EFFECTS OF CHEMICAL CHAPERONES AND DETERGENTS ON THERMOSTABILITY OF PEPPER LEAF PROTEINS

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CHAPTER I

INTRODUCTION

Humans acquire a large portion of nutrition directly or indirectly from plants. Plants are affected by environmental conditions. Adverse environmental factors, for example drought, high temperature, salinity, heavy metals and mechanical stresses are all great challenges to plants. It was calculated from data assembled by the U.S. Department of Agriculture (Boyer, 1982) that 44.9 percent of U.S. arable area suffers from water-deficit, and 16.5 and 15.7 percent of soils are too wet or cold. Saline soil, alkaline soil and soil-less areas take up another 7.4 percent. Only 12.1 percent of the land surface is free of unfavorable factors. Heat stress, where temperatures are high enough for sufficient time to cause irreversible damage to plant function or development, is also a major stress factor limiting crop yield. In sum, research focused on understanding plants' mechanisms in responding to environmental stress, including heat stress, is of great significance and has the potential to help improve agricultural practices and greatly benefit world agriculture.

1.1 Factors affecting plant response to high temperature

The extent of the injuries caused by high temperature depends on the type of crop, stage of growth, the tissue exposed to heat, and the existence of other adverse factors. It

is logical that crops grown in tropic areas are much more adaptable to high temperature than temperate plants. Al-Khatib and Paulsen (1999) compared the heat responses among various species and found that photosystem II in wheat, a cool season species, is more sensitive to heat than that of rice and pearl millet. This discovery can at least partially explain why warm season species are more stable under higher temperatures in respect to CO_2 fixation. Moreover, effects of heat stress vary even within one species of crop. Some developmental stages, such as the periods of pollen development, are more susceptible to heat than others. A marked loss of yield and quality can occur when plants suffer from heat stress during the pollen development period, even though temperatures during the rest of the life cycle are optimum. It is true that injuries may occur throughout the entire life of a plant, but those stages of growth that are crucial for yield and quality are more important for applied research.

The timing and duration of heat stress is also of great fundamental importance. This can be revealed in terms of its relation with growth resumption. The longer the heat duration, the longer total time the resumption needs. Death can occur if the high temperature maintains long enough (Hilbrig, 1900). Also, exposure time is exponentially related with the killing temperature, which had been demonstrated as a linear relationship between the log of heat-killing rate and the increase of temperature in Arrhenius plots (Aleksandrov, 1964).

Other kinds of stresses, such as drought and ultraviolet irradiation, can be associated with heat stress and cause much worse injuries; even much more severe than the additive result of separate ones. It was reported by Al-Khatib and Paulsen in 1990 that

photosynthesis was reduced more rapidly and greatly when treated with high air temperature and high irradiance simultaneously than separately.

1.2 Terminology and classification

A relative standard of high temperature is necessary to be established for comparative studies, although there are no absolute values for all plants. Levitt (1980) dynamically considered different defense systems existing in different crops and defined the term high temperature. The temperatures above 50°C were called lethal temperatures, under which death may occur in most crops if the exposure time duration is long enough. The range of 15-32 °C was termed as moderately high temperatures and the range of 32-50 °C was regarded as very high temperature, which are commonly occurring in world's major crop regions.

Moderate and extreme injuries are termed according to their different intensity. The acute and chronic injuries are based on the time factor. The acute injury, induced by the killing temperature referred to before, can cause irreversible injury in a short period of time. In contrast, chronic injury results from a gradual heating process at more moderate temperatures and the injuries can be repaired. The mechanism in each type of injury is discussed below.

1.3 Injury mechanisms

1.3.1 Chronic injury

1.3.1.1 Imbalances between metabolic pathways: photosynthesis/respiration

Photosynthesis is the process whereby plants absorb solar energy and assimilate carbohydrates, while the respiration process consumes sugars and releases the energy. At normal conditions, there is a balance between these two processes, which guarantees the

normal growth and development of plants. The two processes are differentially affected by heat. The temperature compensation point is defined as the temperature at which respiration and photosynthesis have equal rates (Levitt, 1980). Consumption will be greater than assimilation in plants when the temperature is higher than the compensation point. If the duration of heat stress is long enough, starvation and death will occur, caused by the depletion of the plant's reserves.

1.3.1.2 Secondary stresses

Heat can be accompanied by other kinds of abiotic stresses such as water deficit and ultraviolet irradiation. Drought is commonly such a stress, which mainly results from the sharp increase of transpiration induced by high temperatures. A 5 °C increase of leaf temperature can double the gradient of vapor pressure between the leaf and the atmosphere (Curtis, 1936), one of the major forces promoting transpiration. Besides, transpiration can be directly increased by the environmental temperature. Therefore, the balance of water input and output can be disrupted and water deficit occurs (Levitt, 1980). However, the release of heat can be further limited due to the water deficiency. Stoma is formed by a pair of guard cells located in the leaf epidermis. Guard cells regulate the opening and closure of the stoma by adjusting their shape according to the turgor pressure. In the case of water deficiency, turgor pressure in guard cells will decrease, which will lead them to shrink and to be drawn together from the swelling state of bowing apart from one another. Therefore, the stoma is closed and the temperature of the tissue will increase due to the failure of heat release through the process of transpiration.

1.3.2 Acute injury

1.3.2.1 Membranes

Membranes are found to be a site of heat injury by observation using light and electron microscopes. Membrane integrity will be damaged and the permeability to solutes and ions can be changed by heat (Burke and Orzech, 1988). Bernstam and Arndt (1973) detected that substances leaked from myxomycete membranes under continuously rising temperature and found that those substances were specific under different temperatures and durations. As temperature rises, plasmodial pigments leak first, followed by nucleic acids and protein metabolites. They also found that the rate of leakage increases as the temperatures rise. Base on those results, Levitt (1980) concluded that membrane leakage is the first true sign of injury. The mechanism of leakiness can be the physical disruption of membrane structure, damage to membrane transport systems, or phase transitions in membrane lipids. The mobility of membrane lipids will increase as the temperature rises. Hyper-fluidity of lipids can result in the destruction of the lipid layer (Levitt, 1980). Also, membranes such as chloroplast thylakoid membrane and mitochondria membrane, two of the most heat-susceptible components in cells, would leak after lipid oxidization by ROS (see next section). A much worse effect caused by the leakage is that it changes redox potential between major cellular compartments, which is the force to maintain the order of their components, such as proteins, nucleic acid and membrane lipids. The changed redox potential can mix those components, impair metabolic processes and even cause cell death (Klueva et al., 2001).

1.3.2.2 AOS/ROS lipid peroxidation

Reactive or activated oxygen species (ROS/AOS), including oxygen ions, free radicals and peroxides, are of high reactivity and play an important role in cell structure

and signaling. Free radicals, atoms or small molecules with unpaired electrons in the valence shell are common ROS/AOS. Their levels can be much higher when plants suffer from stresses, resulting in the so-called oxidative stress. Oxidative damage is considered to be one of the major damaging factors induced by heat stress. Yeast exposed to severe high temperature will die mainly due to oxidative damage to its cell (Davidson et al., 1996). In plants, the process of photoinhibition induced by heat is caused by ROS. There is a balance between the solar energy captured by pigments and reducing power transported through photosynthetic cytochromes at normal state. Heat shock disrupts the coupling of electron transport and the balance of energy. The excess energy can flow to molecular oxygen, producing and accumulating ROS, commonly in chloroplast and mitochondria, two major locations where electron transportation takes place. The most heat-susceptible component in chloroplasts is the PSII complex (Schuster et al., 1988; Burke, 1990). Heat induces change in its composition and disaggregation of its functional components (Suss and Yordanov, 1986), resulting in imbalance of electron transport and the accumulation of ROS.

1.3.2.3. Protein Denaturation

Proteins can be denatured when exposed to heat stress, which is considered to be one of the earliest explanations of heat injury (Levitt, 1980). The process of denaturation consists of several steps, beginning with the formation of reversible unfolding intermediates, leading to irreversible structural changes as the temperature or duration of exposure increases, eventually resulting in protein aggregation and loss of solubility (Levitt, 1980). Heat damages protein by disturbing the hydrophobic strength and hydrogen bonds between or within the subunits, which are the main forces to maintain

the structure of protein. Under heat treatment, the conformational entropy increases rapidly, even much faster than the rising of the hydrophobic strength, resulting in damage to hydrophobic bonds followed by disruption of tertiary structure of protein, which is mainly held by hydrophobic strength (Brandt, 1967). It is reported that hydrogen bonds are broken even earlier than the breakup of hydrophobic interaction, which leads to disruption of the secondary and tertiary structure held by hydrogen bonds (Boyarchuk and Vol'kenshein, 1967).

Heat can increase the degradation rate and inhibit activity of enzymes for many essential cellular processes (Klueva et al., 2001). Many enzymes are thermolabile. For example, rubisco activase, a key enzyme in photosynthetic carbon fixation, is particularly susceptible to high temperature inactivation (Eckardt and Portis, 1997). In another example, starch accumulation in wheat grain is greatly reduced by the inhibition of soluble-starch synthase induced by heat (Rijven, 1986). The activity of Cu/Zn SOD in maize seedlings is rapidly decreased when the temperature is above 35 ºC, 25 ºC higher than the optimum temperature (Burke and Oliver, 1992).

1.4 Acclimation responses

1.4.1 Membrane stabilization

Membranes are highly susceptible to heat shock because various enzymes for essential cell processes such as photosynthesis and respiration are concentrated on and in them. It is hypothesized that membranes can maintain their stability under high temperature by changing their composition and interacting with protective substances (Klueva et al., 2001). However, whether and how the modification of composition can protect the membrane function is still under discussion. It is reported that the increase of

digalactosyl diacylglycerol content in thylakoid membrane decreases the ratio of monogalactosyl diacylglycerol to digalactosyl diacylglycerol and can stabilize the membrane. In an experiment using cyanobacterium mutants with polyunsaturated lipid membranes, the extent of thermostability induced by the composition of membrane was very small, which indicates that the saturation of membrane lipids may be unrelated to membrane's thermotolerance (Gombos et al., 1994).

1.4.2 AOS/ROS defense

In order to limit oxidative damage under stress conditions, plants have developed a series of detoxification systems that break down the highly toxic ROS. Plants protect cellular and sub-cellular systems from the cytotoxic effects of ROS using antioxidant enzymes such as superoxide dismutase (SOD), catalase, ascorbate peroxidase, cytochrome peroxidase, glutathione reductase and metabolites like glutathione, ascorbic acid, α-tocopherol and carotenoids. Many experiments provide evidence for antioxidant's function in improving thermostability. Yeast mutants that lack genes for catalase, SOD and cytochrome peroxidase are more sensitive to heat shock than the wild type cells (Davidson et al., 1996). In tobacco mutants with high expression of catalase, the photosynthetic activity is much better protected under heat stress than in the wild type (Willekens et al., 1995). Because antioxidants are a very important component in plant's defense system under heat shock, more studies are still needed (Klueva et al., 2001).

1.4.3 Protein stabilization

Molecular chaperones are defined as proteins assisting the folding and the assembly of other macromolecules without occurring in their structures. Heat shock proteins (HSPs) are common molecular chaperones and have long been expected to play a role in

increasing thermotolerance since they were first discovered by Ritossa (1962). HSP accumulation was detected in soybean, barley, wheat, maize and cotton during their thermotolerance development induced by heat shock (Lin et al., 1984; Marmiroli et al., 1996; Nguyen, 1994).

HSPs are helped by HSP cognate proteins, which have structural homology with their correlated HSPs at normal temperature. HSPs consist of various families, each of which has a specific function. For example, HSP70 can increase the acquired thermotolerance in plants but has no effects on improving the inherent thermostability (Lee and Schoffl, 1996). HSP70 is indicated to be the rate-limiting factor in acquiring thermostability of Drosophila by comparing mutants with higher level of HSP70 expression and the normal embryos (Welte et al., 1993). HSP104 can promote disaggregation of proteins misfolded or denatured by heat (Vogel et al., 1995). HSP27 has a close connection with the antioxidant activity. HSP27 can increase the content of glutathione and reduce the level of ROS in murine cells (Mehlen et al., 1996). However, some HSP families have no effect shown in experiments. Yeast mutants without normal levels of HSP30 have no functional deficiency observed when compared with the wild-type strain (Smith and Yaffe, 1991).

1.4.4 Chemical chaperone

In previous research, Back and colleagues (1979) found that sugars and polyols can help stabilize proteins against heat denaturation. The extent of stabilization by different sugars and polyols is related to their different impacts on the structure of water. Later, more additives, such as L-proline, L-serine, γ-aminobutyric acid, sarcosine, taurine, α-alanine, β-alanine, glycine, betaine, and trimethylamine N-oxide were reported by

Arakawa and Timasheff (1985) to stabilize protein structure under heat stress, yet avoid direct binding to protein molecules.

In a series of studies conducted by Timasheff and co-workers, 2-methyl-2, 4-pentanediol (Pittz and Timasheff, 1978), sugars (Lee et al., 1975), salts (Arakawa and Timasheff, 1982), glycerol (Gekko and Timasheff, 1981), glycine, α-alanine and β-alanine (Arakawa and Timasheff, 1983) were tested as protein stabilizers. Preferential hydration of protein in the protein-water-cosolvent system was reported (2002) to account for the structural stabilization of protein. The addition of these protein stabilizers, which increase the surface tension of water by increasing the chemical potential of protein, further induces the preferential exclusion of cosolvents from protein surfaces and the preferential binding of water molecules by proteins. Interacting loci are sites on protein surfaces where water and solvent components exchange. Preferential interaction includes preferential hydration and preferential binding of ligand, which are termed in terms of their relative affinities with interacting loci. When the affinity between water molecules and interacting loci is greater than that with the ligand, the excess of water binds to the protein domain, which is termed as the preferential hydration or the preferential exclusion of ligand. On the contrary, if the relative affinity between interacting loci and ligand is greater, the preferential binding of ligand can occur. In terms of thermodynamics, preferential binding is quantitatively equal to the thermodynamic binding. When stabilizers are added, the interaction between the cosolvent and protein is unfavorable due to the increase of the system free energy, and preferential exclusion of cosolvent and preferential hydration can be induced.

Timasheff's ideas on protein-solvent-cosolvent interactions receive additional support from studies using other systems. Trimethylamine N-oxide (TMAO) was reported to protect chymotrypsin inhibitor 2 against unfolding induced by urea (Bennion et al., 2004). Urea denatures protein by facilitating the hydrophobic side-chain exposure as well as interacting with the main chain. TMAO can stabilize protein structure by ordering the structure of solvent. Sandwich structure was observed between TMAO and water molecules, by which water-water H-bonds in the hydration layer can be strengthened and water-denatured protein and urea-protein interactions can be discouraged. In this way, a hydration layer on the surface of protein occurs and the folded state can be stabilized.

Many studies focusing on the composition of the cosolvent solution have been conducted. Sarcosine, glycine betaine and trimethylamine N-oxide were reported to be able to protect the active and native conformation of α_1-antitrypsin at elevated temperatures in a concentration-dependent manner. High temperature can induce a partial unfolding state of α_1-antitrysin, resulting in the increase of the exposure of its peptide backbones, which cannot interact favorably with the addition of those osmolytes. However, the free energy decreases with this addition, which can force proteins to sequester and maintain a folded and a more compact state. In this solvophobic manner, α_1-antitrysin's conversion to its intermediate state and the following polymerization can be hindered and the native state is stabilized. However, the effect of aiding correct refolding has not been demonstrated (Chow et al., 2001). Another example of concentration-dependent protection was revealed by Ganea in 2005. High concentration of trehalose and 6-aminohexanoic acid (AHA) helps maintain the activity of glucose-6-phosphate dehydrogenase (G6PD) against glycation-induced inactivation. They can also

help restore the activity; however, the reactivation effect is less efficient than its stabilization effect, due to irreversible processes. Both protection effects by those osmolyte stabilizers exhibited during the folding and refolding processes show a non-specific manner. The unfolded state of protein has high free energy that is thermodynamically unflavored. The addition of osmolytes lowers the system's free energy and drives the folding of the denatured proteins. However, if a protein's native conformation is less thermodynamically stable than its misfolded state, osmolyte stabilizers may have no effective function in helping proteins refold correctly.

However, dimethylsulfoxide, glycerol, proline and sucrose are reported to provide effective protection for proteins not only from the inhibition of aggregation, but also help the refolding of protein's denatured state in a concentration-dependent way (Kim et al., 2006). All of those osmolytes can help reduce the exposure of the protein's hydrophobic core, but their efficiencies are different. Glycerol was detected to provide the best stabilizing and refolding aid for aminoacylase.

A significant connection between stabilizing effects of chemical chaperones and their physical properties has been established. However, studies using different, single proteins to assay chemical chaperone effects are not always in agreement. This can be explained based on the large range of physical properties found in different proteins. One way to address this difficulty is to use a complex mixture of proteins that represents an average of the properties of individual proteins. In a study of pepper leaf thermostability, the relationships between heat stability and leaf extract concentration, buffer pH, chelating agent, nonionic detergent and calcium were established (Anderson, 2006).The thermostability of leaf extracts decreases with its increasing concentrations. High

concentrations of pepper leaf protein generate more opportunities for intermolecular interactions between protein molecules during high temperature denaturation. The pH can affect the thermostability by adjusting the ionization state of protein side chains, or changing their conformation and electrostatic potential. Fixed ions on the surface of protein molecules provide electrostatic repelling forces, by which the solubility can be maintained and aggregation can be hindered. Ethylenediaminetetraacetic acid (EDTA), a chelating agent, was reported to have a small effect in stabilizing proteins over the examined concentration range. In 2007, Anderson focused on chaperone properties such as molecular weight, polarity and hydrogen bond capacity. Through evaluation of various classes of cosolvents differing in properties such as polarity, OH density and molecular weight, it was found that alcohols and polyols can not only have stabilizing or destabilizing effects, but also that those effects are additive. These findings were consistent with chemical chaperone effects on the overall free energy change resulting from the substitution of solvent with cosolvent in the protein-solvent-cosolvent system. To demonstrate the effect of molecular weight, ethylene glycol, methanol, glycerol and mannitol were selected as a series of chemical compounds which have different molecular weight but a fixed carbon atom to OH group ratio. Thermostability, based on the maximum apparent absorbance temperature at 540 nm, increased with increasing molecular weight. Similar results were observed by Davis-Searles and co-workers in 2001 in the study to assess the stabilization of horse heart ferricytochrome *c* by osmolytes. By detecting the free energy change associated with the reaction from the folded and the unfolded state of the protein in the presence of osmolyte, they found that polyol osmolytes was able to stabilize proteins and their stabilizing effects were a

function of both the polyol concentration and molecular size. The OH group density, which reveals the hydrogen bond capacity of the compounds, is another property of interest. Compounds with a range in number of OH groups but with the same number of carbon atoms (n-propyl alcohol, propylene glycol and glycerol), or a different number of carbon atoms with fixed OH groups (methanol, ethanol, propanol and butanol) were chosen for study and chemicals with a higher ratio of OH groups to carbon atoms exhibited a higher temperature at the apparent absorbance maximum, which was attributed to the higher polarity contributed by OH groups. Anderson then evaluated mannitol, glycerol, ethylene glycol, propylene glycol and methanol at concentrations yielding the same OH density but different polarity and found that the more hydrophilic the compounds, the greater they stabilized protein under heat treatment. A linear relationship was reported between the maximum apparent absorbance temperature and OH group density or polarity indicated by log Kow (oil/water partition coefficient). More interestingly, when the stabilizing compounds were mixed together, the effect of stabilizing is also additive and the effect can be canceled out when mixing stabilizing and destabilizing compounds. Many researchers are also interested in the combination effect of cosolvents. Similar studies were conducted by Biar and McClements (2003). They reported that a combination of NaCl and sucrose can protect proteins from gelation induced by high temperature, although they have different stabilizing mechanisms. In 2006, the same authors used a mixture of glycerol and sucrose, both of which are protein stabilizers, to treat bovine serum albumin (BSA) under heat stress and found that the thermostability of BSA increased but the net increase varied with the ratio of these two additives.

1.4.5 Detergents

Based on the charge on the head group of surfactant molecules, surfactants can be divided into four categories: nonionic surfactants with no charge, cationic surfactants that are positively charged, negatively charged anionic surfactants, and zwitterions containing oppositely charged groups. Ionic surfactants are commonly used as the denaturant for proteins. The denaturing activity is influenced by pH and ionic strength of the system. However, nonionic detergent can maintain the protein's structure and function due to its neutral charges but may lower the activity of proteins in the process of protein separation. Zwitterionic detergents combine the features of ionic and nonionic ones. They are less likely to denature proteins than ionic ones but can be more efficient than nonionic detergents in disrupting the protein-protein bond. The charge of detergent plays a determining role in the interactions between enzyme and surfactant. In the research conducted by Yang et al. (2007), the activity and stability of mushroom tyrosinase were tested in the presence of cationic CTAB, anionic sodium di-2-ethylhexylsulfosuccinate (Aerosol OT, AOT) and nonionic Brij 52. Both AOT and Brij52 contributed to a higher value of V_{max}/K_m, suggesting enhanced activity of that enzyme. However, CTAB increased the value of K_m, resulting in a decrease in the V_{max}/K_m ratio, showing an inhibitory effect on its activity. Thermostability was also tested. Brij 52 was found to stabilize mushroom tyrosinase, even at a higher temperature. Aerosol OT can moderately stabilize the enzyme. Mushroom tyrosinase showed the least stable effect in the presence of CTAB.

Not limited to the electrostatic interactions referred above, the hydrophobic interactions between the alkyl chains of surfactant molecules and hydrophobic pockets of

protein should also be put into consideration to interpret the effect of detergent-protein interactions (Savelli et al., 2000). The hydrophobic/hydrophilic balance of the surfactant molecule was suggested as a significant factor in explaining the interaction between sulfobetaines, zwitterionic detergents and β-lactamase (Spreti et al., 2001). In addition, the environment of the enzyme-surfactant complex is another significant factor. The pH and ionic strength of the media can determine how surfactant acts when bound with enzyme molecules, which was revealed by Yang (2007). Mushroom tyrosinase, whose isoelectric point is at 4.8, is negatively charged in a pH 6.0 surrounding adjusted by Tris buffer. The addition of AOT, which can bring in the positive sodium, can greatly increase the ionic strength and enhance its interaction with enzyme molecule. Therefore, the interaction is specific for each surfactant-enzyme combination. It is hard to have a precise predication using a rationale up to now.

