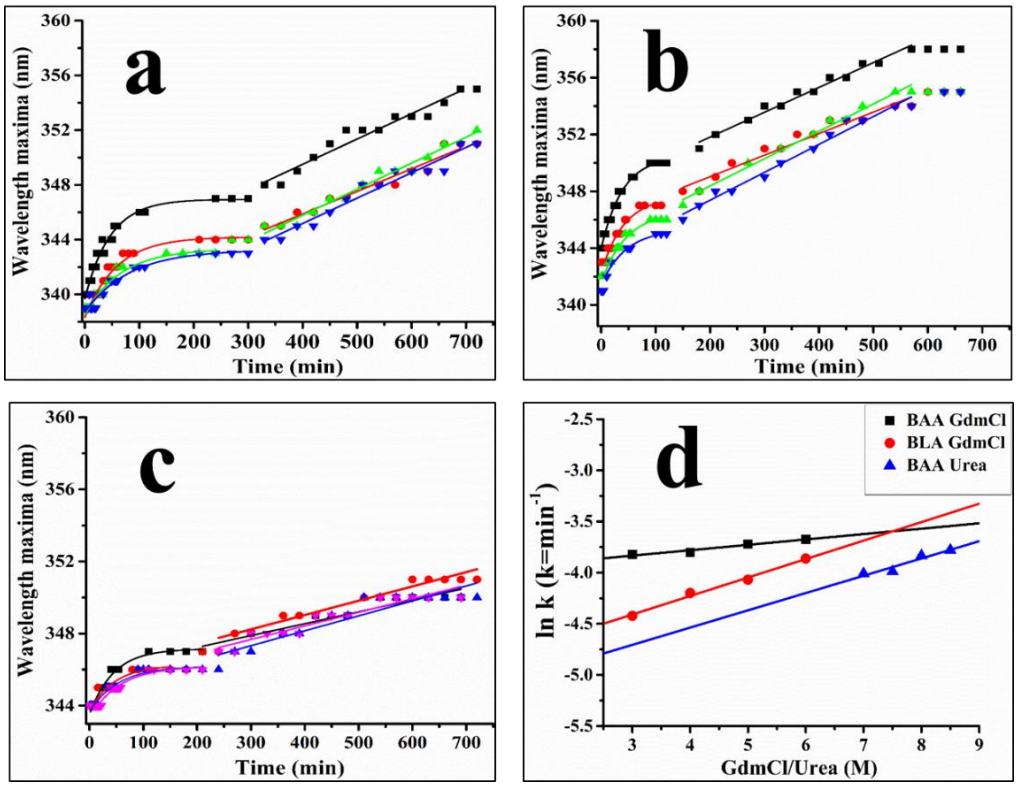

Figure 7: Unfolding kinetics of BLA and BAA in GdmCl and urea. The kinetics of unfolding of BLA (a) and BAA (b) in 3.0 (blue), 4.0 (green), 5.0 (red) and 6.0 M (black) GdmCl were monitored by change in tryptophan wavelength maxima for 12 hours. The fast phases were fitted to single exponential, while slow phases could not be fitted due to linear trend. Similarly, kinetic unfolding of BAA (c) in urea was monitored in 7.0 (pink), 7.5 (blue), 8.0 (red) and 8.5 M (black) urea for 12 hours. The fast phase was fitted to single exponential, while the slow phase could not be fitted due to linear trend. The half Chevron plot of BLA and BAA in GdmCl and urea (Figure d) showed the linear dependence of logarithmic value of unfolding rate constants.

At all the concentration of GdmCl used, kinetic unfolding traces of BLA and BAA were found to exhibit biphasic behaviour with one fast and another slow phase (Figure 7a, b). Although, for a given α-amylase, amplitude of fast and slow phases of unfolding increases with increase in GdmCl concentration, but their duration remain constant. There were significant differences between BLA and BAA, both in the amplitude and the duration of fast and slow phases. At all concentrations of GdmCl, the kinetic unfolding of BLA, has comparatively lower amplitude and longer duration

of both slow and fast phases than BAA. The first phase was fitted to single exponential, while the second phase shows linear tendency and thus could not be fitted. The logarithmic values of unfolding rate constants in 3, 4, 5and 6M GdmCl were -4.42, - 4.19, - 4.07 and -3.86 for BLA and -3.82, - 3.80, - 3.72 and - 3.67 for BAA (Table 3 a). BLA exhibits lower values of rate constants than the BAA at all concentrations. The apparent half-life of BLA and BAA in GdmCl as estimated through half Chevron plot by using unfolding rate constants of the first kinetic phase were 147 and 55 minutes, respectively (Figure 7d).The results of equilibrium unfolding in urea have already showed that the BLA does not unfold even in 8.0M urea at pH 7.0, thus Kinetics of the urea induced unfolding was performed for BAA only. The unfolding rate constant of BAA in 7.0, 7.5, 8.0 and 8.5M urea were -4.01, -3.99, - 3.83 and – 3.78 respectively (Table 3 b). Although similar to GdmCl, unfolding kinetics of BAA in urea also exhibited biphasic nature (Figure 7c) at all of the above concentrations, but duration of fast phases was comparatively longer than GdmCl unfolding at all urea concentrations. Due to same values of burst phase signals at different urea concentration, kinetic unfolding traces of BAA seems to be originated from identical starting points, which is in contrast to the GdmCl unfolding kinetics. The rate of unfolding of BAA in urea was slower in comparison to GdmCl induced unfolding (Table 3 b). In urea, BAA has half-life of 200 minutes which is more than threefold greater than in GdmCl (Figure 7d). Although, calculation of half-life using only first kinetic phase will be giving under estimation during kinetic unfolding in GdmCl and urea.

Table 3. Kinetic parameter of BLA and BAA in GdmCl (a) and urea (b).

(a)

GdmCl (M)	ln k_{obs} BLA	ln k_{obs} BAA
3	-4.42	-3.82
4	-4.19	-3.80
5	-4.07	-3.72
6.	-3.86	-3.67

(b)

Urea (M)	ln k_{obs} BAA
7.0	-4.01
7.5	-3.99
8.0	-3.83
8.5	-3.78

3.3 Discussion

The preservation and maintenance of structural and functional stability of various proteins, especially, industrial enzymes, are of great importance. As most of the reactions at industrial scale carried out at high temperature, so thermal stability is the most desirable property of industrial enzymes. Understanding the factors responsible for the determining the conformational stability of the proteins is of fundamental importance in protein science. A comparative study of thermophilic and mesophilic protein stability and folding should be able to pinpoint the differences in the origin of their stability. Although, several comparative studies have been done but most of them could not reach on a single conclusion. This is because, factors imparting thermal stabilities in different proteins varies from protein to protein. In general, native state stability of proteins can be explained by the thermodynamic hypothesis (Anfinsen, 1973) where, lowest energy state is the most stable, native state. In the kinetic stability, protein's native state is separated from the non-native conformations through large free energy barriers (Baker and Agard, 1994). Thermal stability of thermophilic proteins is attained by the same pool of interactions as the mesophilic proteins like, electrostatic, hydrophobic, hydrogen bonds and van der Waal's interactions. However, relative contributions of each type of interactions towards the conformational stability would be different for different proteins. Although, there is no general mechanism for increased thermal stability of proteins but still there are some structural features like compact protein core, additional hydrogen bonding, salt bridges and their network, disulphide bonds, higher number of proline residues may contribute significantly to the observed differential stability between thermophilic and mesophilic proteins (Jaenicke and Böhm, 1998). While comparing thermophilic proteins like ferreodoxin, glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase with their mesophilic counterparts, it was found that thermophilic proteins have increased content of non-polar and helix stabilizing residues in its protein core than their mesophilic counter parts (Argos et al., 1979). Similarly, the structural comparison between thermophilic alcohol dehydrogenase (TBDAH) with its mesophilic counterpart CBADH, reveals that the presence of additional proline at strategic positions in TBDAH, significantly contributes to its higher thermal stability (Bogin et al., 1998). Different pairs of mesophilic and thermophilic proteins may employ different structural strategies for their conformational stability.

The work carried out in this study was intended towards the understanding of conformational stability differences between homologous bacterial α-amylases, BLA and BAA. It has been shown that the differential thermal stability of BLA and BAA is not related to the Calcium binding (Fitter and Haber, 2004). The neutron scattering analysis has shown that at higher temperature, BLA has higher flexibility than the BAA (Fitter and Heberle, 2000). The unfolding transitions of BLA and BAA in temperature and chemical denaturants are not reversible (Duy and Fitter, 2005; Strucksberg et al., 2007). Structural analysis of BLA showed that the domain B and its interface with the domain A is the most important region for stability determinant. Therefore, any stabilizing (His133Y, A209V) and destabilizing (D204K, K234D) mutations significantly alter their thermal stability (Machius et al., 1995; Bogin et al., 1998). Conard et al. (1995) have proposed four thermal stability determining regions (TSD) in BLA using gene shuffling methods, which may provide hints for differential thermal stability of BAA and BLA but due to the lack of crystal structure of BAA at that time, these regions could not be analyzed in detail (Conard et al., 1995). Crystal structure of BAA showed that among four TSDs regions, only TSD2 plays the defining role in the differential stability of BLA and BAA. Comparing TSD2 in BAA and BLA, it was found that five intra side chain polar interactions are found in BAA but only three such polar interactions are present in BLA, which is in contrast to the thermophlic nature of BLA. However, the role of rest of the region of BLA in its stability cannot be ruled out (Alikhaejh et al., 2010).

In most of the previous studies involving comparison between mesophilic and thermophilic protein stability, major emphasis was given to structural and amino acid sequence comparison, which do not seem sufficient for understanding the conformational stability differences between homologous proteins. However, dynamic information could not be deduced from these kinds of studies. In this study, efforts have been made to dissect the origin of stability differences between BLA and BAA by performing equilibrium and kinetic unfolding in GdmCl and urea. In GdmCl induced unfolding, guanidinium ion which is an arginine mimic, interacts with negatively charged aspartate and glutamate disrupting electrostatic interactions and eventually facilitating unfolding. In the equilibrium unfolding by GdmCl, BLA shows slightly higher C_m values than the BAA (Figure 2a, b and Table 1). In a study by Fitter et al., (2001), it has been shown that the calcium depleted BLA and BAA in GdmCl

show C_m of 0.60 M and 0.24M respectively, which are lower than this study (Fitter et al., 2001). This could be due to fact that, in this study calcium was not depleted. Although BLA and BAA do not show reversibility, however apparent free energy of unfolding (ΔG_{H20}) was calculated, which shows BLA has higher free energy of unfolding (ΔG_{H20}) than BAA (Figure 2e). In light of the structural analysis, even though higher conformational stability of BLA can be attributed to several factors like predicted higher number of possible ionic interactions in BLA than the BAA and more histidine in BLA than BAA (Machius et al., 1995; Janecek, 1997). However, which of the factors plays a dominant role is still not understood by GdmCl unfolding of BLA and BAA. Therefore, equilibrium unfolding of BLA and BAA were also performed in the neutral chaotrope urea. BAA has higher C_m in urea than in GdmCl unfolding (Figure 3a, b and Table 2) and also the higher value of free energy of unfolding, which indicates the it has higher stability in urea than the GdmCl (Figure 2e). Both BLA and BAA are stabilized by number of intra and inter domain electrostatic interactions, which require high free energy input to break them (Machius et al., 1995; Alikhajeh et al., 2010). Urea is a neutral molecule and thus not capable of disrupting electrostatic interactions effectively and in turn BAA shows higher C_m values in urea than GdmCl. However, urea was unable to unfold BLA at pH 7.0 even after 6 days of incubation (Figure 3a). This unusual stability of BLA towards urea denaturation could be due to the additive effects of electrostatic interactions and ten additional histidine residues in its protein core (Alikhajeh et al., 2010). Similar results have also been observed for TBADH which also showed extreme resistance for urea unfolding at neutral pH (Mishra et al., 2008). On the other hand, BLA unfolds easily in urea on lowering the pH to 3.0 (Figure 4a). This is because at low pH, carboxyl groups of Asp and Glu get protonated which results in loss of electrostatic interactions and thus unfolding of BLA in combination with urea becomes easier. The similar effect of combination of urea and low pH was also observed in P22 tailspike protein unfolding (Mishra et al., 2007).

The conformational stability differences between BLA and BAA in GdmCl and urea can be understood through the structural comparison. The domain B and its interface with the domain A is the most important region for thermal stability determinant in BLA (Machius et al., 1995; Alikhajeh et al., 2010). Some of the salt bridges are exclusive to BLA only for e.g. Asp121-Arg127 in domain B and Glu250-Lys251in

domain A, whereas in BAA, corresponding residues are changed to Asn at position 121 and 127 and Ala at 251. Salt bridges which connect domain A and B are between Asp60-Arg146 and Asp204-Lys237 in BLA are also present in BAA (Machius et al., 1995; Declerck et al., 2000). Out of 39 salt bridge in BLA, 5 Histidines are involved in ionic interactions. The ionic interactions between His133-Glu119 and His406-Asp407 reported in BLA which are not possible in BAA due to the replacement of His to Tyr at position 133 and Pro at position 406 in BAA (Hwang et al., 1997). The major difference in the amino acid sequence of BLA and BAA lies in the number of histidine residues. There are 24 histidine in BLA while only 14 in BAA (Alikhajeh et al., 2010). Additional histidine residues in the BLA are scattered throughout the structure. In BAA out of ten histidine, seven are replaced by polar residues. Due to the higher entropic cost on exposure of non-polar residues (in comparison to polar replacement in BAA) folded conformation would be favoured over unfolded one. Although, it has been observed that BLA has more compact unfolded state than BAA during thermal and chemical unfolding, which points towards its entropic stabilization, but still the origin of this entropic stabilization was not clear (Fitter and Haber, 2004). The results of equilibrium in GdmCl and urea along with earlier structural analysis (Machius et al., 1995; Alikhajeh et al., 2010), suggest that more hydrophobic core of BLA is the dominating factor in its entropic stabilization mechanism. This is reflected in GdmCl induced unfolding where BLA has higher C_m than BAA. The same is also true in urea where BLA almost does not unfold.

The structure-function relationship in homologous bacterial α-amylases during chemical unfolding was thoroughly investigated by CD, fluorescence and enzyme activity measurements. During GdmCl induced unfolding of BLA, all three probes show non-coincidental unfolding transitions in GdmCl (Figure 5a). While for BAA, loss of secondary structure occurred slightly earlier than the loss of tertiary structure and enzymatic activity (Figure 5b). These non-coincidental unfolding transitions of BLA in GdmCl indicate the existence of partially unfolded intermediates (Kim and Baldwin, 1990; Khurana et al., 1994; Wani et al., 2006). The very sharp transition observed in BLA and BAA by CD spectroscopy during GdmCl unfolding may be due to the breakdown of interdomain ionic interactions between Asp 60-Arg146, Asp 204-Lys 237 connecting domain A and B and Arg 354 and Asp 401 between domain A and C (Machius et al., 1995). In the case of wheat α-amylase, GdmCl induced

unfolding transitions were also non-coincidental when followed by CD spectroscopy, fluorescence and activity measurement thus indicating the presence of partially folded intermediates during the unfolding pathway (Singh et al., 2015). BLA retains all of its secondary and tertiary structure with only partial loss of enzymatic activity (Figure 5c), which is consistent with what was observed during equilibrium unfolding in urea (Figure 3a). On the other hand, BAA shows sigmoidal coincidental unfolding transitions in urea (Figure 5d) which indicates that intermediates are not populated or detected during urea unfolding.

To confirm the presence of intermediates during unfolding of BLA and BAA, ANS fluorescence was used. ANS is a hydrophobic dye which selectively binds to the hydrophobic patches (Semisontov et al., 1991; Stryer, 1965). The strong binding during equilibrium unfolding of BLA in GdmCl, provided the evidence for the existence of partially unfolded state with exposed hydrophobic residues. ANS fluorescence intensity exhibits fourfold increase in BLA (Figure 6a) and less than twofold increase in BAA (Figure 6b), which shows that the population of intermediate species with exposed hydrophobic residues are more in BLA than in BAA. Unlike GdmCl induced unfolding, no significant ANS binding was observed during equilibrium unfolding of BLA and BAA in urea (Figure 6c, d) which is pointing towards the different unfolding pathways.

To understand the differences in the kinetic stability of BAA and BLA, unfolding kinetics was carried out in GdmCl and urea. As expected, the unfolding rates were slower for BLA than BAA at all the GdmCl concentrations (Figure 7a, b and Table. 3 a). Half Chevron plot extrapolated to 0M GdmCl gave the half-life which is twofold higher for BLA than BAA (Figure 7d). The urea unfolding kinetics of BAA shows slower unfolding than its unfolding in GdmCl (Figure 7c, Table 3 b).

