

1. INTRODUCTION

Enzymes need to meet special requirements in order to be used on industrial scale. In various industrial processes, enzymes are exposed to extreme conditions such as high temperature, high salt concentration (Kuwahara and Konno, 2010), alkaline condition (Favilla *et al.*, 1997), or the presence of surfactant / detergent (Iyer and Ananthanarayan, 2008). Therefore, enzymes which are stabilized against temperature, extreme pH or in the presence of salts and surfactants have significant industrial applications (Iyer and Ananthanarayan, 2008). In view of this, there is a need to study the folding mechanism of industrial enzymes and find possible ways to manipulate their structures for increasing their stability.

Cellulose and starch are two of the most important sources of energy for animals, plants and microorganisms. Both of them contain large number of glucose units linked together by α -glycosidic bonds in starch and β -glycosidic bonds in cellulose. A large group of enzymes known as amylolytic or amylase enzymes, catalyze the hydrolysis of these polymers. Glucoamylases along with α -amylases and β -amylases are the most common amylolytic enzymes. (Machovic and Janecek, 2007). Among industrial enzymes, glucoamylases are second to proteases in worldwide distribution and sales (James and Lee, 1997). Glucoamylases with other amylolytic enzymes play a significant role in the processing of some raw food materials, especially in the production of soft and alcoholic drinks. The conversion of starch into fermentable sugars in the baking industries is achieved by the addition of glucoamylases and fungal α -amylases. Glucoamylase is also necessary for improving the bread crust color (James and Lee, 1997). The combination of glucoamylase with fungal α -amylases is used in chilled or

frozen dough in order to ensure the presence of sufficient quantities of fermentable sugars for yeast during baking. Recently, immobilized glucoamylases have been employed in the modern technologies of low-calorie beer factories (Lewis and Young, 1995). A large amount of starch is converted into non-fermentable dextrans using immobilized glucoamylase under traditional brewing conditions which are carried through to the final product. The break-down of these dextrans to glucose, which is then almost completely transformed into alcohol, is achieved by passing the fermenting beer through a reactor containing immobilized glucoamylase (Ruth *et al.*, 1979).

Glucoamylase (1,4- α -D-glucan glucohydrolase, E.C. 3.2.1.3) catalyzes the production of D-glucose from starch and other gluco-oligosaccharides by cleaving the α -1,6 and α -1,4-glycosidic bonds at the non-reducing ends (McCleary and Anderson, 1980). It is an industrial enzyme, used for the commercial production of glucose which can be further utilized in the production of fructose syrup and ethanol (Hyun and Zeikus, 1985; Shigechi *et al.*, 2004). Although different microorganisms produce glucoamylase (Hayashida, 1975; Miah and Ueda, 1977), those obtained from *Aspergillus niger* and *Rhizopus oryzae* have got preference for industrial use due to their higher stability and activity (Frandsen *et al.*, 1999)

Many studies have been carried out to show the effect of temperature (Christensen *et al.*, 1999) and chemical denaturants, such as GdnHCl and urea on the conformation and activity of glucoamylase (Takahashi *et al.*, 1985; Ghosh *et al.*, 1997). Glucoamylase has been shown to remain active in a wide range of pH (3.0– 11.0), with a maximum activity in the pH range, 3.5–5.0. Furthermore, the enzyme has also been found stable in the above pH range without any detectable conformational change (Negi and Banerjee, 2010). However, the behavior of this enzyme in more acidic range (below pH 3.0) and whether the acid-denatured state represents the molten globule state of the enzyme, are

not known. Since many proteins have been shown to exist in the molten globule state at low pH (Dolgikh *et al.*, 1985; Fink *et al.*, 1993; Muzammil *et al.*, 1999; Nakamura *et al.*, 2011), it would be of interest to investigate the behaviour of glucoamylase at low pH. Furthermore, the presence of the catalytic domain linked to the starch binding domain (SBD) through a linker region in glucoamylase makes it an interesting model for acid denaturation study of multidomain proteins. Such studies would be helpful in understanding the mechanism of folding and structural stability of the enzyme under different denaturing conditions. Various studies have shown the ability of alcohols to induce partially folded states including the molten globule state in the acid-denatured proteins (Sirangelo *et al.*, 2003; Kumar *et al.*, 2004; Dave *et al.*, 2010). Characterization of these partially folded states may provide better insight about the folding mechanism of these enzymes.

Objectives of the study:

This project was aimed to study:

- the conformational stability of *Aspergillus niger* glucoamylase under GdnHCl, pH (acid), and thermal denaturation conditions.
- the characterization of the acid-denatured state at pH 1.0.
- the effect of various alcohols belonging to halogenol and alkanol families on the acid-denatured state of the protein
- Characterization of the alcohol-induced states
- the structural changes and the stabilizing effect of various polyols on the native and the acid-denatured states of the enzyme

2. LITERATURE REVIEW

2.1. Background

Proteins are required to fold into a unique three-dimensional structure in order to be biologically active so as to carry out their functions. Failure of a protein to fold correctly (misfolding) may lead to serious consequences as observed in various diseases such as Alzheimer, prion and Parkinson's diseases (Wood *et al.*, 1996; Zhang *et al.*, 1995; Selkoe, 2004).. Since native structure of a protein represents the expressed version of the genetic information encoded in DNA, understanding the mechanism of protein folding becomes the basic issue in molecular biology. Therefore, protein folding studies have attracted much attention of both industrial and biotechnological researchers, in search of finding ways to increase protein/enzyme stability. Although the mechanism of protein folding is a complex process, identification of intermediates in the protein folding pathway might be helpful in unraveling the folding mechanism (Wu *et al.*, 1994). One of the well-characterized intermediates in the folding pathway of many proteins is the molten globule state which is characterized by a compact denatured form of protein that retained a significant amount of native-like secondary structure, but a largely disordered tertiary structure with the exposure of buried hydrophobic regions of the protein and generally the absence of cooperative thermal transition (Dolgikh *et al.*, 1985; Muzammil *et al.*, 1999; Nakamura *et al.*, 2007). However, some proteins have shown a cooperative thermal transition in their molten globule states (Nakamura *et al.*, 2007; Prajapati *et al.*, 2007)

Molten globule state of proteins has been observed under different experimental conditions such as in presence of moderate concentrations of urea or guanidine hydrochloride (GdnHCl), high temperature, high and low pH and high pressure (Fink *et*

et al., 1993; Wang *et al.*, 1998; Yang *et al.*, 2001; Kristinsson and Hultin, 2003; Nakamura *et al.*, 2011). Molten globule and other non-native states of proteins have been shown to exist in living cells and are involved in many physiological processes (Bychkova *et al.*, 1996). In a recent study (Jiang *et al.*, 2009), structural modifications in the molten globule state of soy protein isolate have shown a markedly improved emulsifying activity of the protein isolate. Both salts and organic solvents have been widely used to generate different states of a protein falling in between the denatured state and the native state (Nakamura *et al.*, 2007; 2011). Furthermore, a well-defined state (alcohol-induced state) in aqueous alcohol characterized by the presence of certain elements of the native structure has been observed with many proteins (Sirangelo *et al.*, 2003; Kumar *et al.*, 2004; Dave *et al.*, 2010; Sen *et al.*, 2010).

One of the popular strategies used to increase the protein stability is the employment of cosolvents, which are small organic molecules such as sugars, polyols, neutral amino acids *etc*, capable to alter the equilibrium either toward the unfolded state (denaturants) or favoring the folded ensemble (osmolytes). Osmolytes such as sugars and polyols are low molecular weight, water-soluble neutral cosolvents. Recently, many studies have shown the increase in the thermal stability, gelation, foaming and emulsion-stabilizing performance of globular proteins in presence of these osmolytes (Baier and McClements, 2001; Dierckx and Huyghebaert, 2002; Davis and Foegeding, 2007).

Glucoamylase plays a significant role in the food industry especially in the saccharification of starch for glucose and high fructose corn syrup as well as ethanol production. Involvement of glucoamylases during industrial processes is advantageous as glucoamylases are inverting exo-acting starch hydrolases releasing β -glucose from non-reducing ends of starch and related substrates, until all the high molecular weight carbohydrate is degraded to glucose (Sauer *et al.*, 2000). Glucoamylases have ability to

cleave both α - 1,4- and α - 1,6 linkages (Pandey, 1995). These enzymes have successfully been evaluated for activity on a number of agricultural raw/ waste materials for the yield of usable products such as high fructose corn syrup.

2.2. Distribution

Glucoamylases are widely distributed in nature and can be obtained from bacteria, yeast, mould and fungi (Table 2.1). Among the microorganisms, various species of *Aspergillus*, *Saccharomyces* and *Clostridium* are the major sources of glucoamylase (Table 2.1). Enzymes from mesophilic microorganisms such as *Saccharomyces*, *Aspergillus*, *Mucor* and *Clostridium* exhibit higher activity in the temperature range, 45–60°C (Machovic and Janecek, 2007). Although these enzymes are derived from a wide variety of microorganisms, glucoamylases produced by various fungi (Table 2.1) remain active at higher temperatures (Jaffar *et al.*, 1993; Norouzzian and Jaffar, 1993; Pandey *et al.*, 2000). Due to higher activity and good thermostability, *Aspergillus niger* and *Rhizopus oryzae* glucoamylases are more popular in the starch processing industries (Frandsen *et al.*, 1999; Reilly, 1999).

2.3. Isolation and purification

Purification strategies of glucoamylase are species specific as the method employed for purification of glucoamylase from one organism does not necessarily work for purification of the enzyme from other sources. Glucoamylases have been generally purified by procedures involving column chromatography such as ion exchange, hydrophobic interaction and gel chromatography (James and Lee, 1997). Glucoamylase was first isolated from *Aspergillus niger* by Pazur and Ando (1959) using DEAE-cellulose column chromatography which yielded two isoforms of the enzyme, namely, glucoamylase I and glucoamylase II. Purification of glucoamylase from contaminating enzymes has also been achieved by adsorption on naturally occurring acid clays, such as

bentonite (Saha and Zeikus 1989). Glucoamylase has been isolated in a homogeneous form, from *Aspergillus oryzae* using extraction with 1 % NaCl, followed by precipitation with ethanol and acarbose affinity chromatography (Ono *et al.*, 1988). Glucoamylase from *Puecilomyces varioti* AIIU 9417 has also been purified by ethanol precipitation, followed by chromatography on DEAE-Sepharose CL-BB and gel filtration columns (Takeda *et al.*, 1985). A simple and rapid method for the purification of glucoamylase from *Aspergillus terreus* based on the affinity for the carbohydrate moiety of the enzyme has been reported by Ali *et al.* (1990).

Glucoamylases have been grouped into five subfamilies of close structural similarities (Coutinho and Reilly, 1994). Glucoamylase from the bacterial genus *Clostridium* is one subfamily. Two subfamilies comprising of two glucoamylases each, are from yeast genera *Saccharomycopsis* and *Saccharomyces*. Another two subfamilies are from filamentous fungi, one comprising of *Aspergillus*, *Humicola*, *Neurospora* and *Humiconis* glucoamylases and another represents a single *Rhizopus* enzyme.

2.4. Genetics

The number of glucoamylase coding genes varies among strains. Three structural genes for glucoamylase have been identified in *Saccharomyces cerevisiae* var. *diastaticus* belonging to DEX gene family or STA gene family (Meaden *et al.*, 1985; Vihinen and Mantsala, 1989). Three genes, namely STA1 (chromosome IV), STA2 (chromosome II) and STA3 (chromosome XIV), responsible for starch fermentation, have been described by Tamaki (1978). Each of these genes encodes one of the glycosylated glucoamylase isozymes, GA I, GAII and GAIII, respectively (Pretorius *et al.*, 1991) Based on dextrin utilization by *Saccharomyces cerevisiae* var. *diastaticus*, Erratt and Stewart (1978) have described DEX1, DEX2 and DEX3 genes which control glucoamylase production. Another sporulation glucoamylase gene, SGA1 has also been

described in *Saccharomyces cerevisiae* which codes for the enzyme during sporulation phase (Pugh *et al.*, 1989). On the other hand, only one structural gene has been found responsible to produce two forms of glucoamylase in *Aspergillus niger* and *Aspergillus awamori*. Four intervening sequences are present in the gene coding for *Aspergillus awamori* glucoamylase, which have to be removed to get the expression in yeast. *Rhizopus oryzae* also has only one structural gene coding for glucoamylase which contains 4 introns and a signal sequence for 25 amino acids. Glucoamylase coding genes of both *Rhizopus* and *Aspergillus* species show close similarity (Ashikari *et al.*, 1985; James and Lee, 1997).

Four introns are also found in the structural gene of *Aspergillus niger* glucoamylase. The synthesis of the two forms of the enzyme is known to occur in two different but closely related mRNAs. Glucoamylase I is synthesized as a precursor of 640 amino acid residues containing a putative signal peptide of 18 residues, a short propeptide of six residues and the 616 residues long mature enzyme. From the nucleotide sequencing data of several glucoamylase-specific cDNA recombinants it has been shown that the glucoamylase I mRNA contains a 169 bp long intervening sequence that can be spliced out to generate GAII mRNA, leaving the 3' part of the glucoamylase I mRNA modified. (Boel *et al.*, 1984). Since glucoamylase II is the isoform without the starch binding domain region of the enzyme, the word 'glucoamylase' used in this study refers to glucoamylase I.

2.5. Physicochemical properties

Table 2.2 summarizes some of the physicochemical properties of *Aspergillus niger* glucoamylase. Different values of the molecular mass ranging from 70–99 kDa have been reported for the enzyme, which can be ascribed to the use of different methods of molecular mass determination. However, both sedimentation velocity and density-

gradient centrifugation have yielded similar values of the molecular mass as 97 kDa and 99 kDa, respectively (Pazur and Kleppe, 1962; Pazur *et al.* 1971). A lesser value of the molecular mass obtained with SDS-PAGE seems understandable due to an experimental error of $\pm 10\%$. A much smaller value (70 kDa) obtained with gel chromatography compared to that obtained with sedimentation velocity (97 kDa) can be attributed to the possible interaction of glucoamylase with the gel media (Lineback and Aira, 1972; Svensson *et al.*, 1982; Kruger and Lineback, 1987). The sedimentation coefficient ($S_{20,w}$) of the enzyme is found to be 5.44 S, whereas a value of $5.05 \times 10^{-7} \text{ cm}^2$ per second has been determined for the diffusion coefficient (Pazur and Kleppe, 1962). Glucoamylase has a partial specific volume of 0.74 ml per gram, similar to the value reported for other globular proteins (Charlwood, 1961; Pazur and Kleppe, 1962). A low value of isoelectric point (4.0) suggested the acidic nature of the enzyme (Svensson *et al.*, 1982) The enzyme shows the maximum activity in the pH range, 4.5–5.0 and at 60 °C (Pazur *et al.*, 1971). Occurrence of the emission maximum of glucoamylase at 340 nm upon excitation at 295 nm suggests the presence of tryptophan. A molar extinction coefficient has been determined as $1.37 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ for *Aspergillus niger* glucoamylase (Clarke and Svensson, 1984). Different theoretical methods, used to calculate the secondary structures of glucoamylase from circular dichroic spectra have yielded 30–35% α -helix and 24–36% β -sheet structures (Shenoy *et al.*, 1985).

2.6. Structural organization

2.6.1. Amino acid composition

Aspergillus niger glucoamylase is a glycoprotein and is comprised of 616 amino acid residues in a single polypeptide chain. Table 2.3 shows the amino acid composition of *Aspergillus niger* glucoamylase as obtained from amino acid sequence as well as amino acid analysis. The total number (69 residues) of the acidic amino acids (aspartic acid

and glutamic acid) exceeds the total number (34 residues) of the basic amino acids (histidine, lysine and arginine). Glucoamylase is characterized by the abundance of the aromatic amino acids as it possesses 18 tryptophan, 27 tyrosine and 21 phenylalanine residues (Svensson *et al.*, 1983). Both hydrophilic amino acids, serine (84 residues) and threonine (73 residues) are widely distributed. Hydrophobic residues such as proline, alanine, valine, leucine, isoleucine and phenylalanine constitute about one third of the total amino acids. About 17–19% carbohydrate content present in the protein is attached to serine and threonine residues (Pazur *et al.*, 1971)

2.6.2. Primary and secondary structures

The primary structure of glucoamylase is characterized by the presence of a catalytic domain (Ala¹–Thr⁴⁴⁰) at the N-terminal side and a starch binding domain (SBD) (Cys⁵⁰⁹–Arg⁶¹⁶) toward the C-terminal side, joined together by glycosylated linker region (Pazur and Kleppe, 1962; Svensson *et al.*, 1983; 1986). The lack of SBD region in glucoamylase II helps to differentiate between the two isoforms of the enzyme, glucoamylase I and II (Svensson *et al.*, 1986). Figures 2.1 and 2.2 show the primary structure of the catalytic domain and the SBD of *Aspergillus niger* glucoamylase, respectively. About 75% and 79% of the total acidic and basic amino acids, respectively, are located in the catalytic domain. The hydrophobic amino acids are distributed as 75%, 16% and 9% in the catalytic domain, SBD and the linker region, respectively. The aromatic amino acids in the catalytic domain follow the distribution as thirteen (13) tryptophan, twenty (20) tyrosine and nineteen (19) phenylalanine residues, whereas SBD contains four (4) tryptophan, six (6) tyrosine and three (3) phenylalanine residues. There is a single residue of each tryptophan and tyrosine residues in the linker region (Svensson *et al.*, 1983).

Using DSSP algorithm (Kabsch and Sander, 1983), different types of secondary structure have been assigned in glucoamylase. Presence of 48% helix (21 helices; 227 residues) and 8% β -sheet (20 strands; 38 residues) characterize the catalytic domain (PDB entry code: 3EQA). Out of twenty one (21) helices, fourteen (14) helices are characterized as α -helices whereas the remaining seven (7) helices represent 3_{10} -helices. Similarly, twenty (20) strands of the β -sheet are equally distributed between β -strands and β -bridges (Figure 2.1). On the other hand, the SBD contains only 2% helix (1 3_{10} -helix; 3 residues) and 23% β -sheet (8 strands; 25 residues) (PDB entry code: 1AC0). Out of eight (8) strands of β -sheet, seven (7) represent β -strands leaving one (1) as the β -bridge (Figure 2.2). In addition, presence of twenty five (25) turns (58 residues) and one (1) turn (2 residues) also characterize the catalytic domain and SBD, respectively (Figures 2.1 and 2.2)

2.6.3. Three-dimensional structure

The complete three-dimensional structure of *Hypocrea jecorina* glucoamylase has been determined at 1.8 Å resolution (Bott *et al.*, 2008). Figure 2.3 shows the location and structural organization of the catalytic domain, SBD and the linker region (PDB entry code: 2VN4). Recently, the crystal structure of the catalytic domain obtained by subtilisin cleavage of *Aspergillus niger* glucoamylase (PDB entry code: 3EQA) has been solved at 1.9 Å resolution (Lee and Paetzel, 2011). The structure shows the presence of thirteen (13) α -helices and six (6) 3_{10} -helices. These numbers are found similar to those obtained from DSSP algorithm (Kabsch and Sander, 1983). Out of the thirteen (13) α -helices, twelve (12) form an α/α barrel involving six (6) parallel helices present in the core surrounded by another six (6) parallel helices aligned on the periphery. The two groups of the six (6) parallel helices are antiparallel to each other. The overall fold of *Aspergillus niger* catalytic domain is found identical to the catalytic

domain of *Aspergillus awamori* (Figure 2.4). About 94% sequence homology is observed in the primary sequence of glucoamylases obtained from *Aspergillus niger* and *Aspergillus awamori* var. *kawachi* (PDB entry code: 1GLM), (Aleshin *et al.*, 1992; Lee and Paetzel, 2011). The crystal structure of the catalytic domain also shows the presence of two disulfide bridges between Cys234(210) and Cys237(213) and between Cys286(262) and Cys294(270). The third disulfide bridge between Cys246(222) and Cys473(449), anchors the heavily glycosylated C-terminal strand to the $\alpha 7$ helix.