Several parameters that can indicate the behavior of detergent include critical micellar concentration (cmc), aggregation number, average micelle molecular weight, critical micelle temperature, cloud point and hydrophile-lipophile balance number. Micelle structure is formed at concentrations of detergent above its cmc. Due to the hydrophilic heads and hydrophobic tails of the surfactant molecules, micelle structure features a hydrophilic surface and hydrophobic core in aqueous solution. Micelle structure lowers the free energy of the system and is thermodynamically favored at higher concentrations. The value of cmc indicates the ability to form the micellar structures and varies with different types of detergents. Critical micellar concentrations may also vary with different measurement techniques and different pH and electrolyte environment. Aggregation number (N) is the average number of monomers per micelle. It is a parameter to

determine the micelle size and varies widely among detergents (Table 1). For example, the aggregation number for sodium glycocholate, N-Lauroylsarcosine, and Sodium cholate is 2 while for Triton X100 it is 100-155 (Anonymous, 2008). Aggregation number can be increased by a larger concentration of detergent due to some micelles exhibiting growth in a concentration-dependent manner when detergent concentration is above the cmc. Another parameter related to micelle size is the average micelle molecular weight, which can be calculated by multiplying the aggregation number by monomer molecular weight. In the process of protein-detergent separation, detergents with a smaller micelle molecular weight value can be easily removed and result in a better separation. A surfactant solution above the cmc is a complex consisting of micelles, free surfactant molecules and hydrated crystals. Critical micelle temperature (cmt) is the temperature at which detergent solution changes into an isotropic micellar state from the hydrated crystalline state (Neugebauer, 1990). Krafft temperature or Krafft point is the temperature at cmc where the monomers, hydrated crystals and micelles are in equilibrium (Englebienne, 1999).Cloud point (CP) phenomenon occurs at elevated temperatures which can induce the dehydration and a phase separation. A rich phase and a poor phase are formed at CP, the temperature triggering the phase separation. The rich phase, where giant micelles gather as temperature increases, has a smaller volume, while the surfactant monomers are located in the poor phase, at a concentration near the cmc, which is much smaller than the micelle's rich phase. Cloud point values vary based on different detergents, for example, the CP for Triton X100 is 65 ºC, for Tween 20 it is 76 ºC and for SDS and CHAPS it is over 100 ºC (Anonymous, 2008). Cloud point is dependent on the properties and concentration of surfactants and electrolytes. Cloud point

has been used in efficient extraction procedures. For example, cobalt which was dissolved in surfactant medium was concentrated by separating the surfactant rich phase from the solution when temperature was above the cloud point (Nascentes, 2003). Hydrophile-lipophile balance number (HLB) can indicate the hydrophilicity of the detergent.

Table 1. Categories and properties of common detergents (Anonymous, 2008)

	Nonionic detergent			Anionic detergent		Cationic	Zwitterionic	
	Tween 20	Triton X 100	Brij 35	SDS	Sodium deoxycholate	CTAB	CHAPS	N-Dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate
CMC (mM)*	0.06	0.2-0.9	91μM	7-10	2-6	1	6	2-4
Aggregation number	----	100-155	20-40	62	3-12	170	10	55
HLB	16.7	13.5	16.9	40	16	----		----
Cloud Point (ºC)	76	65	>100	>100	----	----	>100	----
Average Micellar Wt.	----	80,000	48,000	18,000	1,200-5,000	62,000	6,150	18,500

*CMC in water at 20-25 ºC

Different modes of micelle formation can be predicted by the packing parameter (P). Packing parameter is the ratio of V_0 to a_0l_0, where V_0 and l_0 represent the volume and length of the hydrophobic tail of a surfactant molecule, and a_0 is the area of its hydrophilic head (Israelachvili et al., 1975). The concept of packing parameter is used to predict the relationship between molecular structure of surfactant monomers and their assembly modes. It is determined by the surface free energy of micelles and can indicate

the most favorable and probable structure surfactants form at moderate concentrations. For example, if the p value is smaller than 1/3, spherical micelles are the most probable structures. If the value is between 1/3 and 1/2, such as CTAB, cylindrical micelles can be predicted with high probability (Israelachvili et al., 1975). However, cylindrical micelles, spherical micelles and inverted micelles can co-exist in the same detergent solution.

Otzen (2002) found a phase variety during protein unfolding at different concentrations of SDS. He proposed two modes of denaturation of the monomeric protein S6 by SDS at concentrations above the cmc. At concentrations slightly above the cmc, spherical micelles can be formed and the unfolding rate increased in a ligand-binding manner, which can be saturated. As the concentration is continuously increased, the proportion of cylindrical micelles increases due to the aggregation of spherical micelles. The unfolding rate of S6 increases in a power-law manner, which is concentration dependent and can help micelles bind tightly at protein site in their transition state of denaturation. The cylindrical micelle can also be induced by high content of NaCl.

The interactions between micelles are a topic of interest. In a nonionic surfactant system, the major interactions between micelles are van der Waals attractive forces (Hayter and Zulauf, 1982), which can be weakened by high temperatures. However, the interactions between charged micelles in an ionic system contain the electronic repulsion forces and the solvation effect besides the van der Waals forces, which is more complicated. Kumar and his coworkers (2000) tested the cloud points under different SDS concentrations in addition with a series of organic additives and found that the alkyl chains of the organic compounds can be partially embedded in the micelle core due to hydrophobic effects but the rest of the chains that are left outside the micelle can further

interact with other alkyl chains which are attached to a different micelle. Such interactions bring micelles closer and add a new attractive force. This force can be increased by choosing an organic additive with a longer alkyl chain, increasing the concentration of additives, or lowering the concentration of detergent. This conclusion was confirmed by Musarat in 2007 with Triton X100 and mixed systems using cationic and anionic surfactants.

1.4.5.1 Ionic Detergent

1.4.5.1.1 Anionic Detergent: Sodium Dodecyl Sulfate

Sodium dodecyl sulfate (SDS), a common detergent used in RNA extraction and the solubilization of membrane-associated proteins, is an anionic surfactant with the amphiphilic property. It is a protein denaturant by binding its sulfate group to the positive side chain of proteins and by the interaction between its alkyl chain and hydrophobic chains of proteins. Studies on the interaction of SDS and protein stability have been conducted for many years. The dose sensitivity is of high interest. Moren and Khan (1995) paid attention to the anionic property of SDS. They tested its interaction with the positively charged protein lysozyme. It was reported that when a small amount of SDS was added, precipitation occurred in the aqueous lysozyme solution based on charge neutralization. However, when they increased the concentration of SDS to an SDS: protein ratio of 19:1, where the positive sites of protein were saturated, the precipitate was redissolved. Nanomolar concentrations of SDS can induce the formation of α-helices and facilitate the process of human Calcitonin (hCt) binding with membranes. According to Micelli's interpretation, SDS monomers can shield the hydrophobic sites of Ct and assist the transition from the random coil to the α-helix, avoiding the formation of β-

sheet, which would induce the process of hCt fibrillation and inhibit its binding with membranes.

The anionic property of SDS is another point of interest in the view of some scientists. Enzyme activation by SDS is interpreted as a pH dependent phenomenon (Moore and Flurkey, 1989; Jimenez and Garcia-Carmona, 1996). An acidic environment can eliminate the activity of enzymes in the presence of SDS, while in the neutral environment, SDS can activate enzymes. Several enzymes have been tested, such as cresolase and catecholase. Jimenez and Garcia-Carmona (1996) concluded that this type of activation is independent of substrate.

Morimoto et al. (1978) used hydrophobic interactions in micelle structures to mimic hydrophobic effects in protein molecules. They tested the affinity of antipyrine, 4-aminoantipyrine, and phenylbutazone, all of which are pyrazolone derivatives, for SDS micelles and their ability to stabilize the micelles. Antipyrine and 4-aminoantipyrine showed high affinities to water and great abilities to reduce the hydrophobic interactions in SDS micelles, which mimic the hydrophobic interactions within protein molecules. However, phenylbutazone is less water soluble and has a weak ability to destabilize micelles, which indicates a limited effect in destabilizing protein molecules.

1.4.5.1.2 Cationic Detergent: Cetyltrimethylammonium Bromide

Cetyltrimethylammonium bromide ($(C_{16}H_{33})$ N $(CH_3)_3Br$) is a cationic surfactant. It has been widely usage in industries such as the gold nanoparticles production and hair conditioning products. In aqueous surroundings, it forms micelles with the aggregation number at 170 and cmc at 1 mM in water at 20-25 °C.

Aiming to explain the mechanism of the pH effect, scientists expanded their work to cetyltrimethylammonium bromide (CTAB), a type of cationic surfactant. Sen et al. (1980) set the pH as 6.0, a value greater than the pK_a of gelatin, and tested its affinities with CTAB and SDS. Cetyltrimethylammonium bromide bound to gelatin favorably, due to the electrostatic attraction between its positive charges and the negative charge of gelatin, while the anionic SDS contributed electrostatic repulsion and unfavorable binding. This result illustrates the more complicated interactions between ionic detergents and proteins, compared with nonionic detergents. How proteins can be bound with detergent is dependent on the relative ratio of electrostatic force to the hydrophobic interactions. An equilibrium state can be reached by the balance between the hydrophobic interaction and the electrostatic force. In other words, researchers can adjust the ionic strength of the medium by changing the pH or adding electrolytes to maximize the ionic detergent's binding with proteins. For example, 10 mM of NaBr and pH 6.5 can increase the affinity of CTAB to α-amylase (Bordbar et al., 2005). In regard to hydrophobic interactions, CTAB was found to be more efficient in binding with lysozyme than dodecyltrimethylammonium bromide (DTAB), due to its stronger hydrophobic interactions resulting from its longer hydroxycarbon tail exposed on the surfactant molecule (Subramanian et al., 1984). The micelle structures formed by CTAB exhibit various modes at different concentrations. Choudhury et al. (1994) employed positron lifetime spectroscopy (PLS) to detect the structural transformation in the CTAB/water and CTAB/water/hexanol systems. In the CTAB/water system, the surfactant molecules dispersed homogenously in a monomeric form at concentrations up to 0.7 mM, at which point an abrupt change in physicochemical properties (including surface tension and

viscosity) was detected due to the formation of micelles. With increasing CTAB concentration, the size and number of spherical micelles grew. However, growth had a limitation in that the micelle radius can never exceed the length of the carbohydrate chain of CTAB. As a result, the micelles deformed from spherical structures into prolate shapes beginning at 10 to 15 mM and were highly deformed at 100 mM. At 125 mM, rod-like structures appeared and were transformed into entangled rods at 400 mM. When the concentration of CTAB exceeded 1 M, hexagonal liquid crystalline structures were formed. This result was in agreement with previous studies using small angle neutron scattering (SANS) and small angle X-ray scattering (SAXS) methods. Analysis using PLS also worked well in the CTAB/hexanol/water system but was limited to the concentration range from 0 to 120 mM due to the phase separation of CTAB micelles. Similarly, two cmc values were detected by Guo et al. (2003). One cmc is the concentration when micelles formed from monomers. The other is to reach a state of the spherical micelles mixture. In the water system, cmc_1 for CTAB equals 1 mM and cmc_2 is approximately 10 mM.

Studies of the free energy change in the process of CTAB aggregation in the system with ethanol were conducted by Li et al. (2006). They explained the phenomena of CTAB's increased cmc value and decreased aggregation number exhibited in the CTAB/ethanol system by using the Nagarajan (1991) model. Nagarajan's model considers the free energy change in the formation of the micelle as the sum of the free energy change in transferring tails from solvent to micelle core and in the process of the tails' deformation within the core, interfacial free energy change between micelles and solvent, steric repulsion between surfactant heads, and the electrostatic interactions

between the charged groups. Nagarajan's model worked well in the CTAB/ethanol system and no significant differences were observed between the experimental values and predicted values. The cmc values increase when the carbohydrate tail of CTAB transfers into the micelle. Higher fraction of ethanol in the system can also increase cmc by strengthening the ionic interaction between CTAB's head groups, which contribute to a smaller aggregation number of CTAB micelles. Cetyltrimethylammonium bromide can denature proteins by altering surface tension and influencing hydrophobic interactions, leading to loss of biological activity of proteins. However, CTAB can also facilitate the protein-refolding process. In research aimed at improving the refolding yield of recombinant or native lysozyme, Wang et al. (2005) reported that CTAB is more effective in assisting refolding of urea-denatured lysozyme than the artificial chaperone β-cyclodextrin. Cetyltrimethylammonium bromide can protect denatured lysozyme from aggregation by the formation of CTAB-lysozyme complexes driven by hydrophobic interactions. Dissociation of the complex was accomplished by treatment with refolding buffer containing the reduced and oxidized forms of glutathione which facilitated the formation of disulfide linkages, making it possible for the contact between denatured lysozyme and refolding buffer. The interactions between CTAB and α-chymotrypsin (CHT) can be revealed by means of the solvation speed using the probe dansyl chloride (DC) (Sarkar et al., 2005). The solvation process for the CHT-CTAB complex can be divided into two steps with distinct solvation time constants of 150 ps and 500 ps, both of which are much longer than for CHT in the absence of CTAB and CTAB micelles, indicating the enzyme's structural perturbation in the process of binding with CTAB. A spatial heterogeneity of these two steps can also be revealed and explained as two

different hydrophobicity levels in the CTH-CTAB complex, in which a sandwich structure was suggested. Cetyltrimethylammonium bromide's ability to bind protein and DNA was also studied by Gani et al. (1999). They tested the binding site numbers exposed on CTAB micelles in individual solutions of protein or DNA and their mixture. They found that the CTAB-DNA-protein ternary system can expose more binding sites than any of the protein and DNA individual systems. In addition, the states of protein and DNA have a significant influence on the binding of CTAB. The denatured protein and DNA system has a greater ability to bind CTAB micelles than systems that contain the native protein or DNA molecules.

The number of head groups in cationic surfactants affects their ability to form micelles (Bhattacharya et al., 2004). Although micelle formation by single-tail surfactants with single, double or triple head groups are all thermodynamically favorable, the free energy for each increased as the amount of head groups increased, which indicates a less favored and less spontaneous reaction. They concluded that head groups played negative roles in micelle formation.

The kinetics of CTAB intermolecular interactions is an interesting phenomenon that is of interest to scientists working on industrial applications. It was reported by Ottaviani et al. (2004) that the reaction to form micelle-templated silicas (MTS) from CTAB and silica took 150 minutes. However, the addition of 1, 3, 5-trimethylbenzene (TMB) was able to speed this process into one hour and enlarge the pore size when the ratio of TMB to CTAB equaled 13. More interestingly, they found that the synthesis time could be separated into two distinct steps at a TMB: CTAB ratio of 5. The fast step took place during the first 100 minutes, with a rate and product similar to the reaction at a TMB:

CTAB ratio of 13. The second step was slower, corresponding to the reaction without TMB. Ottaviani et al. (2004) explained their results in light of the polarity changes of the environment brought about by TMB. Trimethylbenzene provided new void spaces by TMB evaporation and lowered the polarity of the environment by locating at CTAB's head groups, which increased the condensation of silanol and produced the large-pore MTS. An interesting experiment was conducted by Ducker and Wanless (1998) to find out the shape of CTAB micelles formed on the surface of muscovite mica. The reaction was lengthy, with the addition of protons or high temperature reported to speed the process. Protons were found to be competitive with CTA^+ on the binding sites of mica, resulting in the detachment of CTA^+ from the mica. Besides, the hydrophobic force provided more drive to the formation of CTAB micelles. Both the competitive effect and hydrophobic effect induced a transition in shape from cylindrical to wormlike.

The micelle structure can be detected by probing with substituted derivatives of the chromophore 2-phenyl-3, 3-dimethyl-3H indole (Sarpal et al., 1994). The structure and properties of the above molecules can determine the binding locations with SDS and CTAB micelles. For example, ester molecules have greater affinity to water, resulting in a surface or a shallow interior binding location. Two solubilization sites in SDS micelles can be recognized by 2-((p-amino) phenyl)-3, 3-dimethyl-5-carboethoxy-3H-indole and 2-((p-methylamino) phenyl) 3, 3-dimethyl-5-carboethoxy-3H-indole, which may due to the rough surface of SDS micelles. In CTAB, only one solubilization site was detected.

1.4.5.1. 3 Zwitterionic Detergent: 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate

3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) is a zwitterionic detergent with the cmc value at 6 mM and the aggregation number of 10 in water at 20-25 ^{o}C. Different from SDS and CTAB discussed above, it is commonly used as a solubilizer and non-denaturing solvent in the process of protein purification, especially for membrane proteins purified from its natively hydrophobic cellular environment. For example, Gall et al. (2003) chose CHAPS to stabilize ST3 (stromelysin-3) before crystallization. They studied the mechanism of how CHAPS molecules act as protein stabilizers and found two sites on ST3 binding CHAPS molecules. At Met-turn, CHAPS' hydrophilic steroid core connects with the polar surface of stromelysin-3, exposing the hydrophobic tail which masks the hydrophobic patch of ST3. Therefore, the recombinant ST3 cannot aggregate with each other during the concentration. The other binding site locates at the hydrophobic pocket formed by the N and C terminals of ST3. This site is the binding site for calcium atoms normally, but is replaced by the CHAPS molecule in this occasion and forms a stronger bond resulting in a more stable structure.

1.4.5.2 Non-ionic detergent

The Tween family is a nonionic polysorbate surfactant family consisting of Tween 20, Tween 40, Tween 80, etc. They also present a stabilizing effect to prevent the process of protein unfolding. In 1998, Bam et al. found that Tween can stabilize recombinant human growth hormone against denaturation. The mechanism is independent of the cmc value of Tween. They proposed a thermodynamic explanation basically saying that Tween molecules block the hydrophobic sites on the surface of proteins, which contribute greatly in the process of aggregation and therefore, inhibit the heat-induced denaturation.

One year later, Arakawa and Kita (1999) chose Tween 80 to interact with bovine serum albumin and reached the same conclusion that Tween 80 can act as a protein stabilizer and the protecting effect is greater at higher concentration, yet lower than the cmc. In addition, they concluded the mechanism of the stabilization is to alter protein's aggregation behavior. In 2005, Chou et al. conducted research on alutropin using Tween 20 and Tween 80. They confirmed not only the above conclusions about Tween's stabilizing effect, but also the explanation in light of thermodynamics. They found that Tween can increase the free energy of the unfolded state of alutropin, which eliminates the destabilizing effect induced by high temperatures.

1.4.5.3 Detergent and Protein Thermostability

The relationship between surfactant and thermostability of proteins are also of interest. Vermeer et al. (2000) tested effects on protein thermostability by different concentrations of individual and mixed detergents. In experiments with SDS, very low concentrations had no influence on thermostability of proteins due to electrostatic binding with specific and limited sites of protein molecules. When the concentration approached cmc, SDS decreased the thermostability of proteins by disturbing their hydrophobicity. However, when micelle structure formed at concentrations above cmc, protein molecules were captured and covered by micelle structure, initiating denaturation of proteins and increasing thermostability. The same phenomena were also described using CHAPS, and a mixture of SDS and Tween 20. In addition, it was reported that the secondary structure was influenced by the addition of detergent, increasing the ratio of α-helix to β-sheet. The ability of detergent micelles to bind the protein can also influence the protein's solubility. The process of increasing solubility of protein by being incorporated into detergent

micelles is referred to as solubilization. Banerjee et al. (1995) tested the ability of different types of detergents in solubilizing protein. They proposed a more detailed classification for detergents based on their structure and charge, including negatively charged detergent, positively charged detergent, zwitterionic detergent, H-bond formation detergent, and hydrophobic detergent with long polyoxyether chains and aromatic rings. There are two sub-classes describing the long flexible chain structure and rigid structure existing in the first three classes referred to above, which offered activity differentiation attributed to structures. For example, in the comparison of membrane protein extraction abilities between CHAPS, a zwitterionic detergent with a rigid structure and Triton X100, a nonionic hydrophobic detergent with a long polyoxyether chain and aromatic rings, Triton X100 extracts more protein and fewer lipids than CHAPS despite similar cmc values. They concluded that extract yield is determined more by the ratio of detergent to proteins instead of the detergent's cmc value. The same principle was also applied by Bennett (1992) in his research to determine the characters of the components in synaptic vesicle membrane proteins. He used three detergents (CHAPS, Triton X100 and octylglucoside) to solubilize and recover the protein complex and distinguish different synaptic vesicle fractions by their different sedimentation rates and recovery efficiency.

Literature Cited

Aleksandrov (Alexandrov), V. Ya.. 1964. Cytophysiological and cytoecological investigations of heat resistance of plant cells toward the action of high and low temperature. Q. Rev. Biol. 39: 35-77.

Al-Khatib, K. and G.M. Paulsen. 1990. Photosynthesis and productivity during high-temperature stress of wheat genotypes from major world regions. Crop Sci. 30: 1127-1132.

Anderson, J.A. 2006. Heat stability of pepper leaf extracts. J. Amer. Soc. Hort. Sci. 131(1): 17-23.

Anderson, J.A. 2007. Additive effects of alcohols and polyols on thermostability of pepper leaf extracts. J. Amer. Soc. Hort. Sci. 132(1): 67-72.

Anonymous. 2008. Detergents and solubilization reagents. Biofiles for Life Science Research (Sigma-Aldrich). 3(3):16-17.

Arakawa, T. and S.N. Timasheff. 1985. The stabilization of proteins by osmolytes. Biophys. J. 47: 411-414.

Arakawa T. and Y. Kita. 2000. Protection of bovine serum albumin from aggregation by tween 80. J. Pharm. Sci. 89(5): 646-651.

Back, J.F., D. Oakenfull, and M. B. Smith. 1979. Increased thermal stability of proteins in the presence of sugars and polyols. Biochemistry. 18: 5191-5196.

Baier, S.K. and D.J. McClements. 2003. Combined influence of NaCl and sucrose on heat-induced gelation of bovine serum albumin. J. Agric. Food Chem. 51: 8107-8112.

Baier, S.K. and D.J. McClements. 2006. The effect of binary cosolvent systems (glycerol–sucrose mixtures) on the heat-induced gelation mechanism of bovine serum albumin. Intl. J. of Food Sci. and Technol. 41: 189–199.

Bam N.B., J.L. Cleland, J. Yang, M.C. Manning, J.F. Carpenter, R.F. Kelley, and J.W. Randolph. 1998. Tween protects recombinant human growth hormone against

agitation-induced damage via hydrophobic interactions. J. Pharm. Sci. 87(12): 1554-1559.

Banerjee, P., J.B. Joo, J.T. Buse, and G. Dawson. 1995. Differential solubilization of lipids along with membrane proteins by different classes of detergents. Chem. and Phys. of Lipids. 77: 65-78.

Barabal' chuck, K.A. and V.N. Chernyavskaya. 1974. Effect of higher temperature on stomatal movement in tradescantia leaves. Tsitologiya. 16: 1481-1487.