The unfolding kinetics of calcium depleted BLA and BAA exhibit single exponential pattern in 8M GdmCl, while our results show biphasic unfolding kinetic of BLA and BAA in 3 to 6M GdmCl (Fitter and Haber, 2004). The single exponential kinetics of calcium depleted form of both enzymes might be due to the additive effect of calcium depletion and higher concentration of GdmCl. Even in urea, BAA showed biphasic unfolding kinetics. This can be attributed to the intrinsic property of both the enzymes which is not originating from GdmCl or urea. The biphasic nature of kinetic unfolding

of BLA and BAA might be related to the differential stability of their individual domains. Differential Scanning Calorimetry (DSC) observed three transitions, at T_m 96°C, 100°C, 104°C for BLA and 79.6°C, 84°C 87°C for BAA (Feller et al., 1999). It may be possible that two closely related domains in terms of T_m may be showing one phase and the other showing the second phase during GdmCl and urea induced unfolding. The first phase is fast unfolding while the second phase is linear and slow unfolding. The domain(s) having lower stability may be represented by fast unfolding phase while higher stability domain may be unfolding in the second linear phase. Different stability of domains was also observed in wheat α-amylase, where domain having catalytic site was found to be more stable than the other two domains (Singh et al., 2015).

In the current objective of this study, dynamic features of BLA and BAA unfolding by equilibrium and kinetic experiments in GdmCl and urea have investigated. This reveals that different nature of non-covalent interactions responsible for unusual stability BLA, as it shows exceptional stability in urea unfolding. Our data also demonstrates that the unfolding pathway of both proteins are different in GdmCl unfolding as shown by the presence of detectable intermediates in BLA. The biphasic nature of kinetic unfolding indicates the differential stability of individual domains of BLA and BAA. The enhanced conformational stability of α-amylase with thermophlic like properties of BLA is the result of its increased hydrophobicity in the protein core and possibly additive effects of electrostatic interactions, relative to its mesophilic counterpart (BAA).

CHAPTER 4

COMPARATIVE EFFECT OF POLYOLS AND SUGARS ON THE REFOLDING OF BACTERIAL ALPHA AMYLASES

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4.1 Introduction

Protein folding is primarily dictated by amino acid sequence however, role of the molecular chaperones during this process also plays an important role (Anfinsen, 1973; Hartl, 1996). Molecular chaperones primarily prevent aggregation during cellular protein folding (Agashe and Hartl, 2000). Apart from molecular chaperones, certain osmolytes or chemical chaperones also play an important role in folding of proteins (Arakawa, 2018; De Marco, 2011). The osmolytes are extensively used in protein stability and formulation of vaccine, antibodies (Roy et al., 2008; Wang and Ohtake, 2019) and explored in preventing the aggregation during amyloid fibril formation (Grudzielanek et al., 2005; Katyal et al., 2018). *in vitro* protein refolding is governed by various factors like concentration of protein, pH, temperature and solvent composition (Kiefhaber et al., 1991; Clark, 1998). Therefore, to improve the refolding of proteins, it is essential to optimize these parameters.

Polyol and sugar osmolytes accumulate under stress conditions in the cells, provide stability and prevent aggregation during protein folding (Yancey, 2005; Sánchez-Fresneda et al., 2013). These molecules are among the best stabilizers of the native protein conformation and their mechanism of action for protein stability has also been extensively studied (Gekko and Timasheff, 1981a; Gekko and Timasheff, 1981b; Gekko, 1981; Kaushik and Bhat, 1998; Kaushik and Bhat, 2003). They exert stabilization effect on proteins through preferential hydration mechanism (Gekko and Timasheff, 1981a; Gekko and Timasheff, 1981b; Gekko and Morikawa, 1981a; Gekko and Morikawa, 1981b). The relative stabilization of proteins by the polyols is depend upon the physicochemical properties of both polyols and proteins (Gerlsma, 1970; Gerlsma and Stuur, 1972). Further, polyols and sugars have also been successfully used in the efficient refolding of proteins under both, *in vitro* and *in vivo* conditions (Singer and Lindquist, 1998; Mishra et al., 2005). These osmolytes are protein-compatible cosolvents; thus, there is no need to remove them after refolding (Yamaguchi et al., 2013; Clark et al., 1999). This has enormous application in the refolding of proteins from inclusion bodies in the protein-based industry. As the protein acquires a more compact conformation in the aqueous solution of polyols and sugars, this observation provides the hint for using polyols for refolding of proteins (Gekko and Timasheff, 1981a; Gekko and Timasheff, 1981b; Xie and Timasheff,

1997; Bolen and Baskakov, 2001; Feng and Yan, 2008). The mechanism of polyols and sugars on the stability of native proteins is well understood. However, their role during refolding is still not clear, although some studies have been done (Mishra et al., 2005). Cosolvent assisted protein refolding requires the interaction of osmolytes with unfolded as well as intermediate species. Therefore, the nature of the unfolded states and the refolding pathways play an important role during cosolvent assisted refolding of proteins.

Several studies have observed the effect of various polyols and sugars during refolding of mesophilic bacterial proteins (Voziyan and Fisher, 2002; Nasrollahi et al., 2012). But, comparatively very few works have been done on polyols and sugars assisted refolding of thermophilic proteins (Stefanova et al., 1999). Comparative effect of acidified glycerol and ethylene glycol on the stability of thermophilic endoglucanases from bacterium *Thermotoga maritima* (Tm-EG) and its mesophilic counterpart from fungus *Trichoderma longibrachiatum* (Tl-EG) has been studied. The results showed that the acidified glycerol and ethylene glycol have a stabilization effect on Tl-EG, while a destabilization effect was observed on Tm-EG (Chong et al., 2014). Hence, one can also expect their comparable effect during the refolding of mesophilic and thermophilic proteins. The comparative effect of cosolvents like TMAO, betaine, glycerol and sucrose was evaluated on the refolding of homologous mitochondrial dehydrogenases from two different sources, porcine (PmMDH) and *E.Coli* (EcMDH). All cosolvents show a positive effect on refolding kinetics of PmMDH, while no such results were obtained during refolding of EcMDH (Tieman et al., 2001). There are some reports where negatively charged osmolytes like mannosylglycerate and diglycerol phosphate, which are found in hyperthermophilic organisms, have been shown to stabilize the proteins (Faria et al., 2003; Pais et al., 2005). TMAO also increases the stability of a hypertherophilic protein (Mukaiyama et al., 2008). However still, there is a lack of systematic study showing the comparative effect of neutral osmolytes like polyols and sugars on the refolding of thermophilic and mesophilic proteins.

In this study, two α-amylases from different bacterial sources, *Bacillus licheniformis* (BLA) and *Bacillus amyloliquefaciens* (BAA) are used to study the effect of polyols and sugars on their refolding. BLA and BAA share 81% sequence identity and 88%

similarity. Both proteins do not have any cysteine residue. Although both, BLA and BAA have a mesophilic origin, but BLA behaves as thermophilic while BAA as mesophilic (Alikhajeh et al., 2010; Brzozowski et al., 2000; Machius et al., 1995). Despite a very high structural and amino acid sequence similarities, both the enzymes follow the different unfolding pathways in GdmCl and urea (Ahmad and Mishra, 2020). It has been observed that both α-amylases undergo irreversible unfolding transition during chemical and thermal denaturation (Fitter and Haber, 2004; Duy and Fitter, 2005). By using cyclodextrin as an additive, the protein aggregation was suppressed during refolding of BAA by masking the exposed hydrophobic surfaces of folding intermediates (Khodarahmi and Yazdanparast, 2004).

The main objective of this work is to unravel the effect of polyols and sugars on refolding of homologous mesophilic (BAA) and thermophilic like protein (BLA). Here, GdmCl induced fully unfolded state of BLA and BAA were refolded in the presence of a series of polyols based on their number of -OH groups like 2C ethylene glycol. 3C glycerol, 4C carbon erythritol, 5C xylitol, 6C sorbitol. Apart from polyols, sugars like trehalose and sucrose have been shown as stabilizer of the native state of the proteins (Chen et al., 2006; Melo et al., 2003). Therefore, being similar to polyols, trehalose and sucrose have also included as cosolvent additive. The results show that among different cosolvents used in this study, glycerol, sorbitol and trehalose were found to be more effective in the refolding of BAA than BLA. Recovery of the secondary structure during refolding was highest in the presence of glycerol, although the extent of recovery was greater for BAA than BLA. The aggregation kinetics show that the cosolvents like glycerol, sorbitol and trehalose successfully inhibit the aggregation during refolding of both BAA and BLA. To the best of our knowledge, this is the first systematic study demonstrating the differential effect of polyols and sugars on refolding of thermophilic and mesophilic protein from the completely unfolded state. The results obtained in this study may facilitate to develop inclusion body refolding protocol, protein formulation and inhibitor of amyloids.

4.2 Results

4.2.1 Effect of concentration and temperature on refolding

Spontaneous refolding of BLA and BAA was carried out at 0.025, 0.05 and 0.1 mg/ml. BAA shows 20 % refolding at 0.025, 0.05 and 0.1 mg/ml protein concentration (Figure 8a). Similar to BAA, BLA also showed 20 % refolding yield at 0.025 mg/ml. But further increases in protein concentration to 0.05 and 0.1 mg/ml completely abolish the refolding (Figure 8a). From this, it can be inferred that at higher protein concentrations, BLA is more aggregation prone than BAA. In addition to protein concentration, temperature also plays a key role in protein aggregation during refolding.

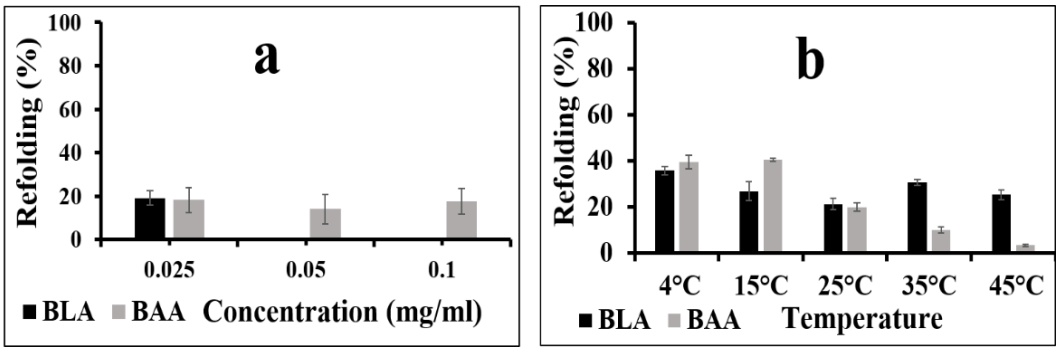


Figure 8. Effect of protein concentration and temperature on spontaneous refolding**.** Refolding of BLA and BAA were carried out at different concentrations (a) and temperatures (b). BLA and BAA were first unfolded in 6M GdmCl for 24 hours and then refolded by 60 fold dilution into three different concentrations, 0.025, 0.05 and 0.1mg/ml. (b) Both, BLA and BAA were refolded at different temperatures at 0.025 mg/ml and enzyme assay were carried out at 25°C.

In this study, the comparative effect of temperature has been investigated on spontaneous refolding of BAA and BLA. The refolding was performed at four different temperatures ranging from 4 to 45°C, at 0.025 mg/ml protein concentration. Before refolding, the buffer was pre-incubated at a given temperature for 30 minutes. The spontaneous refolding yield of both BLA and BAA were 36 and 40% respectively at 4°C (Figure 8b). Although upon increasing the temperature from 4 °C to 15^{o}C, refolding yield of BLA exhibited a sudden decrease to 27 %. But no such effect was observed for BAA and its refolding yield remain unchanged at 15°C. At

25°C, refolding yield of BLA and BAA were 21 and 20% respectively. Further increase in temperature to 35 and 45°C, refolding yield of BAA decreases to 10 and 3% respectively. On the other hand, BLA refolding at 35 and 45°C were 31 and 25% respectively (Figure 8b).

4.2.2 Effect of polyols and sugars on refolding

In this study, the comparative effect of various polyols like 2C ethylene glycol (EG), 3C glycerol, 4C erythritol, 5C xylitol, 6C sorbitol and disaccharide sugars like trehalose and sucrose have been studied for enhancing the refolding yield of homologous bacterial α-amylases BLA and BAA. For control experiment, spontaneous refolding of both the enzymes were chosen at 25°C and 0.025 mg/ml (Figure 8a, b). Surprisingly ethylene glycol shows negligible effects during the refolding of BLA and BAA. The refolding yield of BLA and BAA from 1 to 8M of ethylene glycol remains around 20%, similar to the control. Although at 6M ethylene glycol, the refolding yield of BLA and BAA were 24 and 26%, which were highest refolding yields in ethylene glycol (Figure 9 a). The next polyol in the series is glycerol, a 3C compound, which increases the refolding yield of α-amylases in a concentration-dependent manner. Although the positive effect of glycerol on refolding of α-amylase was evident on as low as 1M, but maximum refolding for BLA and BAA was reached to 36 and 56% respectively in the presence of 6 M glycerol (Figure 9 b). Above this concentration, slight decrease in the refolding yield of both BLA and BAA was observed. At 7M, refolding yield of BLA and BAA were 35 and 53% respectively, which further decreased to 30 and 45 % at 8M. To observe the effect of erythritol on the refolding of BLA and BAA, four different concentrations of 0.5, 1, 1.5 and 2M were used. A maximum refolding of 49% was observed for BAA in 1.5 M erythritol. While for BLA maximum refolding of 24% was observed at 2M. Thus, no significant effect of erythritol was observed in the refolding of BLA (Figure 9 c). Xylitol is a 5 ‘C’ compound that was used from 0.5 to 2M concentration range, but the maximum refolding yield of 39 and 55% for BLA and BAA respectively was observed at 1.5M (Figure 9 d). The refolding yield of both α-amylases exhibits a slight decrease beyond 1.5M of xylitol. The refolding of BLA and BAA decrease to 37 and 51% respectively at 2M xylitol (Figure 9 d). Sorbitol was used in the concentration range from 0.5 to 2M. It successfully enhances the refolding yield of

BAA up to 63%, while BLA refolding was increased only up to 35% at 1.5 M. Upon further increasing sorbitol concentration to 2 M, refolding yield of BLA and BAA was slightly decreased to 34 and 60% respectively (Figure 9 e).

Like polyols, sugars also exert the stabilizing effect on the native conformation of proteins. In sucrose at 0.5 M, maximum refolding for BLA and BAA were obtained 20 and 47 % respectively. Further increase in sucrose concentration leads to a decrease in the refolding yield of both α-amylases. The decrease in the refolding yield was greater in sucrose than the polyols. The maximum decrease was observed at 2M with 12 and 20% for BLA and BAA respectively (Figure 9 f). Another disaccharide, trehalose was used in the concentration range from 0.25 to 1.5 M. Although even at its lower concentration at 0.25M, trehalose exerts an appreciable effect on refolding yield of α-amylases with refolding yield of BLA and BAA increased up to 34 and 40 % respectively (Figure 9 g). The best results were obtained at 0.5 M, trehalose, where it increases the refolding yield of BLA and BAA, up to 35 and 71% respectively. Further increase in trehalose concentration up to 1M, results in an increment in the refolding yield of BAA to 72 %, but BLA exhibits a slight decrease with refolding yield of 31 % (Figure 9 g). Further increase in trehalose concentration has a negative effect on the refolding yield of both α-amylases. The correlation between the number of hydroxyl group in polyols and their effect on refolding yield of BLA and BAA has been shown in Figure 9 (h).