Based on the electron density, nine glycosylation sites have been modeled in the catalytic domain of *Aspergillus niger* glucoamylase. Five (5) serine [Ser467(443), Ser468(444), Ser477(453), Ser483(459) and Ser486(460)] and two threonine [Thr476(452) and 486(462) residues are involved in the attachment of O-linked mannose residues (Figure 2.5). These glycosylation sites agree well with those predicted earlier for *Aspergillus niger* glucoamylase (Svensson *et al.*, 1986). The structure of *Aspergillus awamori* glucoamylase shows three additional O-glycosylation sites at Ser455, Thr457 and Thr464 (Aleshin *et al.*, 1992). Furthermore, two N-acetylglucosamine (NAG) oligosaccharides have been found linked to Asn195(171) and Asn419(395) in *Aspergillus niger* glucoamylase (Figure 2.5). These sites are identical in both *Aspergillus niger* and *Aspergillus awamori* glucoamylase structures except the loss of a single terminal mannose in each oligosaccharide in *Aspergillus niger* glucoamylase.

The three-dimensional solution structure of *Aspergillus niger* glucoamylase SBD, forming the C-terminal domain has been determined by NMR spectroscopy both in the free form (PDB entry code: 1KUL) and its complex with β -cyclodextrin (PDB entry code: 1AC0) (Figure 2.6). Eight (8) β -strands are arranged in the form of two (2) β -sheets. Whereas five (5) strands are arranged in antiparallel fashion forming one (1) β -sheet, one (1) parallel and one (1) antiparallel strand pair form the second β -sheet.

Figure 2.7 shows the direction and alignment of β -strands in the SBD. There are two starch-binding sites, fitted to the starch mimicking β -cyclodextrin, are located on opposite sides, away from the linker attachment point. The O-glycosylated linker region is assumed as a semi-rigid rod (Williamson *et al.* 1992), which ensures the occurrence of the extended peptide backbone, thus keeps a fixed distance between the catalytic and starch binding domains. Furthermore, the O-glycosyl linkers have been found to stabilize the SBD against thermal denaturation (Williamson *et al.*, 1992; Neustroev *et al.*, 1993).

2.7. Denaturation

Due to its importance as an industrial enzyme and in search to improve its stability, glucoamylase stability has been extensively studied under different denaturation conditions. These include exposure to extreme pH and high temperature as well as the effect of chemical denaturants such as guanidine hydrochloride (GdnHCl) and urea.

2.7.1. pH

Glucoamylase from *Aspergillus awamori* has been shown to remain active in a wide range of pH *i.e.* pH 3.0– 11.0, with a maximum activity in the pH range, 3.5–5.0. Shenoy *et al.* (1985) have studied the effect of pH on the structure and activity of glucoamylase obtained from different sources including *Aspergillus niger*. Glucoamylase from *Aspergillus niger* has been found to remain stable and active at pH 2.0 compared to glucoamylases obtained from *Aspergillus candidus* and *Rhizopus*, which lose their conformation and activity at this pH. Far-UV CD spectra of *Aspergillus niger* glucoamylase have shown significant differences in the secondary structures at both acidic (pH 2.0) and alkaline (pH 10.0) pH values compared to those obtained at neutral pH (pH 7.0) (Shenoy *et al.*, 1985). Exposure of *Aspergillus niger* glucoamylase to alkaline pH (pH 10.0) has led to irreversible inactivation of the enzyme due to

disulfide interchange reaction (Donovan, 1967; Nozaki and Tanford, 1967). On the other hand, glucoamylase from *Aspergillus awamori* has shown slight differences in the CD spectral features in the pH range, 9.0–11.0 with retention of significant activity.

2.7.2. Temperature

Many groups have studied the effect of temperature on the structure of glucoamylase (Freedberg *et al.*, 1975; Shenoy *et al.*, 1985; Tanaka *et al.*, 1995; Christensen *et al.*, 1999). Glucoamylase has shown a decrease in the helical content when the temperature is raised from 25°C to 58°C (Freedberg *et al.*, 1975). This has been supported by the data of Shenoy *et al.* (1985) who have determined the secondary structures (α -helix and β -structure) of glucoamylase at different temperatures (Table 2.4). Whereas a decrease in both secondary structures is shown with the increase in temperature, lowering down the temperature to 15.5°C has also produced a decrease in the α -helical content. The enzyme is found to unfold at 60°C. No significant change is observed in the near UV CD spectrum upon increasing the temperature, which indicates that the environment around aromatic residues is not affected with the increase in the temperature (Shenoy *et al.*, 1985). The presence of the disulfide linkages might play an important role to stabilize the structure of the protein (Freedberg *et al.*, 1975; Cassim and Yang, 1967). Based on differential scanning calorimetry (DSC) experiments, Tanaka *et al.* (1995) have demonstrated that thermal unfolding of both glucoamylase I and glucoamylase II at pH 7.0 follows the two-state model and the unfolding of glucoamylase I has been found to be irreversible, while glucoamylase II has shown reversible unfolding. Furthermore, thermal denaturation of the SBD fragment obtained by recombinant DNA techniques as studied by DSC has also displayed reversible unfolding at pH 7.0 and follows the two-state model. In addition, the denaturation temperature of the SBD fragment is found lower than that shown by the SBD in the whole enzyme molecule,

suggesting that SBD might be stabilized by glycosylation of the domain itself or by the glycosylated linker region (Tanaka *et al.*, 1998). Thermal denaturation of three forms of *Aspergillus niger* glucoamylase (Glucoamylase I, containing the full length of the enzyme, glucoamylase II, without the SBD and the proteolytically cleaved glucoamylase containing the catalytic domain and part of the linker region) has also been studied by Christensen and his group using DSC and isothermal titration calorimetry (Christensen *et al.*, 1999). The catalytic domain of all glucoamylase forms, has displayed irreversible thermal unfolding while the unfolding of the SBD has been found reversible. These results are in agreement to those observed by Tanaka *et al.* (1998) on SBD fragment.

2.7.3. Chemical denaturants

The most commonly used chemical denaturants, urea and GdnHCl denature protein when present at high concentrations by weakening the hydrophobic as well as the polar interactions (Dill and Shortle, 1991). Glucoamylases from various sources behave differently towards their stability against urea denaturation. In a previous study (Inokuchi *et al.*, 1981) *Aspergillus saitoi* glucoamylase has shown absence of any change in the CD spectra and activity in 8.0 M urea. *Aspergillus niger* glucoamylase has remained active in the presence of 4.0 M urea, whereas *Aspergillus candidus* and *Rhizopus* glucoamylases lose their activity completely although unfolding of the enzyme is not complete (Shenoy *et al.*, 1985). *Aspergillus niger* glucoamylase has also shown a slight loss in the secondary structure along with a small change in the near UV CD spectrum and the enzyme retains 65% of its activity in 8.0 M urea. The effect of urea and GdnHCl on the conformational stability of *Rhizopus* sp. glucoamylase has shown the start point of the denaturation at 2.0 M GdnHCl or 5.0 M urea and the completion of the denaturation above 4.0 M GdnHCl but has not reached the completion

with urea (Takahashi *et al.*, 1985). These results have suggested high resistance of the enzyme against urea denaturation. GdnHCl denaturation studies on glucoamylase from *Saccharomyces cerevisiae* var. *diastaticus* have also suggested the unfolding of the enzyme in the presence of 2.0–4.0 M GdnHCl (Ono *et al.*, 1996).

2.8. Alcohol / polyol-induced structural changes in proteins

Alcohols are known to induce the formation of the α -helical structure in proteins (Keiderling *et al.*, 1994; Hirota *et al.*, 1997; Luo and Baldwin, 1998; Yoshida *et al.*, 2008). In general, this effect has been ascribed to the decreased polarity of the solvent due to their weak basicity compared to water (Sonnichsen *et al.*, 1992; Chen *et al.*, 1995). However, the degree of effectiveness of these alcohols is found to depend on the type of alcohol as well as protein (Hirota *et al.*, 1998; Khan *et al.*, 2000; Kumar *et al.*, 2004; Yoshida *et al.*, 2008). Both the structural characteristics and the type of substituent(s) in the alcohol have been shown to contribute toward their effectiveness. For example, fluoro alcohols such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and 2,2,2-trifluoroethanol (TFE) have shown greater potential in inducing the α -helical structure in proteins than alkanols (Hirota *et al.*, 1998; Hong *et al.*, 1999; Khan *et al.*, 2000; Yoshida *et al.*, 2008). Although the order of effectiveness of these alcohols seems to be similar with many proteins (Hirota *et al.*, 1998; Hong *et al.*, 1999; Khan *et al.*, 2000; Yoshida *et al.*, 2008), they have shown different behavior in terms of the extent of α -helix induction and the concentration required to acquire the maximum helical structure with different proteins. For example, various alcohols have induced similar percentage of α -helix in melittin and β -lactoglobulin (Hirota *et al.*, 1997; 1998) but have produced different patterns with other proteins (Khan *et al.*, 2000; Kumar *et al.*, 2004; Naeem *et al.*, 2004; Haq *et al.*, 2005). The maximum percentage of the α -helix achieved by various alcohols in human serum albumin (Kumar *et al.*, 2004), α -chymotrypsinogen

A (Khan *et al.*, 2000), stem bromelain (Haq *et al.*, 2005) and papain (Naeem *et al.*, 2004) has been found different but correlates well with the order of their effectiveness. Furthermore, the propensity of an alcohol to induce the α -helix varies from protein to protein (Hirota *et al.*, 1998; Khan *et al.*, 2000; Kumar *et al.*, 2004; Naeem *et al.*, 2004; Haq *et al.*, 2005; Yoshida *et al.*, 2008). Due to such differences, the results of the alcohol effects, obtained with a single protein cannot be generalized and calls for independent studies with other proteins.

Different molecular characteristics of the cosolvents such as size, structure and their interactions with other solvent molecules have been suggested to define the effectiveness of these cosolvents to alter the properties of globular proteins in aqueous solution. Furthermore, the type and the amount of the cosolvent used play a critical role in modulating protein functionality, *i.e.* enhancing the conformational stability of the protein against certain environmental stresses such as exposure to extreme (high and low) temperature, high pressure treatment or dehydration as well as obtaining an appropriate conformational state of the protein (Timasheff, 1998; McClements, 2002; Saunders *et al.*, 2000). Although employment of cosolvents such as salts, amino acids, and polyols including sucrose, glycerol and trehalose is a routinely used strategy to enhancing protein stability, the mechanism by which these cosolvents stabilize native protein conformation is still debatable. Numerous models have been proposed to explain the molecular basis of polyol-induced protein stabilization such as preferential interaction and surface tension (Gekko and Timasheff, 1981), excluded volume effect (Knoll and Hermans, 1983), transfer free energy of protein's chemical groups (Liu and Bolen, 1995) and Wyman linkage function (Tanford, 1969). Preferential interaction theory emphasizes the role of preferential interactions between the protein surface and the cosolvent-solvent molecules (Timasheff, 1998; McClements, 2002; Saunders *et al.*,

2000) through either the steric exclusion or differential interactions (Timasheff, 1998; 2002) in the polyol-induced structural stabilization of globular proteins in aqueous solutions. Most of the studies on polyol-induced protein stabilization have dwelled on the role of polyols in terms of their chemical nature and concentration requirement in inducing protein stability. Importance of protein's intrinsic factors such as size, charge and chemical composition has rarely been studied in polyol-induced stabilization of proteins. In view of the above, there is a need to study the effect of polyols on other proteins in order to generalize the stabilization mechanism / effect of different polyols.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Proteins

Glucosylase from *Aspergillus niger* (Lot 1390149) and bovine serum albumin (BSA), fraction V (Lot 38H1298) were purchased from Sigma-Aldrich Inc., USA. Glucosylase preparation was used as such without further purification.

3.1.2. Reagents used in protein estimation

Analytical grade samples of sodium carbonate, sodium potassium tartrate, copper sulfate, sodium dihydrogen phosphate and disodium hydrogen phosphate were supplied by System, Malaysia. Folin-phenol reagent was purchased from Merck Chemicals, Germany. Copper reagent was prepared by mixing 1 volume of 4% (w/v) sodium potassium tartrate to 100 volume of 4% (w/v) sodium carbonate and finally adding 1 volume of 2% (w/v) copper sulfate.

3.1.3. Reagents used in denaturation studies

Guanidine hydrochloride (GdnHCl), 99% (Lot 078K5425), Glycine (Batch 128K0194), 1-anilinonaphthalene-8-sulfonic acid (ANS) (Lot 104K2510), acrylamide, 99% (Lot 056K2501) and N-acetyl-L-tryptophanamide (NATA) (Lot1399199) were purchased from Sigma-Aldrich Inc., USA. Analytical grade samples of sodium acetate, potassium chloride, sodium hydroxide, acetic acid and hydrochloric acid were obtained from System, Malaysia.

3.1.4. Reagents used in alcohol-induced structural transition studies

Various alcohols, whose structures are shown in Figure 3.1, such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Lot SHBB0681V), 2,2,2-trifluoroethanol (TFE) (Lot

1366086), tert-butanol (Lot BCBD6149V), 2-propanol (Lot 77596MK) and methanol (Lot 42296LJ-199) were purchased from Sigma-Aldrich Inc., USA. 2-Chloroethanol (Lot S6221545), 1-propanol (Lot K42188897) and ethanol (Lot K42113283) were supplied by Merck Chemicals, Germany.

3.1.5. Reagents used in polyol-induced structural transition studies

Various polyols, whose structures are shown in Figure 3.2, such as trehalose (Lot 011M7000V), D-glucose (Lot 080M0175V), glycerol (Lot SHBB4673V) and ethylene glycol (Lot STBB0339H9) were procured from Sigma-Aldrich Inc., USA.

3.1.6. Miscellaneous

Standard buffers of pH 7.0 and pH 4.0 were purchased from Sigma-Aldrich Inc., USA. Millipore filters (pore size 0.45 μm) were obtained from Millipore, Inc., USA, Whatman filter papers (No. 1) were purchased from Whatman International Ltd., England. Parafilm 'M' was supplied by American Can Company, USA. All glass distilled water was used throughout these studies. All the experiments were performed at 25°C unless otherwise stated.

3.2. Methods

3.2.1. Analytical procedures

3.2.1.1. Protein concentration

Protein concentration was determined either spectrophotometrically or by the method of Lowry *et al.* (1951) using BSA as the standard.

Spectrophotometric method: The concentration of glucoamylase stock solution was determined by taking it in a quartz cuvette of 1 cm pathlength followed by absorbance measurements at 280 nm on a Shimadzu double beam spectrophotometer, model UV-2450, using a molar extinction coefficient of $1.37 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 280 nm (Clarke and Svensson, 1984). Scattering corrections, if required, were made by extrapolation of the absorbance values in the wavelength range, 340–360 nm to the desired wavelength.

Method of Lowry et al. (1951): The standard curve for the determination of protein concentration was prepared by taking increasing volumes (0.1–1.0 ml) of the stock protein solution (0.45 mg BSA/ml) in different tubes. The volume in each tube was made to 1.0 ml, if required, with 0.06 M sodium phosphate buffer, pH 7.0, followed by the addition of 5.0 ml of freshly prepared copper reagent. The contents were vortexed and incubated for 10 minutes at room temperature. Then 1 ml of diluted (1:4) Folin-phenol reagent was added, vortexed and further incubated for 30 minutes in dark at room temperature. The color intensity was read at 700 nm against suitable blanks prepared in the same way except that 1 ml of buffer was used instead of the protein solution. A standard curve between the absorbance at 700 nm and the protein concentration yielded the following straight line equation (Figure 3.3):

$$(\text{Absorbance})_{700\text{nm}} = 1.28 \times (\text{protein concentration, mg/ml}) + 0.03 \quad (1)$$

The above equation was used to determine the protein concentration by the method of Lowry *et al.* (1951).

3.2.1.2. ANS concentration

A molar extinction coefficient of $5 \times 10^3 \text{ M}^{-1}.\text{cm}^{-1}$ at 350 nm was employed to determine the concentration of stock ANS solution (Mulqueen and Kronman, 1982).

3.2.1.3. NATA concentration

The concentration of NATA was determined spectrophotometrically using a molar extinction coefficient of $5630 \text{ M}^{-1}.\text{cm}^{-1}$ at 280 nm (Alston *et al.*, 2008).

3.2.1.4. GdnHCl concentration

The method described by Pace *et al.* (1989) was used to determine the concentration of stock GdnHCl solution.

3.2.1.5 Curve fitting

Lines in all curves were drawn as a guide for the eyes using either curve fitting mode of SIGMAPLOT software, version 11, or nonlinear curve fitting mode of ORIGIN software, version 8.5.

Each experiment was repeated at least three times and the percentage error in the observed values was less than $\pm 5\%$.

3.2.2. pH measurements

pH measurements were made at 25°C on a Mettler-Toledo pH meter, model Delta 320 using a BNC's combined electrode, type HA405-K2/120, consisting of glass and reference electrodes in a single entity. The pH meter was routinely calibrated with standard buffers of pH 7.0 and pH 4.0 for pH measurements in the neutral and acidic range, respectively. The least count of the pH meter was 0.01 pH unit.

3.2.3. Spectral measurements

Following spectroscopic techniques were used in this study.

3.2.3.1. Circular dichroism spectroscopy

Circular dichroism (CD) measurements in both near-UV (250–300 nm) and far-UV (200–250 nm) range were carried out on a Jasco spectropolarimeter, model J-815, equipped with a thermostatically-controlled cell holder, attached to a water bath under constant nitrogen flow. The instrument was calibrated with (+)-10-camphorsulfonic acid and all measurements were recorded at 25°C using a scan speed of 50 nm/minute and a response time of 1 second. For far-UV and near-UV CD measurements, protein concentration and path length of the cell used were 1.4 μM; 1 mm and 12 μM; 10 mm, respectively. Each spectrum was the average of three scans and was corrected with suitable blank. The results are expressed as mean residue ellipticity (MRE) in deg.cm².dmol⁻¹, obtained by dividing the molar ellipticity with the number of residues in the protein using the following equation (Bychkova *et al.*, 1992):

$$\text{MRE} = \frac{\theta_{obs}}{(10 \times n \times C_p \times l)} \quad (2)$$

where θ_{obs} is the observed ellipticity in millidegrees; n is the total number of amino acid residues (616) in the protein; C_p is the molar concentration and l is the path length in cm.

The α -helical content was calculated either from the MRE values at 222 nm using the following formula (Chen *et al.*, 1972):

$$\% \text{ Helix} = \left(\frac{\text{MRE}_{222} - 2340}{30300} \right) \times 100 \quad (3)$$

or computed using DICHROWEB software (Whitmore and Wallace, 2008) with the help of K2D algorithm (Andrade *et al.*, 1993).

3.2.3.2. Fluorescence spectroscopy

Fluorescence measurements were performed on a Jasco spectrofluorometer, model FP-6500 equipped with a data recorder and a thermostatically-controlled cell holder, attached to a water bath to maintain the constant temperature at 25°C. Excitation and emission slits were fixed at 10 nm each and the fluorescence spectra were recorded in the wavelength range, 300–400 nm for acid denaturation studies and 310–400 nm for alcohol/polyol-induced structural transition studies or at a fixed wavelength of 340/342 nm upon excitation at 295 nm using a protein concentration of 0.12 µM in a 1 cm path length cell. Each sample was prepared fresh and separately in a single tube and was used once. In titration experiments, various protein samples (10–24) containing similar protein concentration but different solvent composition, were used.

For ANS fluorescence experiments, the excitation wavelength was set at 380 nm and the emission spectra were recorded in the wavelength range, 400–600 nm or at a fixed wavelength of 470/478 nm, using a protein concentration of 0.26 µM. [ANS]/[protein] molar ratio was kept as 70:1 in all experiments. Similar molar ratio has been used in several earlier studies (Fink *et al.*, 1993; Matulis *et al.*, 1999; Haq *et al.*, 2005).

Fluorescence intensity values, obtained at different alcohol concentrations were transformed into relative fluorescence intensity by taking the fluorescence intensity of acid-denatured glucoamylase, in the absence of alcohol as 100.

Three-dimensional (3-D) fluorescence experiments involved the use of a protein concentration of 0.12/0.13 µM and the wavelength range of 220–500 nm for emission spectra. The protein solution was excited at 220 nm with an increment of 10 nm and the

number of scanning curves was kept as 19. In alcohol-induced transition studies, appropriate blanks were prepared and subtracted from the sample spectra.