Bennett, M.K., N. Calakos, T. Kreiner, and R.H. Scheller. 1992. Synaptic vesicle membrane proteins interact to form a multimeric complex. J. of Cell Biol. 116(3): 761-775.

Bennion, B.J. and V. Daggett. 2004. Counteraction of urea-induced protein denaturation by trimethylamine N-oxide: a chemical chaperone at atomic resolution. Proc. Natl. Acad. Sci. USA. 101:6433–6438.

Bernstam, V. A., and S. Arndt. 1973. Effects of supraoptimal temperatures on the myxomycete *Physarum polycephalum*.I. Protoplasmic streaming, respiration and leakage of protoplasmic substances. Arch. Microbiol. 92: 251-261.

Bernstam, V. A., and S. Arndt. 1973. The thermostability of some indices of vitality in *Physarum polycephalum* plasmodium. Tsitologia. 15: 1091-1096.

Bhattacharya S. and J. Haidar. 2004. Thermodynamics of micellization of multiheaded single-chain cationic surfactants. Langmuir. 20: 7940-7947.

Bordbar, A.K., K. Omidiyan, R. Hosseinzadeh. 2005. Study on interaction of α-amylase from *Bacillus subtilis* with cetyltrimethylammonium bromide. Colloids and Surfaces B: Biointerfaces. 40: 67-71.

Boyarchuk, Yu. M., and M.V. Vol'kenshtein. 1967. The effects of protein conformation on hydrogen bonds between peptide groups. Biofizika. 12: 341-343.

Boyer, J. S. 1982. Plant productivity and environment. Science. 218: 443-448.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Brandt, J.F. 1967. Heat effects in proteins and enzymes. P. 25-72. In: A.H. Rose (ed.). Thermobiology. Academic Press, Inc. NY.

Burke, J.J. 1990. High temperature stress and adaptation in crops. P. 295-309. In: R.G. Alscher and J.R. Cumming (ed.). Stress Response in Plants: Adaptation and Acclimation Mechanisms. Wiley-Liss, Inc. NY.

Burke, J.J and M.J. Oliver. 1992. Differential temperature sensitivity of pea superoxide dismutases. Plant Physiol. 100: 1595-1598.

Burke, J.J., and K.A. Orzech. 1988. The heat-shock response in higher plants: A biochemical model. Plant Cell and Environ. 11: 441-444.

Chou D.K., R. Krishnamurthy, T.W. Randolph, J.F. Carpenter and M.C. Manning. 2005. Effects of Tween 20 (®) and Tween 80 (®) on the stability of albutropin during agitation. J. Pharm. Sci. 94(6): 1368-1381.

Choudhury S.R., R. Yadav, A.N. Maitra, and P.C. Jain. 1994. Structural transformations in CTAB aggregated systems investigated by positron lifetime spectroscopy 1: binary-systems. Colloids and Surfaces A-physicochem. and Engineering Aspects. 82(1): 49-58.

Chow, M.K.M., G.L. Devlin, and S.P. Bottomley. 2001. Osmolytes as modulators of conformational changes in serpins. Biol. Chem. 382: 1593-1599.

Curtis, O.F. 1936. Comparative effects of altering leaf temperatures and air humidities on vapor pressure gradients. Plant Physiol. 11: 595-603.

Davidson, J.F., B. Whyte, P.H. Bissinger, and R.H. Shiestl. 1996. Oxidative stress is involved in heat-induced cell death in *Saccharomyces cerevisiae*. Proc. Natl. Acad. of Sci., USA. 93: 5116-5121.

Davis-Searles, P.R., A.J. Saunders, D.A. Erie, D.J. Winzor and G.J. Pielak. 2001. Interpreting the effects of small uncharged solutes on protein-folding equilibria. Annu. Rev. Biophys. Biomol. Struct. 30: 271-306.

Ducker W.A. and E.J. Wanless. 1999. Adsorption of hexadecyltrimethylammonium bromide to mica: Nanometer-scale study of binding-site competition effects. Langmuir. 15: 160-168.

Eckardt, N. A. and A.R. Portis Jr..1997. Heat denaturation profiles of ribulose-1, 5-bisphosphate carboxylase/oxygenase (rubisco) and rubisco activase and the inability of rubisco activase to restore activity of heat-denatured rubisco. Plant Physiol. 113(1): 243–248.

Ellis R.J. and S.M. van der Vies. 1991. Molecular chaperones. Annu. Rev. Biochem. 60: 321-347.

Englebienne, P. 1999. Receptor and antibody production, p. 107-111. In Englebienne, P. (ed.) Immune and Receptor Assays in Theory and Practice. CRC Press. Boca Raton, FL, USA.

Gall, A.L., M. Ruff and D. Moras. 2003. The dual role of CHAPS in the crystallization of stromelysin-3 catalytic domain. Acta Crystallographica Section D-Biol. Crystallography. 59: 603-606.

Ganea, E. and J.J. Harding. 2005. Trehalose and 6-aminohexanoic acid stabilize and renature glucose-6-phosphate dehydrogenase inactivated by glycation and by guanidinium hydrochloride. Biol. Chem. 386: 269–278.

Gani, S.A., D.K. Chattoraj, and D.C. Mukherjee. 1999. Binding of cationic surfactants to DNA, protein and DNA-protein mixtures. Indian J. of Biochem. and Biophysics. 36:165-176.

Gombos, Z., H. Wada, E. Hideg, and N. Murata. 1994. The unsaturation of membrane lipids stabilizes photosynthesis against heat stress. Plant Physiol. 104: 563-567.

Guo, R., X.J. Zhu, and X. Guo. 2003. The effect of β-cyclodextrin on the properties of cetyltrimethylammonium bromide micelles. Colloid Polym. Sci. 281: 876-881.

Hart, G.J., A.E. Russell, and D.R. Cooper. 1971. The effects of certain glycols, substituted glycols and related organic solvents on the thermal stability of soluble collagen. Biochem J. 125(2): 599–604.

Hayter, J.B. and M. Zulauf. 1982. Attractive interactions in critical scattering from non-ionic micelles. Colloid and Polym Sci. 260:1023-1028.

Hilbrig, H. 1900. Ueber den Einfluss supramaximaler Temperatur auf das Wachstum der pflanzen. Inaugural Dissertation, Universitat Leipzig, Leipzig. (Cited from Levitt, J. 1980. High-temperature of heat stress, p.351-353. In: J. Levitt (ed.). Responses of Plants to Environmental Stresses (Volume I). Academic Press, Inc. NY.)

Israelachvili, J. N., Mitchell, D. J. and Ninhem, B. W. 1976. Theory of self-assembly of hydrocarbon amphiphiles into micelles and bilayers. J. Chem. Soc.-Faraday Trans II. 72: 1525-1568.

Jimenez M. and F. Garcia-Carmona. 1996. The effect of sodium dodecyl sulfate on polyphenol oxidase. Phytochemistry. 42(6): 1503-1509.

Kim, S.H, Y.B. Yan, and H.M. Zhou. 2006. Role of osmolytes as chemical chaperones during the refolding of aminoacylase. Biochem. Cell Biol. 84:30-38.

Klueva, N.Y., E. Maestri, N. Marmiroli, and N.T. Nguyen. 2001. Mechanisms of thermotolerance in crops, p. 180-181. In: A.S. Basra (ed.). Crop Responses and Adaptations to Temperature Stress. Food Products Press. Binghamton, NY.

Kovrigin, E.L.and S.A. Potekhin. 1997. Preferential solvation changes upon lysozyme heat denaturation in mixed solvents. Biochemistry 36(30): 9195-9199.

Kumar, S., D. Sharma, and Kabir-ud-Din. 2000. Cloud point phenomenon in anionic surfactant plus quaternary bromide systems and its variation with additives. Langmuir. 16: 6821-6824.

Levitt, J. 1980. High-temperature of heat stress, p. 378-381. In: J. Levitt (ed.). Responses of Plants to Environmental Stresses (Volume I). Academic Press, Inc. NY.

Lee, J.H. and F. Schoffl. 1996. An Hsp70 antisense gene affects the expression of HS70/HSC70, the regulation of HSF, and the acquisition of thermotolerance in transgenic *Arabidopsis thaliana*. Mol. and Gen. Genet. 252: 11-19.

Li W., Y.C. Han, J.L. Zhang, L.X. Wang, and J. Song. 2006. Thermodynamic modeling of CTAB aggregation in water-ethanol mixed solvents. Colloid J. 68(3): 304-310.

Lin, C.-Y., J.K. Roberts, and J.L. Key. 1984. Acquisition of thermotolerance in soybean seedlings. Plant Physiol. 74: 152-160.

Marmiroli, N., M. Gulli, E. Maestri, C. Calestani, A. Malcevschi, C. Perrotta, S.A. Quarrie, K.M. Devos, G. Raho, H. Hartings, and E. Lupotto. 1996. Specific and general gene induction in limiting environmental conditions. P. 171-185. In: S. Grillo and A. Leone (ed.). Physical Stresses in Plants. Springer. Berlin.

Mehlen, P., C. Kretz-Remy, X. Preville, and A.-P. Arrigo. 1996. Human hsp27, Drosophila hsp27 and human αB-crystallin expression-mediated increase in glutathione is essential for the protective activity of these proteins against TNFα-induced cell death. EMBO J. 15: 2695-2706.

Micelli, S., D. Meleleo, V. Picciarelli, M.G. Stoico, and E. Gallucci. 2004. Effect of nanomolar concentrations of sodium dodecyl sulfate, a catalytic inductor of α-helices, on human calcitonin incorporation and channel formation in planar lipid membranes. Biophysical J. 87: 1065-1075.

Moore B.M. and M.H. Flurkey. 1990. Sodium dodecyl-sulfate activation of a plant polyphenoloxidase - Effect of sodium dodecyl-sulfate on enzymatic and physical characteristics of purified broad bean polyphenoloxidase. J. of Biol. Chem. 265(9): 4982-4988.

Moren, A. K. and A. Khan. 1995. Phase equilibria of an anionic surfactant (sodium dodecyl sulfate) and an oppositely charged protein (lysozyme) in water. Langmuir. 11: 3636-3643.

Morimoto, Y., T. Izumi, and T. Arita. 1978. Effect of Pyrazolone derivatives on the critical micelle concentration of ionic surfactants. Chem. Pharm. Bull. 26(8): 2585-2588.

Musarat, J., A.A. Dar, A. Amin, N. Rehman and G.M. Rather. 2007. Clouding behavior of nonionic-cationic and nonionic-anionic mixed surfactant systems in presence of carboxylic acids and their sodium salts. Colloid and Polymer Sci. 285:631-640.

Nagarajan, R. and E. Ruckenstein. 1991. Theory of surfactant self-assembly-a predictive molecular thermodynamic approach. Langmuir. 7(12): 2934-2969.

Nascentes, C.C. and M.A.Z. Arruda. 2003. Cloud point formation based on mixed micelles in the presence of electrolytes for cobalt extraction and preconcentration. Talanta. 61(6): 759-768.

Neugerauer, J.M. 1990. Detergents: an overview. Method in Enzymol. 182: 239-253.

Nguyen, H.T., C.P. Joshi, N. Klueva, J. Weng, K.L. Hendershot, and A. Blum. 1994. The heat-shock response and expression of heat-shock proteins in wheat under diurnal heat stress and field conditions. Aust. J. Plant Physiol. 21: 857-867.

Ottaviani, M.F., A. Moscatelli, D. Desplantier-Giscard, F. Di Renzo, P.J. Kooyman, B. Alonso, and A. Galarneau. 2004. Synthesis of micelle-templated silicas from cetyltrimethylammonium bromide/1, 3, 5-trimethylbenzene micelles. J. Phys. Chem. B 108: 12123-12129.

Otzen, D.E. 2002. Protein unfolding in detergents: Effect of micelle structure, ionic strength, pH, and temperature. Biophys. J. 83(4): 2219-2230.

Rijven, A.H.G.C. 1986. Heat inactivation of starch synthase in wheat endosperm tissue. Plant Physiol. 81: 448-453.

Ritossa, F. 1962. A new puffing pattern induced by temperature shock and DNP. Drosophila Experientia. 18: 571–573.

Sarkar, R., M. Ghosh, A.K. Shaw, and S.K. Pal. 2004. Ultrafast surface solvation dynamics and functionality of an enzyme α-chymotrypsin upon interfacial binding to a cationic micelle. J. of Photochem. and Photobiol. B: Bio. 79: 67-78.

Sarpal, R.S., M. Belletete, and G. Durocher. 1994. Effect of small chemical variation and functionality on the solubilization behavior and recognition capability of 3H-indoles in SDS and CTAB. Chem. Phys. Letter. 221: 1-6.

Savelli G., N. Spreti, and P.D. Profio. 2000. Enzyme activity and stability control by amphiphilic self-organizing systems in aqueous solutions. Current Opinion in Colloid and Interface Sci. 5: 111-117.

Schuster, G., D. Even, K. Kloppstech, and I. Ohad. 1988. Evidence for protection by heat-shock proteins against photoinhibition during heat-shock. EMBO J. 7: 1-6.

Sen, M., S.P. Mitra, and D.K. Chattoraj. 1980. Thermodynamics of binding cationic and anionic detergents to gelatin and gelatin-BSA mixture. Indian J. of Biochem. and Biophysics. 17: 405-413.

Smith, B.J. and M.P. Yaffe. 1991. Uncoupling thermotolerance from the induction of heat shock proteins. Proc. Natl. Acad. of Sci. USA 88: 11091-11094.

Spreti N., S. Reale, G. Amicosante, P.D. Profio, R. Germani, G. Savelli. 2001. Influence of sulfobetaines on the stability of the citrobacter diversus ULA-27 β-lactamase. Biotech. Prog. 17: 1008-1013.

Spreti N., P.D. Profio, L. Marte, S. Bufali, L. Brinchi, and G. Savelli. 2001. Activation and stabilization of α-chymotrypsin by cationic additives. Eur. J. Biochem. 268: 6491-6497.

Subramanian, M., B.S. Sheshadri, and M.P. Venkatappa. 1984. Interaction of cationic detergents, cetyl- and dodecyl- trimethylammonium bromides, with lysozyme. J. of Biochem. 95: 413-421.

Suss, K.-H., and I.T. Yordanov. 1986. Biosynthetic causes of *in vivo* acquired thermotolerance of photosynthetic light reactions and metabolic responses of chloroplast to heat stress. Plant Physiol. 81: 192-199.

Timasheff, S.N. 2002. Protein-solvent preferential interactions, protein hydration, and the modulation of biochemical reactions by solvent components. Proc. Natl. Acad. Sci. USA 99: 9721-9726.

Vermeer, A.W.P., and W. Norde. 2000. The influence of the binding of low molecular weight surfactants on the thermal stability and secondary structure of IgG. Colloids and Surfaces A: Physicochemical and Eng. Aspects. 161(1): 139-150.

Vogel, J.L., D.A. Parsell, and S. Lindquist. 1995. Heat-shock proteins Hsp104 and Hsp70 reactivate mRNA splicing after heat inactivation. Current Biol. 5: 306-317.

Wang, J., D. Lu, Y. Lin, and Z. Liu. 2005. How CTAB assists the refolding of native and recombinant lysozyme. Biochem. Eng. J. 24: 269-277.

Welte, M.A., J.M. Tetrault, R.P. Dellavalle, and S.L. Lindquist. 1993. A new method for manipulating transgenes: Engineering heat tolerance in a complex, multicellular organism. Current Biol. 3: 842-853.

Weis, E. 1981. The temperature-sensitivity of dark-inactivation of the ribulose-1, 5,-biphosphate carboxylase in spinach chloroplasts. FEBS Lett. 129: 197-200.

Willekens, H., D. Inze, M. van Montagu, and W. van Camp. 1995. Catalases in plants. Mol. Breeding. 1: 207-228.

Wormuth, K.R. and E.W. Kaler. 1987. Amines as microemulsion cosurfactants. J. Phys. Chem. 91(3): 611-617.

Xu, Q. and T.A. Keiderling. 2004. Effect of sodium dodecyl sulfate on folding and thermal stability of acid-denatured cytochrome c: a spectroscopic approach. Protein Sci. 13: 2949-2959.

Yang, Z., J. Deng and L.F. Chen. 2007. Effect of ionic and non-ionic surfactants on the activity and stability of mushroom tyrosinase. J. of Mol. Catalysis B-Enzymatic. 47(1-2): 79-85.

CHAPTER II

THERMOSTABILITY OF PEPPER LEAF PROTEINS

Abstract

Thermostability of pepper leaf proteins was evaluated at pH 6.0 and 6.5. For the solubility-based thermostability assay, the apparent absorbance measured at 540 nm accurately indicated the content of the soluble protein measured by Bradford quantitation. Protein solubility was maintained to a higher temperature at pH 6.5, compared to pH 6.0. An activity-based thermostability assay indicated better maintenance of catalase activity at pH 6.5 (53.5 ^{o}C) than at pH 6.0 (47.0 ^{o}C). The activity trend of catalase in solutions exposed to elevated temperatures was consistent with the change in solubility of the total MES buffer-extractable pepper leaf proteins.

Introduction

Protein stability

Among the indicators of protein thermostability, solubility and activity are the most important terms. Protein solubility can be maintained in an aqueous environment with the hydrophobic groups buried inside of the molecules and the hydrophilic groups exposed on the surface of the protein. Changes in protein solubility can be used to indicate

changes in protein structure under unfavorable conditions. Adverse environmental factors such as denaturants and high temperatures induce denaturation and unfolding of the protein. The solubility changes accordingly with the exposure of the hydrophobic groups and the formation of disordered structures during protein denaturation. Under these conditions, protein solutions become turbid due to the formation of small aggregates, followed by precipitation of large aggregates. Activity is a function-based term for protein thermostability which provides information about the activity change in accordance with the conformation shift. However, solubility and activity are not always in agreement. The activity is sensitive to conformational changes, with even minor changes in conformation having the potential to inhibit the function of the protein, especially for enzymes.

Protein solubility is highly related to the pH of the solution. The amino acids on the surface of the protein carry different charges at different pHs. For example, when the pH of the protein solution is 7, the arginine and lysine residues located at the surface of the protein carry positive charges, while aspartic acid and glutamic acid carry negative charges. The net charge or overall charge on the surface of the protein is based on the composition of amino acids with different electrostatic properties. The isoelectric point (pI) is the term that describes the solution with the sum of the positive and negative charges of the amino acids as zero. At pHs above pI, protein surfaces are predominantly negatively charged. Similarly, at pHs lower than pI, protein surfaces mainly carry positive charges. In both cases, repulsive forces can be induced between protein molecules and thus the aggregation of protein molecules can be prevented. However, at the pH equal to pI, the repulsive electrostatic forces are reduced due to the cancellation of

the positive and negative charges on the surface of proteins, which decreases the dispersive forces and induces aggregation and precipitation of the protein solution. In addition to the direct interaction between protein molecules at pH values higher or lower than the isoelectric point, proteins carry charges on the surface and the interaction with water molecules is more favorable than with other protein molecules. In other words, protein-protein interaction and precipitation are favorable when the net charge of the protein is zero, at the isoelectric point (Alberts et al., 1998), which explains the phenomenon that protein reaches its lowest solubility at the isoelectric point.

In the research conducted by Anderson (2006) to systematically test the protein stabilizing and destabilizing factors in pepper leaf protein solutions exposed to high temperatures, pH was included as a variable. It was demonstrated that in a narrow range of pH increase from 6.0 to 7.0, the thermostability of pepper leaf proteins, in terms of the solubility, increased accordingly. This observation is in agreement with greater repulsive interactions between negatively charged protein surfaces at higher pH.

The pI of the protein solution can be calculated as the average of the pKa's of the amino acids in the polypeptide chain exposed on the surface of the protein. The pKa of a protein is determined by the ionizable groups of the polypeptide chain. It is the equilibrium constant for the acid-base reactions of the ionizable group. Larger pKa values indicate a smaller extent of dissociation. Protein solutions have complex titration curves due to the large number of ionizable groups and the covalent and three-dimensional structure of the protein molecule. The influence of nearby charged groups makes the pKa of the ionizable group shift by several pH units from its value in the free amino acid. In addition, hydrogen bonds and salts shield the side chain charges from one

another, decreasing the interaction between the charged groups (Voet and Voet, 2004). The Henderson-Hasselbach equation, $pI=1/2\ (pk_i+pk_j)$, suggests a precise way to measure the pI, where k_i and k_j are the dissociation constants of the two ionizations of the neutral species. The electronic interaction also depends on the distance between the charged groups. The electrostatic interactions between the functional groups attenuate rapidly as the distance increases (Voet and Voet, 2004).

Electrostatic interaction is a significant indicator to predict protein structure and activity. Electrostatic desolvation energy is the energy required to break the molecule-solvent interactions at the binding site when molecules bind in an aqueous environment. The most stable state occurs at the minimum free energy (Mandell et al., 2001). In computational methods to predict the most stable mode of association on protein surfaces, the electrostatic interaction potential and electrostatic desolvation energy are estimated in simulation models. For example, it was reported by Yoont et al. (1992) that between a positively charged ribonuclease A molecule and a negatively charged surface at various orientations and separations, ribonuclease A showed the strongest electrostatic attraction with its active site facing the surface. Electrostatic interaction is highly related to the pH of the system. As studied by Zhang and Yu (2005), the electrostatic interaction energy, electrostatic desolvation free energy and hydrophobic desolvation free energy were measured to indicate the hydrophobic and electrostatic interactions between two monomers of the SARS 3CL proteinase dimer at different pH conditions. The electrostatic interaction was demonstrated to be the key factor to the instability of SARS 3CL proteinase dimer under acidic or alkali conditions. Similarly, Yang and Hoing (1993) discussed the distribution of the charged groups and the destabilizing location and

concluded that the destabilizing effect was not distributed evenly on the surface of the protein and was more prominent at locations concentrated with the destabilizing groups. For example, ionizable groups located on the surface of a protein with anomalous pKa, such as the carboxylic groups with low pKa's, determine the titration end point. Yang and Hoing (1993) also pointed out that the individual ionizable groups contribute differently to the stability of the protein even though the proteins have the same set of pKa's and the same relative free energies as a function of pH. For example, when there is an n pKa unit shift in a single ionizable group, the contribution to the change of free energy can be 1.36 n if the n pKa units shift is entirely in its intrinsic pKa (Yang and Hoing, 1993). The contribution could also be 1.36 n/2 if the pKa shift results from a pair wise interaction.