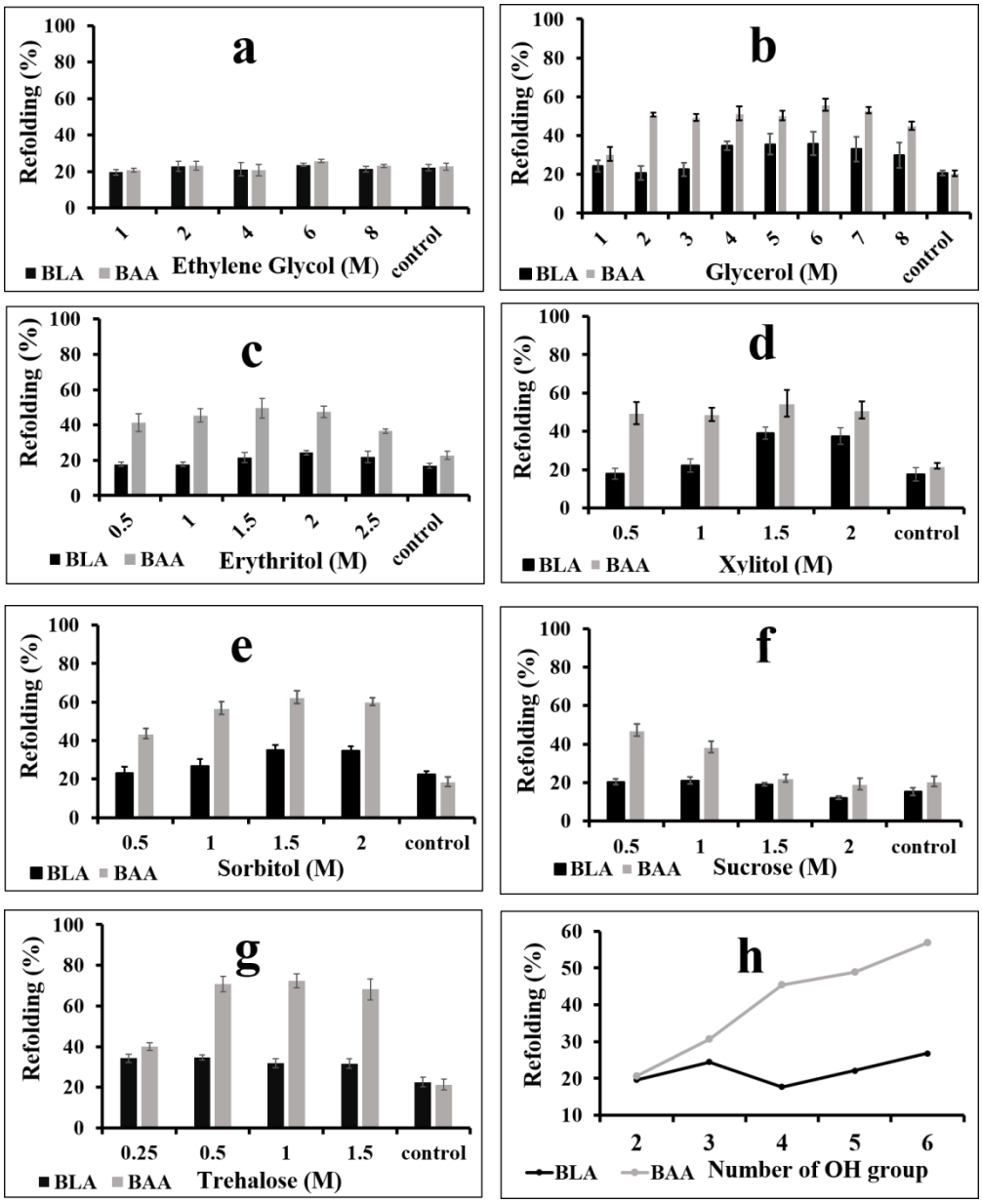


Figure 9. Refolding of BLA and BAA in different polyols and sugars as monitored by enzymatic activity assay. Homologous α-amylases BLA (black) and BAA (grey) were first unfolded in 6M GdmCl for 24 hours and then refolded by 60 fold (0.025mg/ml protein and 0.1 M GdmCl) dilution in various concentrations of ethylene glycol, glycerol, erythritol, xylitol, sorbitol, sucrose and trehalose (a, b, c, d, e, f and g respectively). The correlation between the number of hydroxyl groups and effect of 1M polyols on the refolding yield of BLA and BAA is given in Figure h.

In BAA, refolding yield increases with an increase in the hydroxyl groups of polyols at their equimolar concentration (1M). In contrast, no such correlation was observed in the refolding of BLA as shown in Figure 9 (h).

4.2.3 Aggregation kinetics

The aggregation kinetics of BLA and BAA was measured in the presence and absence of the glycerol, sorbitol and trehalose which have shown promising effect for enhancing the refolding yield (Figure 9). The reactions were initiated by manual mixing and the extent of aggregation was monitored by static light scattering at 400 nm. As per our results, though the aggregation suppression effect of glycerol was evident at 1M, but this effect becomes more prominent upon further increase in glycerol concentration and complete suppression was observed at 5M in both enzymes (Figure 10a, b). However, it is not correlated with activity measurement, where refolding yield of BLA and BAA were 35 and 56%, respectively. In comparison to glycerol, sorbitol was relatively less effective for inhibiting the aggregation of BLA than BAA. At 0.5 M, slight suppression of aggregation was detected in both α-amylases. However, with increasing concentrations of sorbitol, aggregation decreases but this effect is more pronounced in BAA than BLA (Figure 10c, d). The similar trend was also observed in the activity assay where BLA refolding was only 35%, while 63% for BAA. Like glycerol, trehalose also suppresses aggregation in a concentration dependent manner and at 1.5M complete inhibition of aggregation was observed for both BLA and BAA (Figure 10e, f). Thus, it seems that the aggregation suppression effect of trehalose was similar for BLA and BAA. Contrary results were observed while monitoring the enzymatic activity where 71% refolding was observed in BAA and only 36% in BLA.

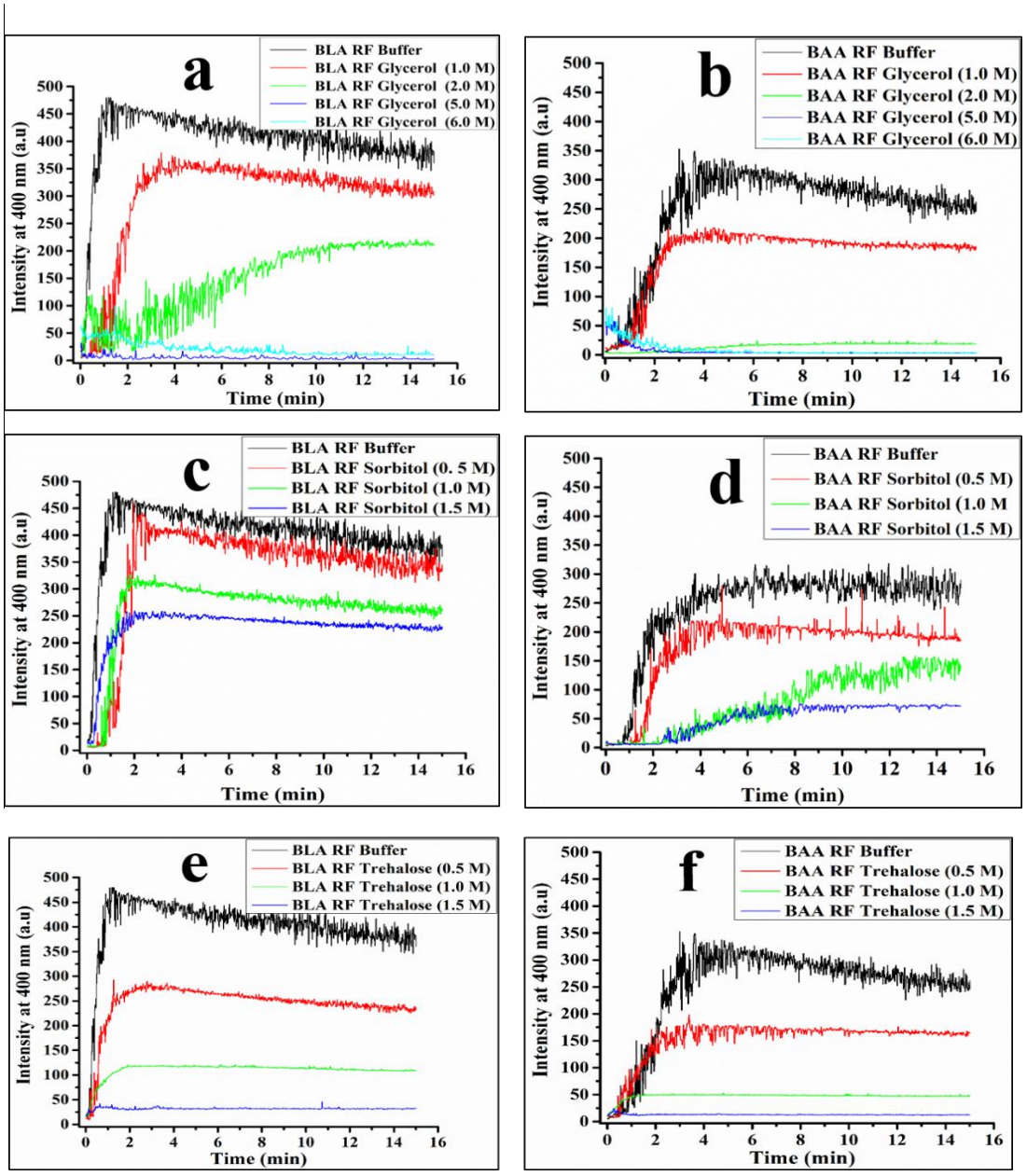


Figure 10. Aggregation kinetics of BLA and BAA by static light scattering at 400 nm. BLA (a, c and e) and BAA (b, d and f) were first unfolded in 6M GdmCl for 24 hours and then refolded by diluting 60 fold (0.025mg/ml protein and 0.1 M GdmCl) in buffer (black) and various concentrations of cosolvents. The refolding buffer consist of 1M (red), 2M (green), 5M (blue) and 6M (cyan) of glycerol (a and b), 0.5 (red), 1.0 (green), 1.5 M(blue) of sorbitol (c and d) and 0.5 (red), 1.0 (green), 1.5M (blue) of trehalose (e and f).

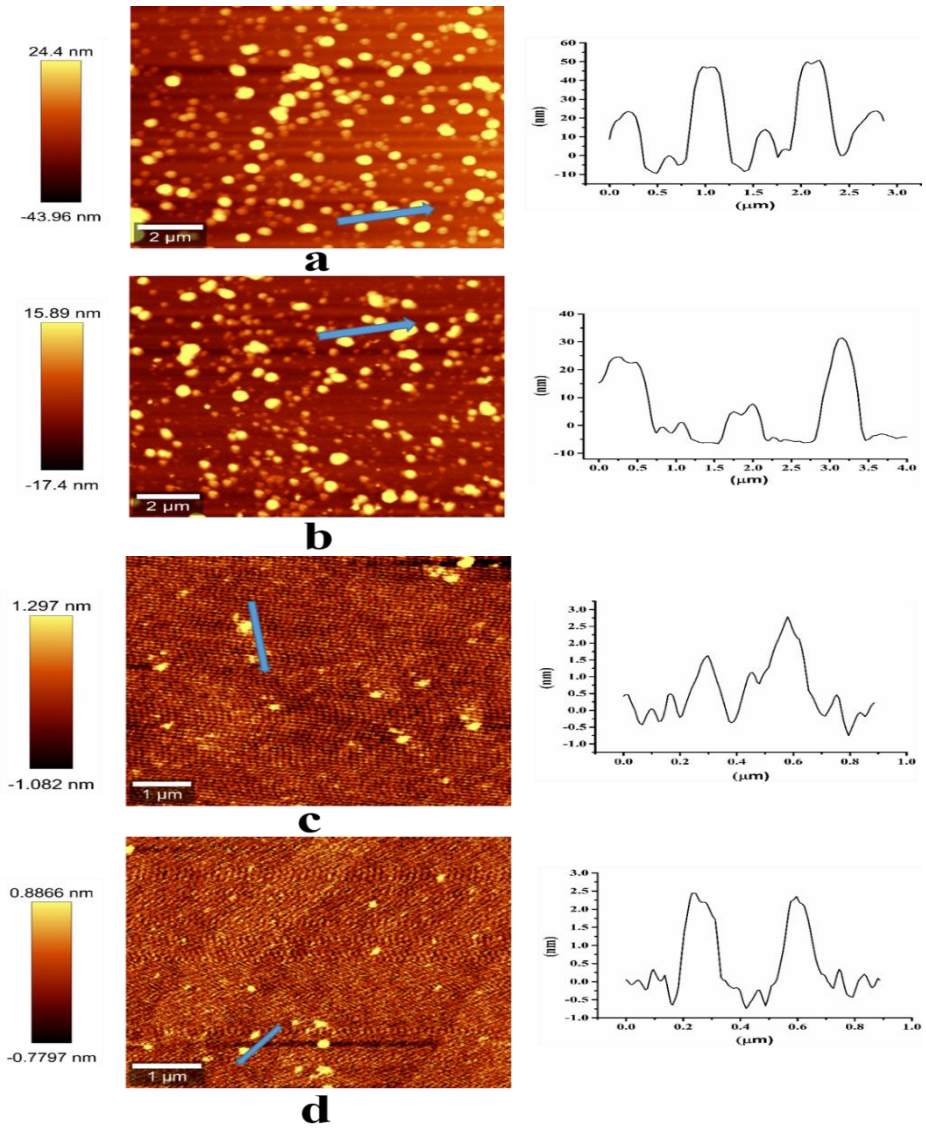
The lack of correlation between enzyme activity and light scattering could be due to the fact that former measure folded monomer while later gives the information about particular aggregate size. Because static light scattering is not always a quantitative method for determining aggregation kinetics, therefore for quantification, refolded samples were centrifuged at 14000xg for 30 minutes to allow the larger aggregates to settle down. The soluble fraction was quantified by measuring the absorbance at 280 nm with the native protein as a control. During this experiment, concentration of cosolvents were chosen based on the refolding yield (Figure 9). Therefore, 2.0M Glycerol, 1.0M sorbitol and 0.5M trehalose were used. Our results showed that BAA has higher soluble fraction than its refolding yield at the same cosolvent concentrations. The similar trend was also observed in the case of BLA (Table 4). In all the cosolvents used, soluble fraction of BAA was higher than BLA which is also in agreement with higher refolding yield of BAA. The cosolvent refolded samples may contain not only folded monomer but also misfolded monomer and soluble aggregates, which may account for the higher soluble proportion.

Table 4. Measurement of soluble fraction in refolded samples by taking absorbance at 280 nm.

Sample	Soluble fraction of BLA (%)	Soluble fraction of BAA (%)
Native	100	100
Refolded in Buffer	25	28
Refolded in 2.0 M glycerol	50	73
Refolded in 1.0 M sorbitol	58	82
Refolded in 0.5M trehalose	45	80

4.2.4 Morphology of Protein Aggregates

To see the morphology of protein aggregates formed during protein refolding of α-amylases in the absence as well in the presence of 6M glycerol, 1.5 M sorbitol and 1.0M trehalose, AFM was carried out. Control sample of BLA without any cosolvent showed protein aggregates of spherical shape with 20-50 nm height (Figure 11a), while comparatively smaller height aggregates of 20-30 nm were observed in BAA (Figure 11b). In the presence of 6M glycerol, height of aggregates decreases and thus, spherical aggregates of smaller size with height ranging from 1-3 nm resembling oligomeric structures were visualized in both BLA and BAA (Figure 11c, d). This observation is also in agreement with the aggregation kinetics, where maximum aggregation suppression was observed in 6M glycerol (Figure 10a, b).