3.2.4. Viscosity measurements

Determination of viscosity was made on a Brookfield digital viscometer, model DV-II+ Pro, MA, USA, attached to UL adaptor. The sample chamber was fitted into a water jacket in order to achieve the desired temperature by using the Brookfield circulating temperature bath. The viscosity was determined using 15 ml of the protein solution and the values were recorded in centipoise (cP) in the shear rate range, 40–75 s⁻¹ at 25°C using a protein concentration of 13.75 μM. Blank solutions were also treated in the same way and their viscosity values were subtracted from the viscosity values of the corresponding protein solutions to get the viscosity of the protein. Experiments were performed in triplicate and the average values of the viscosity, thus obtained, were plotted against the shear rate.

3.2.5. Acrylamide quenching

In the acrylamide quenching experiments, increasing volumes (0.03–0.40 ml) of the stock (5.0 M) acrylamide solution were added to a constant volume (0.5 ml) of the stock (1.2 μM) protein solution, taken in different tubes in a total volume of 5.0 ml. The excitation wavelength was set at 295 nm and the emission spectra were recorded in the wavelength range, 300–400 nm. The decrease in the fluorescence intensity at emission maximum was analyzed according to the Stern-Volmer equation (Eftink and Ghiron, 1981).

$$F_0/F = 1 + K_{sv} [Q] \quad (4)$$

where F_0 and F are the fluorescence intensities at an appropriate wavelength in the absence and the presence of quencher (acrylamide) respectively; K_{sv} is the Stern-

Volmer constant and $[Q]$ is the concentration of the quencher. K_{sv} values were calculated by the least squares analysis of the initial linear points of the Stern-Volmer plots.

3.2.6. Denaturation studies

3.2.6.1. Acid denaturation

To 0.5 ml of protein solution, prepared in distilled water, equal volumes (4.5 ml) of different buffers of similar molarity (10 mM) but different pH in the range of pH 0.5–7.0 [*i.e.* KCl-HCl mixture (pH 0.5–0.9), glycine-HCl buffer (pH 1.0–3.0), sodium acetate buffer (pH 3.5–5.3), sodium phosphate buffer (pH 6.0–7.0)] were added and incubated for 12 hours at 4°C [sufficient for equilibrium attainment (Devaraj *et al.*, 2011)]. The pH values of the protein samples were checked both before and after CD and fluorescence measurements and were found to lie within ± 0.1 pH unit. Both far-UV CD and fluorescence spectra were recorded in the same way as described in section 3.2.3. for ANS binding experiments, a 70 fold molar excess of ANS to protein concentration (0.26 μ M) was mixed in different buffers.

3.2.6.2. Thermal denaturation

Thermal transition studies in the temperature range, 0–100°C were carried out using far-UV CD spectroscopy in the same way as described in section 3.2.3.1. except that samples in the cell were incubated at the desired temperature for 6 minutes for equilibration (Ahmad and Rao, 2009) before recording the spectra.

3.2.6.3. GdnHCl denaturation

Denaturation of glucoamylase in the presence of increasing GdnHCl concentrations was studied both in 10 mM glycine-HCl buffer (pH 1.0) and in 10 mM sodium phosphate buffer (pH 7.0). Stock protein (14 μ M) and GdnHCl (7.8 M) solutions were

prepared separately in each buffer. Increasing volumes of GdnHCl stock solution were added to a constant volume (0.5 ml) of the protein solution to get the desired GdnHCl concentration in the range, 0.0–7.0 M in a total volume of 5.0 ml and the mixture was incubated for 12 hours at 25°C [sufficient for equilibrium attainment (Devaraj *et al.*, 2011)] before far-UV CD measurements.

3.2.6.4. Data analysis

Denaturation curves obtained with thermal and GndHCl denaturation studies were analyzed using two-state model. The unfolding curves for the N \leftrightarrow D transition were normalized into the apparent fraction of the unfolded form, F_D , using the following equation (Tanford, 1968):

$$F_D = (Y - Y_N) / (Y_D - Y_N) \quad (5)$$

where Y is the observed variable parameter and Y_N and Y_D represent the values of the variable characteristics of the folded (native) and unfolded (denatured) states, respectively, of the protein. The equilibrium constant of the unfolding, K , was obtained using the following formula

$$K = F_D / (1 - F_D) \quad (6)$$

The difference in the free energy between the folded and unfolded states, ΔG was calculated using the following equation:

$$\Delta G = -RT \ln K \quad (7)$$

where R is the gas constant (1.987 cal / deg / mol) and T is the absolute temperature.

A least squares analysis of ΔG obtained in Eq. (7) as a function of denaturant concentration, $[D]$ was used to fit the data to the following equation for the

determination of $\Delta G_D^{H_2O}$, the free energy change in the absence of denaturant (Pace *et al.*, 1989).

$$\Delta G = \Delta G_D^{H_2O} - m [D] \quad (8)$$

where m is a measure of the dependence of ΔG on denaturant concentration in $\text{cal.mol}^{-1}.\text{M}^{-1}$. Values of $\Delta G_D^{H_2O}$ and m were obtained from the y-intercept and the slope of the linear plot (ΔG versus $[D]$), respectively.

The midpoint concentration of unfolding (C_m) was determined using the following equation (Yagi *et al.*, 2003):

$$C_m = \Delta G_D^{H_2O} / m \quad (9)$$

Alternatively, C_m values were obtained from F_D versus GdnHCl concentration curves *i.e.* the denaturant concentration at which F_D is equal to 0.5.

In thermal denaturation studies, the midpoint temperature, T_m was determined from the plot of ΔG versus temperature as the temperature at which ΔG is equal to zero. The value of T_m was also obtained from the F_D versus temperature curve as the temperature at which F_D is equal to 0.5.

The enthalpy change (ΔH) was determined using van't Hoff plot ($\ln K_D$ versus $1/T$) according to the following equation.

$$d(\ln K_D) / d(1/T) = -\Delta H/R \quad (10)$$

The value of ΔH was also obtained from the plot of ΔG versus temperature (T) using the following equation:

$$\Delta G = \Delta H - T\Delta S \quad (11)$$

where ΔS is the entropy change of the reaction which was obtained from the slope of the linear plot (ΔG versus T). Since ΔG is zero at T_m , ΔH_m was obtained as the product of T_m and ΔS_m (Pace *et al.*, 1989).

Values of T_m and ΔH were used to estimate ΔG at 25°C with the help of the following equation after assuming the heat capacity change (ΔC_p) to be zero (Pace *et al.*, 1989; Iida *et al.*, 2008).

$$\Delta G(T) = \Delta H(T_m) (1 - T/T_m) - \Delta C_p [(T_m - T) + T \ln (T/T_m)] \quad (12)$$

3.2.7. Preparation of the acid-denatured and the native forms of glucoamylase

Acid-denatured form of glucoamylase was prepared by dissolving the protein in 10 mM glycine-HCl buffer, pH 1.0 and incubating it at 4°C for 12 hours [sufficient for equilibrium attainment (Devaraj *et al.*, 2011)]. For the preparation of the native form of the enzyme, the protein was dissolved in 10 mM sodium phosphate buffer, pH 7.0. These preparations were stored at 4°C.

3.2.8. Alcohol-induced structural transition studies

The conformational transitions in glucoamylase from the acid-denatured state at pH 1.0 to the alcohol-induced state were studied by taking increasing volumes of various alcohols in different tubes and the volume was made to 4.5 ml in each tube with 10 mM glycine-HCl buffer, pH 1.0. This was followed by the addition of a constant volume (0.5 ml) of the stock protein solution, prepared in the same buffer. The contents in each tube were mixed well and the mixture was incubated for 30 minutes at 25°C before CD / fluorescence measurements. Alcohol-induced transitions are rapid and complete within a few minutes (Hirota *et al.*, 1997). Appropriate blanks were prepared in the same way except that 10 mM glycine-HCl buffer, pH 1.0 was used instead of protein solution. Secondary structural changes in the acid-denatured glucoamylase upon addition of

increasing concentrations of various alcohols were monitored using far-UV CD spectral signal, whereas the change in the tertiary structure was studied by near-UV CD spectroscopy, Trp fluorescence and ANS fluorescence.

The transition curves obtained by both MRE_{222nm} and ANS fluorescence measurements were analyzed using two-state model. Both ΔG_H and m (dependence of ΔG_H on alcohol concentration) were determined in the same way as described in section 3.2.6.4. The concentration of alcohol required to achieve 50% helix-induced state of glucoamylase (midpoint concentration or C_m value) was obtained from the transition curves by taking the alcohol concentration required to induce 50% helix transition as well as from ΔG_H versus alcohol concentration plots as used earlier (Haq *et al.*, 2005; Hirota *et al.*, 1997).

3.2.9. Polyol-induced structural transition studies

The structural changes in native and acid-denatured states of glucoamylase in the presence of various polyols were studied following the method described by Devaraneni *et al.* (2012). To 0.5 ml protein solution dissolved in water, 4.5 ml of the buffer (10 mM glycine-HCl buffer, pH 1.0 or 10 mM sodium phosphate buffer, pH 7.0) containing the desired polyol concentration was added. The contents in each tube were mixed gently and the mixture was incubated for 12 hours at 25°C [sufficient for equilibrium attainment (Yadav and Prakash, 2009)] before CD / fluorescence measurements. Blank solutions were prepared in the same way except that the protein solution was replaced with suitable buffer. Far-UV CD spectral signal was employed to monitor the secondary structural changes of glucoamylase in the presence of various polyols, whereas the change in the tertiary structure was studied by Trp fluorescence and ANS fluorescence measurements.

The effect of temperature on native glucoamylase both in the absence and presence of different polyols was studied using CD spectral signal, by measuring the MRE_{222nm} in the temperature range, 20–100°C. A scan rate of 1°C min⁻¹ was used throughout the temperature range, while other experimental conditions were kept same as described in section 3.2.3.1. Thermal denaturation curves were analyzed using the two-state model as described in section 3.2.6.4.

4. RESULTS AND DISCUSSION

4.1. Acid denaturation of glucoamylase

4.1.1. pH titration

Acid denaturation of glucoamylase was studied in the pH range, 7.0–0.5, using far-UV CD spectral signal at 222 nm (MRE_{222nm}), ANS fluorescence intensity at 470 nm and Trp fluorescence intensity at 340 nm as probes.

4.1.1.1. Far-UV CD

Far-UV CD spectrum was used to monitor the backbone conformation of the polypeptide (Georgescu *et al.*, 1998). Figure 4.1 shows pH titration results of glucoamylase as monitored by ellipticity measurements at 222 nm. As can be seen from the figure, MRE_{222nm} remained unchanged within the pH range, 7.0–3.0. Lowering down the pH below pH 3.0 produced a marked decrease in the MRE_{222nm} value, being minimum at pH 1.0. Although a decrease in the MRE_{222nm} was noticed in the pH range, 3.0–1.0, it was more pronounced between pH 1.5 and pH 1.0, suggestive of a two-step transition. Further decrease in pH below pH 1.0 led to a significant increase in the MRE_{222nm} . The absence of any change in the MRE_{222nm} within the pH range, 7.0–3.0 was suggestive of the pH stability of the enzyme within this pH range. This result was in accordance with the previous report showing relatively similar far-UV CD spectra of *Aspergillus niger* glucoamylase II at pH values 7.0 and 2.0 (Shenoy *et al.*, 1984). Significant decrease in the MRE_{222nm} value within the pH range, 3.0–1.0, indicated denaturation of the enzyme as reflected from the loss of the α -helical structure. Since the catalytic domain of glucoamylase contains 13 α -helical segments against a single α -helical segment in SBD (Sorimachi *et al.*, 1996; Lee and Paetzel, 2011), such a decrease

in the MRE_{222nm} can be ascribed to major structural changes in the catalytic domain within this pH range. However, a slow change in the beginning may account for the separation of the two domains. Considering the MRE_{222nm} value at pH 3.0 as 100, about 32% change in the ellipticity was observed on lowering down the pH from pH 3.0 to pH 1.0. Increase in the MRE_{222nm} value of glucoamylase upon further decreasing the pH from pH 1.0 to pH 0.6 suggested the re-formation of secondary structure due to acid-induced refolding, as observed earlier for a number of proteins (Muzammil *et al.*, 1999; Ahmad *et al.*, 2010; Varshney *et al.*, 2010). A large increase in the chloride ions below pH 1.0 might be responsible for acid-induced refolding.

4.1.1.2. ANS fluorescence

ANS, a hydrophobic dye is known to bind hydrophobic regions of the protein. Any exposure of hydrophobic regions upon unfolding of proteins can be studied by the increase in ANS binding as reflected by the increase in its fluorescence intensity at 470 nm (Engelhard and Evans, 1995). Acid titration results of glucoamylase as monitored by ANS fluorescence intensity at 470 nm are shown in Figure 4.2. Native glucoamylase did not produce any significant fluorescence intensity at 470 nm, which was suggestive of the lack of significant ANS binding sites in the native protein. The absence of any change in the ANS fluorescence intensity within the pH range, 7.0–1.5, suggested maintenance of protein's hydrophobic sites (tertiary structure) within this pH range. A marked increase in the ANS fluorescence intensity was observed on decreasing the pH from pH 1.5 to pH 1.0, where it showed the maximum fluorescence intensity. These results were suggestive of protein denaturation, which led to the exposure of hydrophobic regions of the protein to the solvent within this pH range. In view of the presence of several β -strands in the SBD region which is rich in the hydrophobic residues (Sorimachi *et al.*, 1996), it appears that structural deformation in the SBD

occurred within the pH range, 1.5–1.0.

Taken together, both far-UV CD and ANS fluorescence results suggested the start of the structural changes in the catalytic domain followed by the SBD upon lowering down the pH from pH 3.0 to pH 1.0. The decrease in the ANS fluorescence intensity at 470 nm below pH 1.0 indicated burial of some hydrophobic patches in the protein interior. Although these results were similar to our far-UV CD data, showing pH-induced transition in the pH range, 3.0–1.0, earlier start of the transition as monitored by the far-UV CD spectral signal compared to ANS fluorescence signal suggested greater sensitivity of secondary structure to pH change compared to tertiary structure. Anion-induced refolding (Goto *et al.*, 1990) can account for the re-formation of secondary structure as evident by the increase in MRE_{222nm} (Figure 4.1), as well as burial of the hydrophobic sites of the protein leading to lesser ANS binding below pH 1.0 (Figure 4.2).

4.1.1.3. Tryptophan fluorescence

A protein gives fluorescence intensity in the wavelength range, 300–400 nm due to Trp fluorescence upon excitation at 295 nm. In native protein, Trp residues are buried in the protein interior in a non-polar environment. Movement of charged groups along with hydrophobic changes in the microenvironment are known to affect the fluorescence intensity whereas change in the wavelength maxima is uniquely sensitive to hydrophobic changes in the microenvironment (Khanna *et al.*, 1986). Figure 4.3 shows acid titration results of glucoamylase when monitored by Trp fluorescence. Fluorescence intensity at 340 nm remained unchanged within the pH range, 7.0–3.0, suggesting stability of glucoamylase within this pH range. Decreasing the pH below pH 3.0 led to a significant decrease in the fluorescence intensity, being more marked within the pH range, 1.5–1.0. Such decrease in the fluorescence intensity indicated protein

denaturation below pH 3.0 and can be ascribed to the exposure of Trp residues to the polar environment (Khanna *et al.*, 1986).

No significant change in the wavelength maxima of glucoamylase was observed during pH-induced structural changes. There was a small red shift of 2 nm during the pH transition from pH 7.0 to pH 1.0. Since there are 18 Trp residues in glucoamylase, distributed as 3, 8 and 7 in three different CNBr fragments, CB1, CB2 and CB3, respectively (Svensson *et al.*, 1983), it seems possible that a few Trp residues might have moved from nonpolar to polar region viz.-a-viz. the movement of other Trp residues from polar to nonpolar region in the protein molecule during pH transition. This may account for the absence of any major shift in the wavelength maxima. Fluorescence intensity results were in agreement with those observed with far-UV CD measurements in the pH range, 7.0–1.0 (Figure 4.1) as well as ANS fluorescence measurements in the pH ranges, 7.0–3.0 and 1.5–1.0 (Figure 4.2) as described above. However, the acid-induced refolding could not be observed below pH 1.0 with this probe, as the fluorescence intensity continued to decrease down to pH 0.5. Interestingly, the decrease in the fluorescence intensity below pH 1.0 was relatively slower with a smaller slope value compared to the decrease observed within the pH range, 1.5–1.0. Considering the presence of a large number of Trp residues in this protein, it seems quite possible that below pH 1.0, a partially folded state could have been generated, characterized by the presence of higher secondary structure, decreased exposure of hydrophobic segments but increased exposure of Trp residues to a polar environment.

4.1.2. Characterization of the acid-denatured state at pH 1.0

As revealed by both far-UV CD spectral signal (Figure 4.1) and ANS fluorescence signal (Figure 4.2), glucoamylase was maximally denatured at pH 1.0. However, calculation of α -helical content using Eq. (3) suggested a decrease in the α -helical

content from ~27% (at pH 7.0) to ~17% (at pH 1.0). Significant increase in ANS binding and significant retention of α -helical content of acid-denatured state at pH 1.0 (Table 4.1) prompted us to characterize this state (at pH 1.0) in detail using the following probes and compare it with the native state as well as 6.0 M GdnHCl-denatured state.

4.1.2.1. Far-UV CD spectra

Figure 4.4 shows far-UV CD spectra of glucoamylase in the native state (pH 7.0), the acid-denatured state (pH 1.0) and 6.0 M GdnHCl-denatured state. As can be seen from the figure, far-UV CD spectrum of glucoamylase in the native state (pH 7.0) was characterized by the presence of two negative peaks at 210 and 219 nm which were suggestive of the presence of α -helical structure (Georgescu *et al.*, 1998). A calculation of α -helical content using Eq. (3) suggested the presence of ~27% α -helix in native glucoamylase (Table 4.1). This was in accordance with a previous report (Freedberg *et al.*, 1975), suggesting the presence of 15–25% α -helix in glucoamylase. Based on 13 α -helical segments, involving a total of 224 residues as revealed by X-ray crystallographic data (Aleshin *et al.*, 1992), percentage of α -helix was calculated to be ~36%, which agreed well with the calculated value obtained in this study. Far-UV CD spectrum of the acid-denatured glucoamylase at pH 1.0, although showed a significant decrease in MRE at both wavelengths compared to the far-UV CD spectrum of the native enzyme, but retained all spectral characteristics to a significant extent, showing elements of the secondary structure of the native protein. Only 30% decrease in the MRE_{222nm} was observed in the acid-denatured state compared to that obtained with the native state (Table 4.1). On the other hand, glucoamylase in 6.0 M GdnHCl showed a major loss in the secondary structural characteristics as evident by the ~83% decrease in the

MRE_{222nm} (Table 4.1). This was in line with other reports showing the random coil conformation of proteins in the presence of 6.0 M GdnHCl (Tanford *et al.*, 1967).

4.1.2.2. Near-UV CD spectra

The CD spectra of glucoamylase in the native, acid-denatured and 6.0 M GdnHCl-denatured states in the near-UV region (250–300 nm) are shown in Figure 4.5. This probe is normally used to monitor the changes in the environment of the aromatic chromophores of the protein (Kamen *et al.*, 2000). Near-UV CD spectrum of the native glucoamylase showed the presence of two negative peaks at 278 and 285 nm with a small trough around 270 nm. These characteristics were similar to an earlier report, showing the presence of negative signals at 275, 278 and 285 nm in the near-UV CD spectrum of glucoamylase (Freedberg *et al.*, 1975). In the acid-denatured state, the protein showed a loss of the major signal at 278 nm but retained significant negative ellipticity (72%) in the near-UV CD spectrum (Table 4.1). Contrary to it, 6.0 M GdnHCl-denatured state of glucoamylase showed complete loss of spectral signals throughout the near-UV range. Although glucoamylase contains a significantly large number of aromatic amino acids (18 Trp and 27 tyrosine (Tyr)) residues (Svensson *et al.*, 1983), these results indicated partial unfolding of the protein at pH 1.0 in comparison to the spectrum of the completely unfolded state of the protein, obtained in 6.0 M GdnHCl (Table 4.1).