Similar results were published by Spencer et al. (2005).The pH dependence of ΔG; the free energy change in the unfolded and folded states of human FK 506-binding protein (FKBP12) was concluded to be governed by the pKa's of titratable groups. The residual charge-charge interaction is also negligible due to the lack of sequentially neighboring positive charges caused by the salt screening. Furthermore, Spencer et al. (2005) reported the dual influences on protein stability by salt. The dual response can be explained as two opposing types of protein-salt interactions. Small amounts of salt induce the Debye- Hückel interaction (Debye and Hückel, 1923), in which ions interact with protein charges and favor the unfolded state of protein. As salt concentration increases, the unfolded state is unfavorable due to the induced Kirkwood interaction (Kirkwood, 1943), in which the ions move toward the exposed low-dielectric protein cavity of the unfolded proteins from the bulk solvent. The macromolecular crowding was concluded to

be thermodynamically unfavorable based on the free energy increase in both folded and unfolded states of FKBP12.

Similar to the solubility of protein, the activity is highly related to the pH, but the activity change is more complex at different environment pHs. For example, Khurana et al. (1995) tested the stability of barstar at pHs ranging from 2-13 and concluded that the change in stability of barstar is more complex than its solubility change, which is determined by the net charges on protein surfaces indicated by the pI value. Barstar is the intracellular inhibitor to barnase in *Bacillus amyloliquefaciens* and its pI value is 5.0. At pH ranges from 3-5, the stability of barstar decreases, which results from the protonation of one or more of the Asp or Glu residues in barstar. At pHs of 5-7, the stability increases. The stability increase was not observed in a mutant in which the His residue on barstar was replaced with Gln. The stabilization rationale was based on the protonation of the His residue at pH below 7. When the pH is above 7, the stability of barstar decreases, which is due to one or both of the two cysteine residues being deprotonated. Khurana (1995) also tested the effects of the denaturants guanidine hydrochloride (GdnHCl) and urea, as well as high temperatures on the unfolding transition of barstar. The free energy change between the unfolded and the folded barstar was found to be identical among the denaturation induced by GdnHCl, urea and high temperatures.

Catalase and heat stress defense

Reactive or activated oxygen species (ROS/AOS), including oxygen ions, free radicals and peroxides, are of high reactivity and play an important role in cell structure and signaling. Free radicals, atoms or small molecules with unpaired electrons in the

valence shell, are common ROS/AOS. Their levels can be much higher when plants suffer from stresses, resulting in the so-called oxidative stress. Oxidative damage is considered to be one of the major deleterious factors induced by heat stress. Yeast exposed to severe high temperature will die mainly due to oxidative damage to its cell (Davidson et al., 1996). In plants, the process of photoinhibition induced by heat is a source of ROS. There is a balance between the solar energy captured by pigments and reducing power transported through the photosynthetic cytochromes and quinones at normal state. Heat shock disrupts the coupling of electron transport and the absorption of energy. The excess energy can flow to triplet oxygen, and other ROS, commonly accumulating in chloroplasts and mitochondria, two major locations where electron transport takes place. The most heat-susceptible component in chloroplasts is the PSII complex (Schuster et al., 1988; Burke, 1990). Heat induces change in its composition and disaggregation of its functional components (Suss and Yordanov, 1986) resulting in the imbalance of electron transport and the accumulation of ROS.

In order to limit oxidative damage under stress conditions, plants have developed a series of detoxification systems that break down the highly toxic ROS. Plants protect cellular and sub-cellular systems from the cytotoxic effects of ROS using antioxidant enzymes such as superoxide dismutase (SOD), catalase, ascorbate peroxidase, cytochrome peroxidase, glutathione reductase and metabolites like glutathione, ascorbic acid, α-tocopherol and carotenoids. Many experiments provide evidence for antioxidant's function in improving thermostability. Yeast mutants that lack genes for catalase, SOD and cytochrome peroxidase are more sensitive to heat shock than the wild type cells (Davidson et al., 1996). In tobacco mutants with high expression of catalase, the

photosynthetic activity is much better protected under heat stress than in the wild type (Willekens et al., 1995). Because antioxidants are a very important component in plant's defense system under heat shock, more studies are still needed (Klueva et al., 2001).

Previous research has shown that solubility-based protein thermostability was greater in heat tolerant vinca (*Catharanthus roseus* L. G. Don 'Little Bright Eyes'), compared with heat susceptible sweet pea (*Lathyrus odoratus* L. 'Explorer Mix') (Anderson and Padhye, 2004). An activity-based assay using catalase exhibited the same trend between species as the solubility result. In a study examining thermostability of pepper leaf proteins by their solubility, it was found that stability increased as pH increased from 6 to 7 (Anderson, 2006). However, whether environmental pH changes that affect solubility-based protein thermostability will have corresponding effects on activity-based assays is not clear. Therefore, our primary objective was to determine whether solubility and an activity based protein thermostability assay exhibited corresponding changes with the changing pH. A secondary objective was to evaluate whether the apparent absorbance measured at 540 nm wavelength can accurately indicate changes in the concentration of the soluble proteins.

Materials and Methods

PLANT CULTURE AND LEAF EXTRACT. 'Early Calwonder'(*Capsicum annuum* L.) pepper plants were grown in 24 cm diameter pots in a commercial potting mix (BM-1; Saint-Modeste, Quebec) amended with dolomite (3.6 g/L), triple superphosphate (0.7 g/L), Micromax (The Scotts Co., Marysville, Ohio) (0.6 g/L), and KNO_3 (0.6 g/L). Plants were maintained in a controlled-environment chamber (model PGW36; Conviron,

Winnipeg, Man., Canada), at 24/20 °C day/night temperatures with a 14 hour photoperiod and a photosynthetic photon flux density at canopy height of about 400 $\mu \cdot mol \cdot m^{-2} \cdot s^{-1}$. Plants were watered with 0.7 g/L 20N-8.6P-16.6K soluble fertilizer as needed based on media color and the weight of the pot. Fully expanded leaves from 10-week-old plants were collected as the plant material.

Deionized water at room temperature was used to hydrate the leaves after collection and during transfer from the growth chamber to the lab. Fifteen grams of leaves without the midrib were blended in the homogenization buffer consisting of 225 ml MES [2-(N-morpholino) ethanesulfonic acid] buffer (50 mM, pH=6.0, with 1 mM EDTA) (Thermo-Fisher Scientific, Fair Lawn, NJ) with 1.25 g PVPP (polyvinylpolypyrrolidone) (Sigma-Aldrich, St. Louis, MO). Then, the leaf slurry was filtered through Miracloth (Calbiochem, Madison, WI) and collected in a beaker containing an additional 1.25 g PVPP with 15 ml MES buffer. Leaf extract was generated by collecting the supernatants from centrifugation at 16k g_n at 21 ºC for 20 min. Extracts were mixed with an equal volume of MES buffer and stirred for 20 min. After mixing, pH of the solutions was adjusted to 6.0 in the first series of experiments, and to 6.5 in the second series of experiments. An aliquot of solution at pH 6.0 was retained in the second series of experiments and exposed to high temperatures to allow limited comparisons.

HEAT TREATMENT. The treatment temperature range for experiments conducted at pH 6.0 was 21, 33, 48, 50, 52, and 54 ºC. Experiments at pH 6.5 had temperature exposures of 21, 36, 48, 50, 52, 54, 56, 58, 60 and 62 ºC. Three subsamples of 3.4 ml solution were pipetted to16 mm test tubes for each temperature treatment. Test tubes

were held in water baths for 15 min at target temperatures and then cooled to 21 ºC for four hours.

PROTEIN QUANTIFICATION. Protein concentrations in leaf extracts were determined using the Bradford (1976) assay with ovalbumin as a standard. Samples were diluted 1:5, 1:10 and 1:20 with MES buffer (50 mM, pH 6.0 or 6.5, containing 1 mM EDTA). Bradford reagent (3 ml) was added to each cuvette containing 100 µl of diluted extract or standard and mixed by repeated inversion before absorbance measurements at 595 nm were conducted at ambient temperature.

SOLUBILITY MEASUREMENT. A spectrophotometer (DU640B, Beckman) was used to measure apparent absorbance at 540 nm 4 hr after heat treatment. Un-heated solutions (21 ºC) were filtered through 0.2 µm polyethersulfone membrane syringe filters for use as blanks. As treatment temperatures increased, light scattering by aggregated proteins increased apparent absorbance values until a maximum value was reached. Further temperature increases yielded apparent absorbance values similar to controls due to precipitate formation. Only the clear solution over precipitates was sampled in experimental units with insoluble proteins.

CATALASE ASSAY. Catalase activity was determined spectrophotometrically (Aebi, 1983). Samples from each temperature treatment were diluted 1:20 with MES buffer (50 mM, pH 6.0 or 6.5, with 1 mM EDTA). After pipetting 1.5 ml of the diluted solution to a quartz cuvette, hydrogen peroxide (30 mM, 1.5 ml) was added and mixed by multiple inversions. Absorbance at 240 nm was monitored in kinetic mode at 10 s intervals. Substrate depletion rate was calculated from 10 to 30 sec based on a hydrogen peroxide

standard curve generated on each experimental date. Linear response curves were verified throughout the monitoring period.

Results

Turbidity and soluble protein content under high temperature and pH treatments

At pH 6.0, the temperature at the turbidity peak was 50 ^{o}C, which was close to the inflection point in the relationship between soluble protein content and temperature (Figure 1). The turbidity measured by the apparent absorbance at 540 nm increased gradually as temperature reaches 50 ^{o}C. At temperatures higher than 50 ^{o}C, the turbidity curve starts to drop down due to the formation of precipitation. The soluble protein content based on the Bradford assay remained relatively constant at the temperature range from 33 ^{o}C to 50 ^{o}C. The amount of soluble protein decreased sharply at temperatures higher than 50 ^{o}C.

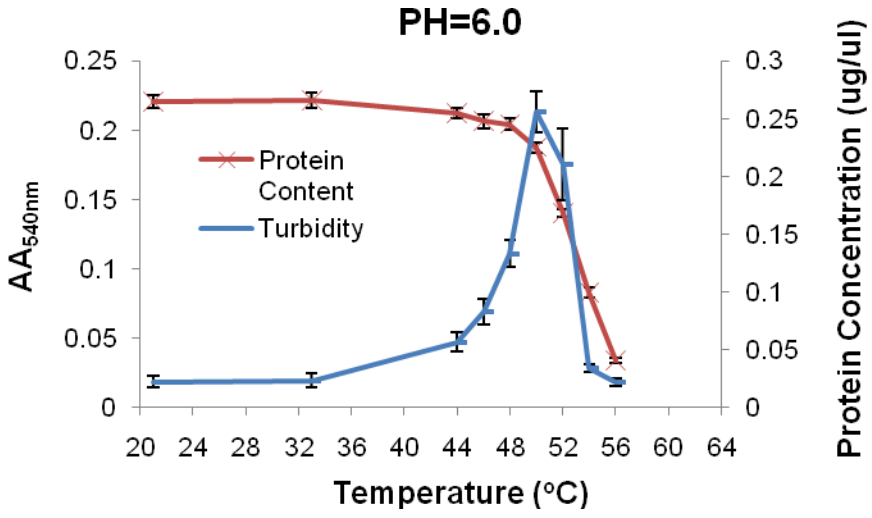

Figure 1. The apparent absorbance at 540 nm vs. Bradford quantified soluble protein content vs. temperature variables at pH 6.0. Means ± SE from 3 subsamples by 5 replications were included (n=15).

At pH 6.5, the temperature at the turbidity peak was 56 ^{o}C, which was close to the inflection point in the soluble protein content curve (Figure 2). The turbidity measured by the apparent absorbance at 540 nm increased gradually as temperature reached 56 ^{o}C. At temperatures higher than 56 ^{o}C, the turbidity curve starts to drop down due to the formation of precipitation. The soluble protein content remained relatively constant at the temperature range from 36 ^{o}C to 54 ^{o}C. The amount of soluble protein decreased sharply at temperatures higher than 54 ^{o}C.

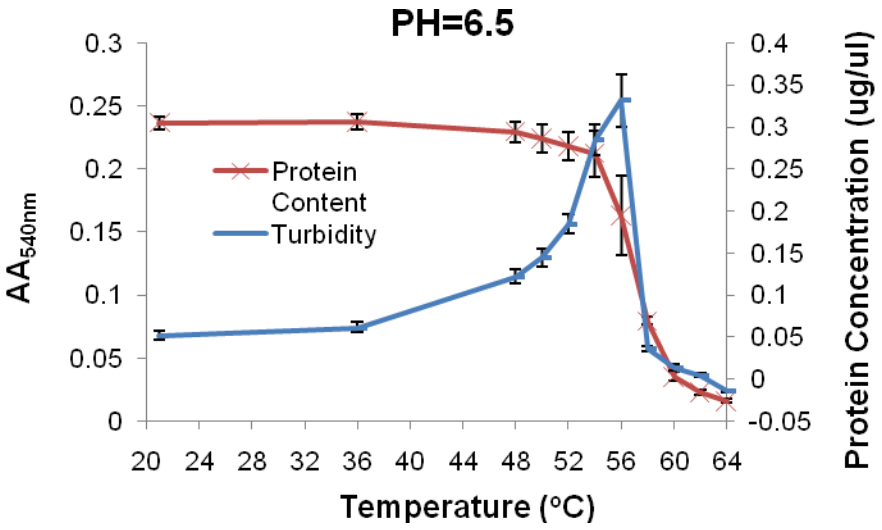

Figure 2. The apparent absorbance at 540 nm vs. Bradford quantified soluble protein content vs. temperature variables at pH 6.5. Means ± SE from 3 subsamples by 5 replications were included (n=15).

Catalase activity under high temperature and pH treatments

Catalase activity can be indicated by measuring the rate of H_2O_2 decomposed catalyzed by catalase. At pH 6.0, the rate of H_2O_2 consumed in 20 seconds decreased as temperatures increase from 33 ^oC to 56 ^oC as Figure 3. The midpoint is estimated as 47 ^oC by SAS PROC NLIN procedure. At pH 6.5 (Figure 4), the catalase activity follows the same trend as the pH 6.0 treatment, but shifted toward higher temperatures. The temperature range tested was from 36 ^oC to 62 ^oC. The midpoint is estimated as 53.5 ^oC by SAS PROC NLIN procedure.

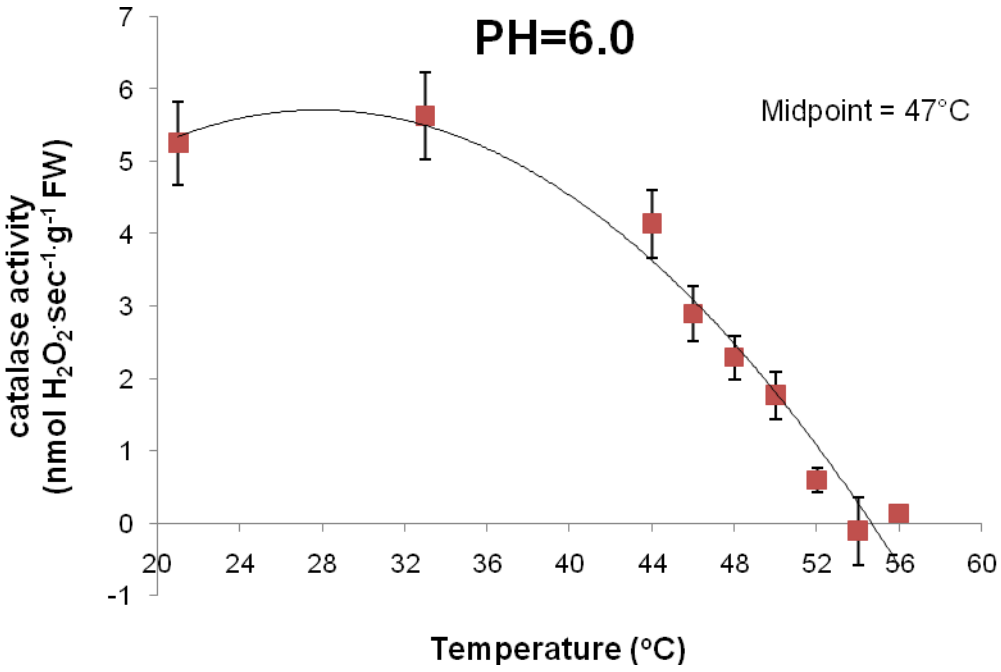

Figure 3. Catalase activity plotted against temperatures at pH 6.0. Means ± SE from 3 subsamples by 5 replications were included (n=15).

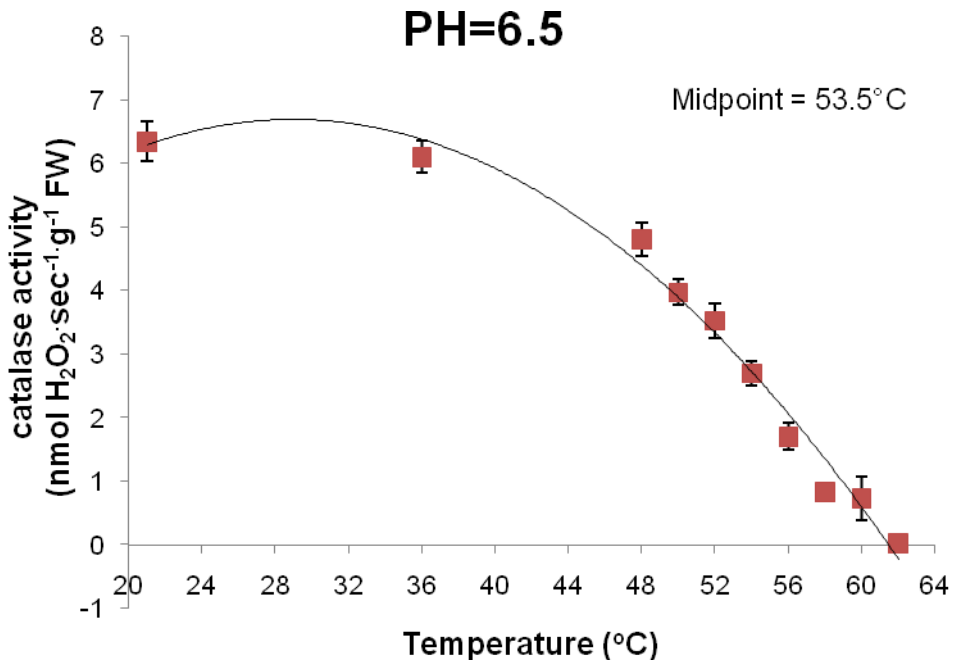

Figure 4. Catalase activity plotted against temperatures at pH 6.5. Means ± SE from 3 subsamples by 5 replications were included (n=15).

Discussion

Turbidity changes determined by apparent absorbance measurement were consistent with changes in the soluble protein content measured by Bradford quantification. As the turbidity of the solution became more intense due to the formation of small aggregates, the soluble protein content measured by Bradford quantification remained fairly constant. Turbidity and soluble protein content decreased when precipitates formed due to severe heat aggregation. The close correspondence between changes in turbidity and soluble protein content allows us to predict and examine the soluble protein content change by the trend of the turbidity measured at 540 nm wavelength after the high temperature treatment.

The correspondence between measurements of turbidity changes and soluble protein content changes exhibited at both pH 6.0 and 6.5 verifies the association between the two solubility indicators we used in the experiment: turbidity and Bradford-quantified soluble protein concentration. Within the range of 6.0 to 6.5, the higher pH environment has a greater ability to maintain the solubility of protein against high temperature aggregation, which is in agreement with the conclusion drawn by Anderson in 2006. The mechanism suggested that the negatively charged environment induced by a higher pH can introduce stronger repulsive forces between the negatively charged surfaces can also apply to our result.

Moreover, based on the result that higher pH is more favorable to pepper leaf protein thermostability than lower pH, we conclude that the molecular surfaces of the mixed proteins are dominated by negative charges. The more intense negative charges at the higher pH yield more repulsive electrostatic interaction between surfaces and attenuate high temperature aggregation.

Catalase in our study undergoes two denaturation challenges. High temperatures increase the free energy of the protein-solution system and make the unfolding process and further aggregation of the unfolded proteins favorable. Defined as the negative logarithm of the hydrogen ion activity in an aqueous solution (IUPAC, 1997), pH can also affect the stability of proteins by influencing the electrostatic interactions between active binding sites on proteins and charges in the environment. At a pH of 6.5, catalase activity declined with increasing temperature with a midpoint of 53.5 ^{o}C. By comparing the midpoint values at pHs of 6.0 and 6.5, catalase activity at pH 6.5 was better maintained against high temperature decline than at pH 6.0 (47.0 ^{o}C). This may be

because the average pKa of the residues at active binding sites on catalase is much closer to 6.5 than to 6.0. In addition, the activity trend of catalase under high temperature denaturation is consistent with the change in the solubility of the total pepper leaf proteins in our study. We can also conclude that maintenance of solubility by stronger repulsive interactions between protein molecules also contributes to maintenance of the conformation of the catalase enzyme, so the activity of pepper leaf catalase is more tolerant against high temperatures at the higher pH of 6.5.

Testing the activity changes and the solubility changes of pepper leaf proteins at pH 6.0 and 6.5, it was found that catalase exhibited a good correspondence between the activity and solubility at both pHs. We consider catalase a representative enzyme. It is expected that other enzymes may have different Tmid values and that their activities may fail to be closely associated with the solubility changes of total pepper leaf proteins. The enzymatic activity can be more sensitive than the solubility and the loss of activity may start earlier than the solubility responses under adverse environmental factors. This may be explained by minor conformational changes that trigger loss of activity although solubility is maintained. Maintenance of solubility results from the repulsive forces driven by the same net charges on the surface of the protein. However, the activity of the protein is determined by the conformational status of the protein and the protonation or deprotonation of the charged residues, not only the net charge on protein surface. Thereby, our conclusions about leaf pepper catalase may not be generally applied to other enzymes to predict their solubility and activity under high temperatures or denaturing treatments.

Literature Cited

Aebi, H.E. 1983. Catalase, p. 273-286. In: H.V. Bergmeyer (ed.). Methods of enzymatic analysis. Verlag, Weinheim.

Alberts, B., K. Roberts, J. Lewis, K. Hopkin, A. Johnson, P.Walter, M. Raff, and D. Bray.1998. Essential Cell Biology: An Introduction to the Molecular Biology of the Cell. New York: Garland.

Anderson, J.A. and S.R. Padhye. 2004. Protein aggregation, radical scavenging capacity and stability of hydrogen peroxide defense systems in heat-stressed vinca and sweet pea leaves. J. Amer. Hort. Sci. 129(1): 54-59.

Anderson, J.A. 2006. Heat stability of pepper leaf extracts. J. Amer. Soc. Hort. Sci., 131: 17-23.

Baker, N.A., D. Sept, S. Joseph, M.J. Holst, and J.A. McCammon. 2001. Electrostatics of nanosystems: Application to microtubules and the ribosome. Proc. Natl. Acad. Sci. 98: 10037-10041.