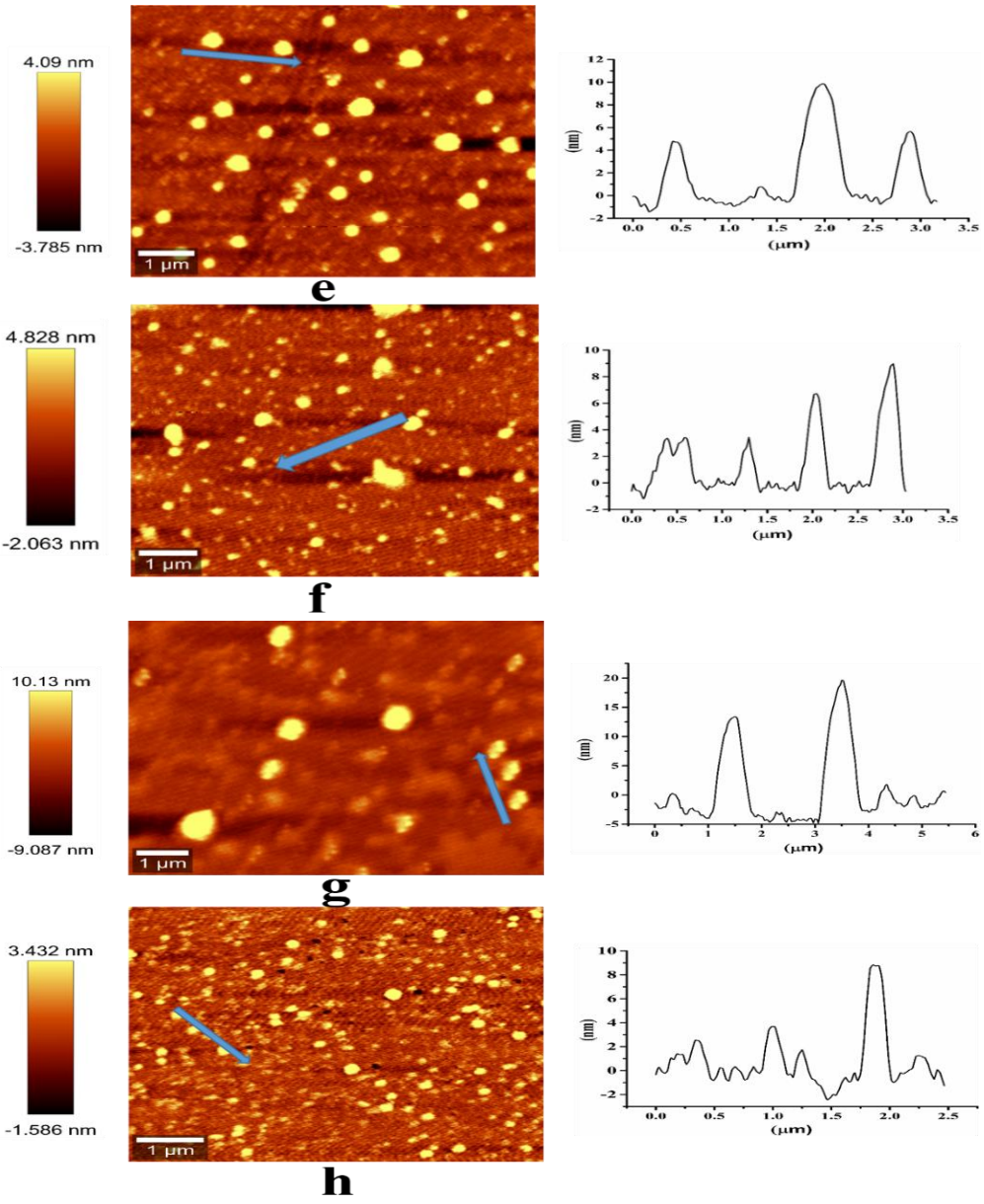


Figure11: Morphology of protein aggregates by AFM which were formed during refolding of BLA (a, c, e, g) and BAA (b, d, f, h) in buffer (a, b), 6M glycerol (c, d), 1.5M sorbitol (e, f) and in 1.0M trehalose (g, h). Height distribution of protein aggregates of each image has been given in right side of the image and the blue arrow in each image indicates the area selected for the height distribution.

In 1.5M sorbitol, which was found less effective during aggregation kinetics (Figure 10c d), relatively larger size spherical aggregates of height 4-10 nm were observed in both BLA and BAA (Figure 11e, f), although number of larger size aggregates comparatively higher in BLA than BAA. Similar to the aggregation kinetics, differential effect of 1.0 M trehalose was also observed by AFM. In BLA, spherical aggregates of height 10-20 nm were detected in 1.0 M trehalose. While in BAA, comparatively smaller spherical aggregates of height 2-9 nm were seen (Figure 11g, h).

4.2.5 Effect of cosolvents on secondary structure

Following a cosolvent screening, glycerol, trehalose, and sorbitol were chosen for the recovery of secondary structure of refolded proteins by far-UV CD spectroscopy at 0.18 mg/ml protein. The background spectra of the cosolvents did not interfere in the far-UV CD range up to 205 nm and high tension (HT) voltage was between 350-470. CD spectra of unfolded BLA and BAA showed the loss of the native structure. Refolding can lead to the formation of native, misfolded, and aggregate structures. As a result, the resulting spectra comprise an ensemble of different species, making it difficult to correctly estimate the native species contribution. Nonetheless, ellipticity at 222 nm provides information regarding protein α-helical structure. Because BLA and BAA are largely α- helical, thus relative gain of ellipticity at 222 nm can provide the estimate of the secondary structure during their refolding.

Our results showed that at 6 M glycerol, recovery of ellipticity at 222 nm for BLA and BAA were 75 and 100 %, respectively (Figure 12a, b). But the CD spectra of refolded sample of both, BLA and BAA, clearly demonstrate that the alpha helical signature at 208 nm was not prominent in both the enzymes. This suggests that the refolded states lack properly folded secondary structure compare to the native states. This outcome is also in agreement with the activity results where refolding yield of BLA and BAA were 35 and 56% respectively, lower than the gain of ellipticity at 222 nm. In comparison to glycerol, sorbitol has less effect on recovering the secondary structure of BAA. At 1.5 M sorbitol, gain of ellipticity at 222 nm for BLA and BAA was 75 and 83%, respectively. Trehalose, unlike its effect on enzymatic activity, where it was found to be effective only for BAA (Fig. 9 g), significant recovery of ellipticity was observed with 70 and 95% gain for BLA and BAA respectively (Figure

12a, b). Gain of ellipticity at 222 nm for both BLA and BAA in sorbitol and trehalose was higher than their refolding yield at corresponding concentrations, which is similar to glycerol. Overall, increased secondary structure content in 6.0 M glycerol, 1.5M sorbitol, and 1.0M trehalose, as well as inhibition of aggregation by light scattering, show that although native-like structures are formed, not all of them are properly folded, resulting in low refolding as evaluated by activity assay in both enzymes.

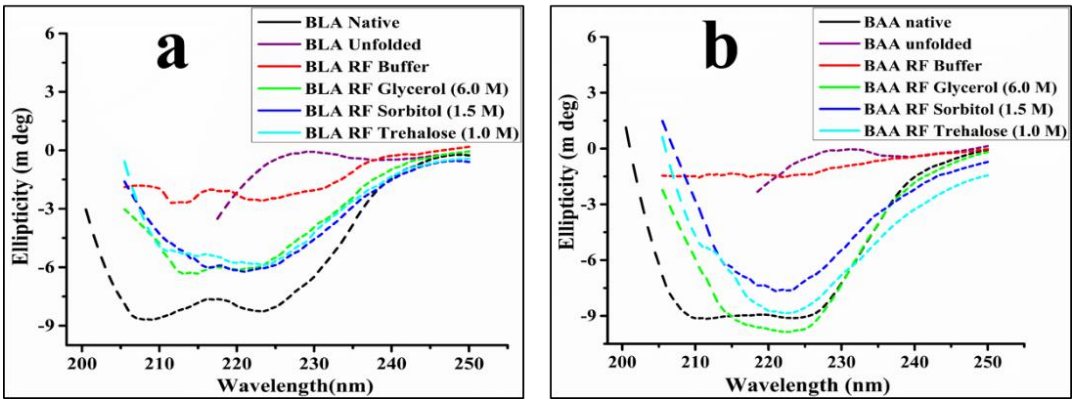


Figure 12. Formation of secondary structure by far-UV CD spectroscopy during refolding of BLA (a) and BAA (b). The far-UV spectrum of native (black), unfolded state (purple), and refolded state in buffer (red), glycerol (green), sorbitol (blue) and trehalose (cyan) were collected at 0.18 mg/ml of protein, 25°C and pH 7.0.

4.2.6 Effect of cosolvents on tertiary structure

Formation of the tertiary structure during BLA and BAA refolding in the presence of glycerol, sorbitol, and trehalose has been measured using tryptophan fluorescence (Figure 13). Both, BLA and BAA, have 17 tryptophan which are distributed uniformly throughout the structure (Alikhajeh et al., 2010; Machius et al., 1995). Fluorescence intensity is easily affected by environmental conditions, which may result in quenching. Therefore, a more robust approach, wavelength shift, was utilised to monitor the formation of tertiary structure during refolding. Fluorescence spectra, like CD, are also altered by the presence of protein aggregates and refolded samples containing aggregates have been shown native-like spectra (Mishra et al., 2005). The wavelength maxima of native BLA and BAA were 338 and 340 nm, respectively. While denatured states of BLA and BAA exhibit a red shift of 22 and 20 nm, respectively, relative to the wavelength maxima of their native states. This is in

agreement with the loss of secondary structure in the unfolded states as shown by CD spectroscopy (Figure 12). The refolded states of BLA and BAA in buffer showed 5 and 2 nm red shift respectively, compared to the emission maxima of their native states. The evident red shift in buffer refolded state may account for the lower refolding yield of BLA and BAA as measured by activity assay (Figure 9). But, during refolding in 6M glycerol, fluorescence emission spectrum of the refolded states of BLA and BAA showed wavelength maxima similar to their respective native states. The blue shift in fluorescence emission maxima during refolding of BLA and BAA in 6M glycerol indicates the formation of compact globular structures of their refolded states (Figure 13a, b) which is in agreement with CD spectrum and complete suppression of aggregation by light scattering (Figure 10).

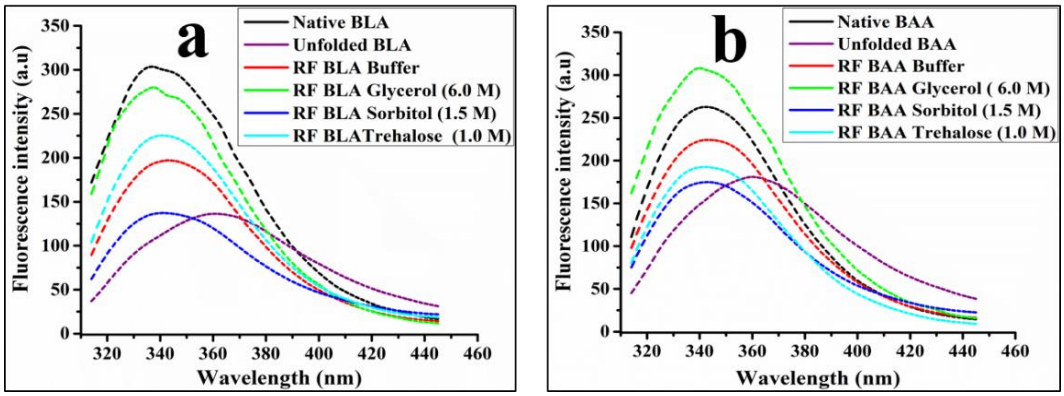


Figure 13. Conformational change by fluorescence spectroscopy during refolding of BLA and BAA in polyols and sugars. The fluorescence spectrum of native (black), unfolded state (purple), and refolded state in buffer (red), glycerol (green), sorbitol (blue) and trehalose (cyan) were collected at protein concentration of 0.025 mg/ml, 25°C and pH 7.0.

However, the activity assay showed lower refolding yield which might be due to the presence of misfolded monomer and soluble aggregates. Like glycerol, refolded state of BAA in sorbitol also showed wavelength maxima similar to its native state. But in the case of refolding of BLA in sorbitol, its refolded state exhibits a 3nm red shift in wavelength maxima with respect to its native state (Figure 13 a). Trehalose behaves like sorbitol during refolding of BLA and BAA, thus, the wavelength maxima of refolded state of BLA also show red shift of 3 nm in the presence of 1.0 M of trehalose (Figure 13 a). In contrast to this, BAA exhibit a clear blue shift in its

wavelength maxima with respect to its native state at the same concentration of trehalose (Figure 13 b). The results of enzymatic activity, Far-UV CD spectroscopy and static light scattering all, accord with the blue shift in refolded state of BAA in sorbitol and trehalose. BLA's apparent red shift in sorbitol and trehalose is consistent with its lower refolding yield than BAA (Figure 13 b).

4.2.7 Addition of trehalose at different time points during refolding of BAA

Trehalose showed the maximum effect on increasing the refolding yield of BAA while less effect on BLA, therefore only BAA was taken into the consideration while studying the mechanistic effect of trehalose on refolding. According to the enzymatic activity, BAA has only a 20% refolding yield in buffer (Figure 14). This low refolding yield could be attributed to substantial aggregation, as seen by greater intensity of static light scattering during aggregation kinetics in buffer (Figure 10). While there was significant decrease in light scattering intensity during refolding of BAA in the presence of trehalose (Figure 10). Which indicates that the extent of aggregation reduces drastically as shown by increase in the refolding yield of BAA to more than 60 % (Figure 14). Therefore, to understand whether osmolytes acts during early stage or late stage of refolding, trehalose was added at different time points. There was no positive effect of trehalose addition, as early as 30 seconds after refolding initiated. The addition of trehalose was extended up to 90 minutes, but no positive effect was observed (Figure 14). This shows that the role of trehalose is required since beginning of the refolding. It seems that essential steps of refolding are completed within 30 seconds. Far-UV CD spectroscopy at 222nm was used to follow BAA refolding within 30 seconds in order to better comprehend it. Refolding in buffer showed only -1.1 m deg ellipticity compared to the native state (-9.7 m deg) which suggests that aggregation supersedes over refolding. However, refolding in the presence of trehalose showed -4.0 m deg ellipticity, which is approximately 40% of the native state. This indicates that fast refolding with significant secondary structure formation occurring before 30 seconds. Thus, trehalose is necessary during the early stages of refolding to improve the refolding yield. This outcome was further confirmed by the addition of trehalose at 30 seconds which showed only -1.0 m deg ellipticity. This is

consistent with enzymatic activity data which reveals no substantial increase in the refolding when trehalose added at 30 seconds of refolding (Table 5).

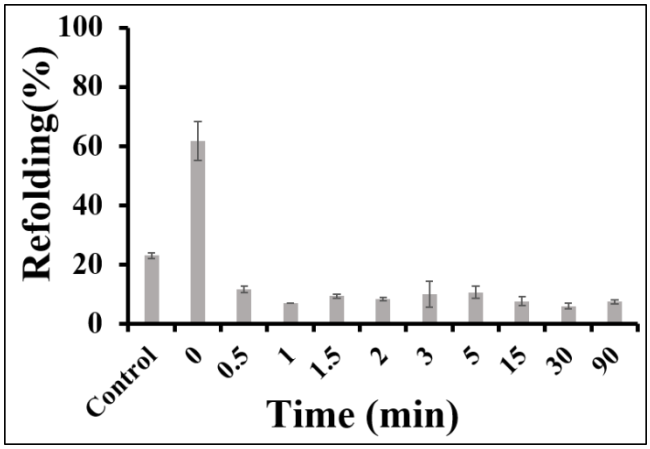


Figure. 14: Effect of trehalose at different time points during refolding of BAA. The enzymes were unfolded in 6M GdmCl and refolded by 60 fold dilution in refolding buffer. Addition of 1.5 M trehalose at different time intervals leads to final protein concentration of 0.0125mg/ml and 0.75 M of trehalose.

Table 5. Refolding of BAA at 30 seconds by Far-UV CD spectroscopy

Sample	Ellipticity at 222 nm (m deg)
Native	-9.7
Refolded in buffer	-1.1
Refolded in trehalose	-4.0
Refolded in trehalose (added at 30 sec of refolding)	-1.0

4.2.8 Effect of trehalose at different temperature during refolding of BLA and BAA

The effect of trehalose at different temperatures was also studied for BLA and BAA. The refolding of BLA and BAA were carried out at 4°C, 15°C, 35°C and 45°C in the presence of 1.0 M of trehalose. The results show that, at 4°C, refolding yields of BLA and BAA were increases to 40 and 50% respectively (Figure 15). Upon increasing the temperature of refolding to 15^{o}C, refolding of BLA and BAA slightly decreases to

35 and 48 % respectively. At 35°C, refolding yield further decreases to 33% for BLA and 45% for BAA. But significant decrease in the refolding yield of BLA (26%) and BAA (25%) was observed at 45 ^{o}C (Figure 15). Thus, trehalose shows comparable effect on refolding yields of BLA and BAA at higher temperatures.

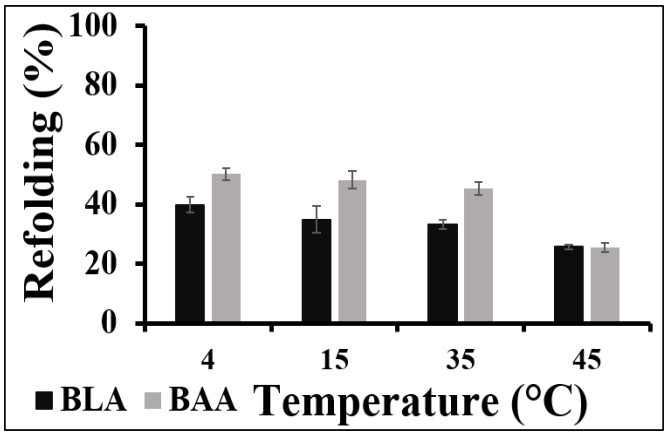


Figure. 15: Effect of temperature during refolding of BLA and BAA. The refolding of BLA and BAA was carried out with 0.025 mg/ml at different temperatures in the presence of 1M of trehalose.