4.1.2.3. Fluorescence spectra

Figure 4.6 shows Trp fluorescence spectra of glucoamylase in the native state (pH 7.0), the acid-denatured state (pH 1.0) and in the presence of 6.0 M GdnHCl in the wavelength range, 300–400 nm upon excitation at 295 nm. The fluorescence spectrum of native glucoamylase was characterized by the presence of an emission maximum at 340 nm, a characteristic of Trp fluorescence (Muzammil *et al.*, 2000). A marked

decrease (36%) in the fluorescence intensity at the emission maximum along with a 2 nm red shift were observed in the fluorescence spectrum of the acid-denatured state (pH 1.0) of glucoamylase (Table 4.1). Both these characteristics indicated the exposure of Trp residues to the polar environment (Khanna *et al.*, 1986) in the acid-denatured state. On the other hand, fluorescence spectrum of glucoamylase in the presence of 6.0 M GdnHCl showed a marked red shift of 13 nm and significant decrease (32%) in the fluorescence intensity (Table 4.1). In view of the complete denatured state of glucoamylase obtained in the presence of 6.0 M GdnHCl, the acid-denatured state with a small red shift of only 2 nm against 13 nm observed with 6.0 M GdnHCl-denatured state, seems to retain significant amount of the tertiary structure. This was in agreement with our near-UV CD spectral data, suggesting partial unfolding of the protein at pH 1.0.

4.1.2.4. ANS fluorescence spectra

ANS binding to proteins has been generally employed to detect the molten globule states (Engelhard and Evans, 1995). ANS fluorescence spectra in the wavelength range, 400–600 nm, obtained in the presence of native and acid-denatured glucoamylases are shown in Figure 4.7. Glucoamylase in the native state produced a weak ANS fluorescence spectrum with an emission maximum at 470 nm, suggesting burial of the hydrophobic regions in the protein interior in its native state. Several proteins have been shown to produce a weak ANS fluorescence signal in their native states (Tatsumi and Hirose 1997; Muzammil *et al.*, 1999). A pronounced increase in the fluorescence intensity accompanied by 8 nm red shift (from 470 to 478 nm) was observed in the fluorescence spectrum of ANS in the presence of the acid-denatured glucoamylase (Table 4.1). These results suggested a large increase in the exposure of the hydrophobic regions of the protein to the solvent at pH 1.0. Various reports have shown different

results (blue shift, no shift or red shift) with respect to the change in the emission maximum in ANS binding experiments of the molten globule states (Muzammil *et al.*, 1999; Fatima *et al.*, 2007; Varshney *et al.*, 2010). The nature of the shift in the emission maximum can be attributed to experiencing a more / less polar microenvironment by the probe in the molten globule state of the protein (Khanna *et al.*, 1986). In view of these findings, it appears that the acid-denatured glucoamylase although retained significant secondary and tertiary structures but possessed a large number of solvent-exposed hydrophobic sites.

4.1.2.5. Thermal transition

Figure 4.8 shows thermal denaturation curves of the native and the acid-denatured glucoamylases as monitored by ellipticity measurements at 222 nm. As can be seen from the figure, MRE_{222nm} value of the native glucoamylase showed smaller variations in the temperature range, 5–45°C. A sharp transition, characterized by a marked decrease in the MRE_{222nm} value occurred in the temperature range, 55–80°C, beyond which no significant change in the MRE_{222nm} was noticed up to 100°C. In an earlier study, Shenoy *et al.* (1985) have also shown unfolding of different glucoamylases at 60°C. The thermal denaturation of the native glucoamylase, showing loss in the secondary structure was found to be a cooperative process (Table 4.1). On the other hand, the acid-denatured glucoamylase showed smaller variations in the MRE_{222nm} value throughout the temperature range, suggesting thermal stability with reference to the secondary structures of the enzyme in the acid-denatured state. In other words, absence of any significant change in the MRE_{222nm} value of the acid-denatured enzyme within the temperature range, 0–100°C indicated more or less similar stability / denatured states obtained by thermal and acid denaturations. Intermolecular disulfide pairing involving free cysteine residue, Cys 320 (Svensson *et al.*, 1983), which may be

easily accessible in the acid-denatured glucoamylase as a result of conformational change, might explain the unusual thermostability of the acid-denatured state. In an earlier study (Sasvari and Asboth, 1998), increased thermostability of glucoamylase resulting from disulfide-bridged dimer formation has also been reported. Due to the absence of any change in the spectral signal with increasing temperature, it is difficult to comment on the cooperativity of the thermal transition of the acid-denatured glucoamylase.

Thermal unfolding curve of the native glucoamylase (Figure 4.8) was normalized into F_D curve as shown in Figure 4.9, whereas the inset of Figure 4.9 shows the variation of ΔG with temperature. Values of the midpoint temperature, T_m and the enthalpy change, ΔH for the thermal unfolding of the native glucoamylase were determined following the methods described in section 3.2.6.4. and both methods yielded similar values, *i.e.* 65.8°C and 54.5 kcal mol⁻¹ for T_m and ΔH_{vH} , respectively (Table 4.2). Similar value of ΔH_m (54.54 kcal mol⁻¹) was obtained from the ΔG versus temperature plot. The T_m value, obtained in this study was in agreement with previously reported T_m values of *Aspergillus niger* glucoamylase based on differential scanning calorimetry data (Williamson *et al.*, 1992; Christensen *et al.*, 1999; 2002; Wang *et al.*, 1996). Interestingly, glucoamylase from a thermophilic mold, *Thermomucor indicaeseudaticae* was found to possess a similar T_m value (67.1°C) (Kumar *et al.*, 2010), suggesting similar thermal stabilities of these enzymes. A value of 6.57 kcal mol⁻¹ was obtained for ΔG at 25°C using Eq. (12) (Table 4.2).

4.1.2.6. GdnHCl denaturation

In order to investigate the unfolding behavior of the acid-denatured enzyme, we studied GdnHCl denaturation of both native and acid-denatured glucoamylases using MRE_{222nm} as a probe and the results are shown in Figure 4.10. Although both native and

acid-denatured enzymes showed cooperative transition, differences were noticed in the start-point of the transition curve. Whereas the native glucoamylase showed a start-point at 2.0 M GdnHCl, similar to the one reported earlier (Christensen *et al.*, 2002), denaturation of the acid-denatured enzyme started at 4.0 M GdnHCl. Both transition curves merged on to each other in the GdnHCl concentration range, 4.25–7.0 M. It appears that the acid-denatured glucoamylase acquired a conformation, similar to the one achieved by the native enzyme in the presence of ~4.25 M GdnHCl. Furthermore, these results also showed that the acid-denatured state of the enzyme was not the fully denatured state, as shown for a number of proteins (Muzammil *et al.*, 1999; Ahmad *et al.*, 2010; Varshney *et al.*, 2010).

Normalization of GdnHCl denaturation data of both native and acid-denatured states of glucoamylase into the fraction denatured (F_D) as well as ΔG_D values were made using the two-state model and the plots are shown in Figures 4.11 and 4.12, respectively. Values of $\Delta G_D^{H_2O}$, m and C_m were obtained from the ΔG_D versus GdnHCl concentration plot (Figure 4.12) and are given in Table 4.3. The value of $\Delta G_D^{H_2O}$ was found in agreement with the ΔG (25°C) estimated from the thermal unfolding curve (Table 4.2). The values of C_m obtained from the F_D curves shown in Figure 4.11 (4.26 M and 4.76 M for the native and the acid-denatured enzymes, respectively) were found similar to those obtained from the ΔG_D versus GdnHCl concentration plot shown in Figure 4.12 (Table 4.3). As can be seen from Figure 4.11 and Table 4.3, the acid-denatured state of glucoamylase was found to be relatively more stable compared to the native state based on their C_m and $\Delta G_D^{H_2O}$ values. It should be recalled that the acid-denatured state had already undergone structural changes and matched with the GdnHCl-denatured state obtained at 4.25 M GdnHCl (Figure 4.10) in terms of the helical content. In other words, structures of these two states (native and acid-denatured)

of glucoamylase are very different from each other and therefore, a direct conclusion about their stabilities from GdnHCl denaturation data may be misleading. Possible explanation for the increased stability of the molten globule-like state at pH 1.0, compared to that shown by the native state of the enzyme can be ascribed to differential effects of GdnHCl toward different conformational states of the protein (Hagihara *et al.*, 1994).

4.1.2.7. Acrylamide quenching

For detailed information about the microenvironment around Trp residues in the native, the acid-denatured and the 6.0 M GdnHCl-denatured glucoamylases, quenching experiments were performed using uncharged molecule of acrylamide, as a quencher (Eftink and Ghiron, 1981). Figure 4.13 shows the Stern–Volmer plots of the fluorescence quenching results along with the theoretical curves by fitting the data to Eq. (4). For getting the complete accessibility to the quencher, Trp analog, NATA was used as a standard. The values of the Stern–Volmer constant were determined from the slope of initial linear parts of the Stern–Volmer plots and are listed in Table 4.4. Stern–Volmer plots showed upward deviation at higher acrylamide concentrations, which was also observed with other proteins (Ray *et al.*, 2005). Increase in the K_{sv} value was suggestive of the exposure of the Trp residues to the solvent due to the conformational change in the protein (Chakraborty *et al.*, 2001). As shown in Figure 4.13 and Table 4.4, K_{sv} value of glucoamylase in the acid-denatured state (5.2 M^{-1}) was found to be relatively higher than that (4.2 M^{-1}) obtained with the native state, but lower than the K_{sv} value of the 6.0 M GdnHCl-denatured glucoamylase (10.2 M^{-1}). On the other hand, NATA, showing complete accessibility, was found to possess a K_{sv} value of 21.0 M^{-1} (Table 4.4), which was in agreement with the previously published value (Muzammil *et al.*, 1999; Christov *et al.*, 2004). These results showed that the Trp residues of

glucoamylase in the acid-denatured state (pH 1.0) were relatively more accessible to the solvent than that in the native state.

4.1.2.8. Three-dimensional fluorescence spectra

Three-dimensional fluorescence spectroscopy has been recently employed as an additional probe to monitor the conformational changes in proteins (Li *et al.*, 2011). In order to verify the results obtained with the far- and the near-UV CD data with respect to the secondary and the tertiary structural changes, 3-D fluorescence spectroscopy was used to compare the native and the acid-denatured states of the enzyme. Figures 4.14 and 4.15 show 3-D fluorescence spectra (A) and corresponding contour maps (B), representing a bird's eye view of the fluorescence spectra, of glucoamylase in the native state (Figure 4.14) and the acid-denatured state at pH 1.0 (Figure 4.15). The 3-D fluorescence spectral characteristics in terms of the peak position and the intensity of various peaks, obtained with the native and the acid-denatured glucoamylases are given in Table 4.5. Two peaks (*a* and *b*) were common in all spectra, representing the Rayleigh scattering peak ($\lambda_{\text{ex}} = \lambda_{\text{em}}$) and the second-order scattering peak ($\lambda_{\text{em}} = 2\lambda_{\text{ex}}$), respectively. As shown in Table 4.5, these scattering peaks were also visible in the 3-D fluorescence spectra of buffers (figures omitted for brevity). A comparison of Figures 4.14 and 4.15 clearly shows significant differences in the 3-D fluorescence maps of the native and the acid-denatured glucoamylases. The presence of protein in the buffer enhanced the scattering effect, thus led to the increase in the fluorescence intensity of peaks *a* and *b*. On the other hand, both peaks 1 and 2 represented typical fluorescence peaks of the protein. As shown in Figures 4.14 and 4.15 and Table 4.5, peak 1 ($\lambda_{\text{ex}} = 280$ nm, $\lambda_{\text{em}} = 338$ nm) was related to the fluorescence spectral characteristics of Trp and Tyr residues due to $\pi \rightarrow \pi^*$ transitions, showing changes in the tertiary structure (Kang *et al.*, 2004), whereas peak 2 ($\lambda_{\text{ex}} = 230$ nm, $\lambda_{\text{em}} = 338$ nm) represented the

fluorescence characteristics of the polypeptide backbone as a result of $n \rightarrow \pi^*$ transitions, a probe for the secondary structural change (Glazer and Smith, 1961). There was a significant decrease in the intensity of both peaks 1 and 2 accompanied by a 3 nm red shift in the 3-D fluorescence spectra of the acid-denatured glucoamylase. The decrease in the fluorescence intensity of the two peaks suggested conformational changes in the protein resulting in the exposure of some buried sites (Trp / Tyr / peptide groups). These results suggested disruption of both tertiary and secondary structures of the protein in the acid-denatured state and were in accordance with our CD data.

In summary, glucoamylase was found stable in the pH range, 7.0–3.0, showed domain separation and structural changes in the catalytic domain as characterized by the disruption of the α -helical structure and exposure of the Trp residues to polar environment within the pH range, 3.0–1.0, structural deformation in the SBD as characterized by the exposure of hydrophobic segments from pH 1.5 to pH 1.0, formation of the molten globule-like state at pH 1.0 and acid-induced partially folded state below pH 1.0 (Figure 4.16). The acid-denatured state of glucoamylase at pH 1.0 may be characterized as the molten globule-like state, based on the retention of significant amount of secondary structure, deformed tertiary structure, more exposed hydrophobic segments showing increased ANS binding and lack of cooperative thermal transition. The molten globule-like state of glucoamylase remained stable, showed no significant structural change within the temperature range, 55–80°C where the native glucoamylase produced a single-step cooperative transition. Furthermore, GdnHCl denaturation of the molten globule-like state of glucoamylase showed a single-step cooperative transition within GdnHCl concentrations, 4.0–6.0 M against a single-step cooperative transition shown within 2.0–6.0 M GdnHCl concentration range by the native enzyme (Figure 4.16). These structural features along with the stability

characteristics obtained from the unfolding transitions may give us an insight to understand the folding mechanism of this enzyme.

The molten globule state of several proteins has been characterized in detail and the mechanism that governs the conformational change of the molten globule state to the unfolded state of such proteins is not very clear (Goto *et al.*, 1993). Different proteins have shown different behavior in thermal and denaturant-induced unfolding of their molten globule states. Whereas cytochrome c, myoglobin and apomyoglobin have been found to unfold cooperatively through a two-state transition mechanism from their molten globule states to the unfolded states (Nishii *et al.*, 1994; Nakamura *et al.*, 2007), many proteins including α -lactalbumin have shown a gradual decrease in their structural organization (noncooperative transition) with the increase in temperature (Dolgikh *et al.*, 1985; Kuwajima, 1989). On the other hand, denaturant-induced unfolding of the molten globule state of α -lactalbumin was found to be cooperative although its thermal unfolding represented a non-cooperative transition. In view of the above, folding mechanism of cytochrome c seems to be similar to that of myoglobin and apomyoglobin, whereas, α -lactalbumin and other proteins showing non-cooperative thermal transition may follow a different folding mechanism. Therefore, it appears that the molten globule state as well as folding mechanism differs from protein to protein. The absence of any significant structural change in the molten globule-like state of glucoamylase with increasing temperature (0–100°C) suggests thermal stability of the molten globule-like state. On the other hand, GdnHCl-induced unfolding of the molten globule-like state of glucoamylase showed cooperative transition. Furthermore, conformation of the molten globule-like state was found similar to that of native glucoamylase obtained in the presence of 4.25 M GdnHCl. Thus, molten globule-like

state of glucoamylase has different structural conformation from molten globule states of other proteins and probably follows a different folding mechanism.

4.2. Alcohol-induced structural transitions

4.2.1. Halogenol- versus alkanol-induced conformational transitions in the acid-denatured glucoamylase

The effect of various alcohols belonging to two different groups namely, halogenols (HFIP, TFE and 2-chloroethanol) and alkanols (tert-butanol, 1-propanol, 2-propanol, ethanol and methanol) on the acid-denatured state of glucoamylase at pH 1.0 was studied using far-UV CD spectral signal, ANS binding and Trp fluorescence.

4.2.1.1. Far-UV CD

Figure 4.17 shows TFE (a halogenol)-induced conformational transition in the acid-denatured glucoamylase at pH 1.0 as monitored by the far-UV CD spectral signal within a concentration range, 0.5–8.0 M. Despite many attempts, the CD spectra could not be obtained below 200 nm due to the high signal to noise ratio. Similar effect was noticed if the experiments were carried out in either KCl-HCl mixture, pH 1.0 or glycine-HCl buffer, pH 1.0. The presence of the chloride ions in the buffer might be responsible for the high signal to noise ratio due to strong absorption of the chloride ions below 195 nm (Kelly and Price, 2000). This seems more likely as the extent of signal to noise ratio was even more pronounced in presence of 2-chloroethanol where the CD spectra could not be obtained below 215 nm (figure not shown). Therefore, the CD spectra in presence of various alcohols except 2-chloroethanol were recorded in the wavelength range, 200–250 nm. The CD spectrum of the acid-denatured glucoamylase at pH 1.0 was characterized by the presence of two minima around 210 and 219 nm, indicative of the presence of the α -helical structure (Tayyab *et al.*, 2003). There was a gradual change, showing increase in the $-\text{MRE}_{222\text{nm}}$ value in the CD spectra upon increasing TFE concentrations up to 5.5 M beyond which smaller changes in the $-\text{MRE}_{222\text{nm}}$ value were noticed. The increase in the $-\text{MRE}_{222\text{nm}}$ value observed in the presence of TFE was

suggestive of the induction of the α -helical structure. In several earlier studies (Hirota *et al.*, 1998; Sen *et al.*, 2010; Anderson *et al.*, 2010), TFE has been shown to induce α -helical structures in proteins. About 98.5% increase in the $-\text{MRE}_{222\text{nm}}$ value was observed in the presence of 8 M TFE, suggesting an increase in the α -helical content from 28% in the acid-denatured state to 41% in the TFE-induced state (Table 4.6), as computed from the DICHROWEB software (Whitmore and Wallace, 2008).

Treatment of the acid-denatured glucoamylase at pH 1.0 with increasing concentrations of tert-butanol (an alkanol) in the concentration range, 0.5–6.0 M produced qualitatively similar CD spectra (Figure 4.18). However, the CD spectra obtained at the highest tert-butanol concentrations were characterized by the presence of a single minimum at ~ 216 nm, characteristic of β -structure (Dave *et al.*, 2010). Since glucoamylases possess significant amounts of both α -helix (30–35%) and β -structure (24–36%) (Shenoy *et al.*, 1985), formation of more β -structure in the presence of tert-butanol was not surprising. In order to compare the effectiveness of tert-butanol in terms of the helix-inducing potential, $\text{MRE}_{222\text{nm}}$ values obtained with different tert-butanol concentrations were used. A gradual increase in the $-\text{MRE}_{222\text{nm}}$ value was observed up to 3.0 M tert-butanol, beyond which it sloped off. About 52% increase in the $-\text{MRE}_{222\text{nm}}$ value was noticed at the highest (6.0 M) tert-butanol concentration, which corresponded to an increase in the α -helical content to 37% in the alcohol-induced state (Table 4.7).

A comparison of these results suggested greater potential of a halogenol (TFE) in inducing the α -helical structure compared to an alkanol (tert-butanol). Therefore, we studied the effect of two additional halogenols (HFIP and 2-chloroethanol) and four alkanols, namely, 1-propanol, 2-propanol, ethanol and methanol on the acid-denatured state of glucoamylase at pH 1.0 and the results are shown in Figures 4.19 and 4.20. As can be seen from these figures, both halogenols and alkanols showed structural

transition from the acid-denatured state to an alcohol-induced state, marked by the increase in the $-\text{MRE}_{222\text{nm}}$ value within the selected alcohol concentration range. However, significant differences were noticed in between the two groups of alcohols as well as among the alcohols within each group. To get further insight, the transition curves obtained with these alcohols (Figures 4.19 and 4.20) were analyzed assuming the two-state hypothesis in the same way as described in section 3.2.6.4. Tables 4.6 and 4.7 show the values of C_m (alcohol concentration required to achieve 50% of the total structural change), m (slope of the ΔG_H versus alcohol concentration plot) and the maximum $-\text{MRE}_{222\text{nm}}$ achieved at the highest alcohol concentration for halogenol- and alkanol-induced structural transitions, respectively. The values of the percentage helical content as computed from DICHROWEB software are also included in the last column of Tables 4.6 and 4.7. Due to high signal to noise ratio below 215 nm observed in the presence of 2-chloroethanol, the α -helix content could not be obtained as the K2D algorithm of the DICHROWEB software requires the CD spectra in the wavelength range, 200–250 nm (Andrade *et al.*, 1993). A comparison of the maximum $-\text{MRE}_{222\text{nm}}$ values along with the percentage α -helical content obtained with different alkanols suggested the formation of similar alcohol-induced state (in terms of the secondary structure) in the presence of various alkanols (Table 4.7). On the other hand, different alcohol-induced states, differing in the α -helical content seem to be induced by different halogenols (Table 4.6). Overall, halogenols appeared to be more effective in inducing the α -helical structure than alkanols (with similar chain length) as evident from the lower C_m and higher m values (Tables 4.6 and 4.7). In view of the formation of different alcohol-induced states by these alcohols, it would be an over simplification to compare the effectiveness of halogenols with that of alkanols. Among halogenols, HFIP was proved to be highly effective whereas 2-chloroethanol as the least effective in inducing

the α -helical structure. These results were similar to earlier reports, suggesting strong helix-inducing potential of fluoro alcohols (Hirota *et al.*, 1998; Khan *et al.*, 2000; Kumar *et al.*, 2004; Yoshida *et al.*, 2008). On the other hand, tert-butanol was found to be most effective in inducing the helical structure among various alkanols, based on the lowest C_m value and highest m value. Contrary to it, methanol showed the highest C_m value and lowest m value and appeared to be the weakest α -helix inducer among all the alkanols studied (Table 4.7). Overall, the effectiveness shown by alkanols was found to follow the order: tert-butanol > 1-propanol > 2-propanol > ethanol > methanol.