Burke, J.J. 1990. High temperature stress and adaptation in crops. P. 295-309. In: R.G. Alscher and J.R. Cumming (ed.). Stress Response in Plants: Adaptation and Acclimation Mechanisms. Wiley-Liss, Inc. NY.

Davidson, J.F., B. Whyte, P.H. Bissinger, and R.H. Shiestl. 1996. Oxidative stress is involved in heat-induced cell death in *Saccharomyces cerevisiae*. Proc. Natl. Acad. of Sci., USA 93: 5116-5121.

Debye, P. and E. Hückel. 1923. The theory of electrolytes I. The lowering of the freezing point and related occurrences. Physikalische Zeitschrift 24: 185–206.

IUPAC. 1997. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A.Wilkinson. Blackwell Scientific Publications, Oxford.

Klueva, N.Y., E. Maestri, N. Marmiroli, and N.T. Nguyen. 2001. Mechanisms of thermotolerance in crops, p. 180-181. In: A.S. Basra (ed.). Crop Responses and Adaptations to Temperature Stress. Food Products Press. Binghamton, NY.

Khurana, R., A.T. Hate, U. Natu, and J.B. Udgaonkar. 1995. pH dependence of the stability of barstar to chemical and thermal denaturation. Protein Sci. 4:1133-1144.

Kirkwood, J.G. 1943. The theoretical interpretation of the properties of solutions of dipolar ions. P. 586–622. In: E.J. Cohn and J.T. Edsall (ed.). Proteins, Amino Acids and Peptides as Ions and Dipolar Ions, Reinhold Publishing Co. NY.

Mandell, J.G., V.A. Roberts, M.E. Pique, V. Kotlovyi, J.C. Mitchell, E. Nelson, I. Tsigelny, and L.F. Ten Eyck. 2001. Protein docking using continuum electrostatics and geometric fit. Protein Eng. 14: 105-113.

Schuster, G., D. Even, K. Kloppstech, and I. Ohad. 1988. Evidence for protection by heat-shock proteins against photoinhibition during heat-shock. EMBO J. 7: 1-6.

Spencer, D.S., K. Xu, T.M. Logan and H.X. Zhou. 2005. Effects of pH, salt, and macromolecular crowding on the stability of FK506-binding protein: An integrated experimental and theoretical study. J. Mol. Biol. 351: 219-232.

Suss, K.-H., and I.T. Yordanov. 1986. Biosynthetic causes of *in vivo* acquired thermotolerance of photosynthetic light reactions and metabolic responses of chloroplast to heat stress. Plant Physiol. 81: 192-199.

Voet D. and Voet J.G. 2004. Biochemistry, 3rd Edition. New York: J Wiley. P.45-55.

Willekens, H., D. Inze, M. van Montagu, and W. van Camp. 1995. Catalases in plants. Mol. Breeding. 1: 207-228.

Yang, A.-S. and B. Honig. 1993. On the pH dependence of protein stability. J. Mol. Biol. 231: 459-474.

Yoont, B.J. and A.M. Lenhoff. 1992. Computation of the electrostatic interaction energy between a protein and a charged surface. J. Phys. Chem. 96: 3130-3134.

Zheng, K., Q. Yu, Y. Jiang, G. Ma, W. Zhao, and M. Zeng. 2005. Effects of electrostatic and hydrophobic interaction on the stability of SARS coronavirus 3CL proteinase dimer. Chemical Journal of Chinese Universities. 26(1): 55-60.

CHAPTER III

EFFECTS OF CHEMICAL CHAPERONES ON THERMOSTABILITY OF PEPPER LEAF PROTEINS

Abstract

Effects of chemical chaperones on solubility-based thermostability of pepper leaf proteins were evaluated using mixtures of stabilizers and mixtures of stabilizers and destabilizers. Glucose and glycine (stabilizers) with similar polarity exhibited an equivalent stabilizing effect. Destabilization of pepper leaf proteins by CHAPS was significantly weakened by the stabilization provided by glycine. Destabilization by CHAPS and stabilization by glycine were numerically additive for commercial ovalbumin.

Introduction

Sugars and polyols can stabilize proteins against heat denaturing (Back et al., 1979). The extent of stabilization by different sugars and polyols was related to different influences on the structure of water. Additional solutes, such as L-proline, L-serine, γ-aminobutyric acid, sarcosine, taurine, α-alanine, β-alanine, glycine, betaine, and

trimethylamine N-oxide were subsequently reported to stabilize protein structure under heat stress by avoiding direct binding to the protein (Arakawa and Timasheff, 1985). Timasheff et al. (2002) studied the interaction between sugars, polyols and proteins, and theorized that preferential hydration of protein in the protein-water-cosolvent system accounted for the structural stabilization of proteins. The addition of these protein stabilizers, which increased the surface tension of water by changing the chemical potential of proteins, further induced the cosolvent's preferential exclusion from the protein surface and water molecule's preferential binding. Interacting loci are sites on protein surfaces where water and solvent components exchange. Preferential interaction includes preferential hydration and preferential binding of ligand, which considers their relative affinities for interacting loci. When the affinities between water molecules and interacting loci are greater than for the ligand, an excess of water will bind in the protein domain, which is called preferential hydration or a preferential exclusion of ligand. On the contrary, if the relative affinity between interacting loci and ligand is greater, preferential binding of ligand will occur. In terms of thermodynamics, preferential binding is quantitatively equal to the thermodynamic binding. When stabilizers are added, the perturbation of chemical potential of the protein is positive; the interaction between the cosolvent and protein is unfavorable due to the increase in the system's free energy, and preferential exclusion of cosolvent and preferential hydration will result.

Timasheff's (2002) ideas on protein-solvent-cosolvent interactions received additional support from studies of other systems. Sarcosine, glycine betaine and trimethylamine N-oxide were reported to protect the active and native conformation of α_1-antitrysin at elevated temperatures in a concentration-dependent manner (Chow et al.,

2001). High temperature can induce a partial unfolding state of α_1-antitrysin, resulting in increasing exposure of its peptide backbone, which cannot interact favorably with the addition of those osmolytes. However, the free energy is increased with this addition, which will force proteins to sequester, maintaining a folded and more compact state. In this solvophobic manner, a_1-antitrysin's conversion to its intermediate state and the following polymerization can be hindered and the native state stabilized. However, these osmolytes have no effect on aiding correct refolding (Chow et al., 2001). Another example of concentration-dependent protection was revealed by Ganea and Harding (2005). The activity of glucose-6-phosphate dehydrogenase (G6PD) was more stable against glycation-induced inactivation at higher concentration of trehalose and 6-aminohexanoic acid (AHA). Those compounds can also help restore activity; however, the reactivation is less efficient than the protection due to irreversible processes occurring. The different protective effects between folding and refolding processes in both experiments suggest a non-specific effect of those osmolyte stabilizers. Osmolytic forces drive the folding of the denatured proteins to lower the system's free energy because the unfolded state is thermodynamically unfavorable. However, if a protein's native conformation is less thermodynamically stable than the misfolded state, osmolyte stabilizers may be ineffective in helping proteins refold correctly.

A significant connection between stabilizing effects of chemical chaperones and their physical properties has been established. Guiavarc'h et al. (2003) applied different sugars (sucrose and trehalose) and polyols (mannitol, sorbitol, lactitol and glycerol) on purified tomato pectinmethylesterase (PME) and tested the remaining enzyme activity after heat treatment. The decimal reduction time D (time course for one log reduction

from the initial enzyme activity to the response value after exposure to a certain temperature) was selected to reflect the inactivation of PME. Both sugars and polyols exhibited a protective effect on thermal stability of tomato PME. The stabilizing effect introduced by polyols was greater than that by sugars tested in their experiment. Glycerol exhibited the greatest stabilizing effect among all stabilizers tested and provided the highest D value both at 65 ^{o}C and 70 ^{o}C. In addition, the function between the D values for PME and numbers of OH groups per unit volume of enzyme provided by stabilizers was found to be exponential (Guiavarc'h et al., 2003). OH groups were interpreted as the only factor determining their stabilizing effect. They also tested the ability of polymers with different molecular weight in stabilizing PME from heat denaturation. The polyvinyl alcohol with lower molecular weight showed a greater stabilizing effect (higher D value at 65 ^{o}C) than higher molecular weight ones. Guiavarc'h et al. (2003) pointed out that the number of OH groups affects the hydrophilicity of the protein-solute system. The access of OH groups of the osmotyles weakens the interaction of water with both the hydrophilic surface and the hydrophobic core of the protein and replaces it with a more hydrophilic environment, by which the protein's hydrophilic surface-hydrophobic core structure can be stabilized against heat unfolding. Similarly, OH groups possessed by polymers with lower molecular weight have a smaller size and less steric limitation to access the protein, which benefits the interaction between osmolyte OH groups and protein structure. The explanation is different from the preferential hydration theory suggested by Timasheff (2002). Timasheff explained the stabilizing effect as a result of preferential hydration, which results from the greater affinity between water and protein than between ligand and protein at the interacting loci. According to the preferential hydration theory, it is the

hydration layer on the surface of the protein structure that stabilizes the protein from high temperature unfolding, and not the OH groups attracted directly to the structure as suggested by Guiavarc'h (2003).

Polyols were also tested by Anderson in his research in 2007. Pepper leaf protein mixtures were studied aiming to address the divergent conclusions drawn by using different, single proteins in chemical chaperone assays. Polyol OH group density is defined as the number of OH groups polyols provide per molar volume of solution. Compounds with a range in number of OH groups but with the same number of carbon atoms (n-propyl alcohol, propylene glycol and glycerol), or a different number of carbon atoms with fixed OH groups (methanol, ethanol, propanol and butanol) were studied. The higher maximum apparent absorbance temperature, expressing the solubility of pepper leaf protein mixture after heat treatment, was reached with stabilizers with higher OH density. The polarity and hydrophilicity of stabilizers were also tested with mannitol, glycerol, ethylene glycol, propylene glycol and methanol at concentrations yielding the same OH density but different polarity. The more polar and hydrophilic the compounds, the more they stabilized the protein under heat treatment. A linear or quadratic relationship with the maximum apparent absorbance temperature was reported both at various OH group densities and various log Kow (oil/water partition coefficient) at fixed OH density. In contrast with Guiavarc'h et al. (2003), it was concluded that not only the amount of OH groups but also the source of OH, which covers the properties of the non-OH portion such as the polarity and hydrophilicity, determines the polyol stabilizing effect. The colligative effect of the number of OH groups supported by Guiavarc'h et al. (2003) was not satisfied. Anderson's (2007) result, which emphasized the interaction

between solutes and cosolutes, was in agreement with Timasheff's (2002) preferential hydration theory and the thermodynamics rule about the free energy change.

Molecular weight of polyols was also the focus of Anderson (2007) aiming to reveal the relationship between the size of cosolvent molecules and their effect on stabilizing proteins against high temperature aggregation. Ethylene glycol, methanol, glycerol and mannitol were selected as a series of chemical compounds with different molecular weight but a fixed carbon atom to OH group ratio. Thermostability, based on the maximum apparent absorbance temperature at 540 nm, increased with increasing molecular weight. The greater thermostability introduced by larger cosolvent molecules can be a result of greater amount of OH groups possessed by larger cosolvent molecules than that of smaller ones at the fixed carbon to OH group ratio. Anderson's (2007) conclusion is in agreement with the result reported by Davis-Searles et al. (2001). The stability of horse heart ferricytochrome *c* was tested by the addition of osmolytes. By detecting the free energy change associated with the transition between the folded and the unfolded state of the protein in the presence of osmolytes, they found that the polyol osmolytes were able to stabilize proteins, and their stabilizing effects were a function of both the polyol concentration and molecular size.

In addition to test treatments with individual stabilizers, the additive effect between and within stabilizers and destabilizers was observed in Anderson's (2007) research, by mixing stabilizing and destabilizing compounds in a pepper leaf protein system. The additive effect was consistent with chemical chaperone effects on the overall free energy change resulting from the substitution of solvent with cosolvent in the protein-solvent-cosolvent system. The combination effect was consistent with Baier and McClements

(2003), who reported that a combination of NaCl and sucrose can protect proteins from gelation induced by high temperature, although they have different stabilizing mechanisms. Later, Baier and McClements (2006) used a mixture of glycerol and sucrose, both of which are protein stabilizers, to treat bovine serum albumin under heat stress and found that the thermostability of BSA increased but the net increase varied with the ratio of these two additives.

Although many theories and discussions have already been well developed, more work is in need. One problem is that most of the research focused on an individual protein, which provides information specific to that single protein, such as commercial ovalbumin. Divergent conclusions between studies using different proteins may be drawn due to the specific properties and characteristics of the individual proteins. Protein mixtures can be used to address the problem, and in addition, better represent the real plant tissue which is much more complex than a single protein. Not limited to theoretical significance, the information about the additive effects is of value in chemical applications of protein stabilization. Optimal stabilization can be obtained by adjusting the ratio of stabilizing and destabilizing chaperones based on their effects on thermostability of proteins. Previous work has focused primarily on a single class of compounds or a simple solution of a single stabilizer/destabilizer. Limited information is available on the additive effects of mixtures, especially involving compounds from different chemical classes. Specifically, my objectives were to: 1) determine whether the effects of the stabilizers glucose and glycine on pepper leaf proteins are additive under heat stress; 2) determine whether the effects of the stabilizing compound glycine and the destabilizing compound CHAPS are additive with respect to the thermostability of pepper

leaf proteins; and 3) determine whether the effects of the stabilizing compound glycine and the destabilizing compound CHAPS are additive with respect to the thermostability of commercial ovalbumin.

Materials and Methods

PLANT CULTURE AND LEAF EXTRACT. 'Early Calwonder' pepper plants were grown in 24 cm diameter pots in a commercial potting mix (BM-1; Saint-Modeste, Quebec) amended with dolomite (3.6 g/L), triple superphosphate (0.7 g/L), Micromax (The Scotts Co., Marysville, Ohio) (0.6 g/L), and KNO_3 (0.6 g/L). Plants were maintained in a controlled-environment chamber (model PGW36; Conviron, Winnipeg, Man., Canada), at 24/20 °C day/night temperatures with a 14 hour photoperiod and a photosynthetic photon flux density at canopy height of about 400 $\mu \cdot mol \cdot m^{-2} \cdot s^{-1}$. Plants were watered with 0.7 g/L 20N-8.6P-16.6K soluble fertilizer as needed based on the medium darkness and the weight of the pot. Fully expanded leaves from 10-week-old plants were collected as the plant material. Deionized water at room temperature was used to hydrate the leaves after collection and during the transfer to the lab. Fifteen grams of leaves without midrib was blended in the homogenization buffer consisting of 225 ml MES buffer (50 mM, pH=6.0, with 1 mM EDTA) with 1.25 g PVPP. Then, the leaf solution was filtered through Miracloth (Calbiochem, Madison, WI) and collected in a beaker containing an additional 1.25 g PVPP in 15ml MES buffer. Leaf extract was generated by collecting the supernatants from centrifugation at 16k $g_{n,}$ 21 ºC for 20 min.

CHEMICALS. Glycine, glucose, CHAPS {3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate}, and PVPP (polyvinylpolypyrrolidone) from Sigma-Aldrich Co. (St.

Louis, MO) were used throughout this experiment. EDTA (ethylenediaminetetraacetic acid) and MES [2-(N-morpholino) ethanesulfonic acid] were purchased from Fisher Scientific (Fair Lawn, N.J.).

SOLUTION TREATMENT. A 2x concentration of the cosolvent dissolved in MES buffer at 21 ºC was mixed with an equal volume of plant extract. In the experiments testing the glucose and glycine additive effects, 5 treatments were set as buffer (control), 0.5 M glucose, 0.5 M glycine, 1 M glucose, 1 M glycine, and 0.5 M glucose + 0.5 M glycine. Treatments in the experiment testing additive effects of glycine and CHAPS on pepper leaf proteins were buffer (control), 2 M glycine, 10 mM CHAPS, and 2 M glycine +10 mM CHAPS. Concentration of CHAPS in testing glycine and CHAPS additive effect for commercial ovalbumin was adjusted to 15 mM. Treatments were buffer (control), 2 M glycine, 15 mM CHAPS, and 2 M glycine +15 mM CHAPS. The solution of extract and compound was stirred using a magnetic stir bar for 30 minutes.

HEAT TREATMENT. Temperature ranges, which cover the transition from clear state to cloudy state and then the precipitate state, were established according to preliminary experiments. In experiments of glucose and glycine, the temperature range was from 48 to 58 ºC with 1 ºC interval. Temperature ranges from 44 ºC to 58 ºC, and from 56 ºC to 75 ºC, with 1 ºC interval, were used in experiments testing the effect of glycine and CHAPS on pepper leaf proteins and on ovalbumin, respectively. A 21 ºC treatment was included in each concentration group as the control temperature treatment. Three subsamples of 3.4 ml solution from each group at each temperature were pipetted to 16-mm diameter test tubes. Due to the limited volume of pepper leaf extract, 2 subsamples were used in the experiment studying glycine and CHAPS effects on pepper leaf proteins.

Test tubes were held in water baths at target temperatures for 15 min and then cooled to 21 ºC.

PROTEIN QUANTIFICATION. Protein concentrations in leaf extracts were determined using the Bradford assay (1976) with ovalbumin as a standard. Non-heated leaf extract solutions were diluted to 1:5, 1:10, and 1:20 with MES buffer. Bradford reagent (3 ml) was added to each protein-containing cuvette and mixed by inversion before absorbance measurements at 595 nm were conducted at ambient temperature.

SPECTROPHOTOMETRIC MEASUREMENT. A spectrophotometer (DU640B, Beckman) was used to measure apparent absorbance at 540 nm 4 hr after heat treatments. Unheated solutions from each treatment were filtered through 0.2 µm polyethersulfone membrane syringe filters for use as blanks. Apparent absorbance at 436, 540, and 679 nm of samples of each treatment before heat exposure were conducted using MES buffer as the blank to provide are reference across treatments. Readings at 436 nm and 679 nm corresponded with the absorbance maxima for chlorophyll, complementing nonspecific apparent absorbance at 540 nm.

DATA ANALYSIS. Each experiment was conducted with five independent replications (dates) with three subsamples per treatment combination in each replication. Analysis of variance was conducted using PROC GLM (SAS Institute, Cary, N.C.) to analyze the treatment effects with treatment by date as the error term. The interaction effect between treatment and temperature was analyzed with the error term of treatment by temperature by date. When a significant treatment by temperature interaction was observed, Duncan's New Multiple Range Test was conducted at the critical value of $P \leq 0.05$ to group the treatment effects at each temperature with treatment by date as the error term.

In a separate analysis of variance, treatment and interaction effects were tested at P≤0.05 using PROC GLM for the apparent absorbance maxima temperature and precipitation temperature response variables. Duncan's New Multiple Range Test was conducted as appropriate to group treatment means using treatment by date as the error term at the critical value of P≤0.05.

Results

GLUCOSE AND GLYCINE. The control pepper leaf extract without chemical chaperones exhibited a maximum apparent absorbance temperature of 51.7 °C (Fig. 5). Glycine and glucose at 0.5 M stabilized leaf proteins at high temperatures, resulting in maximum apparent absorbance temperatures of 53.3 °C and 53.7 °C, respectively. Maximum apparent absorbance temperatures for glucose and glycine at 0.5 M were not significantly different from each other, but were significantly higher than the control (Table 2). At 1 M, glycine and glucose solutions reached a maximum apparent absorbance at 55.5 °C. A mixture of glycine and glucose, both at 0.5 M, exhibited a maximum apparent absorbance at 55.0 °C, which was not significantly different from the compounds used singly at 1 M, but was significantly higher than the compounds at 0.5 M.

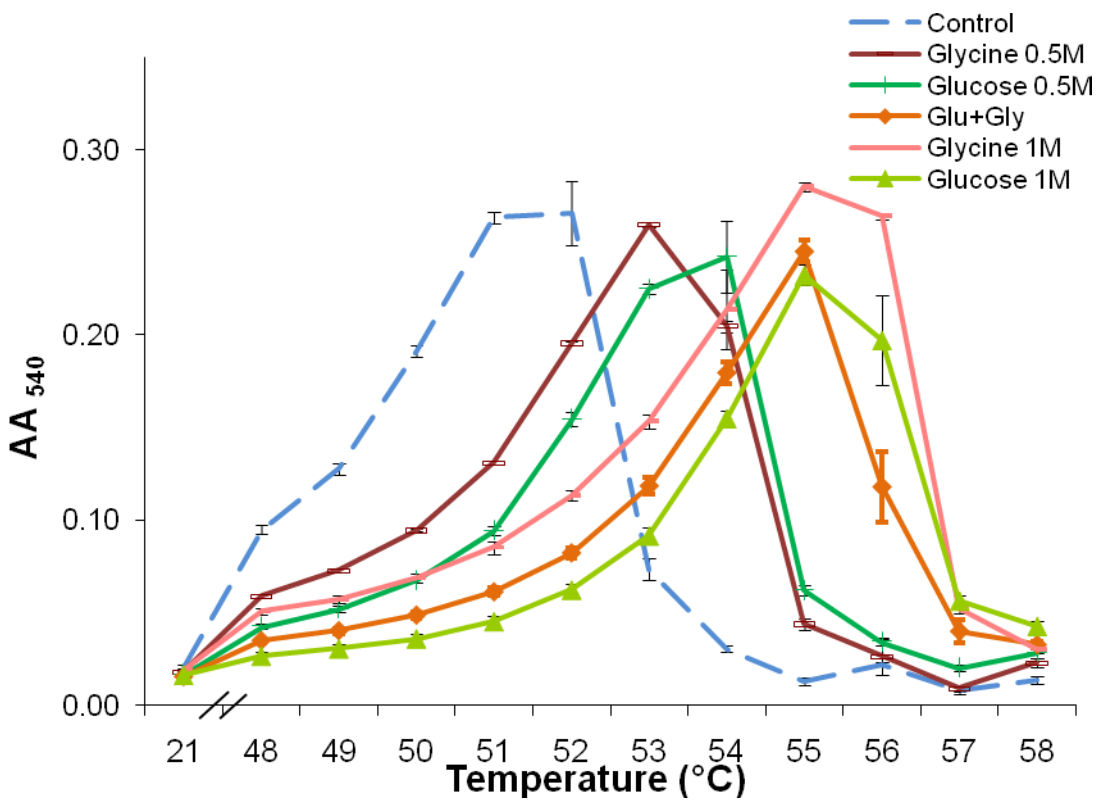

Figure 5. Additive effect of glucose and glycine on thermostability of pepper leaf proteins. Mean apparent absorbance values (± SE) at 540 nm ($AA_{540\ nm}$) versus temperature (˚C) are presented for five replications with two subsamples (n=10).