4.2.9 Compensation effects of cosolvents

As shown previously, both urea and GdmCl, at low concentrations, act as refolding additives (Hevehan et al., 1997; Orsini and Goldberg, 1978). The native conformation of BAA and BLA is highly sensitive towards even sub denaturation concentration of GdmCl (Ahmad and Mishra, 2020). Thus, in this study, only urea has been used as an additive in refolding of BLA and BAA. The refolding of BLA and BAA was performed in the presence of various urea concentrations, from 0.5 to 2 M (Figure 16). The results showed that 0.5 M of urea increases the refolding yield from 20 to 33%. While in the case of BLA it did not. But, further increase in urea concentration exerts a negative effect on refolding of BLA, and its refolding yield decreases from 21 to 12% upon increasing the urea concentration from 0.5 to 0.7 M. In contrast to BLA, increase in urea concentration exhibits a positive effect during refolding of BAA and refolding yield increases from 33 to 37 % as urea concentration increases from 0.5 to 0.7 M (Figure 16). However, an increase in urea concentration above 0.7 M shows a negative effect on refolding of both BLA and BAA. The refolding yield of BLA at

1.0, 1.5 and 2.0 of urea were 9, 6 and 5 %, respectively, while for BAA, 30, 21 and 11 %, respectively (Figure 16). To find out whether stabilizing effect of cosolvents can counter the effect of urea on the refolding of BLA and BAA, refolding was carried out in the presence of both urea and urea with cosolvents. For this purpose, 2.0 M urea with

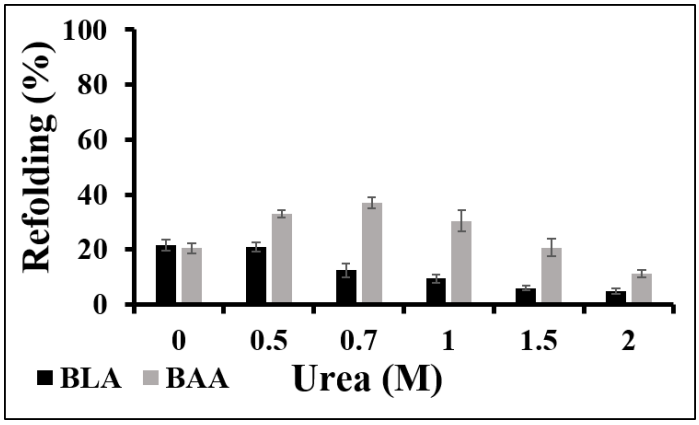


Figure. 16: Effect of urea during refolding of BLA and BAA as observed by enzymatic activity. The enzymes were unfolded in 6M GdmCl and refolded by 60fold dilution in the refolding buffer containing various concentrations of urea. All the measurements were performed at protein concentration of 0.025mg/ml, 25°C and pH 7.0.

Table 6. Effect of cosolvents without and with urea during refolding of BLA and BAA.

Name of Additive	Refolding BLA (%)	Refolding BAA (%)
Glycerol 6M	32	54
Trehalose 1.0 M	32	67
Sorbitol 1.5 M	37	56
2M Urea + 6.0 M glycerol	23	42
2M Urea + 1.5 M sorbitol	14	47
2M Urea + 1.0 M trehalose	6	46

different glycerol, trehalose and sorbitol concentrations were employed during refolding of BLA and BAA. At 2.0 M urea, BAA refolding was reduced to 11%, while in combination with 2.0 M urea with 6M glycerol, the refolding yield increased up to 42% (Table 6). For BLA, spontaneous refolding at 2.0 M urea was reduced to 5 %. In contrast to BAA, less increase in the refolding yield of BLA was observed in the presence of 2M urea with 6 M glycerol and only 23% refolding was observed. Like glycerol, sorbitol was also used in combination with 2.0 M urea, and the refolding yield for BLA and BAA were 14 and 47 %, respectively. Similarly, in the presence of 2.0 M urea and 1.5M trehalose, the refolding yield of BLA and BAA were 6 and 46%, respectively (Table 6).

4.3 Discussion

In this work, emphasis has been given to the comparative effect of polyols and sugars on refolding of two homologous bacterial α-amylases behaving as thermophilic (BLA) and mesophilic (BAA). Our investigation suggests that except ethylene glycol, all the polyols and sugars show more positive effect on refolding of BAA than BLA. Ethylene glycol has been shown as a mild destabilizer of the chymotripsinogen (Gekko and Morikawa, 1981 b), but this effect of ethylene glycol is not general, and it depends upon the surface characteristics of proteins (Vagenende et al., 2009). Because of its smaller side chain, ethylene glycol seems unable to preferential hydrate the unfolded states and the folding intermediates of BLA and BAA. Thus, indifferent behaviour of ethylene glycol during refolding of α-amylases (Figure 9a) might be due to its similar interaction with unfolded states and partially folded intermediate states of BLA and BAA. In contrast to ethylene glycol, glycerol acts as a mild protein stabilizer, although at higher concentrations than the other larger polyols. Although protein stabilization effect of glycerol is due to its preferential exclusion from the surface of protein (Gekko and Timasheff, 1981a; Gekko and Timasheff, 1981b). But in a recent study, polyols and sugar have been shown to shorten the protein backbone hydrogen bonds thus stabilizing the proteins (Li et al., 2020). In addition to protein stabilization, glycerol has also been used for enhancing the refolding of proteins (Mishra et al., 2005; Feng and Yan, 2008; Sharma and Singh, 2017; Meng et al., 2001). Glycerol increases the refolding yield of proteins by stabilizing the partially folded intermediates, thus inhibiting protein aggregation (Gorovits et al., 1998;

Mishra et al., 2007). Glycerol favours compact state by strengthening hydrophobic interactions (Kamiyama et al., 1999). In the presence of glycerol, enhancement in refolding yield was higher for BAA than BLA (Figure 9 b). This differential behaviour of glycerol might be due to the different nature of refolding intermediates of BLA and BAA as both have different unfolded states (Ahmad and Mishra, 2020). Being multidomain proteins, existence of refolding intermediates is very likely in both BLA and BAA. The refolding intermediates may have different nature, which is also supported by compensation experiments where both enzymes behave differently in urea alone as well as in the presence of urea and cosolvents (Figure 16 and Table 6). Considering the fact that BLA is more hydrophobic than BAA, BLA intermediates are expected to be more prone to aggregation than BAA (Shokri et al., 2006). Therefore, interaction of glycerol with folding intermediates may play an important role in their stabilization. Preferential interaction of glycerol with native and folding intermediates would depend on its affinity towards hydrophobic surfaces (Vagenende et al., 2009), which may vary in BLA and BAA. Electrostatic interaction of glycerol is also reported to be an important factor for preferential hydration of proteins which orients glycerol on the surface of the protein in such a way that it gets further excluded (Vagenende et al., 2009). The less hydrophobic surface of BAA folding intermediates might orient glycerol so that it gets excluded and favours preferential hydration.

Same might be true in case of other polyols including erythritol, xylitol and sorbitol, as they also enhance protein stability through the same mechanism as glycerol (Gekko and Morikawa, 1981 a; b, Xie and Timasheff, 1997). Thus, similar to glycerol, all other polyols have also been found less effective for refolding of BLA than BAA (Figure 9). Apart from glycerol, sorbitol also shows the pronounced effect on refolding yield of BAA (Figure 9e). Sorbitol also acts as a protein stabilizer and increases the thermal stability of proteins by several degrees (Kaushik and Bhat, 1998; Xie and Timasheff, 1997). Similar to their effect on protein thermal stability, where stabilizing effect of polyols increases with increasing carbon chain length and number of the hydroxyl group (Kaushik and Bhat, 1998; Gekko and Morikawa, 1981 b) at their equimolar concentrations, polyols enhance the refolding of BAA depending on the number of the hydroxyl groups but this trend was not followed during the refolding of BLA (Figure 9h). Increase in the number of -OH groups increase the polarity of polyols making them less likely to reside at the surface of proteins.

Therefore, the less hydrophobic BAA undergoes higher degree of preferential hydration than BLA in the presence of polyols and varies with number of –OH groups (Alikhajeh et al., 2010; Shokri et al., 2006).

Like polyols, disaccharides sucrose and trehalose also show more effect on the refolding yield of BAA than BLA. Among polyols and sugars, trehalose was the most effective for increasing the refolding of BAA. Trehalose has been shown to be the best stabilizer of BAA among various polyols and sugars used (Yadav and Prakash, 2009). In addition to its role as an exceptional stabilizer of protein native conformation (Kaushik and Bhat, 2003), trehalose has also been used for improving the folding of proteins, under both *in vivo* and *in vitro* conditions (Singer and Lindquist, 1998). It also shows positive effect during temperature dependent refolding of BLA and BAA by reducing the aggregation even at higher temperatures indicating the stabilization of folding intermediates against thermal denaturation (Figure 15). To get mechanistic insights of the role of cosolvents during protein refolding, trehalose was added at different time intervals. Low refolding yield was obtained when trehalose was added at 30 seconds of refolding or late, thus indicates its role at early stages of refolding (Figure 14). Confirming the same, CD spectroscopy showed significant secondary structure formation with trehalose present since beginning. When trehalose was added at 30 sec in the refolding in buffer, extensive aggregation is considered to prevent the formation of secondary structure (Table 5). The enzyme activity and CD data together indicate the rapid kinetics of BAA refolding. Hence the possibility of trehalose interaction with early species formed during refolding is an important step in enhancing the refolding yield. Sucrose also acts as a protein stabilizer and has also been found to increase the refolding yield of several proteins including arginine kinase, porcine mitochondrial dehydrogenase (Lee and Timasheff, 1981; Yu and Li, 2003; Tieman et al., 2001). However, in this study, it acts as an enhancer of refolding only at 0.5M and a further increase in its concentration decreases the refolding yield of BAA.

It is observed in case of glycerol, erythritol and sucrose that the refolding yield decreases at higher concentrations and the decrease was highest in sucrose. This trend is similar to that of polyol assisted refolding of citrate synthase at higher concentrations (Mishra et al., 2005). Although, decrease in the refolding yield is not

in agreement with the effect of polyols and sugars on the stability of proteins where increasing polyol concentration enhance their thermal stability (Kaushik and Bhat, 1998). Since at higher concentrations of polyols and sugars, viscosity may also play an important role, the increase in viscosity might slow down the kinetics of protein refolding resulting in the formation of off-pathway protein aggregates. Thus, depending on the physico-chemical nature of cosolvent, an optimal concentration of polyols and sugars is required for maximum refolding yield of a protein.

The aggregation kinetics indicate that unlike their effect on enzymatic activity, glycerol and trehalose decrease the aggregation of BLA and BAA in a concentration dependent manner (Figure 10). While sorbitol is comparatively less effective for inhibiting the aggregation during refolding of α-amylases, the lack of correlation between activity and prevention of aggregation indicates that it is not only larger aggregates that decreases the refolding yield of BLA but also misfolded monomer and soluble aggregates which may not be detected by light scattering, can also reduce the refolding yield. The aggregation kinetics data is further supported by AFM results where larger size, spherical protein aggregates were observed in the buffer (Figure 11a, b) while in the presence of cosolvents, smaller aggregates resembling oligomeric structures were observed. Glycerol has prominent effect on the morphology of protein aggregates where aggregates with decreased heights were visualized in both BLA and BAA (Figure 11c, d).

The secondary structure comparison of refolded samples in glycerol, sorbitol and trehalose was carried out (Figure 12). Glycerol at 6M was the most successful among the three cosolvents in helping to regain the secondary structure which was higher for BAA than BLA. In another study, comparable results of regaining the secondary structure in BLA was also observed at 7M glycerol (Strucksberg et al., 2007). The refolded states of both enzymes acquire native like tertiary structures in the presence of glycerol and trehalose as shown by tryptophan fluorescence (Figure 13). In presence of sorbitol, refolded BAA acquires native like tertiary structure while BLA adopts less compact tertiary structure. Taken together, enzyme activity, aggregation kinetics, secondary and tertiary structure analysis suggest that in case of BAA the effect of cosolvents does correlate qualitatively. However, different techniques measure different properties of proteins and therefore, it is difficult to compare the

results obtained quantitatively. For example, increasing concentrations of glycerol, sorbitol and trehalose result in enhanced refolding yield of BAA. Similar observations were found during aggregation kinetics, Far-UV CD and fluorescence spectroscopy, where higher concentration of cosolvents successfully inhibited the aggregation, regain of secondary and tertiary structure, respectively. In the case of BLA, cosolvents do exhibit some degree of correlation as measured by aggregation kinetics, far-UV CD and fluorescence spectroscopy but to a lesser extent than BAA. On the other hand, enzymatic activity data show lower refolding yield of BLA, which is not in agreement with other techniques.

To get mechanistic insight into cosolvents assisted refolding, BLA and BAA were refolded in the presence of low concentrations of urea in the absence and the presence of cosolvents. Because of the aggregation suppression property of low concentration of urea, it has long been used as a refolding additive (Clark et al., 1999; Arora and Khanna, 1996). Low concentration of urea (0.7M) shows appreciable effect on the refolding of BAA (Figure 16). This behaviour may be explained by the solubilizing effect of low concentration of urea on aggregation prone species formed during refolding. But further increase in urea concentration results in the decrease of refolding yield due to destabilization of folding intermediates (Mishra et al., 2005). Unlike BAA, low urea concentration (0.7M) exhibit inhibitory effect on refolding yield of BLA (Figure 16), which could be due to the destabilization of folding intermediates Differential effect of 0.7M urea on BLA and BAA refolding indicates the different surface properties of their folding intermediates. As suggested by Vagenende et al. (2009), stabilization or destabilization of folding intermediates will depend on their surface properties. In the case of BAA, deleterious effect of urea was compensated by glycerol, sorbitol and trehalose, but in the case of BLA, only glycerol has some protective effect. (Table 6). Trehalose being disaccharide might be interfering with folding intermediates during refolding in the presence of urea. Only glycerol has some stabilization effect on BLA but that also lesser than BAA. The different behaviour of urea during refolding of both enzymes, points towards different nature of their refolding intermediates. The similar results have been obtained in glycerol assisted refolding of P22 tailspike protein (Mishra et al., 2007). In a recent study, glycerol has also been shown to stabilize the protein in *E. Coli* cells (Song et al., 2021).

All the polyols and sugars were found less effective during the refolding of thermophilic like α-amylase (BLA), than the mesophilic α-amylase (BAA). It has been proposed that homologous proteins from a thermophilic and mesophilic organism may have similar, partially similar or entirely different folding pathways (Glyakina and Galzitskaya, 2010). As we have shown earlier that both, BLA and BAA, have different unfolding pathways in GdmCl and urea (Ahmad and Msihra, 2020). The different unfolded states should lead to different refolding pathways as well. In another study, mesophilic and thermophilic homologs of heptameric co-chaperonin proteins fold via different routes (Luke et al., 2007). Intermediates formed during refolding of BLA and BAA, may have different surface properties, so the different interactions with osmolytes. As the protein core of BLA is more hydrophobic than BAA (Alikhajeh et al., 2010; Machius et al., 1995), refolding intermediates of BLA may have more hydrophobic surfaces than BAA. Thus, BLA refolding pathway could be more prone to aggregation and misfolding than BAA. When acid induced molten globule state of BLA and BAA at pH 4.0 were refolded in the presence of various polyols, it successfully increases the refolding yield of BAA, but was less effective for the refolding of molten globule of BLA (Shokri et al., 2006). The refolding of thermostable pullulanase from *Bacillus acidopullulyticus,* in various cosolvents including polyols, showed similar results. Here also, sugars and polyols were failed to enhance the refolding of thermostable pullulanase (Stefanova et al., 1999).

The differential effect of polyols and sugars on thermophilic and mesophilic α-amylases might be due to the differential weaker interactions between these compounds and hydrophobic surfaces of unfolded state and refolding intermediates. Thus, critical balance of weaker interactions between protein and osmolyte is required for the refolding of proteins. This study may help to design an efficient refolding protocol and formulation of various thermophilic and mesophilic aggregation prone proteins of industrial importance. It may further help to design compatible, non-toxic inhibitors of highly aggregation prone amyloidogenic proteins involved in several amyloid diseases.