4.2.1.2. ANS binding

To study the change in the tertiary structure of the acid-denatured glucoamylase at pH 1.0 accomplished in the presence of different alcohols, ANS binding experiments were performed. ANS in free solution form showed an increase in its fluorescence upon addition of alcohols which became more pronounced with the decrease in the polarity of alcohols. In other words, a lesser increase in the ANS fluorescence was observed with methanol while tert-butanol produced the maximum increase in the ANS fluorescence among all the alkanols studied. On the other hand, presence of halogenols produced only a small increase in the ANS fluorescence, which was much lesser than the increase noticed with methanol. This can be clearly seen from the insets of Figures 4.21 and 4.22 showing ANS fluorescence intensity in the presence of increasing concentrations of TFE and 1-propanol, respectively. The hydrophobicity of alcohols has been suggested to contribute toward their binding to ANS molecules, thus leading to an increase in the ANS fluorescence (Alexandrescu *et al.*, 1994). Contrary to it, presence of glucoamylase in the reaction mixture showed a decrease in the ANS fluorescence at lower alcohol concentrations which became more or less similar to that shown by free ANS at higher alcohol concentrations (insets of Figures 4.21 and 4.22). Subtraction of the free ANS

fluorescence spectra from those obtained in the presence of glucoamylase yielded the transition curves of the acid-denatured glucoamylase in the presence of different halogenols and alkanols as shown in Figures 4.21 and 4.22, respectively. As evident from these figures, the ANS fluorescence intensity at 478 nm decreased steadily on increasing the alcohol concentration and became minimal at higher alcohol concentrations, suggesting lesser ANS binding to glucoamylase. The decrease in the ANS binding indicated re-burial of the hydrophobic regions of the protein, which were initially exposed in the acid-denatured state and this can be interpreted as the structure retrieval of glucoamylase in the presence of these alcohols. In several earlier studies, decrease in the ANS fluorescence intensity has been reported with different proteins in the presence of increasing concentrations of various alcohols (Alexandrescu *et al.*, 1994; Khan *et al.*, 2000; Haq *et al.*, 2005).

Both HFIP and TFE seem to induce a similar state, characterized by the lack of ANS binding but required different concentrations, being lower for HFIP than TFE (Figure 4.21). The absence of ANS binding, shown in the presence of higher alcohol concentrations indicated formation of a compact structure of the protein, with least exposure of the nonpolar hydrophobic clusters, required for ANS binding (Dave *et al.*, 2010). A closely similar state, characterized by the absence of ANS binding was also produced by other alkanols (Figure 4.22). This can be explained on the basis of similar surface hydrophobic make-up in the alcohol-induced states, thus formed. On the other hand, alcohol-induced state produced by 2-chloroethanol was different from the state produced by HFIP and TFE, as it showed a little ANS binding (Figure 4.21). Although the TFE-induced state was different from the HFIP-induced state in terms of the α -helical content (Figure 4.19), these states were indistinguishable with respect to surface hydrophobicity or tertiary structural make-up (Figure 4.21). Except methanol, all other

alcohols showed the completion of transition within the selected alcohol concentration range among various alkanols and halogenols. Therefore, these transition curves were treated in the same way as described above. The transition curves showed a similar pattern as that obtained with MRE_{222nm} measurements in terms of the effectiveness of each alcohol toward structure retrieval in the acid-denatured glucoamylase. For example, HFIP with a C_m value of 0.8 M was found to be most effective than TFE ($C_m = 2.1$ M) and 2-chloroethanol ($C_m = 2.6$ M). Similarly, tert-butanol ($C_m = 2.4$ M) and 1-propanol ($C_m = 2.5$ M) showed greater effectiveness than 2-propanol ($C_m = 3.0$ M) and ethanol ($C_m = 4.0$ M), among various alkanols studied.

4.2.1.3. Tryptophan fluorescence

Figures 4.23 and 4.24 show titration curves of the acid-denatured glucoamylase obtained with increasing concentrations of halogenols (Figure 4.23) and alkanols (Figure 4.24), when studied using Trp fluorescence at 342 nm. Whereas presence of alkanols led to an increase in the Trp fluorescence intensity, two halogenols showed an anomalous behavior. Both fluoro alcohols quenched the Trp fluorescence, being more significant with HFIP than TFE (Figure 4.23). This anomalous behavior can be ascribed to the presence of trifluoromethyl groups in the fluorinated alcohols compared to the nonfluorinated one. Both high electronegativity of F atoms and large field effect of trifluoromethyl groups tend to increase the acidity of the OH group, consequently, making these alcohols as active proton donors (Roberts *et al.*, 1950; Dyatkin *et al.*, 1965; Chen *et al.*, 1995; Othon *et al.*, 2009). Therefore, Trp fluorescence is quenched as a result of excited-state proton transfer from fluorinated alcohols to the indole ring of the Trp residues. A previous study has also shown quenching of indole fluorescence in presence of TFE using excited-state proton transfer (Chen *et al.*, 1995). The increase in the Trp fluorescence intensity with increasing alkanol concentrations can be ascribed to

the internalization of Trp residues in the protein interior (hydrophobic environment), suggesting protein structural re-arrangement (Dave *et al.*, 2010). Glucoamylase has 18 Trp and 27 Tyr residues with a distribution of 13 Trp and 20 Tyr residues in the catalytic domain; 4 Trp and 6 Tyr residues in the SBD and a single residue of each in the linker region (Svensson *et al.*, 1983). Accordingly, any change around the Trp environment mainly reflects conformational changes in the catalytic domain. Except tert-butanol, other alkanols were found to induce a similar state based on the Trp environment (Figure 4.24). However, they differ in the mid-point of the transition, which followed the order: tert-butanol > 1-propanol > 2-propanol > ethanol > methanol. This order of effectiveness was similar to the one observed with MRE_{222nm} and ANS fluorescence measurements.

4.2.2. Characterization of the alcohol-induced states

Alcohol-induced states produced by TFE (a halogenol) and tert-butanol (an alkanol) were characterized using far- and near-UV CD, Trp fluorescence, ANS fluorescence and 3-D fluorescence spectral probes as well as viscosity measurements.

4.2.2.1. Far-UV CD spectra

Far-UV CD spectra were used to characterize the presence of secondary structures in different states of glucoamylase, obtained under different conditions. Figure 4.25 shows the far-UV CD spectra of glucoamylase in the native state (pH 7.0) and the acid-denatured state (pH 1.0) in the absence and the presence of 7.0 M TFE (halogenol-induced state) as well as 5.5 M tert-butanol (alkanol-induced state). Two minima around 210 and 219 nm (characteristic of the α -helix) characterized the far-UV CD spectrum of the native glucoamylase. Far-UV CD spectrum of the acid-denatured glucoamylase also showed the presence of these minima but with reduced MRE values, suggesting ~6% loss in the α -helical structure in the acid-denatured state. These results were similar to

our previous results, showing the presence of 17% α -helix in the acid-denatured glucoamylase at pH 1.0 against 27% present in the native glucoamylase (Zaroog and Tayyab, 2012). The difference in the calculation of the α -helix content produced in different states of the protein can be attributed to the different theoretical methods used (Chen *et al.*, 1972; Whitmore and Wallace, 2008). A marked increase in the MRE values was observed in the presence of 7.0 M TFE as well as 5.5 M tert-butanol, implying an increase in the α -helical content of the acid-denatured glucoamylase. However, the increase in the α -helical content was more pronounced with TFE (13%) compared to tert-butanol (9%). These results suggested higher potential of the TFE for inducing the α -helical structure in the protein than the tert-butanol and agreed well with earlier reports (Hirota *et al.*, 1998; Khan *et al.*, 2000; Kumar *et al.*, 2004; Yoshida *et al.*, 2008).

4.2.2.2. Near-UV CD spectra

The information about the protein's tertiary structure under different conditions was obtained from the near-UV CD spectra. These spectra of the native glucoamylase and the acid-denatured glucoamylase in the absence and the presence of 7.0 M TFE or 5.5 M tert-butanol are shown in Figure 4.26. As shown in the figure, the near-UV CD spectrum of the native glucoamylase was characterized by the presence of three negative signals around 270, 278 and 285 nm, in agreement with a previous report (Freedberg *et al.*, 1975). There was a significant reduction in the MRE values in the near-UV CD spectrum of the acid-denatured glucoamylase, suggesting partial unfolding of the protein in the acid-denatured state (Naeem *et al.*, 2004). Presence of 7.0 M TFE in the acid-denatured glucoamylase showed further changes in the tertiary structure of the protein as evident from the significant loss in the MRE values at all these wavelengths. On the other hand, addition of 5.5 M tert-butanol to the acid-denatured

glucoamylase produced favorable changes in the protein's tertiary structure, showing significant increase in the $-MRE$ values along with the abolishment of 285 nm signal. Previous studies also showed the change in the protein's tertiary structure in the presence of higher alcohol concentrations (Kamatari *et al.*, 1996; Khan *et al.*, 2000). Although both halogenols and alkanols were found to change the protein's tertiary structure, their effects were opposite to each other. Since the near-UV CD spectrum monitors the change in the aromatic amino acids' environment (Wong and Tayyab, 2012), it can be assumed that both these alcohols changed the environment around aromatic chromophores in a different manner.

4.2.2.3. Tryptophan fluorescence spectra

In order to get more insight about the tertiary structural changes in the alcohol-induced states of glucoamylase, Trp fluorescence spectra of different glucoamylase states were recorded in the wavelength range, 310–400 nm upon excitation at 295 nm. Figure 4.27 shows Trp fluorescence spectra of the native and the acid-denatured glucoamylase in the absence and the presence of 7.0 M TFE or 5.5 M tert-butanol. The fluorescence spectrum of the native glucoamylase showed an emission maximum at 340 nm, which was red shifted by 2 nm, accompanied by a marked decrease (37%) in the fluorescence intensity at the emission maximum in the acid-denatured state. This was in accordance with an earlier report (Zaroog and Tayyab, 2012), suggesting exposure of Trp residues to a relatively polar environment due to the partial unfolding of the protein in the acid-denatured state at pH 1.0. Presence of 7.0 M TFE in the acid-denatured glucoamylase led to a further decrease (24%) in the fluorescence intensity accompanied by 4 nm red shift in the emission maximum, which was suggestive of more exposure of Trp residues to polar environment. Contrary to it, acid-denatured glucoamylase in the presence of 5.5 M tert-butanol showed a marked increase in the fluorescence intensity at

the emission maximum, suggesting movement of Trp residues especially in the catalytic domain to a more nonpolar environment (Haq *et al.*, 2005). These results were similar to our near-UV CD spectral results, showing opposite effects of TFE and tert-butanol with respect to tertiary structural changes in the acid-denatured glucoamylase, in which aromatic chromophores were placed in relatively nonpolar environment in the presence of tert-butanol and more polar environment in the presence of TFE.

4.2.2.4. ANS fluorescence spectra

Proteins in the molten globule state have been found to show higher ANS binding than in the native state, suggesting exposure of the buried hydrophobic regions in the protein to the solvent (Alexandrescu *et al.*, 1994; Muzammil *et al.*, 1999). Since the acid-denatured glucoamylase at pH 1.0 was found to represent the molten globule state of the enzyme (Zaroog and Tayyab, 2012), a comparison of the ANS fluorescence spectrum of the acid-denatured state of glucoamylase with those of the alcohol-induced states would be of interest to study the conformational differences between these states. The ANS fluorescence spectra of the native and the acid-denatured glucoamylase in the absence and the presence of 5.0 M TFE or 5.0 M tert-butanol were recorded in the wavelength range, 400–600 nm upon excitation at 380 nm and the results are shown in Figure 4.28. Native glucoamylase showed a very weak ANS fluorescence signal with an emission maximum at 470 nm, which was suggestive of the burial of the hydrophobic ANS binding sites in the protein interior. Marked increase in the ANS fluorescence intensity and a red shift of 8 nm characterized the ANS fluorescence spectrum of the acid-denatured glucoamylase, indicating exposure of the buried hydrophobic regions to the solvent. This result was similar to the one reported earlier (Zaroog and Tayyab, 2012). Addition of 5.0 M TFE to the acid-denatured glucoamylase led to nearly complete quenching (~99%) of the ANS fluorescence. Similarly, presence of 5.0 M tert-

butanol in the acid-denatured glucoamylase also showed significant decrease (~92%) in the ANS fluorescence intensity along with a 14 nm red shift. However, this decrease was relatively lesser than that observed in the presence of 5.0 M TFE. The small difference in the ANS fluorescence intensity of the acid-denatured glucoamylase observed in the presence of 5.0 M TFE and 5.0 M tert-butanol might be attributed to the greater extent of energy transfer from the Trp residues to the ANS fluorophore via the Förster mechanism in the presence of tert-butanol than TFE (Sirangelo *et al.*, 2003). This was in agreement with our Trp fluorescence results (Figure 4.27) where the presence of tert-butanol showed increase in the Trp fluorescence while TFE quenched it. Hence, the available energy that can be transferred from Trp to ANS will be more in presence of tert-butanol compared to TFE. Overall, the quenching of ANS fluorescence observed in the presence of these alcohols can be ascribed to the burial of the hydrophobic regions in the protein interior, suggesting structure retrieval in the acid-denatured glucoamylase. This was supported by far- and near-UV CD spectral results, showing structural reformation in the protein in the presence of these alcohols. Presence of the charged groups of the protein side chains in the vicinity of ANS binding sites may account for the observed red shift in the emission maximum (Khanna *et al.*, 1986). Although, these alcohol-induced states seem to be similar in terms of the overall three-dimensional conformation, showing burial of the hydrophobic clusters (Figure 4.28), they might be different with respect to the aromatic amino acids' microenvironment as reflected from the near-UV CD (Figure 4.26) and the Trp fluorescence (Figure 4.27) results.

4.2.2.5. Three-dimensional fluorescence spectra

To validate the results obtained by the far- and near-UV CD, Trp fluorescence and ANS fluorescence regarding secondary and tertiary structural changes, 3-D fluorescence

spectroscopy was employed to characterize different conformational states of glucoamylase. The 3-D fluorescence spectra (A) and corresponding contour maps (B) of glucoamylase in the native state, the acid-denatured state and the acid-denatured state in the presence of 5.5 M tert-butanol are shown in Figures 4.29, 4.30 and 4.31, respectively. Table 4.8 represents the 3-D fluorescence spectral characteristics of these conformational states in terms of the fluorescence intensity and peak position. As shown in these figures and Table 4.8, peaks *a* and *b* corresponded to the Rayleigh scattering peak ($\lambda_{\text{ex}} = \lambda_{\text{em}}$) and the second-order scattering peak ($\lambda_{\text{em}} = 2\lambda_{\text{ex}}$), respectively, whereas peak 1 ($\lambda_{\text{ex}} = 280 \text{ nm}$) and peak 2 ($\lambda_{\text{ex}} = 230 \text{ nm}$) represented the fluorescence spectral characteristics of Trp and Tyr residues due to $\pi \rightarrow \pi^*$ transition (a probe for tertiary structural change) (Feroz *et al.*, 2012) and the polypeptide backbone due to $n \rightarrow \pi^*$ transition (a probe for secondary structural change) (Kang *et al.*, 2004), respectively. In a comparison to the native glucoamylase, the acid-denatured glucoamylase showed a significant decrease in the fluorescence intensity along with a 3 nm red shift in both peaks 1 and 2 (Table 4.8), indicating exposure of fluorophores (Trp and Tyr) and peptide groups, respectively, as a result of partial unfolding of the polypeptide chain. These results suggested disruption of both tertiary and secondary structures of the protein in the acid-denatured state and were in line with our previous results (Zaroog and Tayyab, 2012). However, addition of 5.5 M tert-butanol led to a significant increase in the fluorescence intensity along with a shift in the emission maximum as ~60%, 2 nm blue shift and ~48%, 4 nm red shift for peaks 1 and 2, respectively (Table 4.8). The fluorescence intensity of peak 1 was even more than that shown by the native state, suggesting burial of fluorophores in the nonpolar environment. On the other hand, formation of more secondary structure in the presence of 5.5 M tert-butanol was evident from the increase in the fluorescence intensity of peak 2, which was slightly higher than

the native state. All these results suggested structure retrieval in the protein and agreed well with other spectral results.

4.2.2.6. Viscosity

The viscosity of the protein solution was employed to qualitatively characterize the overall dimension of glucoamylase under different conditions. Figure 4.32 shows viscosity of glucoamylase in the native state and the acid-denatured state in the absence and the presence of 8.0 M TFE, obtained at different shear rates. As evident from the figure, the viscosity was found to be independent of the shear rate, suggesting the Newtonian behavior of glucoamylase solution under different conditions (Sharma *et al.*, 2011). Furthermore, absence of any change in the viscosity with the increase in the shear rate also ruled out the possibility of any effect of the shear rate on the protein structure. A comparison of the average viscosity values obtained for different states of glucoamylase, clearly showed increased viscosity of the acid-denatured glucoamylase (0.115 cP) compared to its native state (0.024 cP). Increase in the viscosity of the acid-denatured state was suggestive of more extended conformation of the acid-denatured form of glucoamylase than its native counterpart. Interestingly, the viscosity of TFE-induced state (0.057 cP) was found close to the value obtained with the native state, suggesting structure reformation toward the globular conformation. The viscosity of tert-butanol-induced state could not be obtained due to the appearance of turbidity in the protein solution. Furthermore, the viscosity of the tert-butanol solvent (3.78 cP) was also found higher than the other solvents used. However, TFE results clearly demonstrated the reversal of the extended structure of the acid-denatured glucoamylase toward the globular form.

Overall, similar order of effectiveness of these alcohols to drag the acid-denatured glucoamylase into a partially folded state was observed with both MRE_{222nm}

measurements and ANS binding, based on the α -helix induction and tertiary structure reorganization, respectively. This order of effectiveness was akin to that observed with other proteins (Hirota *et al.*, 1998; Kumar *et al.*, 2004; Yoshida *et al.*, 2008). Among the alcohols used, halogenols were found more effective than alkanols in inducing both α -helical and tertiary structures in the acid-denatured glucoamylase. Although the exact mechanism of the alcohol-induced changes in the protein conformation is still unclear, two main mechanisms have been suggested, which include stabilization of the α -helices by direct binding of fluorinated alcohols to the protein or indirect mechanism involving stabilization of the peptide hydrogen bonds (H-bonds) by weakening the H-bonds of water molecules to the peptide carbonyl (CO) and imino (NH) groups (Jasanoff and Fersht, 1994; Cammers-Goodwin *et al.*, 1996; Luo and Baldwin, 1997). Due to their nonpolar property, alcohols have been found to decrease the polarity of the solvent which can be accounted as the transfer of the protein groups from water to alcohol solvent (Tanford, 1968; Liu and Bolen, 1995; Yanagi *et al.*, 2011). In low polarity solvents, local H-bonds are strengthened, resulting in the induction of more α -helical structures. Furthermore, various observations favoring this mechanism have been described, such as reduction in the dielectric constant (Uversky *et al.*, 1997; Yanagi *et al.*, 2011) as well as enthalpic and entropic effects (Walgers *et al.*, 1998; Andersen *et al.*, 1999; Othon *et al.*, 2009).