Table 2. Temperatures (°C) yielding apparent absorbance maxima (AA_{max}) at 540 nm for pepper leaf extracts containing glucose or glycine singly or in combination. Solutions were exposed to elevated temperatures for 15 min in circulating water baths, then held at ambient temperature for 4 h before assay. Mean values for five replications with two subsamples are presented.

Treatment	AA_{max} (^{o}C)
Glycine (1.0 M)	55.5 a [z]
Glucose (1.0 M)	55.5 a
Glycine (0.5 M)+ Glucose (0.5 M)	55 a
Glucose (0.5 M)	53.67 b
Glycine (0.5 M)	53.33 b
Control	51.67 c

[z] Means followed by the same letter are not significantly different based on Duncan's new multiple range test at $P \leq 0.05$.

GLYCINE AND CHAPS: PEPPER LEAF PROTEINS. The control pepper leaf extract without chemical chaperones reached the maximum apparent absorbance at a temperature of 51.2 °C (Fig. 6). Glycine (2 M) and CHAPS (10 mM) exhibited maximum apparent absorbance temperatures at 54.8 °C and 46.4 °C, 3.6 °C and 4.8 °C greater and smaller compared with the control, respectively. A mixture of glycine (2 M) and CHAPS (10 mM) showed a maximum apparent absorbance at 48.2 °C, which is between the apparent absorbance maxima temperature of CHAPS and the control. The Duncan's multiple range test grouped the four treatments as individual groups (Table 3).

Figure 6. Additive effect of glucose and CHAPS on thermostability of pepper leaf proteins. Mean apparent absorbance values (±SE) at 540 nm ($AA_{540\ nm}$) versus temperature (˚C) are presented for five replications with two subsamples (n=10).

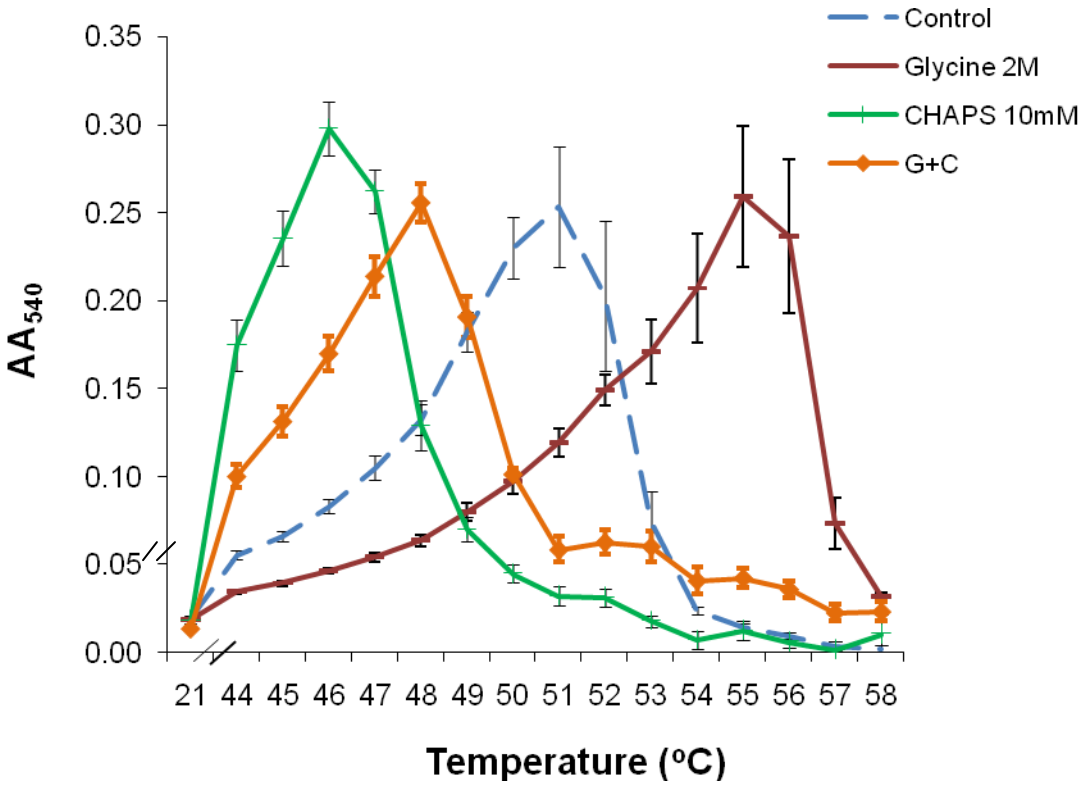

Table 3. Temperatures (°C) yielding apparent absorbance maxima (AA_{max}) at 540 nm for pepper leaf extracts containing CHAPS or glycine singly or in combination. Solutions were exposed to elevated temperatures for 15 min in circulating water baths, then held at ambient temperature for 4 h before assay. Mean values for five replications with two subsamples are presented.

Treatment	AA_{max} (°C)
Glycine (2 M)	54.8 a [z]
Control	51.2 b
Glycine (2 M)+CHAPS (10 mM)	48.2 c
CHAPS (10 mM)	46.4 d

[z] Means followed by the same letter are not significantly different based on Duncan's new multiple range test at $P \leq 0.05$.

GLYCINE AND CHAPS: OVALBUMIN. The ovalbumin control without chemical chaperones reached the maximum apparent absorbance at a temperature of 64 °C (Fig. 7). Glycine (2 M) and CHAPS (15 mM) exhibited maximum apparent absorbance temperatures at 68 °C and 61 °C, respectively. A mixture of glycine (2 M) and CHAPS (15 mM) showed a maximum apparent absorbance at 64 °C, which is significantly different from the glycine and CHAPS treatments, but the same as the control (Table 4).

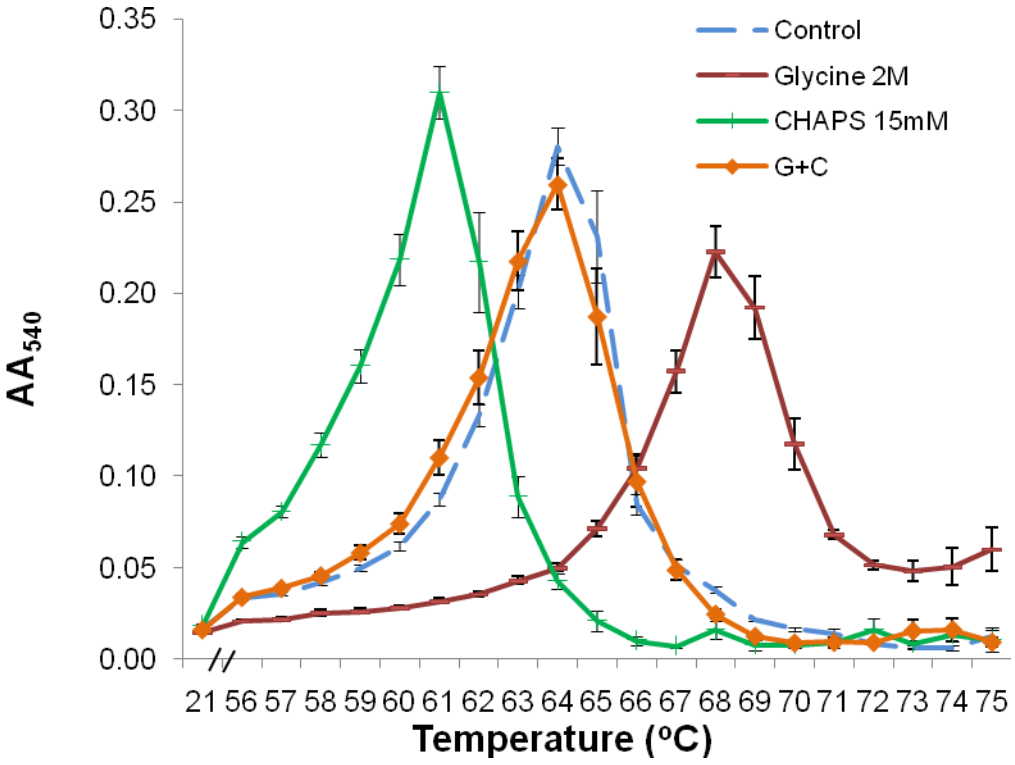

Figure 7. The additive effect of glucose and CHAPS on thermostability of ovalbumin.

Mean apparent absorbance values (± SE) at 540 nm ($AA_{540\ nm}$) versus temperature (˚C) are presented for five replications with three subsamples (n=15).

Table 4. Temperatures (°C) yielding apparent absorbance maxima (AA_{max}) at 540 nm for ovalbumin containing CHAPS or glycine singly or in combination. Solutions were exposed to elevated temperatures for 15 min in circulating water baths, then held at ambient temperature for 4 h before assay. Mean values for five replications with three subsamples are presented.

Treatment	AA_{max} (^{o}C)
Glycine (2 M)	68 a [z]
Glycine (2 M)+CHAPS (15 mM)	64 b
Control	64 b
CHAPS (15 mM)	61 c

[z] Means followed by the same letter are not significantly different based on Duncan's new multiple range test at $P \leq 0.05$.

Discussion

MECHANISM OF ADDITIVE EFFECT BETWEEN GLYCINE AND GLUCOSE. Glycine and glucose provided equivalent protection at the same concentration, and their effect was additive when combined in a mixture of the two chemical chaperones. Although chemicals of different classes were tested, the principle that the polarity of the compound is a key indicator for the stabilizing effect was supported by our study.

Glucose and glycine share the similar log Kow values of -3.24 and -3.21, respectively, by which similar hydrophilicity can be concluded and the same ability to induce the preferential hydration layers for protein molecules can be suggested.

The enhanced thermostability of proteins results from the hydration layer on the surface of the protein molecules induced by the cosolvent in the system. The preferential hydration theory was introduced by Timasheff (2002), which focuses on the relationship of affinities to protein molecules between cosolvent and water molecules. The mechanism behind the equivalent stabilizing effect is based on their equivalent ability to increase the surface tension of water in the protein-solvent-cosolvent system. The high surface potential drives the exclusion of the cosolvent from the protein surface, as well as the binding of water molecules to proteins. Also, in terms of hydrophilicity and polarity of the cosolvent, compounds of high polarity are favored to bind to water molecules instead of to protein molecules, favoring formation of a hydration layer. Therefore, the hydration layer can be induced by the addition of polar compounds, and the corresponding stabilizing effect on protein molecules results. In our study, the equivalent stabilizing effect by glycine and glucose on pepper leaf proteins likely resulted from their similar polarity and hydrophilicity, and therefore equivalent preferential hydration.

The preferential hydration effects can be further explained by similar abilities between glucose and glycine to be replaced at the interacting loci on proteins. Interacting loci are sites on protein surfaces where water and cosolvent components exchange. Preferential interaction includes preferential hydration or preferential binding of ligand, which is determined by their relative affinities to interacting loci. In the process of preferential hydration, the affinities between water molecules and interacting loci are

greater than that for the cosolvents and an excess of water binds to the protein domain. Therefore, the same stabilizing effect introduced by the glycine and glucose can reveal their same ability to stabilize the hydration layer of protein molecules and similar affinities to interacting loci on protein molecules. Furthermore, a more important conclusion can be drawn from their additive effects. The additive effect resulting from the additive affinity at interacting loci on protein molecules reveals a non-competitive interaction between glucose and glycine when they are replaced by water molecules on the interacting loci of proteins.

ADDITIVE EFFECT OF GLYCINE AND CHAPS. The additive effects of the stabilizer glycine and destabilizer CHAPS were tested on pepper leaf proteins and commercial ovalbumin. The amino acid glycine is considered to be a general stabilizer since it can bind with water efficiently and increase the surface tension of the solution, which induces the exclusion of the cosolvent and therefore preferential hydration (Timasheff, 2002). Zwitterionic CHAPS possesses both a hydrophobic region and a charged area on the monomeric molecule. The addition of CHAPS reduces preferential hydration by the disfavored water binding of the hydrophobic part of the monomers. The monomeric CHAPS molecule has a hydrophobic tail as well as a hydrophilic head which contains both positively and negatively charged groups at physiological pH. Both the electrostatic interactions with the charged surface of the protein molecules and hydrophobic interactions with the hydrophobic core of proteins can be induced. Thus, under the combination treatment of CHAPS and glycine, the destabilizing effect caused by CHAPS can be counter-acted by the stabilization of the hydration layer induced by glycine. In the same way, the stabilizing effect of glycine can be disturbed by the direct

interaction with protein molecules resulting from CHAPS. Although the additive effects of glycine and CHAPS were demonstrated with both the pepper leaf proteins and commercial ovalbumin in our study, the results are quantitatively different. In the commercial ovalbumin system, which is the single purified protein, the additive effect is numeric. It can be interpreted that the destabilizing effect by binding of CHAPS on ovalbumin can be cancelled out by the hydration layers induced by the addition of the glycine. The affinity of CHAPS to ovalbumin maintains a numeric balance with the preferential exclusion force provided by glycine.

However, in the case of the pepper leaf protein system, which contains all soluble proteins as well as other unpurified soluble compounds, the additive effect of glycine and CHAPS is not numeric. The numeric balance of the CHAPS binding with the exclusion force in the case of ovalbumin fails to hold for pepper leaf proteins, suggesting that the properties of ovalbumin are not identical to the average properties of pepper leaf proteins. The affinity of CHAPS to a pepper protein mixture is greater than the exclusion force by the generated hydration layer induced by glycine. Subsequently, the destabilizing effect is more dominant than the preferential hydration. The additive effect of glycine and CHAPS is likely to be highly related to the composition and properties of the proteins tested.

Literature Cited

Anderson, J.A. 2006. Heat stability of pepper leaf extracts. J. Amer. Soc. Hort. Sci. 131(1): 17-23.

Anderson, J.A. 2007. Additive effects of alcohols and polyols on thermostability of pepper leaf extracts. J. Amer. Soc. Hort. Sci. 132(1): 67-72.

Arakawa, T. and S.N. Timasheff. 1985. The stabilization of proteins by osmolytes. Biophys. J. 47: 411-414.

Back, J.F., D. Oakenfull, and M. B. Smith. 1979. Increased thermal stability of proteins in the presence of sugars and polyols. Biochemistry 18: 5191-5196.

Baier, S.K. and D.J. McClements. 2003. Combined influence of NaCl and sucrose on heat-induced gelation of bovine serum albumin. J. Agric. Food Chem. 51: 8107-8112.

Baier, S.K. and D.J. McClements. 2006. The effect of binary cosolvent systems (glycerol–sucrose mixtures) on the heat-induced gelation mechanism of bovine serum albumin. Intl. J. of Food Sci. and Technol. 41: 189–199.

Bennion, B.J. and V. Daggett. 2004. Counteraction of urea-induced protein denaturation by trimethylamine N-oxide: a chemical chaperone at atomic resolution. Proc. Natl. Acad. Sci. USA. 101:6433–6438.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Chow, M.K.M., G.L. Devlin, and S.P. Bottomley. 2001. Osmolytes as modulators of conformational changes in serpins. Biol. Chem. 382: 1593-1599

Davis-Searles, P.R., A.J. Saunders, D.A. Erie, D.J. Winzor, and G.J. Pielak. 2001. Interpreting the effects of small uncharged solutes on protein-folding equilibria. Annu. Rev. Biophys. Biomol. Struct. 30: 271-306.

Ganea, E. and J.J. Harding. 2005. Trehalose and 6-aminohexanoic acid stabilize and renature glucose-6-phosphate dehydrogenase inactivated by glycation and by guanidinium hydrochloride. Biol. Chem. 386: 269–278.

Hart, G.J., A.E. Russell, and D.R. Cooper. 1971. The effects of certain glycols, substituted glycols and related organic solvents on the thermal stability of soluble collagen. Biochem J. 125(2): 599–604.

Kim, S.H, Y.B. Yan, and H.M. Zhou. 2006. Role of osmolytes as chemical chaperones during the refolding of aminoacylase. Biochem. Cell Biol. 84:30-38.

Kovrigin, E.L.and S.A. Potekhin. 1997. Preferential solvation changes upon lysozyme heat denaturation in mixed solvents. Biochemistry 36(30): 9195-9199.

Timasheff, S.N. 2002. Protein-solvent preferential interactions, protein hydration, and the modulation of biochemical reactions by solvent components. Proc. Natl. Acad. Sci. USA 99: 9721-9726.

CHAPTER IV

EFFECTS OF CATIONIC, ANIONIC, ZWITTERIONIC AND NONIONIC DETERGENTS ON THERMOSTABILITY OF PEPPER LEAF PROTEIN

Abstract

Effects on solubility-based thermostability of pepper leaf proteins by detergents representing four classes were studied. For the cationic detergent CTAB, thermostability of pepper leaf proteins decreased with increasing CTAB concentration up to about 1 mM. However, CTAB at 2 and 5 mM prevented turbidity and precipitation to 100 °C. The transition from CTAB monomers to micelles occurred at approximately 0.7 mM. The anionic detergent SDS reduced pepper protein thermostability at concentrations up to 0.15 mM. Higher SDS concentrations increased thermostability with 11 °C protection provided by 0.35 mM. Unlike CTAB, the transition from destabilization to stabilization occurred over a lower concentration range than the transition from monomers to micelles (2 mM). In contrast with CTAB and SDS, the nonionic detergent Tween 20 and the zwitterionic detergent CHAPS showed no significant stabilization of pepper leaf proteins at elevated temperatures. Protein thermostability was not significantly different from controls at Tween 20 concentrations up to 0.1 mM and up to 1 mM CHAPS. Higher detergent concentrations reduced protein thermostability. Destabilization approached

maxima at concentrations near the critical micelle concentrations for Tween 20 (1.1 mM) and CHAPS (4.4 mM).

Introduction

Detergents are widely used in solubilization, purification, extraction and denaturation of proteins, both in industrial areas and biological research. Studies on the interaction between detergent and protein have been conducted for many years and are not only of practical importance but also of theoretical significance. Detergents can be classified into four categories based on the nature of the hydrophilic head group: nonionic detergents with no charge, cationic detergents that are positively charged, negatively charged anionic detergents, and zwitterions containing oppositely charged groups. The different detergent groups interact differently with proteins. The denaturing activity of ionic detergents is influenced by pH and ionic strength of the system. However, nonionic detergents can maintain the protein's structure and function due to their neutral charges but may lower the activity of proteins in the process of protein separation (Jimenez and Garcia-Carmona, 1996). Zwitterionic detergents combine the features of ionic and nonionic ones. They are less likely to denature proteins than ionic ones and can be more efficient than nonionic detergents in disrupting protein-protein bonds. For example, the stability of mushroom tyrosinase after treatment with different classes of detergents was tested by Yang et al. (2007). In the presence of anionic sodium di-2-ethylhexylsulfosuccinate (Aerosol OT, AOT) and nonionic polyoxyethylene (2) cetyl ether (Brij52), a higher value of V_{max}/K_m of tyrosinase was observed, suggesting enhanced activity of that enzyme. However, cationic CTAB increased the value of K_m,

resulting in a decrease in the V_{max}/K_m ratio, showing an inhibitory effect on its activity. Thermostability was also tested. Brij 52 was found to stabilize mushroom tyrosinase even at a higher temperature. AOT can moderately stabilize the enzyme. Mushroom tyrosinase was least stable in the presence of CTAB.

The relationship between protein stability and properties of monomeric detergents is complex. Besides the electrostatic interactions between the surfactant head group and the charged amino acid residues of a protein, the hydrophobic interactions between the alkyl chains of surfactant molecules and hydrophobic pockets of the protein should also be considered (Savelli et al., 2000), as well as other properties of monomeric surfactants such as the head group size and the hydrophobic/hydrophilic balance. For ionic detergents, the environment of the enzyme-detergent complex is also a significant factor. The mushroom tyrosinase used in Yang's (2007) research was negatively charged because the surrounding pH was adjusted to 6.0 by Tris, higher than its isoelectric point at 4.8. In that case, the addition of AOT that brings in the Na^+ can greatly increase the ionic strength and enhance its interaction with the enzyme molecule.

Micelle structure, the key feature of a detergent, exists when the critical micelle concentration (cmc) of the detergent is reached. In solution, micelle structure is thermodynamically favored by exposing the hydrophilic heads of the surfactant molecules at the surface of the micelle to contact with water and burying the hydrophobic tails, which can bond with the hydrophobic surface of a protein inside the micelle structure to avoid the contact with water. However, types of micelles, such as spherical, cylindrical and inverted micelles, are not fixed and can exist simultaneously at certain concentrations and system compositions. Micelle structures formed by

cetyltrimethylammonium bromide (CTAB) are of different types at different concentrations. Choudhury and co-workers (1994) employed positron lifetime spectroscopy (PLS) to detect structural transformations in the CTAB/water and CTAB/water/hexanol systems. In the CTAB/water system, the surfactant molecules dispersed homogenously in a monomeric form at concentrations up to 0.7 mM, at which point an abrupt change in physicochemical properties (including surface tension and viscosity) was detected due to the formation of micelles. With increasing CTAB concentration, the size and number of spherical micelles grew. However, growth had a limitation in that the micelle radius can never exceed the length of the carbohydrate chain of CTAB. As a result, the micelles deformed from spherical structures into prolate shapes beginning at 10 to 15 mM and were highly deformed at 100 mM. At 125 mM, rod-like structures appeared and were transformed into entangled rods at 400 mM. When the concentration of CTAB exceeded 1000 mM, hexagonal liquid crystalline structures were formed. This result is in agreement with previous studies using small angle neutron scattering (SANS) and small angle X-ray scattering (SAXS) methods (Choudhury et al., 1994). Analysis using PLS also worked well in the CTAB/hexanol/water system but was limited to the concentration range from 0 to 120 mM due to the phase separation of CTAB micelles. Similarly, two cmc values were detected by Guo et al. (2003). One cmc was the concentration when micelles formed from monomers. The other was when a mixture of spherical micelles appeared. In the water system, cmc_1 for CTAB equaled 1 mM and cmc_2 was approximately 10 mM.

Interactions among micelles have been studied by several researchers. In a nonionic surfactant system, the major interactions between micelles are van der Waals attractive

forces (Hayter and Zulauf, 1982), which can be weakened by high temperatures. However, the interactions between charged micelles in an ionic system contain the electronic repulsion forces and the solvation effect besides the van der Waals forces, which is more complicated. Kumar et al. (2000) tested the cloud point, the temperature above which the micelle-rich phase and monomer-rich phase separate, under different SDS concentrations and with a series of organic additives. It was reported that the alkyl chains of the organic compounds were embedded in the micelle core due to hydrophobic effects but the rest of the chains that are left outside the micelle can further interact with other alkyl chains which are attached to a different micelle. Such interactions bring micelles closer and add a new attractive force. This force can be enhanced by adding a longer alkyl chain-organic compound, increasing the concentration of additives, or lowering the concentration of detergent. This conclusion was confirmed by Musarat (2007) with Triton X100 and mixed systems using cationic and anionic surfactants.