CHAPTER 5

SCREENING OF AMINO ACIDS AND DERIVATIVES FOR ENHANCED REFOLDING OF BLA AND BAA

5.1 Introduction

Osmolytes are naturally occurring small molecules that accumulate in living organisms under stress circumstances such as heat, salt, dehydration etc. The most common osmolytes are polyols, sugars, amino acids like arginine, proline, glycine and glycine's natural derivative like sarcosine, betaine, TMAO etc. (Yancey and Somero, 1979; Yancey, 1982). Amino acids are next to polyols and sugars in the row of cosolvents being used for improving *in vitro* refolding of aggregation prone proteins (Arakawa et al., 2007). The osmolytes increase refolding yield either by acting as folding enhancer or as aggregation suppressor by reducing the side chain-side chain interactions, thus decreasing the extent of aggregation (Tsumoto et al., 2003; Wang and Engel, 2009). Unlike polyols, which improve the refolding yield of proteins by their preferential exclusion from the surface of unfolded state (Gekko and Timasheff, 1981), certain amino acids like arginine and proline, work differently during refolding process. One of the most commonly used amino acid, arginine reduces the intermolecular association of aggregation prone folding intermediates and thus act as aggregation suppressor during protein refolding (Arakawa et al., 2007; Ravi et al., 2005; Arakawa and Tsumoto, 2003). However, arginine has been found less effective in refolding of the disulfide bonded proteins like green fluorescent protein (GFP) and recombinant human colony stimulating hormone (rhCSH) (Chen et al., 2008). Although, in some cases, proteins having disulfide bonds have been discovered to be refolded in the presence of arginine (Rudolph et al., 1992; Schaffner et al., 2001). Thus, arginine is unusual in affecting stability, refolding and solubilization of proteins depending upon the physio-chemical properties of proteins (Tsumoto et al., 2004). Proline, like arginine, works as an aggregation suppressor and since it is hydrophobic in nature, it may interacts with the hydrophobic surfaces of folding intermediates and prevent them from being implicated in protein aggregation during refolding (Meng et al., 2001; Schobert and Tschesche, 1978; Bruździak et al., 2016).

In addition to arginine and proline, glycine and its naturally occurring methyl derivative sarcosine, as well as trimethyl derivative betaine, have also been shown to exhibit preferential exclusion like polyols and sugars and hence stabilizes the native state of protein (Arakawa and Timasheff, 1985). Similar to polyols and sugars, they are also found to enhance the refolding yield of proteins (Yancey and Somero, 1979).

As in the previous chapter of this study, where the effects of polyols and sugars on the refolding of BLA and BAA were explored, the effects of amino acids such as arginine, proline, glycine, and methyl derivatives of glycine on the refolding of BLA and BAA are investigated here. The goal of employing amino acids as a refolding additive is to get mechanistic insights into how they affect the refolding of two proteins with structural similarities but differential thermal stabilities. Furthermore, how amino acids behave differently with respect to polyols and sugars during BLA and BAA refolding may be elucidated. Our results showed that among all the amino acids and derivatives, betaine showed positive effect on the refolding yield of α-amylases. Although, arginine and proline enhance the refolding yield of BAA at their lower concentrations, but complete inhibition of refolding was observed at their higher concentrations. Betaine, like polyols and sugars, had a more positive effect on BAA than BLA, but surprisingly, sarcosine and glycine both inhibited BLA and BAA refolding at all concentrations.

5.2 Results

5.2.1 Effect of amino acids and derivatives on the refolding of BLA and BAA

The comparative effect of amino acids and derivatives on refolding yield of BLA and BAA has been investigated by enzymatic activity measurements. The amino acids used in this study include arginine, proline, glycine and methyl derivatives of glycine like sarcosine and betaine. A range of concentrations of different amino acids and derivatives were used on the basis of their solubility.

Arginine was used from 0.125 to 0.5M during the refolding of BLA and BAA. The results showed that 0.125 M of arginine has no significant effect on the refolding of BLA, where refolding yield remains unchanged with respect to the control, but at the same concentration refolding yield of BAA was increased from 20 to 42%. (Figure 17 a).

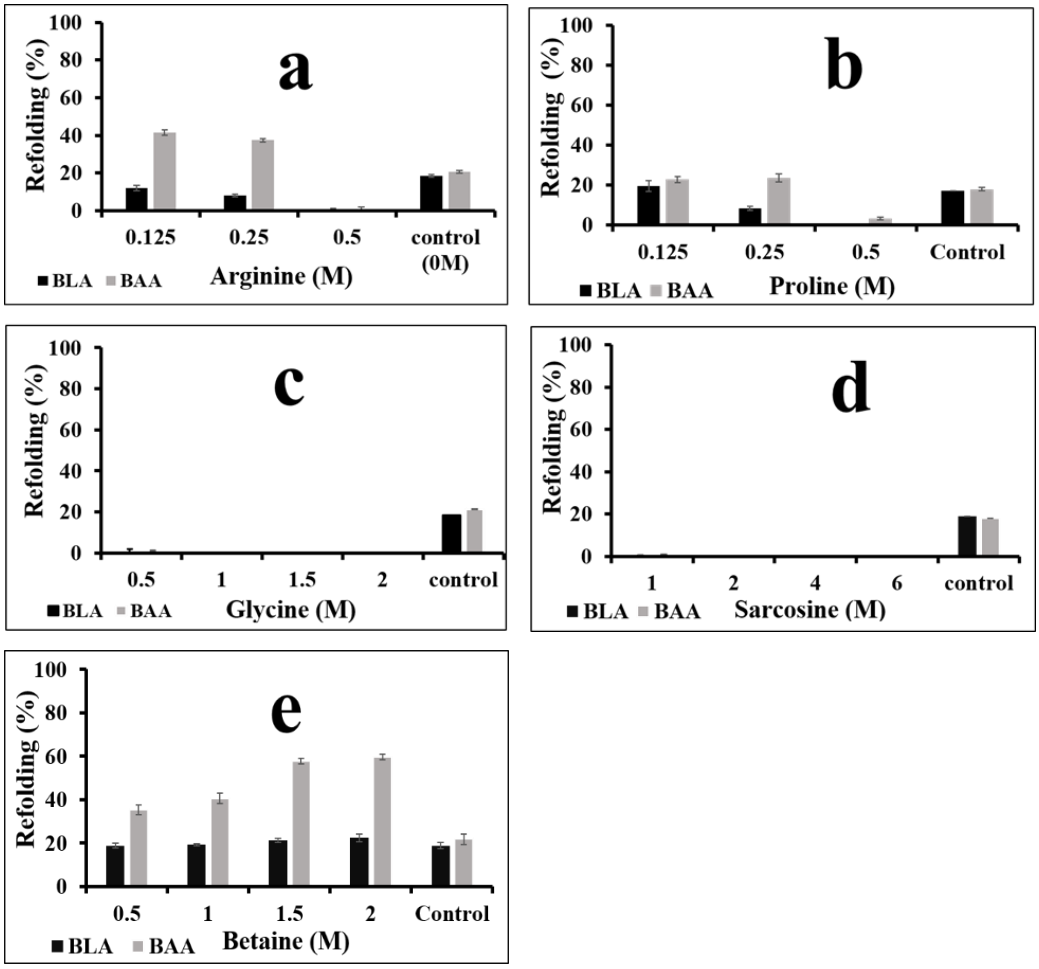


Figure 17. Refolding of homologous α-amylases, BLA (black) and BAA (grey) as monitored by enzymatic activity assay. Both enzymes were first unfolded in 6M GdmCl for 24 hours and then refolded by 60 fold (0.025mg/ml protein and 0.1M GdmCl) dilution in various concentrations of arginine, proline, glycine, sarcosine and betaine (a,b,c,d and e respectively). Control represents the spontaneous refolding of α-amylases in 20 mM phosphate buffer pH 7.0 without any additive.

Further increase in arginine concentration to 0.25M exhibited negative effect on the refolding of BLA and BAA and refolding yields decreased to 12 and 38% respectively. While at 0.5M arginine, the refolding of both enzymes was completely inhibited (Figure 17a). Proline was also used in the concentration range from 0.125M to 0.5M to enhance the refolding yield of BLA and BAA. Although, 0.125M proline has no effect on the refolding yield of BLA and BAA, but at 0.25M, the refolding yield of BLA exhibited 10% decrease compared to the control, while BAA refolding remains unchanged (Figure 17b). Upon increase in proline concentration to 0.5M, negative effect was more evident and refolding yields of BLA and BAA were reduced

to 0 and 3% respectively (Figure 17b). In this study, four different concentrations of glycine from 0.5 to 2M were used. Surprisingly, glycine did not show positive effect on refolding yield of BLA and BAA, rather, it showed inhibitory effect on the refolding of both α-amylases (Figure 17c). Unlike glycine, which has limited solubility in water, sarcosine has higher solubility in water, and therefore up to 6M was used. Similar to glycine, sarcosine was also found to inhibit the refolding yield of both, BLA and BAA at the all concentrations employed during this study (Figure 17d).

Betaine (Trimethyl glycine) is a methyl derivative of glycine with three methyl groups, has also been explored as refolding additive at concentration from 0.5 to 2M. In contrast to glycine and sarcosine, betaine exhibited positive effect on the refolding of α-amylases. At 0.5M, refolding yield of BAA increased to 38% with respect to the control (Figure 17e). Maximum refolding of BAA was observed at 2M, with refolding yield of 60%. While in the case of BLA, no significant increase in the refolding yield was observed even at 2M betaine with net yield of 20% (Figure 17e).

5.2.2 Effect of amino acids and derivatives on the native state of BLA and BAA

As described in the previous section, except betaine, all the amino acids and their derivatives exhibited negative effect on the refolding yield of BLA and BAA in a concentration dependent manner (Figure 17).

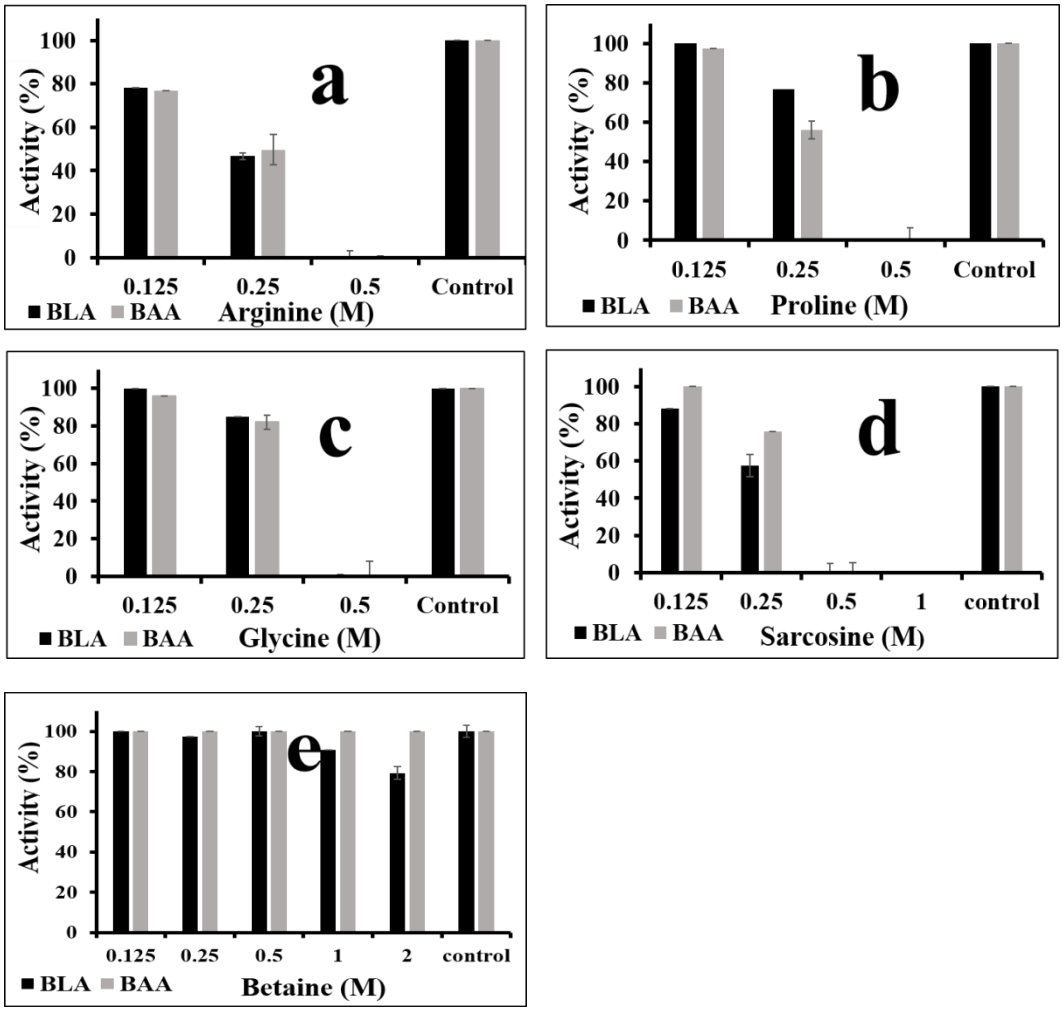


Figure 18. Effect of amino acids and their derivatives on the native states of BLA and BAA as monitored by enzymatic activity assay. Both enzymes (0.025mg/ml) were dissolved in various concentrations of arginine, proline, glycine, sarcosine and betaine (a, b, c, d, and e respectively). Control represents enzymatic activity of enzymes in 20 mM Phosphate buffer, pH 7.0, 25°C.

To understand this, effect of amino acids and derivatives on the native state stability of BLA and BAA was also examined by monitoring the change in enzymatic activity at their various concentrations. Our results showed that the native state of BLA and BAA behaves similarly in different concentrations of arginine. At 0.125M, activity of native states of BLA and BAA were decreased to 77 and 78% respectively, relative to their control. Upon increasing the concentration to 0.25M, activity of BLA and BAA reduced to 49 and 46% respectively, while no enzymatic activity was observed at 0.5M of arginine for both enzymes (Figure 18a). In the case of proline, a lower concentration (0.125M) had a mild inhibitory effect on BLA stability, resulting in a

3% drop in enzymatic activity compared to the control. On the other hand BAA exhibits no change in activity with respect to its control. But at 0.25M, enzymatic activity was reduced to 76 and 56% for BLA and BAA respectively and complete loss of the activity was observed at 0.5M (Figure 18b).

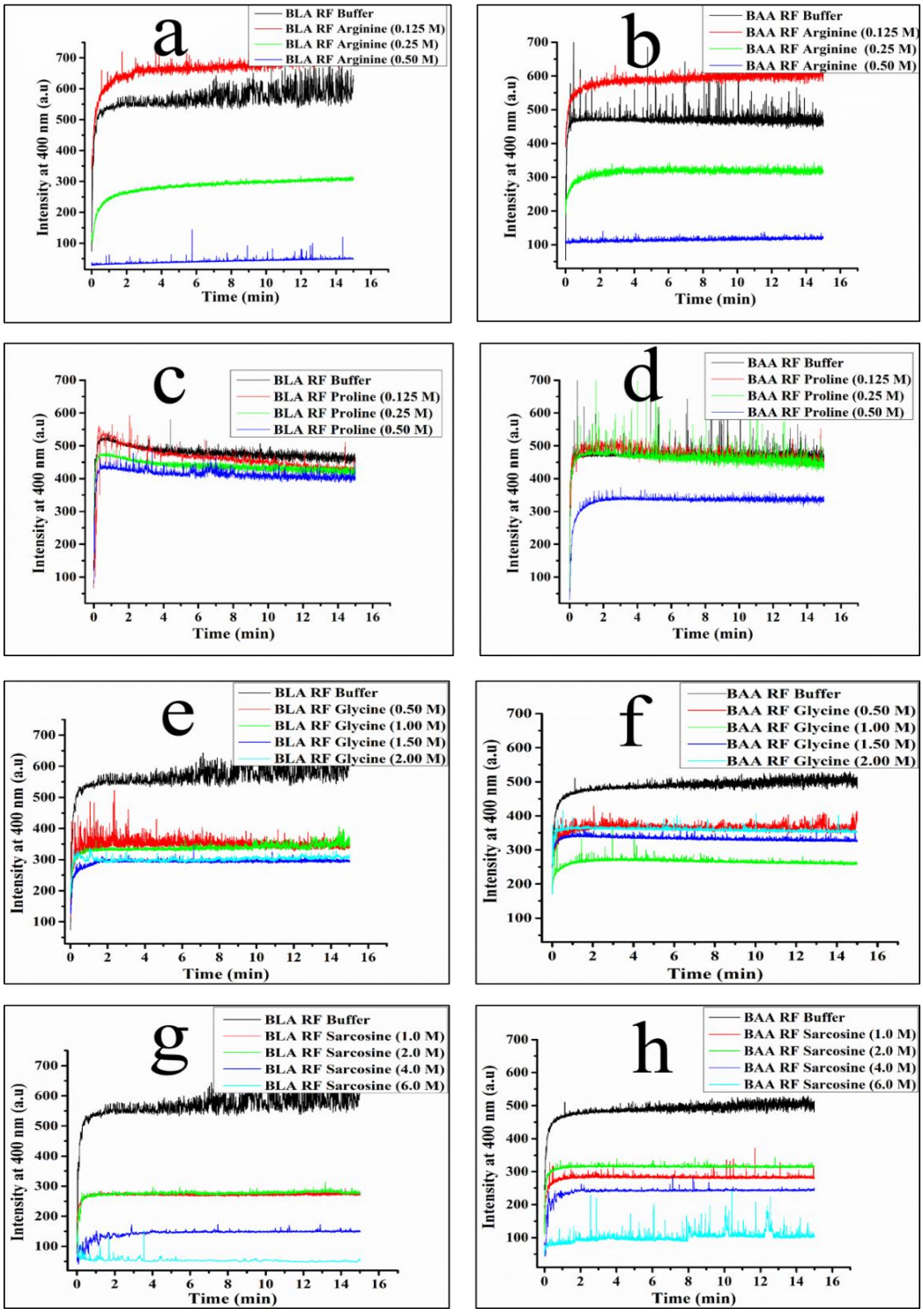
Like arginine, native state of BLA and BAA has similar behaviour in glycine and no enzymatic activity was observed above 0.25M glycine. But decrease in the enzymatic activity of both the enzymes at 0.25M glycine was less as compared to 0.25M arginine (Figure 18c). Sarcosine has a varied effect on BLA and BAA native state stability, where even lower concentration of sarcosine (0.125M) showed mild inhibitory effect on the enzymatic activity of BLA while BAA activity remains unchanged. At 0.25M, reduction in enzyme activity was observed for both BLA and BAA, but this effect was more for BLA than BAA with net activity of 57 and 74 respectively (Figure 18d). Further increase in sarcosine concentration results in complete loss of enzymatic activity for both BLA and BAA (Figure 18d). Betaine showed the least inhibitory effect on the native of BLA and BAA, where up to 0.5M, activity of both BLA and BAA remains identical to that of their respective control. While at 1 and 2M, enzymatic activity of BLA decreased to 91 and 79% with respect to its controls (Figure 18e). Unlike BLA, the native state of BAA has the same enzymatic activity as the control at all the concentrations of betaine (Figure 18e).