According to Hirota *et al.* (1998), alcohol effect for inducing α -helical structure in proteins can be viewed as the additive function of the contribution of all the constituent groups present in these alcohols. They noticed the positive contribution of hydrocarbon groups and halogen substituent's (if any) and a negative contribution of the hydroxyl group to this effect. Furthermore, they observed a linear correlation between the number of carbon atoms as well as the accessible surface area and the effectiveness of alcohols.

Our results also supported this finding as the effectiveness of alcohols decreased from 1-propanol to methanol in inducing the α -helical structure. Accessible surface area has also been found greatly correlated with the effectiveness of alcohols with the same number of carbon atoms but different structures. This was holding true in our study where 2-propanol produced relatively lesser effect compared to 1-propanol. On the other hand, the marked effectiveness of fluoro alcohols over their alkanol counterparts seems to involve some other factors in addition to the number of carbon atoms and accessible surface area. Although isolated fluorine (F) atom in fluoro alcohol has been reported to have a weak potential to induce alcohol effect, presence of multiple F atoms has been shown to markedly increase this potential among other halogenols (Hirota *et al.*, 1998; Khan *et al.*, 2000; Kumar *et al.*, 2004; Yoshida *et al.*, 2008). Indeed, the high electronegativity of the F atoms in fluorinated alcohols has been suggested to increase the acidity of the OH group, driving alcohol to act as a proton donor (Chen *et al.*, 1995; Othon *et al.*, 2009). In an earlier study of HFIP/water mixture by small-angle X ray scattering, HFIP was found to have a high tendency to form micelle-like assemblies (Kuprin *et al.*, 1995). Furthermore, aggregation of HFIP around the peptide has been reported based on molecular dynamic simulation, suggesting the displacement of water at the peptide surface as the main contribution of HFIP in stabilizing the secondary structure of melittin (Roccatano *et al.*, 2005). In a recent study of solvation in folding/unfolding of melittin tetramer-monomer transition in presence of TFE, large conformational changes in the protein were found to be regulated by the local solvent hydrophobicity and bulk solvent viscosity (Othon *et al.*, 2009). However, the existence of multiple F atoms in fluoro alcohols remains the key contributing factor that increases their effectiveness for α -helix induction compared to nonfluoro alcohols.

4.3. Polyol-induced structural transitions

4.3.1. Polyol-induced conformational changes in the native and the acid-denatured glucoamylases

The effect of various cosolvents (polyols) including a monosaccharide (glucose), a disaccharide (trehalose), a dihydric alcohol (ethylene glycol) and a trihydric alcohol (glycerol) on the conformation of the native and the acid-denatured states of glucoamylase was studied using different probes such as far-UV CD, Trp fluorescence and ANS fluorescence.

4.3.1.1. Far-UV CD spectra

Polyol-induced secondary structural changes in the native and the acid-denatured states of glucoamylase were studied using far-UV CD spectroscopy. The far-UV CD spectra of the native and acid-denatured glucoamylases, obtained at 25°C in the absence and the presence of various polyols are shown in Figures 4.33 (glucose), 4.34 (trehalose), 4.35 (glycerol) and 4.36 (ethylene glycol). As can be seen from these figures, the CD spectrum of the native state was characterized by the presence of two negative signals around 210 and 219 nm, a characteristic of the α -helical structure of the protein (Ramos *et al.*, 2004). On the other hand, the far-UV CD spectrum of the acid-denatured state showed a significant decrease in the MRE values along with a shift in the wavelength of the negative signals, which occurred at 212 and 216 nm. Both these changes indicated a different conformation of the acid-denatured state of glucoamylase compared to native state. In a previous section, we have characterized the acid-denatured state of glucoamylase at pH 1.0 as the molten globule-like state (Zaroog and Tayyab, 2012). Addition of polyols to the native and the acid-denatured states of glucoamylase produced structural changes in both states as reflected from the increase in the MRE values, being more pronounced in the acid-denatured state than the native

state (Figures 4.33–4.36). A quantitative analysis of the results shown in Figures 4.33–4.36 in terms of the MRE_{222nm} values of the two states of glucoamylase obtained in the absence and the presence of various polyols along with the percentage increase in the MRE_{222nm} value (ΔMRE_{222nm}) in the presence of polyols are given in Table 4.9. Whereas the presence of glycerol produced a maximum increase in the MRE_{222nm} of ~47% in the acid-denatured state, only ~14% increase in the MRE_{222nm} was observed in the native state. These results were similar to earlier reports on other proteins where the effect of various polyols on the native state was found comparatively lesser than that observed with the acid-denatured state (Xie and Timasheff, 1997; Kaushik and Bhat 1998). A comparison of polyol-induced conformational changes in the native and the acid-denatured state of glucoamylase, based on ΔMRE_{222nm} showed greater effectiveness of glycerol in both states. On the other hand, ethylene glycol and trehalose were found least effective in altering the secondary structures in the native state and the acid-denatured state, respectively. Quantitatively, various polyols followed the order of effectiveness in increasing MRE_{222nm} in the acid-denatured state as glycerol > glucose > ethylene glycol > trehalose (Table 4.9).

In addition to the increase in the MRE_{222nm} values of both states of glucoamylase in the presence of different polyols, change in the shape of the CD spectrum was also noticed. Although the characteristic shape of the α -helical structure was retained in the CD spectra of the native state in presence of polyols, shape of the CD spectrum of the acid-denatured state was transformed into a CD spectrum showing characteristics of β -structure in presence of all polyols except glucose (Figures 4.33–4.36). Since the catalytic domain of glucoamylase is rich in the α -helical segments while β -structure is more populated in the starch binding domain, SBD (Sorimachi *et al.*, 1996; Lee and Paetzel, 2011), these polyols seem to induce structural changes in both domains to a

different extent in the acid-denatured state. On the other hand, polyols might have produced structural changes restricted to the catalytic domain of glucoamylase in its native state.

4.3.1.2. Tryptophan fluorescence spectra

In order to study the tertiary structural changes occurred in the native and the acid-denatured states of glucoamylase in the presence of various polyols, Trp fluorescence was employed to monitor the change in the environment around Trp residues. The Trp fluorescence spectra of the native and acid-denatured glucoamylases in the absence and the presence of various polyols are shown in Figures 4.37 (glucose), 4.38 (trehalose), 4.39 (glycerol) and 4.40 (ethylene glycol). As evident from these figures, the spectrum of the native glucoamylase was characterized by the presence of an emission maximum (E_m) at 340 nm when excited at 295 nm. About 36% decrease in the fluorescence intensity (FI) along with 2 nm red shift in the E_m were noticed in the Trp fluorescence spectrum of the acid-denatured state (Table 4.10). These results were in agreement with our previous results on the acid-denatured glucoamylase (Zaroog and Tayyab, 2012). A marked increase (46–94%) in the FI at 342 nm (FI_{342nm}) of the acid-denatured glucoamylase was observed in the presence of polyols, following the order: ethylene glycol > glycerol > glucose > trehalose (Table 4.10), which was suggestive of the transfer of Trp residues from a polar environment to a nonpolar environment. In addition to the increase in the FI_{342nm} , a slight red shift (1-2 nm) in the E_m was also observed in the presence of ethylene glycol and glycerol whereas glucose and trehalose showed a small blue shift (1-2 nm) in the E_m (Table 4.10). These results suggested partial refolding of the acid-denatured glucoamylase in the presence of these polyols, marked by the burial of the Trp residues in the nonpolar interior of the protein. In view of the distribution of the Trp residues in the catalytic domain (13 Trp) and SBD (4Trp)

(Svensson *et al.*, 1983), change in the FI_{342nm} of the protein mainly reflected the structural changes in the catalytic domain. Furthermore, these results were supported by the far-UV CD spectral results, showing increased α -helical structure in the acid-denatured glucoamylase in the presence of polyols (Table 4.9).

On the other hand, presence of glycerol, glucose or trehalose in the incubation mixture containing native glucoamylase produced a small quenching (2-12%) in the Trp fluorescence intensity, suggesting partial exposure of the Trp residues to the polar environment. Contrary to it, ethylene glycol showed similar behavior with the native state as that shown with the acid-denatured state by producing an increase (15%) in the FI_{340nm} (Table 4.10). The increase in the Trp fluorescence intensity of the native state in presence of ethylene glycol against the decrease shown in the presence of the other polyols indicated different conformational structures acquired by the catalytic domain in the presence of these polyols.

4.3.1.3. ANS fluorescence spectra

Binding of the hydrophobic dye, ANS to the acid-denatured glucoamylase in the absence and the presence of various polyols was studied to get insight about the tertiary structural changes in the acid-denatured state induced by these polyols and the results are shown in Figure 4.41. Native glucoamylase produced a weak ANS fluorescence spectrum with an emission maximum at 470 nm (figure omitted for brevity), indicating burial of the hydrophobic regions in the protein interior in its native state (Biswas and Kayastha 2004; Muzammil *et al.*, 1999). The acid-denatured state showed a marked increase in the ANS fluorescence intensity along with 8 nm red shift (Figure 4.41), suggesting exposure of the hydrophobic regions of the protein to the solvent at pH 1.0. These results agreed well with those reported earlier for acid-denatured proteins (Yadav and Prakash, 2009; Devaraneni *et al.*, 2012). Presence of 2.6 M glucose or 1.3 M

trehalose in the incubation mixture led to a further increase in the ANS fluorescence intensity at 478 nm by 14% and 15%, respectively, accompanied by 2 nm red shift in the E_m for glucose and 1 nm blue shift in the E_m for trehalose, suggesting the availability of more hydrophobic clusters to the solvent. Interestingly, 8.0 M glycerol or 8.0 M ethylene glycol completely quenched the ANS fluorescence, similar to that found with the native state, indicating burial of the hydrophobic clusters, which were thought to be exposed to the solvent in the acid-denatured state. As more hydrophobic residues are predominantly distributed in the SBD region (Svensson *et al.*, 1983), burial or exposure of these residues mainly reflected structural alterations in the SBD. In view of the burial of the hydrophobic segments upon addition of glycerol or ethylene glycol and more exposure in presence of glucose or trehalose, it appears that different conformational make-up was acquired in SBD in the presence of these polyols. These results agreed well to our far-UV CD spectral results where both glycerol and ethylene glycol induced more β -structural features (Figures 4.35 and 4.36).

4.3.2. Polyol-induced thermal stabilization of glucoamylase

4.3.2.1. Thermal transition

Figures 4.42A and B show thermal transition curves and corresponding normalized curves (F_D plots), respectively of the thermal denaturation of glucoamylase in the absence and the presence of various polyols as studied by ellipticity measurements at 222 nm. As evident from the figure, MRE_{222nm} of glucoamylase remained unchanged within the temperature range, 20–47°C, decreased markedly between 53°C and 77°C and became constant thereafter up to 100°C. The thermal denaturation of glucoamylase, showing the loss of secondary structure with increasing temperature, followed a cooperative transition. Several proteins have shown a cooperative thermal transition (Tripathi *et al.*, 2008; Yadav and Prakash, 2009; Borges *et al.*, 2012). The transition

curves of glucoamylase obtained in the presence of glucose, trehalose or glycerol were found shifted toward the higher temperature range, suggesting increase in the thermal stability of the enzyme in the presence of these polyols. Contrary to it, presence of ethylene glycol in the incubation mixture shifted the thermal transition curve toward the lower temperature range, indicating destabilization of glucoamylase. Previous studies have also shown stabilization of many proteins in presence of glucose, trehalose and glycerol (Gangadhara *et al.*, 2008; Poddar *et al.*, 2008; Kumar *et al.*, 2012) and destabilization in presence of ethylene glycol (Kumar *et al.*, 2011; Devaraneni *et al.*, 2012).

Thermodynamic analysis of the thermal transition curves was made following the methods described in section 3.2.6.4 and the values of T_m , ΔH_{vH} and ΔG (25°C), thus obtained are presented in Table 4.11. The midpoint temperature, T_m (64.3°C) obtained for glucoamylase was found to be similar to an earlier report (Zaroog and Tayyab, 2012). As can be seen from the table, presence of glucose, trehalose or glycerol in the incubation mixture led to an increase in the stability of glucoamylase whereas ethylene glycol destabilized it. Glucose was found to be the strongest cosolvent in increasing the thermal stability of glucoamylase among the polyols studied, as it increased the T_m value by 13°C up to 77°C along with 80% increase in the ΔG (25°C). In general, a comparison of different polyols based on the thermodynamic parameters shown in Table 4.11 suggested the order: glucose > trehalose > glycerol for glucoamylase stabilization. This order was found similar to those reported earlier for yeast hexokinase A (Devaraneni *et al.*, 2012) and α -amylase (Yadav and Prakash, 2009).

4.3.2.2. Effect of polyols on the thermal-denatured glucoamylase at 71°C

4.3.2.2.1. Far-UV CD spectra

In order to verify the thermal stabilizing effect of these polyols, the far-UV CD spectra of the thermal-denatured glucoamylase at 71°C were obtained in the absence and the presence of polyols (Figure 4.43). The far-UV CD spectrum of the native glucoamylase (pH 7.0, 25°C) has also been included in the figure for comparison. As can be seen from the figure, the far-UV CD spectrum of the thermal-denatured state of glucoamylase showed ~48% loss in the MRE_{222nm} value compared to the native glucoamylase (Tables 4.9 and 4.12), along with a shift in the CD spectral signals, suggesting denaturation of the enzyme at high temperature as observed with other proteins (Kaushik and Bhat, 1998; Ortbauer and Popp, 2008; Gangadhara *et al.*, 2008). Interestingly, addition of glucose or trehalose to the incubation mixture markedly increased the MRE_{222nm} value of the thermal-denatured glucoamylase by ~110% and ~91%, respectively (Table 4.12), showing significant reversal in the CD spectral characteristics close to the native glucoamylase. However, slight change in the position of the minima was observed with trehalose. On the other hand, no increase in the MRE_{222nm} was observed in the presence of ethylene glycol rather it showed a ~6% decrease in the MRE_{222nm} (Figure 4.43, Table 4.12), suggesting no stabilizing effect of ethylene glycol on the secondary structural characteristics of the thermal-denatured glucoamylase. Glycerol was able to induce ~22% regain in the MRE_{222nm} value. Thus, both glucose and trehalose were able to induce the native-like secondary structure in the thermal-denatured glucoamylase.

4.3.2.2.2. Tryptophan fluorescence spectra

Figure 4.44 shows Trp fluorescence spectra of the thermal-denatured glucoamylase at 71°C in the absence and the presence of polyols. Tryptophan fluorescence spectrum of the native glucoamylase is also included in the same figure. Thermal-denatured glucoamylase showed a significant decrease (54%) in the FI_{340} accompanied by 9 nm red shift in the E_m compared to native glucoamylase (Tables 4.10 and 4.13). Both decrease in the FI_{340} and significant red shift in the fluorescence spectrum of the thermal-denatured glucoamylase were suggestive of the exposure of Trp residues to the polar solvent (Halim *et al.*, 2013), indicating protein denaturation. Except ethylene glycol, other polyols (glucose, trehalose and glycerol) produced a significant blue shift (5-9 nm) and increase in the FI_{340} . Both these changes in the fluorescence characteristics of the thermal-denatured glucoamylase suggested significant refolding in the protein characterized by the burial of Trp residues in the nonpolar environment. Both glucose and trehalose were found more effective in inducing the native-like tertiary structure as reflected from the retrieval of the emission maximum, similar to the E_m of native glucoamylase. These results were similar to the far-UV CD spectral results (Figure 4.43) where both glucose and trehalose were found to induce secondary structure similar to that present in the native glucoamylase. Presence of a polar/charged group in the vicinity of the Trp residues might account for the lesser increase in the FI_{340} in the presence of glucose and trehalose (Khanna *et al.*, 1986). On the other hand, addition of ethylene glycol to the incubation mixture produced 2 nm red shift in the E_m along with a marked increase (45%) in the FI_{340} , indicating a different tertiary structural make-up compared to the one obtained with other polyols. The far-UV CD spectral signal of the thermal-denatured glucoamylase in the presence of ethylene glycol also showed no significant change in the CD spectral characteristics (Figure 4.43).

Taken together, all polyols appeared to protect the native-like structure (to a greater extent) in the acid-denatured glucoamylase. This was evident from the higher MRE_{222nm} and FI_{342nm} values of the acid-denatured glucoamylase in presence of polyols compared to those obtained in their absence (Tables 4.9 and 4.10). Polyols were also found to stabilize the native state against the thermal denaturation. On the other hand, ethylene glycol produced the reversed effect.

5. REFERENCES

- Ahmad E., Fatima S., Khan M.M. and Khan R.H. (2010) More stable structure of wheat germ lipase at low pH than its native state. *Biochimie* 92:885–893.
- Ahmad S. and Rao N.M. (2009) Thermally denatured state determines refolding in lipase: Mutational analysis. *Protein Sci.* 18:1183–1196.
- Aleshin A., Golubev A., Firsov L.M. and Honzatko R.B. (1992) Crystal structure of glucoamylase from *Aspergillus awamori* var. X100 to 2.2-Å resolution. *J. Biol. Chem.* 267:19291–19298.
- Alexandrescu A.T., N.g. Y-L. and Dobson C.M. (1994) Characterization of a trifluoroethanol-induced partially folded state of α -lactalbumin. *J. Mol. Biol.* 235:587–599.
- Ali S., Hossain Z., Mahmood S. and Alam R. (1990) Purification of glucoamylase from *Aspergillus terreus*. *World J. Microbiol. Biotechnol.* 6:431–433.
- Ali S. and Hossain Z. (1991) Characteristics of glucoamylase from *Aspergillus terreus*. *J. Appl. Microbiol.* 71:144–146.
- Alston R.W., Lasagna M., Grimsley G.R., Scholtz J.M., Reinhart G.D. and Pace C.N. (2008) Peptide sequence and conformation strongly influence tryptophan fluorescence. *Biophys. J.* 94:2280–2287.
- Andersen N.H., Dyer R.B., Fesinmeyer R.M., Gai F., Liu Z., Neidigh J.W. and Tong H. (1999) Effect of hexafluoroisopropanol on the thermodynamics of peptide secondary structure formation. *J. Am. Chem. Soc.* 121:9879–9880.
- Anderson V.L., Ramlall T.F., Rospigliosi C.C., Webb W.W. and Eliezer D. (2010) Identification of helical intermediate in trifluoroethanol-induced alpha-synuclein aggregation. *Proc. Natl. Acad. Sci., USA* 107:18850–18855.
- Andrade M.A., Chacón P., Merelo J.J. and Morán F. (1993) Evaluation of secondary structure of proteins from UV circular dichroism using an unsupervised learning neural network. *Protein Eng.* 6:383–390.
- Ashikari T., Nakamura N., Tanaka Y., Kiuchi N., Shibano Y., Tanaka T., Amachi T. and Yoshizumi H. (1985) Cloning and expression of the *Rhizopus* glucoamylase gene in yeast. *Agric. Biol. Chem.* 49:2521–2523.
- Baier S. and McClements J. (2001) Impact of preferential interactions on thermal stability and gelation of bovine serum albumin in aqueous sucrose solutions. *J. Agric. Food Chem.* 49:2600–2608.
- Bender H. (1981) A bacterial glucoamylase degrading cyclodextrins. *Eur. J. Biochem.* 115:287–291.