The interaction between micelle structure and protein molecules is another important topic in research. For example, the interaction between sodium dodecyl sulfate (SDS) and protein stability has been studied for many years. Sodium dodecyl sulfate is an anionic surfactant that can be used as protein denaturant by binding its sulfate group to the positive side chain of proteins and by the interaction between its alkyl chain and hydrophobic parts of the protein. Moren and Khan (1995) evaluated the anionic property of SDS. They tested its interaction with the positively charged protein lysozyme. When a small amount of SDS was added, precipitation occurred in the aqueous lysozyme solution based on charge neutralization. However, when they increased the concentration of SDS

to an SDS: protein ratio of 19:1, where the positive sites of the protein were saturated, the precipitate was redissolved.

The interaction between SDS and proteins can also be studied in terms of its anionic property. Enzyme activation by SDS is a pH dependent phenomenon (Moore and Flurkey, 1989; Jimenez and Garcia-Carmona, 1996). An acidic environment can eliminate the activity of enzymes in the presence of SDS, while in the neutral environment, SDS can activate enzymes. Several enzymes have been tested, such as cresolase and catecholase. Jimenez and Garcia-Carmona (1996) concluded that this type of activation is independent of substrate.

Aiming to explain the mechanism of the pH effect, scientists expanded their work to CTAB, a type of cationic surfactant. Sen et al. (1980) set the pH at 6.0, a value greater than the pK_a of gelatin, and compared the interactions with CTAB and SDS. Cetyltrimethylammonium bromide bound to gelatin favorably due to the electrostatic attraction between its positive charges and the negative state of gelatin, while the anionic SDS contributed electrostatic repulsion rather than binding. These results illustrate the more complicated interactions between ionic detergents and proteins, compared with nonionic detergents. How proteins can be bound to detergents is dependent on the relative ratio of electrostatic forces to hydrophobic interactions. An equilibrium state is reached by the balance between the hydrophobic interaction and the electrostatic force. In other words, one can adjust the ionic strength of the medium by changing the pH or adding electrolytes to maximize the ionic detergent's binding to proteins. For example, 10 mM NaBr at pH 6.5 can increase the affinity of CTAB to α-amylase compared with pH 9.7 (Bordbar et al., 2005). In regard to hydrophobic interactions, CTAB was found to be

more efficient in binding with lysozyme than dodecyltrimethylammonium bromide (DTAB), due to its stronger hydrophobic interactions resulting from its longer hydroxycarbon tail exposed on the surfactant molecules (Subramanian et al., 1984).

Studies of the free energy change during CTAB aggregation in the system with ethanol were conducted by Li et al. (2006). They explained the phenomena of CTAB's increased cmc value and decreased aggregation number exhibited in the CTAB/ethanol system by using Nagarajan's (1991) model. This model defined the free energy change in the formation of the micelle as the sum of the free energy changes in transferring tails from solvent to micelle core and in the process of the tails' deformation within the core, interfacial free energy change between micelles and solvent, steric repulsion between surfactant heads, and the electrostatic interactions between the charged groups. Nagarajan's (1991) model worked well in the CTAB/ethanol system and no significant differences were noted between the experimental values and predicted values. The cmc values increase when the carbohydrate tail of CTAB transfers into the micelle. Higher fraction of ethanol in the system can also enhance the value of cmc by strengthening the ionic interaction between CTAB's head groups, which contributes to a smaller aggregation number of CTAB micelles.

Cetyltrimethylammonium bromide can denature proteins by altering surface tension and influencing hydrophobic interactions, leading to a loss of biological activity of proteins. However, CTAB can also facilitate the protein-refolding process. In research aiming at improving the refolding yield of recombinant or native lysozyme, Wang et al. (2005) reported that CTAB was more productive in assisting refolding of urea-denatured lysozyme than the artificial chaperone β-cyclodextrin. This was caused by protecting

denatured lysozyme from aggregation with the CTAB-lysozyme complexes formed by hydrophobic interactions. Dissociation of the complex was accomplished by treatment with refolding buffer containing the reduced and oxidized forms of glutathione which facilitated the formation of disulfide linkages, making contact possible between denatured lysozyme and refolding buffer. The interactions between CTAB and α-chymotrypsin can be revealed by means of the solvation speed using the probe dansyl chloride (Sarkar et al., 2005). Cetyltrimethylammonium bromide's ability to bind protein and DNA was also studied by Gani et al. (1999). They tested the binding site numbers exposed on CTAB micelles in individual solutions of protein or DNA and their mixture. They found that the CTAB-DNA-protein ternary system can release more binding sites than any of the individual protein and DNA systems. In addition, the states of protein and DNA have a significant influence on the binding of CTAB. The denatured protein and DNA systems have a greater ability to bind CTAB micelles than systems that contain the native protein or DNA molecules.

The relationship between surfactant and thermostability of proteins is also of interest. In experiments with SDS, very low concentrations had no influence on thermostability of proteins due to electrostatic binding with specific and limited sites of protein molecules. When the concentration approached cmc, SDS decreased the thermostability of protein by disturbing the hydrophobicity of the protein. However, when micelle structure formed at concentrations above cmc, protein molecules were captured and covered by micelle structure, inhibiting denaturation of protein and increasing thermostability (Moren and Khan, 1995). The same phenomena were also described using CHAPS and a mixture of SDS and Tween 20 in a study conducted by

Vermeer et al. (2000). It was reported that the secondary structure was influenced by the addition of detergent, increasing the ratio of α-helix to β-sheet. The ability of detergent micelles to bind the protein can also influence the protein's solubility. The process of increasing solubility of protein by incorporation into detergent micelles is referred to as solubilization. Banerjee et al. (1995) tested the ability of different types of detergents in solubilizing protein. They proposed a more detailed classification for detergents based on their structure and charge, including negatively charged detergent, positively charged detergent, zwitterionic detergent, H-bond formation detergent, and hydrophobic detergent with long polyoxyether chains and aromatic rings. There are two sub-classes describing the long flexible chain structure and rigid structure existing in the first three classes referred to above, which offer activity differentiation attributed to these structures. For example, in the comparison of membrane protein extraction abilities between CHAPS, a zwitterionic detergent with a rigid structure and Triton X100, a nonionic hydrophobic detergent with a long polyoxyether chain and aromatic rings, Triton X100 extracts more protein and fewer lipids than CHAPS despite similar cmc values. It was concluded that extract yield is determined more by the ratio of detergent to proteins instead of the detergent's cmc value. The same principle was also applied by Bennett (1992) in his research to determine the characters of the components in synaptic vesicle membrane proteins. He used three detergents (CHAPS, Triton X 100 and octylglucoside) to solubilize and recover the protein complex and distinguish different synaptic vesicle fractions by their different sedimentation rates and recovery efficiency.

Although many theories and discussions have attempted to explain specific pair-wised combinations among various single proteins and different types of detergents,

considerations of interactions between detergent and protein mixtures have seldom been taken into account. Protein mixtures can reflect the average properties of the proteins and are also significant in practice. The information available so far does not give a clear interpretation of the interaction between protein mixtures and surfactant types (nonionic, anionic, and cationic). To extend the previous research done by Vermeer (2000) in testing the relationship between SDS and Ig G, the surfactants tested were extended to nonionic Tween 20, anionic SDS, and the cationic CTAB, interacting with a pepper leaf protein mixture. My objectives were to: 1) determine the relationship between thermostability of pepper leaf proteins and detergent treatments of different classes and various concentrations; and 2) define the relationship between micelles and stabilizing effect on pepper leaf proteins.

Materials and Methods

PLANT CULTURE AND LEAF EXTRACT. 'Early Calwonder' pepper plants were grown in 24 cm diameter pots in a commercial potting mix (BM-1; Saint-Modeste, Quebec) amended with dolomite (3.6 g/L), triple superphosphate (0.7 g/L), Micromax (The Scotts Co., Marysville, Ohio) (0.6 g/L), and KNO_3 (0.6 g/L). Plants were maintained in a controlled-environment chamber (model PGW36; Conviron, Winnipeg, Man., Canada), at 24/20 °C day/night temperatures with a 14 hour photoperiod and a photosynthetic photon flux density at canopy height of about 400 $\mu \cdot mol \cdot m^{-2} \cdot s^{-1}$. Plants were watered with 0.7 g/L 20N-8.6P-16.6K soluble fertilizer as needed based on the darkness of the soil surface and the weight of the pot. Fully expanded leaves from 10-week-old plants were collected as the plant material.

Deionized water at room temperature was used to hydrate the leaves after collection and during the process of transfer to the lab. Fifteen grams of leaves without midrib was blended in the homogenization buffer consisting of 225 ml MES buffer (50 mM, pH=6.0, with 1 mM EDTA) with 1.25 g PVPP. Then, the leaf solution was filtered through Miracloth (Calbiochem, Madison, WI) and collected in a beaker containing an additional 1.25 g PVPP with 15 ml MES buffer. Leaf extract was generated by collecting the supernatants from centrifugation at 16k $g_{n,}$ 21 ºC for 20 min.

CHEMICALS. Tween 20 (Polysorbate 20), SDS (Sodium dodecyl sulfate), CTAB (Cetyltrimethylammonium bromide), and PVPP (polyvinylpolypyrrolidone) from Sigma (St. Louis, MO) were used throughout this experiment. EDTA (ethylenediaminetetraacetic acid) and MES [2-(N-morpholino) ethanesulfonic acid] were purchased from Fisher Scientific (Fair Lawn, N.J.). Eosin Yellowish was purchased from the Coleman & Bell Company (Norwood, MA). Cadmium red light paint was produced by Duro Art Supply Company (Chicago, IL).

SOLUTION TREATMENT. A 2x concentration of the cosolvent dissolved in MES buffer at 21 ºC was mixed with an equal volume of plant extract. Groups were set based on different concentrations of SDS or CTAB. In the experiments testing the SDS dose effect, groups were MES buffer without cosolvent as the control, 0.01, 0.05, 0.10, 0.15, 0.20, 0.25 0.30, and 0.35 mM SDS. In CTAB experiments, the groups were MES buffer as control, 0.005, 0.05, 0.5, 1, 2 and 5 mM. The combination of leaf extract and detergent was stirred for 45 minutes. The extended stirring time, compared with previous

experiments, was used because certain concentrations of CTAB-extract mixtures (2, 5 and 10 mM) were initially cloudy, but cleared over time at ambient temperature.

HEAT TREATMENT. The whole temperature range for SDS was from 44 ºC to 67 ºC with 1 ºC interval. Due to the limit of the extract volume, temperature ranges for each individual solution treatment varied, based on the state transition temperature exhibited in preliminary experiments. In experiments with CTAB, for example, temperatures from 42 ºC to 54 ºC with 1 ºC intervals were tested but the range was different for other detergent treatments. The 21 ºC treatment was included as the control temperature. Three subsamples of 3.4 ml solution from each group at each temperature were pipetted to16-mm diameter test tubes. Test tubes were held in water baths for 15 min at target temperatures and then cooled to 21 ºC.

PROTEIN QUANTITATION. Protein concentrations in leaf extracts were determined using the Bradford assay (1976) with ovalbumin as a standard. Non-heated leaf extract solutions were diluted to 1:5, 1:10, and 1:20 with MES buffer. Bradford reagent (3 ml) was added to each protein-containing cuvette and mixed by inversion before absorbance at 595 nm was measured at ambient temperature.

SPECTROPHOTOMETRIC MEASUREMENT. A spectrophotometer (DU640B, Beckman) was used to measure apparent absorbance at 540 nm 4 hr after heat treatment. Un-heated solutions from each treatment were filtered through 0.2 μm syringe filter at 21 ºC as blanks. Apparent absorbance at 436, 540, and 679 nm of samples of each treatment before heat exposure were conducted using MES buffer as the blank.

CMC MEASUREMENT. The cmc value of CTAB in pepper leaf protein solutions was measured using a modification of the dye solubility procedure (Courtney et al., 1986).

Briefly, 2 mg cadmium red light paint (Duro, Chicago) was added to the bottom of 16-mm diameter test tubes. CTAB at 0, 0.005, 0.05, 0.1, 0.5, 1, 2, 5or 10 mM was added and vortexed every 10 min for 2 hours. Percent transmission at each CTAB concentration was measured at 540nm. For SDS, the absorbance of chlorophyll in the SDS-extract mixture was measured at wavelengths of 415, 437, 671, and 679 nm to detect spectral shifts associated with micelle formation (Harris, 1958). The cmc for SDS was also estimated using the paint solubility assay. Eosin Y was used to measure the cmc value for both CHAPS and Tween 20 (Patist et al., 2000). Spectral shifts were detected by measuring absorbance at 518 and 528.5 nm.

DATA ANALYSIS. Each experiment was conducted with five independent replications (dates) with three subsamples per treatment combination in each replication, except the SDS experiment, which had two subsamples due to the limitation of the pepper leaf extract. Analysis of variance was conducted using PROC GLM (SAS Institute, Cary, N.C.) to analyze the treatment effects with treatment by date as the error term. The interaction effect between treatment and temperature was analyzed with the error term of treatment by temperature by date. When a significant treatment by temperature interaction was observed, Duncan's new multiple range test was conducted at the critical value of $P \leq 0.05$ to group the treatment effects at each temperature with treatment by date as the error term. In a separate analysis of variance, treatment and interaction effects were tested at $P \leq 0.05$ using PROC GLM for the apparent absorbance maxima temperatures in the SDS experiment and precipitation temperature response variables in the CTAB experiment. Duncan's new multiple range test was conducted as appropriate to group treatment means using treatment by date as the error term at the critical value of $P \leq 0.05$.

Results

DOSE EFFECT OF CTAB ON THERMOSTABILITY OF PEPPER LEAF PROTEIN. Dual effects of CTAB in stabilizing pepper leaf protein under high temperatures were exhibited. A destabilizing effect on protein thermostability was observed as concentrations increased from 0.005 mM to 1 mM (Table 5) with precipitation temperatures decreasing from 53.0 ºC to room temperature (21 ºC). Lower precipitation temperature which is beyond our temperature range can be expected. No turbidity or precipitation was observed at the concentrations of 2 mM and 5 mM, indicating a strong stabilizing effect at these concentrations. In our experiments, 100 ^{o}C was the highest temperature treatment. Higher precipitation temperatures in high concentration treatments can be expected beyond our experimental temperature range. The transition state was between the concentrations of 1 mM and 2 mM, which corresponded with CTAB's cmc value of 0.7 mM in this system.

Table 5. Temperatures (°C) yielding apparent absorbance maxima (AA_{max}) at 540 nm for pepper leaf extracts containing CTAB. Solutions were exposed to elevated temperatures for 15 min in circulating water baths, and then held at ambient temperature for 4 h before assay. Mean ± SE from 3 subsamples and 5 replications are presented.

CTAB (mM)	Temperature (^{o}C)
0	52.4±0.2 a^{z}
0.005	53.0±0.3 a
0.05	45.6±0.4 b
0.5	X
1	X
2	Y

5	Y

X= precipitation was observed at all temperatures, including the control temperature of 21 ^{o}C, the lowest temperature in this experiment.

Y= no turbidity or precipitation formed at all temperatures, included 100 ^{o}C, the highest temperature in this experiment.

[Z] Means in the column with the same letter are not significantly different by Duncan's new multiple range test with $P \leq 0.05$.

DOSE EFFECT OF SDS ON THERMOSTABILITY OF PEPPER LEAF PROTEIN. Dual effects of SDS on pepper leaf protein thermostability were exhibited. A destabilizing effect on pepper leaf proteins under high temperature treatment was observed as concentrations increased from 0.005 mM to 0.15 mM (Fig. 8). The greatest destabilizing effect was observed at 0.15 mM with a mean AA_{540nm} temperature 3.6 ºC lower than the control. A stabilizing effect was observed at SDS concentrations from 0.25 mM to 0.35 mM, with the concentration range from 0.15 to 0.25 mM as transition concentrations (Table 6). The stabilizing effect increased with the continually increasing SDS concentrations. The highest value was observed at 0.35 mM SDS with 11 ºC higher than control in AA_{540nm} temperature. The cmc measured was 2 mM in the system.

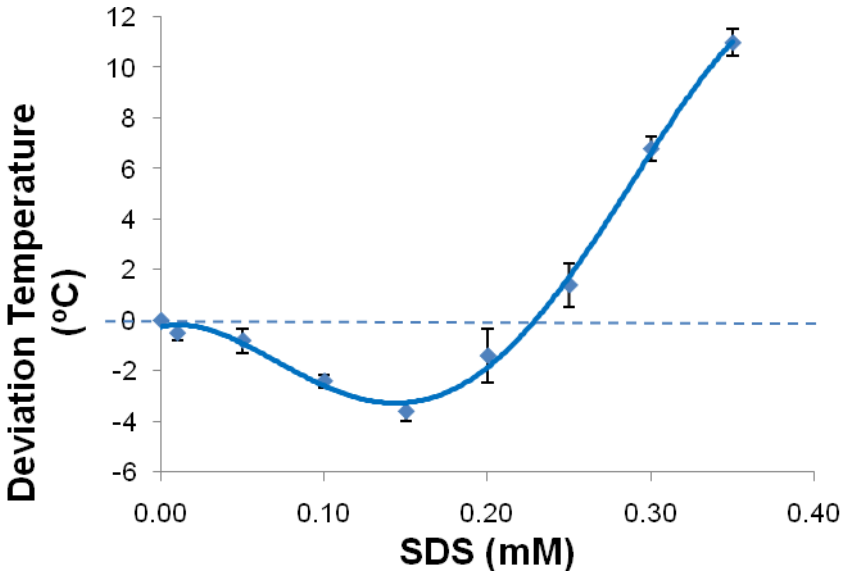

Figure 8. Dose- response of SDS on thermostability of pepper leaf proteins. Deviation temperatures from the control at mean maximum apparent absorbance values (± SE) at 540 nm ($AA_{540\ nm}$), versus SDS concentration (mM) are presented for five replications with two subsamples (n=10).

Table 6. Temperatures (°C) yielding apparent absorbance maxima (AA_{max}) at 540 nm for pepper leaf extracts containing SDS. Solutions were exposed to elevated temperatures for 15 min in circulating water baths, and then held at ambient temperature for 4 h before assay. Mean ± SE from 3 subsamples and 5 replications are presented.

SDS (mM)	Temperature (^{o}C)
0	51.8 c d
0.01	51.25 d
0.05	51 d e
0.1	49.4 e f
0.15	48.2 f

0.2	50.4 d e
0.25	53.2 c
0.3	58.6 b
0.35	62.8 a [z]

[z] Means followed by the same letter are not significantly different based on Duncan's new multiple range test at $P \leq 0.05$.

DOSE EFFECT OF TWEEN 20 ON THERMOSTABILITY OF PEPPER LEAF PROTEIN. Dual effects of Tween 20 in stabilizing pepper leaf protein under high temperatures were exhibited (Fig. 9). No significant stabilizing effects on pepper leaf protein under high temperature treatment were observed (Table 7). Within the concentration range from 0.1 mM to 1 mM, a significant destabilizing trend was observed. There were no significant differences between 1 mM and 10 mM treatments in destabilizing effects. Transition exhibited at Tween 20 concentrations between 0.01 and 0.1 mM. Critical micellar concentration was measured as 1.1 mM in the system.

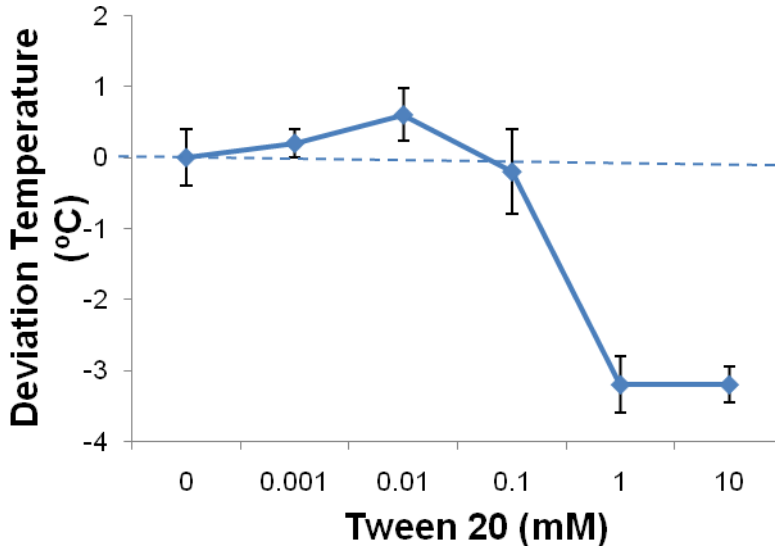

Figure 9. Dose- response of Tween 20 on thermostability of pepper leaf proteins. Deviation temperatures from the control at maximum mean apparent absorbance values (± SE) at 540 nm ($AA_{540\ nm}$), versus Tween 20 concentration (mM) are presented for five replications with three subsamples (n=15).

Table 7. Temperatures (°C) yielding apparent absorbance maxima (AA_{max}) at 540 nm for pepper leaf extracts containing Tween 20. Solutions were exposed to elevated temperatures for 15 min in circulating water baths, and then held at ambient temperature for 4 h before assay. Mean ± SE from 3 subsamples and 5 replications are presented.

Tween 20 (mM)	Temperature (^oC)
0	52.6 a
0.001	52.8 a
0.01	53.2 a [z]
0.1	52.4 b
1	49.4 b
10	49.4 b

[z] Means followed by the same letter are not significantly different based on Duncan's new multiple range test at $P \leq 0.05$.

DOSE EFFECT OF CHAPS ON THERMOSTABILITY OF PEPPER LEAF PROTEIN. A significant destabilizing effect by CHAPS on pepper leaf proteins under high temperatures was exhibited (Table 8). As concentrations increased from 1 mM to 5 mM, the $AA_{540\ nm}$ temperatures decreased with increasing concentration of CHAPS and reached a minimum 5.6 ºC lower than the control at 5 mM (Fig. 10). There were no

significant differences between 3 mM and 5 mM treatments. Within the concentration range from 0 mM to 1 mM, no significant changes were observed, although a minor increasing trend was observed. Critical micellar concentration was measured as 4.4 mM in the system.