5.2.3 Aggregation kinetics

Aggregation suppressor amino acids such as arginine and proline, are known to improve the protein refolding yield (Samuel et al., 2000; Shiraki et al., 2002). Surprisingly, with the exception of betaine, none of them were found to be able of increasing the refolding yield of BLA and BAA at the concentrations used in this work. One possible explanation for this unusual result is that amino acids may increase the degree of aggregation rather than decrease it. To test this, aggregation kinetics during the refolding of BLA and BAA was monitored in the presence of amino acids and its derivatives. The aggregation kinetics was followed by measuring static light scattering at 400 nm, which measures the aggregates of a particular size. Arginine, was used at 0.125, 0.25 and 0.5M during aggregation kinetics of BLA and BAA. During aggregation kinetics, arginine exhibited unusual behaviour for BLA and BAA, where the amount of aggregation at 400 nm was increased for both BLA and

BAA in the presence of 0.125M arginine, compared to their controls. While, upon increasing the arginine concentration to 0.25M, significant decrease in aggregation was observed for both BLA and BAA. At 0.5M arginine, the aggregation was completely suppressed for BLA and BAA (Figure 19a, b). Proline has limited effect on the aggregation during refolding of both α-amylases. At 0.125M proline, extent of aggregation during refolding of BLA was similar to the control. While in the presence of 0.25 and 0.5M, aggregation was reduced to some extent. (Figure 19c, d). Although, aggregation during refolding of BAA remain unaltered at 0.125 and 0.25M, but it was significantly reduced in the presence of 0.5M proline. (Figure 19c, d).

Glycine, like proline, has a mild effect on aggregation kinetics during α-amylase refolding. Although 0.5M glycine exhibited appreciable aggregation inhibition effect during refolding of BLA and BAA. But at 1M, there was no change in the aggregation of BLA, while in the case of BAA, it showed highest aggregation suppression effect (Figure 19e, f). On the other hand, for BLA, maximum aggregation suppression effect was observed in the presence of 1.5M, which remains the same at 2M glycine. Surprisingly, 1.5 and 2M glycine showed less effect on aggregation kinetics of BAA and their effects on aggregation suppression was less prominent than 1M glycine (Figure 19e, f). In contrast to glycine, sarcosine, a methyl derivative of glycine, has a greater effect on the aggregation kinetics of both α-amylases. Sarcosine was used from 1 to 6M, but even 1M showed strong anti-aggregation effect during refolding


a
BLA RF Buffer
BLA RF Arginine (0.125 M)
BLA RF Arginine (0.25 M)
BLA RF Arginine (0.50 M)
b
BAA RF Buffer
BAA RF Arginine (0.125 M)
BAA RF Arginine (0.25 M)
BAA RF Arginine (0.50 M)
c
BLA RF Buffer
BLA RF Proline (0.125 M)
BLA RF Proline (0.25 M)
BLA RF Proline (0.50 M)
d
BAA RF Buffer
BAA RF Proline (0.125 M)
BAA RF Proline (0.25 M)
BAA RF Proline (0.50 M)
e
BLA RF Buffer
BLA RF Glycine (0.50 M)
BLA RF Glycine (1.00 M)
BLA RF Glycine (1.50 M)
BLA RF Glycine (2.00 M)
f
BAA RF Buffer
BAA RF Glycine (0.50 M)
BAA RF Glycine (1.00 M)
BAA RF Glycine (1.50 M)
BAA RF Glycine (2.00 M)
g
BLA RF Buffer
BLA RF Sarcosine (1.0 M)
BLA RF Sarcosine (2.0 M)
BLA RF Sarcosine (4.0 M)
BLA RF Sarcosine (6.0 M)
h
BAA RF Buffer
BAA RF Sarcosine (1.0 M)
BAA RF Sarcosine (2.0 M)
BAA RF Sarcosine (4.0 M)
BAA RF Sarcosine (6.0 M)
Intensity at 400 nm (a.u)
Time (min)

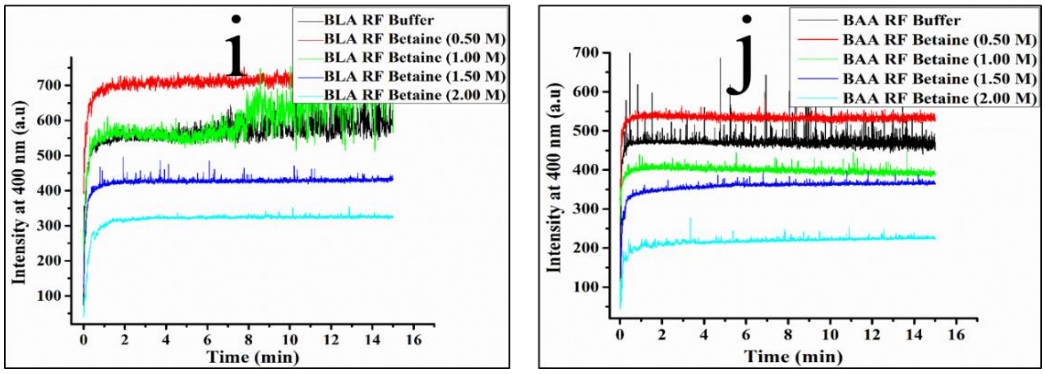


Figure 19. Aggregation kinetics of BLA and BAA by static light scattering at 400 nm. Homologous α-amylases BLA (a, c, e,g, and i) and BAA (b,d,f,h and j), were first unfolded in 6M GdmCl for 24 hours and then refolded (RF) by diluting 60 fold (0.025mg/ml protein and 0.1 M GdmCl) in 20mM phosphate buffer, pH 7.0 refolding buffer containing various concentrations of arginine (a and b), proline (c and d), glycine (e and f), sarcosine (g and h) and betaine (i and j). The aggregation kinetics were monitored at 400 nm at 25°C.

of BLA and BAA and thus their aggregation get reduced significantly. The aggregation kinetics of BLA remains unchanged at 2M sarcosine, while slight increase in aggregation was detected for BAA. At 4M sarcosine, aggregation decreases significantly during refolding of both BLA and BAA. For BLA, aggregation was completely abolished at 6M sarcosine although some aggregation was still visible during refolding of BAA (Figure 19g, h). Sarcosine was found to be relatively more effective in suppressing aggregation for BLA than BAA. Despite the positive effect on both enzymes' refolding yields, lower concentration of betaine (0.5M) was observed to promote aggregate formation during BLA and BAA refolding. Thus, at 0.5M betaine, extent of aggregation during refolding of BLA and BAA was greater than their buffer counterparts (Figure 19i, j). However, when the concentration of betaine was increased to 1M, the extent of aggregation for BLA equals that of buffer. While 1M betaine showed more anti-aggregation effect during refolding of BAA.

5.2.4 Effect of amino acids and derivatives on tertiary structure

There are seventeen uniformly distributed tryptophan residues are present in BLA and BAA (Machius et al. 1995, Alikhajeh et al 2010). Therefore, formation of the tertiary structure upon refolding was monitored by tryptophan fluorescence measurement. Shift in wavelength maxima was followed to detect the conformational changes in the refolded states of enzymes compared to their native state (Figure 20).

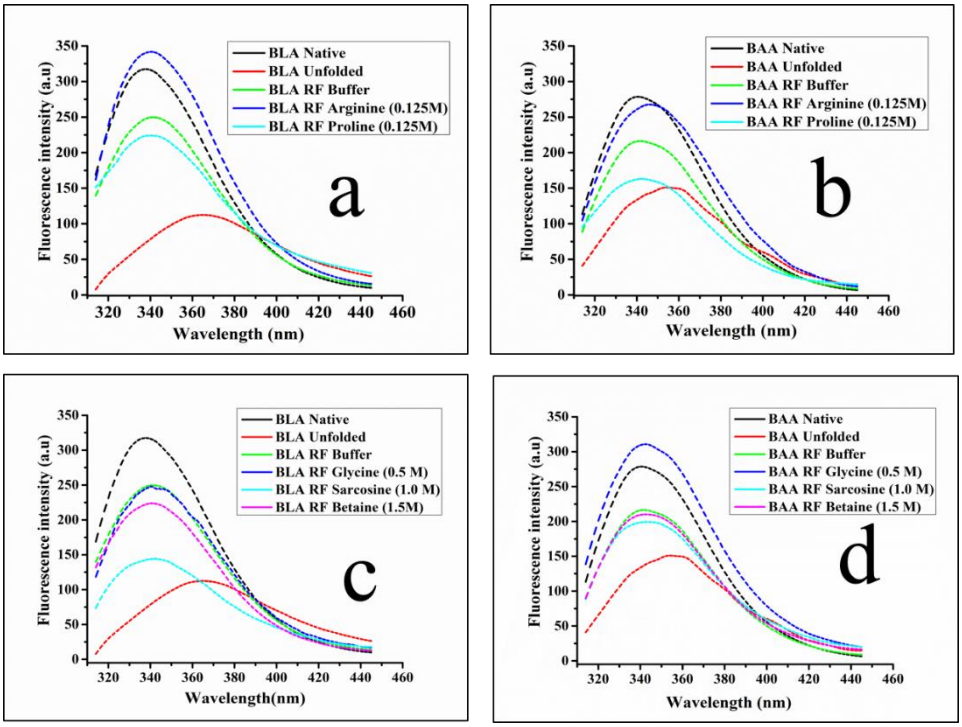


Figure 20. Conformational change by fluorescence spectroscopy during refolding of BLA and BAA in amino acids and derivatives. Homologous α-amylases BLA and BAA were first unfolded in 6M GdmCl for 24 hours and then refolded by diluting 60 fold (0.025mg/ml protein and 0.1M GdmCl) in the 20 mM phosphate buffer pH 7.0 containing 0.125M arginine and proline (a, b) and 0.5M glycine, 1M sarcosine and 1.5M betaine (c, d). All the measurements were performed at 0.025mg/ml, pH 7.0 and 25°C.

But in some cases, fluorescence spectrum of refolded states may be biased by aggregation, thus the resultant spectrum might have the contribution of both, the refolded and the aggregated species. Herein, effect of selected concentrations of amino acids like arginine, proline, glycine and glycine derivatives like, sarcosine and betaine was observed on the tertiary structure of refolded state. As per the results,

fluorescence spectrum of unfolded states of both, BLA and BAA showed 22 and 18 nm red shift respectively, with respect to their native states. In buffer, the fluorescence spectra of the refolded state of BLA showed a 3 nm red shift compared to its native state, however the refolded state of BAA showed no red shift (Figure 20). The fluorescence spectrum of the refolded state of BLA showed a 4 nm red shift in the presence of 0.125M arginine, while that of BAA showed a 5 nm red shift in the presence of 0.125M arginine (Figure 20a, b). The apparent red shift of 4 nm in the case of BLA, is also in agreement with enzymatic activity results, where decrease in refolding yield was observed at 0.125M of arginine (Figure 17a), but this correlation is lacking in the case of BAA. Although, fluorescence intensity of refolded state of BLA showed slight increase with respect to its native state, but BAA refolded state showed slight reduction in fluorescence intensity in arginine. During refolding of α-amylases in 0.25M proline, unlike arginine, the refolded states of BLA and BAA showed no red shift in their fluorescence spectra, compared to their native states. (Figure 20a, b). These results are not in agreement with the enzymatic activity results, where only 20% refolding yield was observed for both BLA and BAA (Figure 17b). Wavelength maxima of the refolded state of BLA and BAA in 0.125M proline were 339 and 340 nm respectively (Figure 20a, b). During proline-induced refolding, the refolded state of BAA revealed a significantly lower fluorescence intensity than BLA (Figure 20 a, b). In 0.5M glycine, fluorescence spectra of refolded states of both BLA and BAA showed 2 nm red shift with respect to their native states. Unlike this, fluorescence intensity showed differential behaviour during refolding of BLA and BAA in glycine. In comparison to their native states, BLA's fluorescence intensity decreased, while BAA's fluorescence intensity increased in 0.5M glycine. During refolding in the presence of betaine (1.5M) and sarcosine (1.0M), fluorescence spectrum of refolded state of BLA exhibit red shift of 2 and 3 nm respectively, relative to its native state (Figure 20c). While BAA refolded state showed identical red shift of 2 nm in both, betaine (1.5M) and sarcosine (1M) (Figure 20d). Although both BLA and BAA exhibited decrease in fluorescence intensity during refolding in betaine and sarcosine, but this decrease was relatively greater for BLA than BAA (Figure 20c, d). The fluorescence spectroscopy results of glycine, sarcosine and betaine in the case of BLA and BAA show no correlation with enzymatic activity.

5.3 Discussion

Correct folding of proteins under *in vitro* condition is affected by several factors like protein concentration, temperature, pH, composition of refolding buffer, physico-chemical properties of proteins etc. Efficient *in vitro* refolding has a significant impact on commercial protein production using recombinant DNA technology. Although, major road barriers for both *in vivo* and *in vitro* protein refolding are misfolding and aggregation, but protein aggregation is the common cause of low refolding yield during recombinant protein production (Yamaguchi and Miyazaki 2014; Clark, 1998). A wealth of literature available that details several ways for improving protein refolding yield, such as artificial chaperone assisted refolding, use of cosolvents, and molecular chaperones etc. (Rozema and Gellman, 1995; Tsumoto et al., 2003; Lee et al., 2006; Aguado et al., 2015; Balchin et al., 2020). But use of the refolding additives like polyols, sugars, amino acids, detergents, denaturants etc. during protein refolding is simple, inexpensive, effective, and reliable way to increase refolding yield (Alibodani and Mirzahoseini, 2011; Clark, 1998). In this chapter, different amino acids like arginine, proline, glycine and glycine derivatives sarcosine and betaine were employed for enhancing the refolding yield of BLA and BAA. Like polyols and sugars, amino acids also act as protein stabilizer and known to increase the protein stability towards temperature, chemical denaturants and other harsh conditions (Tsumoto et al., 2004; Arakawa et al., 2007). Amino acids like arginine, glycine, sarcosine, exert their stabilizing effect through preferential exclusion mechanism (Bruzdziak et al., 2016; Tsumoto et al., 2004). However, some amino acids like arginine and proline also show weak binding on protein surface thus reduces the extent of intermolecular interactions and protein aggregation (Arakawa et al., 2007; Samuel et al., 2000). Different amino acids interact differently with the protein surface depending on the nature of their side chains, and hence could have different effects on protein stability and refolding (Chen et al., 2008).