- Biswas S. and Kayastha A.M. (2004) Unfolding and refolding of Leucoagglutinin (PHA-L), an oligomeric lectin from kidney beans (*Phaseolus vulgaris*). *Biochim. Biophys. Acta* 1674:40–49
- Boel E., Hjort I., Svensson B., Norris F., Norris K.E. and Fiil N.P. (1984) Glucoamylases G1 and G2 from *Aspergillus niger* are synthesized from two different but closely related mRNAs. *EMBO J.* 3:1097–1102.
- Borges J.C., Seraphim T.V., Mokry D.Z., Almeida F.C.L., Cyr D.M. and Ramos C.H.I. (2012) Identification of regions involved in substrate binding and dimer stabilization within the central domains of yeast Hsp40 Sis1. *PLoS One* 7:1–15.
- Bott R., Saldajeno M., Cuevas W., Ward D., Scheffers M., Aehle W., Karkehabadi S., Sandgren M. and Hansson H. (2008) Three-dimensional structure of an intact glycoside hydrolase family 15 glucoamylase from *Hypocrea jecorina*. *Biochemistry* 27:5746–5754.
- Bychkova V.E., Berni R., Rossi G.L., Kutysenko V.P. and Ptitsyn O.B. (1992) Retinol-binding protein is in the molten globule state at low pH. *Biochemistry* 31:7566–7571.
- Bychkova V.E., Dujsekina A.E., Klenin S.I., Tiktopulo E.I., Uversky V.N. and Ptitsyn O.B. (1996) Molten globule-like state of cytochrome c under conditions simulating those near the membrane surface. *Biochemistry* 35:6058–6063.
- Cammers-Goodwin A., Allen T.J., Oslick S.L., McClure K.F., Lee J.H. and Kemp D.S. (1996) Mechanism of stabilization of helical conformations of polypeptides by water containing trifluoroethanol. *J. Am. Chem. Soc.* 118:3082–3090.
- Cassim J.Y. and Yang J.T. (1967) Effect of molecular aggregation on circular dichroism and optical rotatory dispersion of helical poly-L-glutamic acid in solution. *Biochem. Biophys. Res. Commun.* 26:58–64.
- Chaga G., Porath J. and Illeni T. (1993) Isolation and purification of amyloglucosidase from *Halobacterium sodomense*. *Biomed. Chromatogr.* 7:256–261.
- Chakraborty S., Ittah V., Bai P., Luo L., Haas E. and Peng Z. (2001) Structure and dynamics of the α -lactalbumin molten globule: Fluorescence studies using proteins containing a single tryptophan residue. *Biochemistry* 40:7228–7238.
- Charlwood P.A. (1961) Ultracentrifugal studies of rat, rabbit and guinea-pig serum albumins. *Biochem. J.* 78:163–172.
- Chen Y., Liu B. and Barkley M.D. (1995) Trifluoroethanol quenches indole fluorescence by excited-state proton transfer. *J. Am. Chem. Soc.* 117:5608–5609.
- Chen Y-H., Yang J.T. and Martinez H.M. (1972) Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry* 11:4120–4131.

- Chojceki A. and Blaschek H.P. (1986) Effect of carbohydrate source on alpha-amylase and glucoamylase formation by *Clostridium acetobutylicum* SA-1. *Ind. Microbiol.* 1:63–67.
- Christensen T., Frandsen T.P., Kaarsholm N.C., Svensson B. and Sigurskjold B.W. (2002) Physicochemical characterisation of the two active site mutants Trp⁵²→Phe and Asp⁵⁵→Val of glucoamylase from *Aspergillus niger*. *Biochim. Biophys. Acta* 1601:163–171.
- Christensen T., Svensson B. and Sigurskjold B.W. (1999) Thermodynamics of reversible and irreversible unfolding and domain interactions of glucoamylase from *Aspergillus niger* studied by differential scanning and isothermal titration calorimetry. *Biochemistry* 38:6300–6310.
- Christov C., Ianev D., Shosheva A. and Atanasov B. (2004) pH-dependent quenching of the fluorescence of tryptophan residues in class A β -lactamase from *E. coli* (TEM-1). *Z. Naturforsch.* 59:824–827.
- Clarke A.J. and Svensson B. (1984) The role of tryptophanyl residues in the function of *Aspergillus niger* glucoamylase G1 and G2. *Carlsberg Res. Commun.* 49:111–122.
- Coutinho P.M. and Reilly P.J. (1994) Structural similarities in glucoamylases by hydrophobic cluster analysis. *Protein Eng.* 7:749–760.
- Dave S., Dkhar H.K., Singh M.P., Gupta G., Chandra V., Mahajan S. and Gupta P. (2010) Hexafluoroisopropanol-induced helix–sheet transition of stem bromelain: Correlation to function. *Int. J. Biochem. Cell Biol.* 42:938–947.
- Davis J.P. and Foegeding E.A. (2007) Comparisons of the foaming and interfacial properties of whey protein isolate and egg white proteins. *Colloids Surf., B* 54:200–210.
- Devaraj K.B., Kumar P.R. and Prakash V. (2011) Comparison of activity and conformational changes of ficin during denaturation by urea and guanidine hydrochloride. *Process Biochem.* 46:458–464.
- Devaraneni P.K., Mishra N. and Bhat R. (2012) Polyol osmolytes stabilize native-like cooperative intermediate state of yeast hexokinase A at low pH. *Biochimie* 94:947–952.
- Dierckx S. and Huyghebaert A. (2002) Effects of sucrose and sorbitol on the gel formation of a whey protein isolate. *Food Hydrocolloids* 16:489–497.
- Dill K.A. and Shortle D. (1991) Denatured states of proteins. *Ann. Rev. Biochem.* 60:795–825.
- Dolgikh D.A., Abaturov L.V., Bolotina I.A., Brazhnikov E.V., Bychkova V.E., Gilmanshin R.I., Lebedev Yu.O., Semisotnov G.V., Tiktopulo E.I. and Ptitsyn O.B. (1985) Compact state of a protein molecule with pronounced small-scale mobility: Bovine α -lactalbumin. *Eur. Biophys. J.* 13:109–121.

- Donovan J.W. (1967) Spectrophotometric observation of the alkaline hydrolysis of protein disulfide bonds. *Biochem. Biophys. Res. Commun.* 27:734–740.
- Dyatkin B.L., Mochalina E.P. and Knunyants I.L. (1965) The acidic properties of fluorine-containing alcohols, hydroxylamines and oximes. *Tetrahedron* 21:2991–2995.
- Eftink M.R. and Ghiron C.A. (1981) Fluorescence quenching studies with proteins. *Anal. Biochem.* 114:199–227.
- Engelhard M. and Evans P.A. (1995) Kinetics of interaction of partially folded proteins with a hydrophobic dye: Evidence that molten globule character is maximal in early folding intermediates. *Protein Sci.* 4:1553–1562.
- Erratt J.A. and Stewart G.G. (1978) Genetic and biochemical studies on yeast strains able to utilize dextrans. *J. Am. Soc. Brew. Chem.* 36:151–156.
- Fatima S., Ahmad B. and Khan R.H. (2007) Native-like tertiary structure in the *Mucor miehei* lipase molten globule state obtained at low pH. *IUBMB Life* 59:179–186.
- Favilla R., Parisoli A. and Mazzini A. (1997) Alkaline denaturation and partial refolding of pepsin investigated with DAPI as an extrinsic probe. *Biophys. Chem.* 67:75–83.
- Feroz S.R., Mohamad S.B., Bujang N., Malek S.N.A. and Tayyab S. (2012) Multispectroscopic and molecular modeling approach to investigate the interaction of flavokawain B with human serum albumin. *J. Agric. Food Chem.* 60:5899–5908.
- Fink A.L., Calciano L.J., Goto Y., Nishimura M. and Swedberg S.A. (1993) Characterization of the stable, acid-induced, molten globule-like state of staphylococcal nuclease. *Protein Sci.* 2:1155–1160.
- Frandsen T.P., Fierobe H.P. and Svensson B. (1999) Engineering specificity and stability in glucoamylase from *Aspergillus niger*. In: Alberghin L., editor. *Protein Engineering in Industrial Biotechnology*. Amsterdam, Harwood Academic Press, p. 189–206.
- Freedberg I.M., Levin Y., Kay C.M., McCubbin W.D. and Katchalski-Katzir E. (1975) Purification and characterization of *Aspergillus niger* exo-1,4-glucosidase. *Biochim. Biophys. Acta.* 391:361–381.
- Gangadhara, Kumar P.R. and Prakash V. (2008) Influence of polyols on the stability and kinetic parameters of invertase from *Candida utilis*: Correlation with the conformational stability and activity. *Protein J.* 27:440–449.
- Gekko K. and Timasheff S.N. (1981) Mechanism of protein stabilization by glycerol: Preferential hydration in glycerol-water mixtures. *Biochemistry* 20:4667–4676.

- Gellissen G., Janowicz Z.A., Merckelbach A., Piontek M., Keup P., Weydemann U., Hollenberg C.P. and Srasser A.W.M. (1991) Heterologous gene expression in *Hansenula polymorpha*: Efficient secretion of glucoamylase. *Biotechnology* 9:291–295.
- Georgescu R.E., Li J-H., Goldberg M.E., Tasayco M.L. and Chaffotte A.F. (1998) Proline isomerization-independent accumulation of an early intermediate and heterogeneity of the folding pathways of a mixed α/β protein, *Escherichia coli* thioredoxin. *Biochemistry* 37:10286–10297.
- Ghosh A.K., Naskar A.K. and Sengupta S. (1997) Characterisation of a xylanolytic amyloglucosidase of *Termitomyces clypeatus*. *Biochim. Biophys. Acta* 1339:289–296.
- Glazer A.N. and Smith E.L. (1961) Studies on the ultraviolet difference spectra of proteins and polypeptides. *J. Biol. Chem.* 236:2942–2947.
- Goto Y., Hagihara Y., Hamada D., Hoshino M. and Nishii I. (1993) Acid-induced unfolding and refolding transitions of cytochrome c: A three-state mechanism in water and deuterium oxide. *Biochemistry* 32:11878–11885.
- Goto Y., Takahashi N. and Fink A.L. (1990) Mechanism of acid-induced folding of proteins. *Biochemistry* 29:3480–3488.
- Haasum I., Ericksen S.H., Jensen B. and Olsen J. (1991) Growth and glucoamylase production by the thermophilic fungus *Thermophilus lanuginose* in a synthetic medium. *Appl. Microbiol. Biotechnol.* 34:656–660.
- Hagihara Y., Tan Y. and Goto Y. (1994) Comparison of the conformational stability of the molten globule and native states of horse cytochrome c. Effects of acetylation, heat, urea and guanidine hydrochloride. *J. Mol. Biol.* 237:336–348.
- Halim A.A.A., Feroz S.R. and Tayyab S. (2013) Does recovery in the spectral characteristics of GdnHCl-denatured *Bacillus licheniformis* α -amylase due to added calcium point towards protein stabilization? *Biosci. Biotechnol. Biochem.* 77:87–96.
- Haq S.K., Rasheedi S., Sharma P., Ahmad B. and Khan R.H. (2005) Influence of salts and alcohols on the conformation of partially folded intermediate of stem bromelain at low pH. *Int. J. Biochem. Cell Biol.* 37:361–374.
- Hayashida S. (1975) Selective submerged productions of three types of glucoamylases by a Black-koji mold. *Agric. Biol. Chem.* 39:2093–2099.
- Hirota N., Mizuno K. and Goto Y. (1997) Cooperative α -helix formation of β -lactoglobulin and melittin induced by hexafluoroisopropanol. *Protein Sci.* 6:416–421.
- Hirota N., Mizuno K. and Goto Y. (1998) Group additive contributions to the alcohol-induced α -helix formation of melittin: Implication for the mechanism of the alcohol effects on proteins. *J. Mol. Biol.* 275:365–378.

- Hong D., Hoshino M., Kuboi R. and Goto Y. (1999) Clustering of fluorine-substituted alcohols as a factor responsible for their marked effects on proteins and peptides. *J. Am. Chem. Soc.* 121:8427–8433.
- Hyun H.H. and Zeikus J.G. (1985) General biochemical characterization of thermostable pullulanase and glucoamylase from *Clostridium thermohydrosulfuricum*. *Appl. Environ. Microbiol.* 49:1168–1173.
- Iida T., Nishimura S., Mochizuki M., Uchiyama S., Ohkubo T., Urade Y., Tanaka A. and Inui T. (2008) Thermal unfolding mechanism of lipocalin-type prostaglandin D synthase. *FEBS J.* 275:233–241.
- Inokuchi R., Takahashi T. and Irie M. (1981) Purification and characterization of a minor glucoamylase from *Aspergillus saitoi*. *J. Biochem.* 90:1055–1067.
- Itoh T., Sakata Y., Akada R., Nimi O. and Yamshita I. (1989) Construction and characterization of mutant glucoamylases from the yeast *Saccharomycopsis fibuligera*. *Agric. Biol. Chem.* 53:3159–3168.
- Iyer P.V. and Ananthanarayan L. (2008) Enzyme stability and stabilization—Aqueous and non-aqueous environment. *Process Biochem.* 43:1019–1032.
- Jaffar M.B, Bharat R.P., Norouzian D., Irani S.D. and Shetty P. (1993) Production of glucoamylase by nematophagus fungi *Arthrobotrys* species. *Ind. J. Exp. Biol.* 31:87–89.
- James J.A. and Lee B.H. (1995) Cultural conditions for production of glucoamylase from *Lactobacillus amylovorus* ATCC 33621. *J. Appl. Bacteriol.* 79:499–505.
- James J.A. and Lee B.H. (1997) Glucoamylases: Microbial sources, industrial applications and molecular biology - a review. *J. Food Biochem.* 21:1–52.
- Jasanofft A. and Fersht A.R. (1994) Quantitative determination of helical propensities from trifluoroethanol titration curves. *Biochemistry* 33:2129–2135.
- Jiang J., Chen J. and Xiong Y.L. (2009) Structural and emulsifying properties of soy protein isolate subjected to acid and alkaline pH-shifting processes. *J. Agric. Food Chem.* 57:7576–7583.
- Kabsch W. and Sander C. (1983) Dictionary of secondary structures: Pattern recognition of hydrogen-bonded and geometrical features. *Biopolymers* 22:2577–2637.
- Kamatari Y.O., Konno T., Kataoka M. and Akasaka K. (1996) The methanol-induced globular and expanded denatured states of cytochrome c: A study by CD fluorescence, NMR and small-angle X-ray scattering. *J. Mol. Biol.* 259:512–523.
- Kamen D.E., Griko Y. and Woody R.W. (2000) The stability, structural organization, and denaturation of pectate lyase C, a parallel β -helix protein. *Biochemistry* 39:15932–15943.

- Kang J., Liu Y., Xie M.X., Li S., Jiang M. and Wang Y.D. (2004) Interactions of human serum albumin with chlorogenic acid and ferulic acid. *Biochim. Biophys. Acta* 1674:205–214.
- Kaushik J.K. and Bhat R. (1998) Thermal stability of proteins in aqueous polyol solutions: Role of the surface tension of water in the stabilizing effect of polyols. *J. Phys. Chem. B* 102:7058–7066.
- Keiderling T.A., Wang B., Urbanova M., Pancoska P. and Dukor R.K. (1994) Empirical studies of protein secondary structure by vibrational circular dichroism and related techniques. *Faraday Discuss.* 99:263–285.
- Kelly S.M. and Price N.C. (2000) The use of circular dichroism in the investigation of protein structure and function. *Curr. Protein Pept. Sci.* 1:349–384.
- Khan F., Khan R.H. and Muzammil S. (2000) Alcohol-induced versus anion-induced states of α -chymotrypsinogen A at low pH. *Biochim. Biophys. Acta* 1481:229–236.
- Khanna N.C., Tokuda M. and Waisman D.M. (1986) Conformational changes induced by binding of divalent cations to calregulin. *J. Biol. Chem.* 261:8883–8887.
- Kleinman M.J., Wilkinson A.E., Wright I.P., Evans I.H. and Bevan E.A. (1988) Purification and properties of an extracellular glucoamylase from a diastatic strain of *Saccharomyces cerevisiae*. *Biochem. J.* 249:163–170.
- Knoll D. and Hermans J. (1983) Polymer-protein interactions. Comparison of experiment and excluded volume theory. *J. Biol. Chem.* 258:5710–5715.
- Kristinsson H.G. and Hultin H.O. (2003) Changes in conformation and subunit assembly of cod myosin at low and high pH and after subsequent refolding. *J. Agric. Food Chem.* 51:7187–7196.
- Kruger J.E. and Lineback D.R. (1987) Carbohydrate degrading enzymes in cereals. In: Kruger J.E., Lineback D.R. and Stauffer C.E., editors. *Enzymes and Their Role in Cereal Technology*. Minnesota, American Association of Cereal Chemists, p. 117–139.
- Kumar A., Attri P. and Venkatesu P. (2012) Effect of polyols on the native structure of α -chymotrypsin: A comparable study. *Thermochim. Acta* 536:55–62.
- Kumar P., Islam A., Ahmad F. and Satyanarayana T. (2010) Characterization of a neutral and thermostable glucoamylase from the thermophilic mold *Thermomucor indicae-seudaticae*: Activity, stability, and structural correlation. *Appl. Biochem. Biotechnol.* 160:879–890.
- Kumar V., Chari R., Sharma V.K. and Kalonia D.S. (2011) Modulation of the thermodynamic stability of proteins by polyols: Significance of polyol hydrophobicity and impact on the chemical potential of water. *Int. J. Pharm.* 413:19–28.

- Kumar Y., Tayyab S. and Muzammil S. (2004) Molten-globule like partially folded states of human serum albumin induced by fluoro and alkyl alcohols at low pH. *Arch. Biochem. Biophys.* 426:3–10.
- Kuprin S., Graslund A., Ehrenberg A. and Koch M.H.J. (1995) Nonideality of water-hexafluoropropanol mixtures as studied by X-ray small angle scattering. *Biochem. Biophys. Res. Commun.* 217:1151–1156.
- Kuwahara K. and Konno K. (2010) Suppression of thermal denaturation of myosin and salt-induced denaturation of actin by sodium citrate in carp (*Cyprinus carpio*). *Food Chem.* 122:997–1002.
- Kuwajima K. (1989) The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure. *Proteins: Struct. Funct. Bioinform.* 6:87–103.
- Lee J. and Paetzel M. (2011) Structure of the catalytic domain of glucoamylase from *Aspergillus niger*. *Acta Cryst.* F67:188–192.
- Lewis M.J. and Young T.W. (1995) *Brewing*. Chapman and Hall, London.
- Li D., Zhang T., Xu C. and Ji B. (2011) Effect of pH on the interaction of baicalein with lysozyme by spectroscopic approaches. *J. Photochem. Photobiol. B.* 104:414–424.
- Lineback D.R. and Aira L.A. (1972) Structural characterization of the two forms of glucoamylase from *Aspergillus niger*. *Am. Assoc. Cereal Chem.* 49:283–298.
- Liu Y. and Bolen D.W. (1995) The peptide backbone plays a dominant role in protein stabilization by naturally occurring osmolytes. *Biochemistry* 34:12884–12891.
- Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
- Luo P. and Baldwin R.L. (1997) Mechanism of helix induction by trifluoroethanol: A framework for extrapolating the helix-forming properties of peptides from trifluoroethanol/water mixtures back to water. *Biochemistry* 36:8413–8421.
- Luo Y. and Baldwin R.L. (1998) Trifluoroethanol stabilizes the pH 4 folding intermediate of sperm whale apomyoglobin. *J. Mol. Biol.* 279:49–57.
- Machovic M. and Janecek S. (2007) Amylolytic enzymes: Types, structures and specificities. In: Polaina J. and MacCabe A.P., editors. *Industrial Enzymes*. Dordrecht, Springer, p. 3–18.
- Malek S.A.S. and Hossain Z. (1994) Purification and characterization of a thermostable glucoamylase from *Myrothecium* isolate. *J. Appl. Bacteriol.* 76:210–215.
- Matulis D., Baumann C.G., Bloomfield V.A. and Lovrien R.E. (1999) 1-Anilino-8-naphthalene sulfonate as a protein conformational tightening agent. *Biopolymers* 49:451–458.