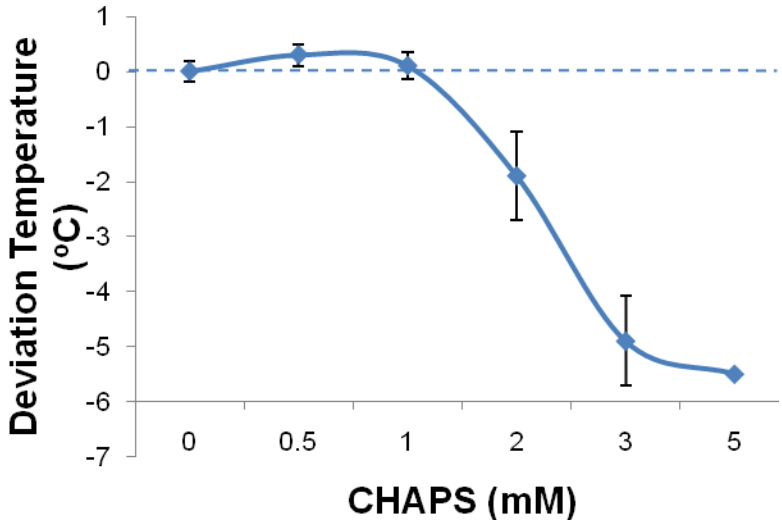

Figure 10. Dose- response of CHAPS on thermostability of pepper leaf proteins. Deviation temperatures from the control at maximum mean apparent absorbance values (±SE) at 540 nm ($AA_{540\ nm}$), versus CHAPS concentration (mM) are presented for five replications with three subsamples (n=15).

Table 8. Temperatures (°C) yielding apparent absorbance maxima (AA_{max}) at 540 nm for pepper leaf extracts containing CHAPS. Solutions were exposed to elevated temperatures for 15 min in circulating water baths, and then held at ambient temperature for 4 h before assay. Mean ± SE from 3 subsamples and 5 replications are presented.

CHAPS (mM)	Temperature (^oC)
0.5	52.8 a ^{z}

1	52.6 a
0	52.5 a
2	50.6 b
3	47.6 c
5	47 c

[z] Means followed by the same letter are not significantly different based on Duncan's new multiple range test at $P \leq 0.05$.

Discussion

CATIONIC CTAB. In the experiment to test the dual effects of CTAB in stabilizing pepper leaf protein under high temperatures, the formation of the CTAB micelle was suggested to be highly related to the transition of the destabilizing and stabilizing effects. The CTAB monomer can be concluded as the destabilizer for pepper leaf proteins and the CTAB micelle acts as the stabilizer. The electrostatic interaction is dominant within the CTAB- protein complex, but between the complexes, the hydrophobic interaction becomes dominant. At low concentrations of CTAB (from 0.005 mM to 0.05 mM), the positively charged heads of monomeric CTAB bind with negatively charged regions of the protein molecules and exposes the hydrophobic tails of CTAB molecules to the exterior. However, the interaction between the CTAB tail and the aqueous environment is disfavored. The exposed hydrophobic tails aggregate to avoid the interaction with aqueous solvent and lower the free energy of the system. As concentrations go higher, the aggregations become greater. Thus, a stronger destabilizing effect is observed at higher CTAB concentrations. Repulsion between the negative charges can also be expected and the aggregation interactions can be weakened, however, the electrostatic repulsion may

be much weaker than the hydrophobic interactions between the proteins so the destabilizing effect will dominate. It is also possible that some monomers penetrate their hydrophobic tails into the protein hydrophobic core to lower the free energy of the system, which can severely disrupt the protein structure. At the concentrations of 0.5 mM and 1 mM, aggregation was observed even at room temperature.

As the CTAB concentration further increases to the cmc, the CTAB micelle forms. The micelle shields the hydrophobic tails of the CTAB monomer and attenuates the hydrophobic aggregations among the CTAB- protein complexes and the hydrophobic penetration into the protein cores, and contributes a less destabilizing effect. At the cmc, the negatively charged sites on proteins are bound to CTAB micelles instead of CTAB monomers. However, the CTAB micelle hides the hydrophobic tails and exposes the hydrophilic and positively charged heads on the surface. The electrostatic repulsion between positively charged complexes and the favorable interaction between hydrophilic heads of CTAB and the aqueous environment are exhibited. In this way, the protein structure is protected against high temperature-induced aggregation.

An interesting phenomenon observed is that the protein solubility is maintained even at 100 ^{o}C. Besides the rationale suggested previously, a strong stabilizing effect can also result from the micelle structure formed, which can enclose the unfolded proteins inside the micelle core driven by the hydrophobic interactions. Therefore, the unfolded proteins are locked up in the confined space and the aggregation and precipitation can be limited.

ANIONIC SDS. Both positive and negative effects on thermostability of pepper leaf protein were induced by SDS in our experiment. Vermeer and Norde (2000) selected

SDS/Tween 20 to treat immunoglobulin G, whose molecular size is relatively larger than the surfactant molecule. A transition effect was observed on protein thermostability with the cut-off concentration much lower than the cmc detected. Vermeer and Norde (2000) interpreted the phenomenon as a dominance shift between the electrostatic interactions and hydrophobic interactions of the detergent-protein complexes, which also would explain our results well. The head of SDS monomers bind with the hydrophilic surface of proteins, exposing the hydrophobic tail of SDS out of the protein. Also, the positively charged area of the proteins can be neutralized by binding to the negatively charged SDS heads. Therefore, the protein- SDS monomer complex with a negatively charged hydrophobic surface is established. Driven by the dominant hydrophobic interactions, the exposed hydrophobic surface is favored to aggregate in order to lower the free energy in aqueous system. Some monomers could also penetrate their hydrophobic tails into the protein hydrophobic core, which can severely disrupt the protein structure. However, the electrostatic interactions will be predominant once the hydrophilic sites of the protein are saturated by the addition of SDS. The aggregation can be attenuated by the electrostatic repulsions as we observed at concentrations of 0.15 mM and 0.20 mM. Under the limitation of the positively charged sites, the more SDS that is added, the more dominant the repulsion forces will be, which is a possible explanation for the continually increasing thermostability as the concentration increases. Within the concentration range from 0.20 mM to 0.35 mM, the stabilizing effect observed can also be attributed to an SDS monomer layer accumulating on the surface of the protein molecule, limiting its unfolding space by a confined environment. The locking effect is so strong that the

precipitation was observed first at 11 ºC higher than the control treatment, and the solution remained clear at 90 ºC in the research of Vermeer and Norde (2000).

The transition concentration around 0.15 mM and 0.30 mM is much lower than the cmc at 2 mM, which indicates that the interaction between SDS and pepper leaf proteins is strong and the micelle structure formed among SDS molecules are not as dominant as observed in SDS and individual protein systems. However, the conclusion drawn by Vermeer and Norde (2000) also takes the appropriate size ratio of protein molecule to SDS molecule, the number of electrostatic binding sites on the surface of the protein and the ratio of SDS hydrophobic tail to the hydrodynamic radius of IgG into consideration. Although the proteins tested in our experiment are a mixture, which represents the average property of the pepper leaf protein and is more complex than the single IgG, the explanation is also sound in our case.

Similar with that of CTAB, the electrostatic interaction is dominant within the SDS monomer- protein complex, and the hydrophobic interaction is dominant between the complexes at low concentrations. However, the transition induced by SDS starts at concentrations much lower than the cmc. The close relationship between micelle formation and stabilization cannot be interpreted in the same way as for CTAB. The mechanism can include a stronger interaction between the SDS monomer and pepper leaf proteins than that with CTAB. Binding of the negatively charged SDS can saturate the positive charges on the surface of proteins before the formation of SDS micelles. The negative charges will accumulate on the surface of proteins and the overall charges of the SDS-protein complex can be dominantly negative. The stabilizing effect results from the induced electrostatic repelling forces. As the concentration goes higher, SDS micelles

form and the unfolding proteins are locked in the micelle structures and aggregation is inhibited. It is likely that negative charges on the surface of the pepper leaf proteins are denser than positive charges, which makes it difficult to neutralize by addition of the cationic detergents. Therefore, the electrostatic repulsion cannot be induced by CTAB concentrations lower than the cmc.

NONIONIC TWEEN 20. For the non-ionic detergent Tween 20, there are no charges on the head of the monomer. Only hydrophobic interactions can be induced, including interactions between the hydrophobic tails of the detergent and the protein core, as well as the interactions between the hydrophilic head of the detergents and protein surfaces. The stabilizing effect that was observed at the low concentrations is likely due to the insertion of the hydrophobic tails of monomer Tween 20 into the protein cores and the exposure of the hydrophilic head outside the protein. An expanded difference in hydrophilicity between the protein surface and the core is induced. Therefore, the hydrophobic force that maintains the structure of the protein is increased and the loss in solubility under high temperatures is attenuated.

Tween 20 monomers can also bind with unfolded protein structures. As a result, the inside exposed hydrophobic sites of the protein can be shielded, and therefore the aggregation among unfolded proteins can be attenuated.

However, the stabilizing effect introduced by Tween 20 is relatively limited compared with that of cationic CTAB and anionic SDS. One possible explanation is the electrostatic repulsive forces that strongly benefit maintenance of solubility under adverse environmental factors cannot be established by the non-ionic Tween 20.

Following on the stabilizing effect was a dominant destabilizing effect at higher Tween 20 concentrations. The explanation may be the saturation of the interior space of the protein molecule by the detergent insertion. When no more Tween 20 monomers can be loaded inside a protein, the preferred conformation is to leave the hydrophobic tails of the Tween 20 monomer outside the protein molecule with the head binding to the hydrophilic surface of the protein. The exposure of hydrophobic tails is thermodynamically disfavored, which drives the aggregation between the Tween 20-protein complexes. The more free Tween 20 monomers added to the system, the greater is the destabilizing effect.

It is noticeable that the destabilizing effect became no more severe when micelles formed at 1 mM Tween 20. Different from the interactions with Tween 20 monomers, micelles shield the hydrophobic tails from exposure to proteins and result in attenuated hydrophobic aggregation. However, due to the saturation of the protein interior space, as well as the less available Tween 20 tails, no more stabilizing hydrophobic forces can be induced by the tail insertion of Tween 20. As a result, the transformation from the monomeric Tween 20 to micelles inhibits further destabilization of proteins but cannot add a stabilizing effect.

ZWITTERIONIC CHAPS. For the zwitterionic detergent CHAPS, there are both positive and negative charges on the head of the monomer. As a member of the ionic detergents, the interaction between CHAPS monomers and protein molecules consists of both hydrophobic interactions and electrostatic interactions. However, different from either anionic or cationic detergents, both positive and negative charges are present and

both positive and negative charges on the surface of proteins can be neutralized, which means no repulsive forces between protein molecules are induced by the addition of CHAPS. Therefore, although the electrostatic interactions can be strong between the charged head of CHAPS and proteins, the solubility of pepper leaf proteins do not benefit significantly from electrostatic repulsions. As opposed to Tween 20, the significant stabilizing effect was observed with CHAPS. Although at low concentrations of CHAPS, the solubility of pepper leaf proteins can be barely protected from the high temperature unfolding, the destabilizing effect induced by high concentrations of CHAPS is much more dominant. The mechanism may involve the charges on the head of the CHAPS monomer, which inhibit the insertion of the hydrophobic tail into the hydrophobic core of the protein. Accumulation of the hydrophobic monomers at the exterior of the protein can only introduce aggregation between protein molecules. Therefore, neither electrostatic repulsion nor hydrophobic interaction can be established to benefit maintenance of solubility.

The destabilizing effect of CHAPS is predominant as concentration increases. The mechanism may involve accumulation of CHAPS at the exterior of the protein by charge-charge interactions and hydrophilic interactions between the hydrophilic heads and the hydrophilic surfaces of the protein. The hydrophobic tails of the CHAPS monomer exposed outside the protein molecules interact directly with the aqueous surroundings, which is thermodynamically unfavorable to binding. The aggregation between the CHAPS- protein complexes is induced spontaneously and loss of protein solubility will be observed.

An interesting phenomenon is that the destabilizing effects were less severe when micelles formed. The interpretation of the interactions between the CHAPS monomers and proteins is clear. CHAPS micelles shield the hydrophobic tails of CHAPS outside proteins, but have no influences on the charges of the CHAPS head. In other words, the electrostatic repulsion fails to be established, but the hydrophobic aggregation is attenuated. As a result, the transformation from monomeric CHAPS to micelles inhibits further destabilization of proteins induced by hydrophobic aggregations, but cannot stabilize proteins by electrostatic repulsion.

THE GENERALITY OF PROTEIN-DETERGENT INTERACTIONS. The relationship between the interaction and effect is complicated. The same interaction can result in different effects. For example, the nonionic detergent Tween 20, which can only induce hydrophobic interactions, also exhibits the dual response of stabilizing effect and destabilizing effect on pepper leaf proteins. At low concentrations, the hydrophobic interaction contributes to the maintenance of the protein hydrophilic surface and hydrophobic core structure by inserting into the protein molecules. As the concentration increases, the extra Tween20 monomers accumulate at the exterior of the protein molecules, inducing the aggregation and destabilization of the proteins. Also, the same effect on thermostability of pepper leaf proteins can be resulted from different interactions. For example, both the low dose of Tween20 and high concentration of CTAB stabilize the pepper leaf proteins. The mechanisms behind are obviously different.

The relationship between the thermostability effect and micelle formation is different. For CTAB, the micelle is concluded to be highly related to the stabilization of

the protein structures by confining the unfolding and aggregation space. However, for SDS, the electrostatic repulsion contributes more to the stabilization effect. The contribution of micelle structures is very limited.

SOLUBILITY AND ACTIVITY. It is impressive that the solubility of the pepper leaf proteins can be maintained even high as 100 ^{o}C by the high dose of CTAB. However, the enzymes activity cannot be maintained in that extreme condition. We can conclude that the solubility based thermostability and the activity based thermostability are not in agreement, which is different from the catalase under different pH environments.

COMPARISON OF THE SOLUBILITY MAINTENANCE BY PH AND DETERGENT. The electrostatic repulsion is a factor that maintains the solubility of proteins under adversary environmental factors treatments as we discussed in chapter 2. The solubility of the pepper leaf protein can be maintained by 6 ^{o}C with the 0.5 unit of increase in pH. However, the solubility can be much better maintained by comprehensive stabilizing factors such as the detergent we tested in chapter 4. Compared with the stabilizing effect induced by the detergent such as the cationic CTAB, the solubility can still be maintained even at 100^{o}C. The rationale can be interpreted as the comprehensive interactions between the detergent and protein molecules which include the micelle confining, hydrophobic interactions as well as the electrostatic repulsions.

Literature Cited

Bennett, M.K., N. Calakos, T. Kreiner, and R.H. Scheller. 1992. Synaptic vesicle membrane proteins interact to form a multimeric complex. J. of Cell Biol. 116(3): 761-775.

Banerjee, P., J.B. Joo, J.T. Buse, and G. Dawson. 1995. Differential solubilization of lipids along with membrane proteins by different classes of detergents. Chem. and Phys. of Lipids. 77: 65-78.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Bordbar, A.K., K. Omidiyan, R. Hosseinzadeh. 2005. Study on interaction of α-amylase from *Bacillus subtilis* with cetyltrimethylammonium bromide. Colloids and Surfaces B: Biointerfaces. 40: 67-71.

Choudhury S.R., R. Yadav, A.N. Maitra, and P.C. Jain. 1994. Structural transformations in CTAB aggregated systems investigated by positron lifetime spectroscopy 1: binary-systems. Colloids and Surfaces A-physicochem. and engineering aspects. 82(1): 49-58.

Courtney H. S., W. A. Simpson, and E.H. Beachey. 1986. Relationship of critical micelle concentrations of bacterial lipoteichoic acids to biological activities. Infection and Immunity. 51(2): 414-418.

Gani, S.A., D.K. Chattoraj, and D.C. Mukherjee. 1999. Binding of cationic surfactants to DNA, protein and DNA-protein mixtures. Indian J. of Biochem. and Biophysics. 36:165-176.

Guo, R., X.J. Zhu, and X. Guo. 2003. The effect of β-cyclodextrin on the properties of cetyltrimethylammonium bromide micelles. Colloid Polym. Sci. 281: 876-881.

Guillemet F. and L. Piculell. 1995. Interaction in aqueous mixtures of hydrophobically modified polyelectrolyte and oppositely charged surfactant: Mixed miceele formation and associative phase separation. J. Phys. Chem. 99: 9201-9209.

Jimenez M. and F. Garcia-Carmona. 1996. The effect of sodium dodecyl sulfate on polyphenol oxidase. Phytochemistry. 42(6): 1503-1509.

Harris, J.C. 1958. Detergent application of the measurement of critical micelle concentration. J. Amer. Oil Chem. Soc. 35: 670-674.

Hayter, J.B. and M. Zulauf. 1982. Attractive interactions in critical scattering from non-ionic micelles. Colloid and Polym Sci. 260:1023-1028.

Kumar, S., D. Sharma, and Kabir-ud-Din. 2000. Cloud point phenomenon in anionic surfactant plus quaternary bromide systems and its variation with additives. Langmuir. 16: 6821-6824.

Li W., Y.C. Han, J.L., Zhang, L.X. Wang, and J. Song. 2006. Thermodynamic modeling of CTAB aggregation in water-ethanol mixed solvents. Colloid J. 68(3): 304-310.

Moren, A. K. and A. Khan. 1995. Phase equilibria of an anionic surfactant (sodium dodecyl sulfate) and an oppositely charged protein (lysozyme) in water. Langmuir. 11: 3636-3643.

Musarat, J., A.A. Dar, A. Amin, N. Rehman and G.M. Rather. 2007. Clouding behavior of nonionic-cationic and nonionic-anionic mixed surfactant systems in presence

of carboxylic acids and their sodium salts. Colloid and Polymer Sci. 285:631-640.

Moore B.M. and M.H. Flurkey. 1990. Sodium dodecyl-sulfate activation of a plant polyphenoloxidase - Effect of sodium dodecyl-sulfate on enzymatic and physical characteristics of purified broad bean polyphenoloxidase. J. of Biol. Chem. 265(9): 4982-4988.

Nagarajan, R. and E. Ruckenstein. 1991. Theory of surfactant self-assembly-a predictive molecular thermodynamic approach. Langmuir. 7(12): 2934-2969.

Patist, A., S.S. Bhagwat and K.W. Penfield. 2000. On the measurement of critical micelle concentrations of pure and technical-grade nonionic surfactants, J. Surfact. Deterg. 3: 53–58.

Piculell, L., F. Guillemet, K. Thurresson, V. Shubin, and O. Ericsson. 1996. Binding of surfactants to hydrophobically modified polymers. Adv. Colloid Interface Sci. 63: 1-21.

Sarkar, R., M. Ghosh, A.K. Shaw, and S.K. Pal. 2004. Ultrafast surface solvation dynamics and functionality of an enzyme α-chymotrypsin upon interfacial binding to a cationic micelle. J. of Photochem. and Photobiol. B: Bio. 79: 67-78.

Savelli G., N. Spreti, and P.D. Profio. 2000. Enzyme activity and stability control by amphiphilic self-organizing systems in aqueous solutions. Current Opinion in Colloid and Interface Sci. 5: 111-117.

Sen, M., S.P. Mitra, and D.K. Chattoraj. 1980. Thermodynamics of binding cationic and anionic detergents to gelatin and gelatin-BSA mixture. Indian J. of Biochem. and Biophysics. 17: 405-413.

Subramanian, M., B.S. Sheshadri, and M.P. Venkatappa. 1984. Interaction of cationic detergents, cetyl- and dodecyl- trimehylammonium bromides, with lysozyme. J. of Biochem. 95: 413-421.

Vermeer, A.W.P., and W. Norde. 2000. The influence of the binding of low molecular weight surfactants on the thermal stability and secondary structure of IgG. Colloids and Surfaces A: Physicochemical and Eng. Aspects. 161(1): 139-150.

Wang, J., D. Lu, Y. Lin, and Z. Liu. 2005. How CTAB assists the refolding of native and recombinant lysozyme. Biochem. Eng. J. 24: 269-277.

Yang, Z., J. Deng and L.F. Chen. 2007. Effect of ionic and non-ionic surfactants on the activity and stability of mushroom tyrosinase. J. of Mol. Catalysis B-Enzymatic. 47(1-2): 79-85.

CHAPTER V

CONCLUSION

The effects of cosolvents on protein thermostability varied with different cosolvents, and with the same cosolvent at different concentrations. Reciprocal effects between cosolvents were based primarily on hydrophobic interactions, electrostatic interactions and micellar effects.

Hydrophobic/hydrophilic interactions are involved in both stabilization and destabilization of pepper leaf proteins by cosolvents. Chemical chaperone-induced stabilization of proteins is attributed to strengthening of the hydration layer on protein surfaces rather than direct binding to protein molecules. The polarity/hydrophilicity of the chemical chaperone determines its ability to strengthen the hydration layers. Similar polarities between different classes of chemical chaperones yielded equivalent and additive stabilizing effects. Hydrophobic and amphipathic molecules can destabilize proteins as discussed in chapter four. Detergent monomers expose their hydrophobic tails at the exterior of proteins when polar head groups bind to hydrophilic protein surfaces. The accumulation of hydrophobic tails is disfavored in aqueous environments and contributes to aggregation of the protein-detergent complex.

Another key property of cosolvents is charge. Chapter two demonstrated that pH has significant effects on both solubility and activity of proteins. At pH 6.5, protein surfaces

had more negative charges and a stronger repulsion force against high temperature aggregation than at pH 6.0. Chapter four suggested that changing protein surface charges by binding ionic detergents can induce electrostatic repulsions, by which protein solubility can be maintained.

Cosolvents, including detergents, can form specific structures such as micelles. The micelle shields the hydrophobic tails of detergent molecules and forms a confined space to limit aggregation of unfolded structures. Micellar effects on protein thermostability are, in part, a function of the nature of the detergent's polar head group. A strong stabilizing effect on protein molecules was observed at CTAB concentrations higher than cmc. For Tween 20 and CHAPS, destabilization reached maxima at the concentrations micelles started to form.

Since various interactions can be involved in a single system, the primary interaction can shift when the system is altered, making interpretation and prediction a challenge. As discussed in chapter four, pepper leaf proteins were destabilized by hydrophobic aggregation induced by CTAB monomers. As CTAB concentration increased, micellar effects dominated the interactions and stabilized proteins against high temperature aggregation. Hydrophobic interactions also destabilized proteins at low concentrations of SDS, while electrostatic repulsions stabilized proteins at higher SDS concentrations. However, the relationship was more complex since the transition from destabilization to stabilization occurred at sub-micellar SDS concentrations.

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The goal of my research project is to explore structure-function relationships between cosolvents and protein thermostability. Effects of cosolvents, including chemical chaperones and detergents, are monitored as changes in protein solubility and activity following high temperature exposure. Additive effects of chaperones such as glucose and glycine have been observed. Additive effect of glycine and CHAPS on both pepper leaf proteins and commercial ovalbumin were tested. The relationship between the observed stabilizing effect and the polarity of the chemical was discussed. Ionic detergents yield a dual response mode on thermostability of pepper leaf proteins as concentrations increase. The relationship between response transition and micelle formation was discussed.

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