Objective of this study is to investigate that how amino acids behave during refolding of two homologous bacterial α-amylase having significant amino acid sequence and structural similarities but different thermal stabilities. For this, five naturally occurring amino acids and their derivatives; arginine, proline, glycine, sarcosine and betaine have been selected. The effect of amino acids on the refolding yield of BLA and BAA

were studied through enzymatic activity measurements at their different concentrations. Our results showed that lower concentration of arginine (0.125 M) exhibited positive effect on the refolding yield of BAA with 20% increase relative to its spontaneous refolding yield in buffer. While at the same concentration of arginine, refolding yield of BLA exhibited substantial decrease with only 12 % refolding yield (Figure 17a). This is similar trend, as we have observed during polyols and sugars assisted refolding of BLA and BAA (Chapter 4). There are several reports where arginine at low concentrations successfully enhances the refolding yield of several proteins like lysozyme, carbonic anhydrase and human p53 tumor suppressor protein (Chen et al., 2008; Bell et al., 2002; Hevehan and Clark, 1997). Although, arginine above 1M, successfully reduce aggregation, but it may have a negative impact on protein refolding yield. At higher concentrations, arginine can interact with the side chains of the amino acid residues through its guanidinium group and these interactions are more favoured with the unfolded state than the folded state (Arakawa et al., 2004.). Therefore, an optimal concentration of arginine becomes prerequisite condition for efficient refolding. For example, during refolding of carbonic anhydrase, almost 100% refolding yield was observed at 0.75M of arginine, but above this concentration refolding yield decreases drastically and reached to 0% at 2M (Chen et al., 2008). But contrasting results were observed in this study, where refolding yield of BLA and BAA showed significant reduction and no enzymatic activity was observed at 0.5M of arginine for both the enzymes (Figure 17a). Unlike other proteins, where inhibition of refolding generally occurs above 1M, BLA and BAA refolding were found to be more prone towards lower arginine concentration and therefore inhibition occurred even at 0.5M arginine (Bell et al., 2002; Chen et al., 2008). To explain this unusual behaviour, effect of different arginine concentrations were examined on the enzymatic activity of native BLA and BAA. Like refolding, arginine also showed negative effect on the native state stability of BLA and BAA, and complete inhibition of enzymatic activity of native BLA and BAA was observed at 0.5M arginine (Figure 18a). This anomalous behaviour of arginine during refolding and native state stability of BLA and BAA can be understood by analyzing its interaction with their amino acids side chains. It has been reported that guanidinium group in arginine interacts with π electron cloud of aromatic amino acid chains (Flocco et al. 1994, Mitchell et al. 1994). The crystal structures of both BLA and BAA have 17 and 23, tryptophan and tyrosine residues respectively (Alikhajeh et al.,

2010; Machius et al., 1995). Due to abundance of aromatic residues in BLA and BAA, it is possible that they may have a stronger affinity for arginine, resulting in a deleterious effect on refolding and native state stability even at low concentrations (Figure 17a). Like arginine, proline has also been used for enhancing the refolding yield of proteins by reducing the formation of aggregation prone intermediates species (Xia et al., 2007; Meng et al., 2001). Although, extraordinary capability of proline to increase the solubility of proteins, makes it highly efficient in suppressing the aggregation during refolding of proteins, and this effect is more prevalent at its higher concentration (Schobart and Tschesche, 1978). But in this study, proline behaves differently with complete inhibition of refolding yield of BLA and BAA above 0.5M (Figure 17b). Similar results were also observed during refolding of hen egg white lysozyme, where the extent of aggregation was significantly decreased at high proline concentration (2M), but activity decreases (Samuel et al., 2000). However in BLA and BAA reduction in the enzymatic activity of refolded protein was observed at lower concentration of proline (0.5M) than lysozyme. Above 0.25M, proline has also been found to inhibit the enzymatic activity of the native state of BLA and BAA (Figure 18b). The possible explanation for this effect could be due to the presence of abundant hydrophobic residues in BLA and BAA, which might have higher affinity for proline (Schobart and Tschesche, 1978). As a result of the increased binding of proline to BLA and BAA, it is probable that it may have deleterious influence on the refolding.

Like arginine and proline, glycine and sarcosine also act as stabilizers of protein's native conformation and thus increases their thermal stability in a concentration dependent manner (Chow et al., 2001; Ratnaparkhi and Varadarajan, 2001; Street et al., 2010; Bruzdziak et al., 2016; McLachalan et al., 2016). In addition, they have also been used in increasing the refolding yield of proteins (Mitchell et al., 2001; Yancey et al., 1979). In the present study, contrasting results were observed during refolding of BLA and BAA, wherein, the refolded states of both enzymes showed no enzymatic activity in the presence of glycine and sarcosine (Figure 17c, d). Like arginine and proline, glycine and sarcosine were also found to inhibit the enzymatic activity of the native state of BLA and BAA above 0.25M (Figure 18c, d). Unlike glycine and sarcosine, betaine which is also a glycine derivative with three methyl groups showed significant enhancement in refolding of BAA, where its refolding yield increased to 60 % at 2M. Although, no appreciable increment in refolding yield was detected

during betaine assisted refolding of BLA (Figure 17 e). The enzymatic activity of native state of BAA remains unaffected even at 2M of betaine, although some loss of activity was observed for BLA (Figure 18 e). Glycine and sarcosine, unlike arginine and proline, are not effective inhibitors of protein aggregation. In the case of citrate kinase, glycine fails to suppress the aggregation during refolding (Ou et al., 2002). Similarly, high concentrations of sarcosine shows negative effect on the biological activity of α_1-antitrypsin during its refolding (Mitchell et al., 2001). However, betaine is more effective than sarcosine during refolding (Yancey et al., 1979).

To delineate the negative effect of glycine and sarcosine during refolding of BLA and BAA, aggregation kinetics were performed at different concentrations. Arginine, which is known as aggregation suppressor, found to increase the scattering intensity at its lower concentration during refolding of both BLA and BAA. This could be due to the increased intermolecular association between the folding intermediates (Figure 19a, b). Although at higher concentrations, arginine successfully inhibited the aggregation. So, the diverse effect of arginine at different concentrations during refolding of BLA and BAA might be due to its different interactions with their unfolded states and intermediates. Arginine might interact with side chains of other amino acids and prevent them to be involved in any kind of intermolecular interactions like hydrogen bonding, salt bridge formation etc. The second possible reason could be that arginine with the help of its guanidinium group may decrease the aggregation by solubilizing and hence stabilizing the partially folded intermediates. It has been reported that arginine has a varying effect on protein refolding, depending on its concentration and protein features such as disulfide bonds, hydrophobicity of amino acid side chains etc. (Samuel et al., 2000). On the other hand, proline failed to show the significant aggregation suppression effect during refolding of BLA and BAA (Figure 19). Although like arginine, proline is supposed to interact with hydrophobic surfaces of folding intermediates and thus block their road to aggregation pathway (Meng et al., 2001; Samuel et al., 2000), but this might be true at its high concentration.

As per our results, glycine exhibited negative effect on refolding of both BLA and BAA, which is also observed during its effect on aggregation kinetics. While, higher concentrations of sarcosine successfully decrease the scattering intensity during aggregation kinetics of BLA and BAA (Figure 19g, h). Similar to glycine, betaine

also found to be less effective for suppressing the aggregation during refolding of BLA and BAA. Like arginine, light scattering intensity was also higher during refolding of BLA and BAA at lower concentrations of betaine as compared to buffer (Figure 19i, j). This increase might be due to the fact that the lower concentration of betaine showing the positive effect on protein-protein association by increasing the extent of inter molecular protein interactions like hydrophobic interaction etc.

To see the effect of amino acids on tertiary structure during refolding of α-amylases, tryptophan fluorescence measurement of osmolyte induced refolded state of BLA and BAA were carried out. Our results showed that even at low concentration of arginine (0.125M), wavelength maxima of refolded state of BAA shifted towards the unfolded state (Figure 20a). Although, wavelength maxima of refolded state of BLA remains similar to its native state, thus observed red shift in refolded state of BAA might be due to its higher susceptibility towards guanidinium group of arginine. During refolding in proline at 0.125M, the fluorescence emission spectrum of refolded states of both BLA and BAA did not show red shift with respect to their native states (Figure 20b). While, refolded states of both BLA and BAA in glycine (0.5M), sarcosine (1.0M) and betaine (1.5M) showed similar red shift of 2 nm in their wavelength maxima with respective to their native states (Figure 20c, d). Although it was evident that the fluorescence data was not fully correlated with activity measurement data of refolding of BLA and BAA. But small red shift in the fluorescence spectrum of refolded states of BLA and BAA indicates that the refolded states have acquired partially folded conformation, lacking the properly folded active sites.

In conclusion, among all the amino acids and derivatives studied here, only betaine showed significant positive effect during the refolding of BAA. This could be due to its hydrophobic nature and preferential exclusion from the surface of unfolded state thus favouring formation of the native state. The loss of enzymatic activity of refolded states of BLA and BAA in glycine, sarcosine and also at higher concentration of arginine and proline might be due to the formation of non-native and enzymatically inactive folded conformations. Aggregation suppression effect at higher concentrations of amino acids like arginine and proline is considered to occur via weak binding to the unfolded states or partially folded states of BLA and BAA.

SUMMARY

Alpha amylases (EC: 3.2.1.1) are hydrolytic enzymes belong to the family of glycosyl-hydrolases and catalyze the hydrolysis of α-1,4 glycosidic bond in starch and other related polysaccharides. Bacterial α-amylases are widely used in industrial applications such as textile, food, pharmaceutical, detergent etc. Although they are ubiquitous in nature, but bacterial α-amylases, especially from *Bacillus* species have drawn the largest attention to the scientific community due to their diverse thermostability and industrial applications. Structural and functional stability of proteins are prerequisite to work in harsh environmental conditions. A stable native conformation of a protein is the net result of its stabilizing and destabilizing non-covalent interactions including H-bonding, electrostatic, hydrophobic interactions and van der Waal's forces. Disruption of non-covalent interactions leads to the unfolded state of a protein and thus understanding the contribution of non-covalent interactions can provide new insights to the mechanistic understanding of protein stability. A careful investigation of the denatured state in addition to the native state is of immense importance for understanding the stability of proteins. Although most of the single domain proteins obey two state folding without any intermediate but this is not true for multidomain proteins. Contrary to the single domain proteins, folding pathway of multidomain proteins involves intermediates. The complex behaviour of multidomain protein folding and unfolding is not very well understood.

To study the unfolding of bacterial α-amylases, we have chosen two homologous α-amylases, one from *Bacillus licheniformis* (BLA) and another from *Bacillus amyloliquefaciens* (BAA). The objective of this study is to address the origin of the stability differences between two homologous bacterial α-amylases (BLA and BAA) through experimental approaches. Beside this, emphasis has been given to explore the differential refolding behaviour of BLA and BAA in the presence and absence of various cosolvents including polyols, amino acids and their derivatives. To answers the above questions, current study has been divided into three objectives:

The first objective of this study is to address the origin of the stability differences between BLA and BAA. To achieve this, conformational stability of both α-amylases were compared in chemical denaturants like GdmCl and urea. Despite the difference in the thermal stability of BLA and BAA, they have structural similarities, like both have no disulfide bonds, similar content of proline and charged residues etc.

Therefore, we have focused on the relative contribution of each type of interactions in the stability of these two α-amylases. For this, we have performed equilibrium unfolding of BLA and BAA in the chemical denaturants, as the equilibrium unfolding has been considered as a robust method and it may provide the realistic measure of conformational stability. In several studies, urea and GdmCl have been used for deciphering the existence of equilibrium and kinetic intermediates during the folding pathway of multidomain proteins. Due to their different chemical nature, urea and GdmCl provide the different estimate of conformational stability of proteins. Moreover, for the conformational stability measurement, chemical denaturants (GdmCl and urea) induced unfolded state of proteins are more favoured over temperature induced denaturation as the former is devoid of any residual secondary and tertiary structure. The results showed that BAA is more susceptible towards GdmCl and urea unfolding than BLA under same experimental conditions. BLA shows extreme resistance towards urea unfolding at pH 7.0. From the current unfolding study by GdmCl and urea, it is clear that both electrostatic and hydrophobic interactions are playing major role towards the higher stability BLA. The structure-function relationship and ANS binding during equilibrium unfolding in GdmCl and urea indicates the existence partially unfolded intermediates (molten globule) during the GdmCl unfolding of BLA. It has been reported that bacterial α-amylases undergo irreversible unfolding transition during chemical and thermal denaturation. It is also of great interest to follow the kinetics of unfolding in order to understand the kinetic origin of stability. Comparatively higher values of unfolding rate constants of BLA than BAA during kinetic unfolding in GdmCl, suggest the kinetic origin of its higher stability.

In the second part of this study, we have tried to optimize the refolding conditions of both α-amylases in term of the protein concentration, buffer composition and environmental factors (pH and temperature). Series of polyols along with few sugars like trehalose and sucrose were used for improving the refolding yield of two homologous α-amylases, BAA and BLA. The results show that among the various cosolvents, glycerol, sorbitol and trehalose were found to be the most effective, which is more for BAA than BLA. Although aggregation kinetics monitored by static light scattering at 400 nm showed that cosolvents like glycerol and trehalose have similar effect on the aggregation of BAA and BLA during refolding. In addition,

AFM results further confirm that number as well size of aggregates decreased in the presence of cosolvents in the case of both BLA and BAA.

Lack of correlation between activity recovery and aggregation suppression effect of cosolvents during refolding of BAA and BLA might be due to their different refolding pathways. Unlike activity, more than 90% recovery of secondary structure of BAA was observed in the presence of 7M glycerol, while 71% for BLA. BAA has maximum activity recovery in the presence of trehalose, thus further mechanistic studies were carried out with trehalose only. Addition of trehalose at different time points of refolding indicates that refolding process is highly spontaneous and completed within seconds. Refolding during non-permissive (urea and high temperature) conditions showed that low urea concentrations have some positive effect on the spontaneous refolding of BAA, while at the same time, negative effect on BLA refolding. This differential behaviour of BLA during refolding with or without cosolvents indicates the different refolding pathways of BLA and BAA. The effect of cosolvents on the refolding of both α-amylase was also analyzed during non-permissive conditions like high temperatures and low concentrations of urea.

Like polyols, amino acids and their derivatives have been also used as stabilizers and enhance the refolding yield of proteins. Most frequently used amino acids for this purpose are arginine, proline, sarcosine, glycine etc. Contrary to polyols, which exert the stabilizing effect on proteins through preferential hydration, amino acids show weak binding on the protein surfaces, although extent of binding may vary from protein to protein depending upon their physicochemical properties. BLA and BAA despite of very high structural similarity, possess some differences in their physicochemical properties like pI, distribution of amino acids in the core and surfaces etc. Thus, it is of significant importance to study the effect of amino acids and their derivative on refolding yield of BLA and BAA. Arginine is one of the most frequently used amino acid for increasing the refolding yield of proteins through its aggregation suppression effect. For BAA, refolding yield increases up to 40% at 0.125M arginine and above this, refolding start decreasing and no refolding was observed at 0.5M arginine for both BLA and BAA. This might be due to the non-native interaction of guanidinium groups of arginine with side chains of BLA and BAA. Like arginine, proline also shows inhibitory effect on the refolding of both

enzymes above 0.25M. Both, BLA and BAA, were unable to refold in glycine and sarcosine. To clarify this, effect of amino acids and derivatives was also analysed on the native state of BLA and BAA through enzymatic activity assay which showed that at higher concentrations, arginine, proline, glycine and sarcosine inhibited the enzymatic activity of both BLA and BAA. Thus, it can be hypothesized that arginine, proline, sarcosine and glycine exhibit weak binding to the protein surface of BLA and BAA thus preventing proper refolding. Betaine was found to increase the refolding yield of BAA up to 60% while no significant effect was observed on the refolding of BLA. Unlike other amino acid derivatives, Betaine might increase the refolding of BAA through preferential exclusion from the protein surface thus allowing it to refold properly.

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LIST OF PUBLICATIONS

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1. Ahmad, A., Mishra, R., 2020. Different unfolding pathways of homologous alpha amylases from Bacillus licheniformis (BLA) and Bacillus amyloliquefaciens (BAA) in GdmCl and urea. Int J Biol Macromol, 159:667-674.

2. Ahmad, A., Mishra, R., 2022. Differential effect of polyol and sugar osmolytes on the refolding of homologous alpha amylases: A comparative study. Biophys Chem, 281:106733.

CONFERENCES

1. Aziz Ahmad and Rajesh Mishra (2021) Differential Effect of Polyols on the Refolding of Microbial Alpha Amylases. International Conference on Biotechnology for Sustainable Agriculture, Environment and Health, Malaviya National Institute of Technology Jaipur and the Biotech Research Society, Jaipur, India. April 04-08.

2. Aziz Ahmad and Rajesh Mishra (2020). Comparative effect of various cosolvents on refolding of homologous bacterial alpha amylases. International conference on Innovation in Biotechnology and Life sciences (ICIBL) Delhi Technology University, Delhi, India. December 18-20.

3. Aziz Ahmad and Rajesh Mishra (2017). Unfolding the stability of Alpha Amylase. Annual Symposium of the Indian Biophysical Society (IBS), IISER Mohali, India. March 23-25.

APPENDICES

APPENDIX 1

APPENDIX 2

Differential effect of polyol and sugar osmolytes on the refolding of homologous alpha amylases: A comparative study

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