- McCleary B.V. and Anderson M.A. (1980) Hydrolysis of α -D-glucans and α -D-gluco-oligosaccharides by *Cladosporium resinae* glucoamylases. Carbohydr. Res. 86:77–96.
- McClements D.J. (2002) Modulation of globular protein functionality by weakly interacting cosolvents. Crit. Rev. Food. Sci. Nutr. 42:417–471.
- Meaden P., Ogden K., Bussey H. and Tubb R.S. (1985) A DEX gene conferring production of extracellular amyloglucosidase on yeast. Gene 34:325–330.
- Miah M.N.N. and Ueda S. (1977) Multiplicity of glucoamylase of *Aspergillus oryzae*. Part 1. Separation and purification of three forms of glucoamylase. Starch 29:191–196.
- Mulqueen P.M. and Kronman M.J. (1982) Binding of naphthalene dyes to the N and A conformers of bovine α -lactalbumin. Arch. Biochem. Biophys. 215:28–39.
- Muzammil S., Kumar Y. and Tayyab S. (1999) Molten globule-like state of human serum albumin at low pH. Eur. J. Biochem. 266:26–32.
- Muzammil S., Kumar Y. and Tayyab S. (2000) Anion-induced stabilization of human serum albumin prevents the formation of intermediate during urea denaturation. Proteins: Struct. Funct. Genet. 40:29–38.
- Naeem A., Khan K.A. and Khan R.H. (2004) Characterization of a partially folded intermediate of papain induced by fluorinated alcohols at low pH. Arch. Biochem. Biophys. 432:79–87.
- Nakamura S., Baba T. and Kidokoro S. (2007) A molten globule-like intermediate state detected in the thermal transition of cytochrome c under low salt concentration. Biophys. Chem. 127:103–112.
- Nakamura S., Seki Y., Katoh E. and Kidokoro S. (2011) Thermodynamic and structural properties of the acid molten globule state of horse cytochrome c. Biochemistry 50:3116–3126.
- Negi S. and Banerjee R. (2010) Study of conformational changes in glucoamylase of *Aspergillus awamori* nakazawa in presence of denaturants through CD-spectroscopy. Bioresource Technol. 101:7577–7580.
- Neustroev K.N., Golubev A.M., Firsov L.M., Ibatullin F.M., Protasevich I.I. and Makarov A.A. (1993) Effect of modification of carbohydrate component on properties of glucoamylase. FEBS Lett. 316:157–160.
- Nishii I., Kataoka M., Tokunaga F. and Goto Y. (1994) Cold denaturation of the molten globule states of apomyoglobin and a profile for protein folding. Biochemistry 33:4903–4909.
- Norouzian D. and Jaffar M.B. (1993) Immobilization of glucoamylase produced by fungus *Arthrobotrys amerospor*. Ind. J. Exp. Biol. 31:680–681.

- Nozaki Y. and Tanford C. (1967) Acid-base titrations in concentrated guanidine hydrochloride. Dissociation constants of the guanidinium ion and of some amino acids. *J. Am. Chem. Soc.* 89:736–742.
- Ohnishi H., Sakai H. and Ohta T. (1991) Purification and some properties of a glucoamylase from *Clostridium sp.* G0005. *Agric. Biol. Chem.* 55:1901–1902.
- Okada G. and Unno T. (1989) A glucodextranase accompanied by glucoamylase activity from *Arthrobacter globiformis* I42. *Agric. Biol. Chem.* 53:223–228.
- Ono K., Shigeta S. and Oka S. (1988) Effective purification of glucoamylase in *Koji*, a solid culture of *Aspergillus oryzae* on steamed rice, by affinity chromatography using an immobilized acarbose (BAY g-542). *Agric. Biol. Chem.* 52:1707–1714.
- Ono S., Tanpa S., Yamazaki I., Yoshimura T., Matsumoto T., Yamaura I., Kato T., Yamashita I. and Shimasaki C. (1996) Comparison of thermal and guanidine hydrochloride denaturation behaviors of glucoamylase from the STA1 gene of *Saccharomyces cerevisiae* var. *diastaticus*. *Biosci. Biotechnol. Biochem.* 60:1543–1545.
- Ortbauer M. and Popp M. (2008) Functional role of polyhydroxy compounds on protein structure and thermal stability studied by circular dichroism spectroscopy. *Plant Physiol. Biochem.* 46:428–434.
- Othon C.M., Kwon O., Lin M.M. and Zewail A.H. (2009) Solvation in protein (un)folding of melittin tetramer-monomer transition. *Proc. Natl. Acad. Sci., USA* 106:12593–12598.
- Pace C.N., Shirley B.A. and Thomson J.A. (1989) Measuring the conformational stability of a protein. In: Greighton T.E., editor. *Protein Structure: A Practical Approach*. New York, Oxford University Press, p. 311–330.
- Pandey A. (1995) Glucoamylase research: An overview. *Starch* 47:439–445.
- Pandey A., Nigam P., Soccol C.R., Soccol V.T., Singh D. and Mohan R. (2000) Advances in microbial amylases. *Biotechnol. Appl. Biochem.* 31:135–152.
- Pazur J.H. and Ando T. (1959) The action of an amyloglucosidase of *Aspergillus niger* on starch and malto-oligosaccharides. *J. Biol. Chem.* 234:1966–1970.
- Pazur J.H. and Kleppe K. (1962) The hydrolysis of α -D-glucosides by amyloglucosidase from *Aspergillus niger*. *J. Biol. Chem.* 237:102–106.
- Pazur J.H., Knull H.R. and Cepure A. (1971) Glycoenzymes: Structure and properties of the two forms of glucoamylase from *Aspergillus niger*. *Carbohydr. Res.* 20:83–96.
- Poddar N.K., Ansari Z.A., Singh R.K.B., Moosavi-Movahedi A.A. and Ahmad F. (2008) Effect of monomeric and oligomeric sugar osmolytes on ΔG_D , the Gibbs energy of stabilization of the protein at different pH values: Is the sum

effect of monosaccharide individually additive in a mixture? *Biophys. Chem.* 138:120–129.

- Prajapati R.S., Indu S. and Varadarajan R. (2007) Identification and thermodynamic characterization of molten globule states of periplasmic binding proteins. *Biochemistry* 46:10339–10352.
- Pretorius I.S., Lambrechts M.G. and Marmur J. (1991) The glucoamylase multigene family in *Saccharomyces cerevisiae* var. *diastaticus*: An overview. *CRC Crit. Rev. Biochem. Mol. Biol.* 26:53–76.
- Pugh T.A., Shah J.C., Magee P.T. and Clancy M.J. (1989) Characterization and localization of the sporulation glucoamylase of *Sacchromyces cerevisiae*. *Biochim. Biophys. Acta* 994:200–209.
- Ramos C.H.I., Lima Jr. M.V., Silva S.L.F., Borin P.F.L., Regis W.C.B. and Santoro M.M. (2004) Stability and folding studies of the N-domain of troponin C. Evidence for the formation of an intermediate. *Arch. Biochem. Biophys.* 427:135–142.
- Ray S., Bhattacharyya M. and Chakrabarti A. (2005) Conformational study of spectrin in presence of submolar concentrations of denaturants. *J. Fluoresc.* 15:61–70.
- Reilly P.J. (1999) Protein engineering of glucoamylase to improve industrial properties: A review. *Starch* 51:269–274.
- Roberts J.D., Webb R.L. and McElhill E.A. (1950) The electrical effect of the trifluoromethyl group. *J. Am. Chem. Soc.* 72:408–411.
- Roccatano D., Fioroni M., Zacharias M. and Colombo G. (2005) Effect of hexafluoroisopropanol alcohol on the structure of melittin: A molecular dynamics simulation study. *Protein Sci.* 14:2582–2589.
- Ruth S., Nielsen T. and Poulsen P.B. (1979) Application possibilities of a novel immobilized glucoamylase. *Starch* 31:333–337.
- Saha B.C and Zeikus J.G. (1989) Microbial glucoamylases: Biochemical and biotechnological features. *Starch* 41:57–64.
- Sasvari Z. and Asboth B. (1998) Formation of disulfide-bridged dimers during thermoinactivation of glucoamylase from *Aspergillus niger*. *Enzyme Microb. Technol.* 22:466–470.
- Sauer J., Sigurskjold B.W., Christensen U., Frandsen T.P., Mirgorodskaya E., Harrison M., Roepstor P. and Svensson B. (2000) Glucoamylase: Structure/function relationships, and protein engineering. *Biochim. Biophys. Acta* 1543:275–293.
- Saunders A.J., Davis-Searles P.R., Allen D.L., Pielak G.J. and Erie D.A. (2000) Osmolyte-induced changes in protein conformational equilibria. *Biopolymers* 53:293–307.

- Selkoe D.J. (2004) Cell biology of protein misfolding: The examples of Alzheimer's and Parkinson's diseases. *Nat. Cell Biol.* 6:1054–1061.
- Sen P., Ahmad B., Rabbani G. and Khan R.H. (2010) 2,2,2-Trifluoroethanol induces simultaneous increase in α -helicity and aggregation in alkaline unfolded state of bovine serum albumin. *Int. J. Biol. Macromol.* 46:250–254.
- Sharma V., Jaishankar A., Wangb Y-C. and McKinley G.H. (2011) Rheology of globular proteins: Apparent yield stress, high shear rate viscosity and interfacial viscoelasticity of bovine serum albumin solutions. *Soft Matter* 7:5150–5160.
- Shenoy B.C., Katwa L.C., Rao A.G.A. and Rao M.R.R. (1985) Fungal glucoamylases. *J. Biosci.* 7:399–419.
- Shenoy B.C., Rao A.G.A. and Rao M.R.R. (1984) Structure and stability of glucoamylase II from *Aspergillus niger*: A circular dichroism study. *J. Biosci.* 6:601–611.
- Shigechi H., Koh J., Fujita Y., Matsumoto T., Bito Y., Ueda M., Satoh E., Fukuda H. and Kondo A. (2004) Direct production of ethanol from raw corn starch via fermentation by use of a novel surface-engineered yeast strain codisplaying glucoamylase and α -amylase. *Appl. Environ. Microbiol.* 8:5037–5040.
- Sills A.M., Saunder M.E. and Stewart G.G. (1984) Isolation and characterization of the amyolytic system of *Schwanniomyces castellii*. *J. Inst. Brew.* 90:311–316.
- Sirangelo I., Piaz F.D., Malmo C., Casillo M., Birolo L., Pucci P., Marino G. and Irace G. (2003) Hexafluoroisopropanol and acid destabilized forms of apomyoglobin exhibit structural differences. *Biochemistry* 42:312–319.
- Sonnichsen F.D., Van Eyk J.E., Hodges R.S. and Sykes B.D. (1992) Effect of trifluoroethanol on protein secondary structure: An NMR and CD study using a synthetic actin peptide. *Biochemistry* 31:8790–8798.
- Sorimachi K., Jacks A.J., Le Gal-Coeffet M-FL, Williamson G., Archer D.B. and Williamson M.P. (1996) Solution structure of the granular starch binding domain of glucoamylase from *Aspergillus niger* by nuclear magnetic resonance spectroscopy. *J. Mol. Biol.* 259:970–987.
- Specka U., Mayer F. and Antranikian G. (1991) Purification and properties of thermoactive glucoamylase from *Clostridium thermosaccharolyticum*. *Appl. Environ. Microbiol.* 57:2317–2323.
- Srivastava R.A.K. (1984) Studies on extracellular and intracellular purified amylases from a thermophilic *Bacillus stearothermophilus*. *Enzyme Microb. Technol.* 6:422–426.
- Svensson B., Larsen K. and Gunnarsson A. (1986) Characterization of a glucoamylase G2 from *Aspergillus niger*. *Eur. J. Biochem.* 154:497–502.

- Svensson B., Larsen K., Svendsen I. and Boel E. (1983) The complete amino acid sequence of the glycoprotein, glucoamylase G1, from *Aspergillus niger*. Carlsberg Res. Commun. 48:529–544.
- Svensson B., Pedersen T.G., Svendsen I.B., Sakai T. and Ottesen M. (1982) Characterization of two forms of glucoamylase from *Aspergillus niger*. Carlsberg Res. Commun. 47:55–69.
- Takahashi T., Iwama M., Tsuchida Y. and Irie M. (1985) Kinetic and circular dichroism spectroscopic comparison among three forms of glucoamylase from a *Rhizopus* sp. Chem. Pharm. Bull. 33:276–281.
- Takeda Y., Matsui H., Tanida M., Tani S. and Chib S. (1985) Purification and substrate specificity of glucoamylase of *Puccinomyces variofi* AHU9417. Agric. Biol. Chem. 49:1633–1641.
- Tamaki H. (1978) Genetic studies of ability to ferment starch in *Saccharomyces*: Gene polymorphism. Mol. Gen. Genet. 164:205–210.
- Tanaka A., Fukada H. and Takahashi K. (1995) Differential scanning calorimetric studies on the domain structure of *Aspergillus* glucoamylase. J. Biochem. 117:1024–1028.
- Tanaka A., Karita S., Kosuge Y., Senoo K., Obata H. and Kitamoto N. (1998) Thermal unfolding of the starch binding domain of *Aspergillus niger* glucoamylase. Biosci. Biotechnol. Biochem. 62:2127–2132.
- Tanford C., Kawahara K., Lapanje S., Hooker T.M. Jr., Zarlengo M.H., Salahuddin A., Aune K.C. and Takagi T. (1967) Proteins as random coils. III. Optical rotatory dispersion in 6 M guanidine hydrochloride. J. Am. Chem. Soc. 89:5023–5029.
- Tanford C. (1968) Protein denaturation. Adv. Protein Chem. 23:121–282.
- Tanford C. (1969) Extension of the theory of linked functions to incorporate the effects of protein hydration. J. Mol. Biol. 39:539–544.
- Tatsumi E. and Hirose M. (1997) Highly ordered molten globule-like state of ovalbumin at acidic pH: Native-like fragmentation by protease and selective modification of Cys367 with dithiodipyridine. J. Biochem. 122:300–308.
- Taylor P.M., Napier E.J. and Fleming I.D. (1978) Some properties of a glucoamylase produced by the thermophilic fungus *Humicola lanuginosa*. Carbohydr. Res. 16:301–308.
- Tayyab S., Khan N.J., Khan M.A. and Kumar Y. (2003) Behavior of various mammalian albumins towards bilirubin binding and photochemical properties of different bilirubin-albumin complexes. Int. J. Biol. Macromol. 31:187–193.

- Timasheff S.N. (1998) Control of protein stability and reactions by weakly interacting cosolvents: The simplicity of the complicated. *Adv. Protein Chem.* 51:355–432.
- Timasheff S.N. (2002) Protein hydration, thermodynamic binding, and preferential hydration. *Biochemistry* 41:13473–13482.
- Tripathi P., Hofmann H., Kayastha A.M. and Ulbrich-Hofmann R. (2008) Conformational stability and integrity of α -amylase from mung beans: Evidence of kinetic intermediate in GdmCl-induced unfolding. *Biophys. Chem.* 137:95–99.
- Uversky V.N., Narizhneva N.V., Kirschstein S.O., Winter S. and Lober G. (1997) Conformational transitions provoked by organic solvents in β -lactoglobulin: Can a molten globule like intermediate be induced by the decrease in dielectric constant? *Folding Des.* 2:163–172.
- Varshney A., Ahmad B., Rabbani G., Kumar V., Yadav S. and Khan R.H. (2010) Acid-induced unfolding of didecameric keyhole limpet hemocyanin: Detection and characterizations of decameric and tetrameric intermediate states. *Amino Acids* 39:899–910.
- Vihinen M. and Mantsala P. (1989) Microbial amylolytic enzymes. *Crit. Rev. Biochem. Mol. Biol.* 24:329–418.
- Walgers R., Lee T.C. and Cammers-Goodwin A. (1998) An indirect chaotropic mechanism for the stabilization of helix conformation of peptides in aqueous trifluoroethanol and hexafluoro-2-propanol. *J. Am. Chem. Soc.* 120:5073–5079.
- Wang C., Eufemi M., Turano C. and Giartosio A. (1996) Influence of the carbohydrate moiety on the stability of glycoproteins. *Biochemistry* 35:7299–7307.
- Wang C., Lascu I. and Giartosio A. (1998) Bovine serum fetuin is unfolded through a molten globule state. *Biochemistry* 37:8457–8464.
- Whitmore L. and Wallace B.A. (2008) Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases. *Biopolymers* 89:392–400.
- Williamson G., Belshaw N.J., Noel T.R., Ring S.G. and Williamson M.P. (1992) O-Glycosylation and stability. Unfolding of glucoamylase induced by heat and guanidine hydrochloride. *Eur. J. Biochem.* 207:661–670.
- Wong Y-H. and Tayyab S. (2012) Protein stabilizing potential of simulated honey sugar cocktail under various denaturation conditions. *Process Biochem.* 47:1933–1943.
- Wood S.J., Maleeff B., Hart T. and Wetzel R. (1996) Physical, morphological and functional differences between pH 5.8 and 7.4 aggregates of the Alzheimer's amyloid peptide A β . *J. Mol. Biol.* 256:870–877.

- Wu L.C., Grandori R. and Carey J. (1994) Autonomous subdomains in protein folding. *Protein Sci.* 3:369–371.
- Xie G. and Timasheff S.N. (1997) Mechanism of the stabilization of ribonuclease A by sorbitol: Preferential hydration is greater for the denatured than for the native protein. *Protein Sci.* 6:211–221.
- Yadav J.K. and Prakash V. (2009) Thermal stability of α -amylase in aqueous cosolvent systems. *J. Biosci.* 34:1–11.
- Yagi M., Sakurai K., Kalidas C., Batt C.A. and Goto Y. (2003) Reversible unfolding of bovine β -lactoglobulin mutants without a free thiol group. *J. Biol. Chem.* 278:47009–47015.
- Yanagi K., Ashizaki M., Yagi H., Sakurai K., Lee Y-H. and Goto Y. (2011) Hexafluoroisopropanol induces amyloid fibrils of islet amyloid polypeptide by enhancing both hydrophobic and electrostatic interactions. *J. Biol. Chem.* 286:23959–23966.
- Yang J., Dunker A.K., Powers J.R., Clark S. and Swanson B.G. (2001) β -Lactoglobulin molten globule induced by high pressure. *J. Agric. Food Chem.* 49:3236–3243.
- Yoshida K., Kawaguchi J., Lee S. and Yamaguchi T. (2008) On the solvent role in alcohol-induced α -helix formation of chymotrypsin inhibitor 2. *Pure Appl. Chem.* 80:1337–1347.
- Zaroog M.S. and Tayyab S. (2012) Formation of molten globule-like state during acid denaturation of *Aspergillus niger* glucoamylase. *Process Biochem.* 47:775–784.
- Zhang H., Kaneko K., Nguyen J.T., Livshits T.L., Baldwin M.A., Cohen F.E., James T.L. and Prusiner S.B. (1995) Conformational transitions in peptides containing two putative α -helices of the prion protein. *J. Mol. Biol.* 250:514–526.

LIST OF PUBLICATIONS / PRESENTATIONS

Publications

1. **Zaroog M.S.** and Tayyab S. (2013) Halogenol- versus alkanol-induced structural transitions of acid-denatured glucoamylase: Characterization of alcohol-induced states. *Process Biochem.* 48:853–862.
2. **Zaroog M.S.** and Tayyab S. (2012) Formation of molten globule-like state during acid denaturation of *Aspergillus niger* glucoamylase. *Process Biochem.* 47:775–784.
3. **Zaroog M.S.**, Kadir H.A. and Tayyab S. (2013) Structural changes and stabilizing effect of various polyols on the native and the acid-denatured states of glucoamylase. *Biosci. Biotechnol. Biochem.* – Communicated.
4. **Zaroog M.S.**, Kadir H.A. and Tayyab S. (2013) Stabilizing effect of various polyols on the native and the denatured states of glucoamylase. *The Sci. World J.* – Accepted.
5. Halim A.A.A., **Zaroog M.S.**, Kadir H.A. and Tayyab S. (2013) A comparative study on the alcohol-induced structural transitions in acid-denatured *Bacillus licheniformis* α - amylase. *Spectrochim. Acta* –Communicated.

Presentations

1. **Zaroog M.S.** and Tayyab S. (2012) Behavior of the molten globule state of *Aspergillus niger* glucoamylase towards halogenols and alkanols. Proceedings of the 13th FAOBMB Congress held at BITEC, Bangkok, Thailand, on November 25-29, 2012. Poster Abstract No. P-B-11, p. 98.
2. **Zaroog M.S.** and Tayyab S. (2012) Fluoro, chloro and alkyl alcohol-induced structural changes in ficin at low pH. Proceedings of the 37th Annual Conference

of the Malaysian Society for Biochemistry and Molecular Biology, held at Sime Derby Convention Centre, Kuala Lumpur, Malaysia, on July 18-19, 2012. Poster Abstract No. 101, p. 155. *[Awarded the Best Poster Award]*

3. **Zaroog M.S.** and Tayyab S. (2011) Does acid-denatured state of *Aspergillus niger* glucoamylase represent molten globule state? Proceedings of the 16th Biological Sciences Graduate Congress, held at NUS, Singapore, on December 12-13, 2011. Poster Abstract No. PP-2-08, p. 72.