

ABSTRACT

SCHNEIDER, PAULA ANNE. Aggregation of α -lactalbumin at pH 3.5-6.0. (Under the direction of Dr. E. Allen Foegeding.)

Heat-induced protein aggregation is an important reaction in food processing because it causes undesirable appearance changes, which may lead the beverage to phase separate. Recently, there has been an increased demand for and growth in the area of sports beverages. Sports beverages can be categorized several different ways; although the category of current interest is that of beverages having a low pH (from 2.8 to 3.5) and representing a good source of protein. Generally these beverages derive a high proportion of their protein from bovine whey, since it is a source for branched chain amino acids which can benefit muscle building and maintenance. These low pH beverages can be astringent. Astringency is positively related to low pH where the degree/intensity of perceived astringency increases as the pH of the beverage decreases. One way to minimize astringency is to increase the pH; although aggregation readily increases the nearer the pH is to the protein's isoelectric point. The selection of a less heat sensitive protein source (ex. α -lactalbumin) and/or the use of molecular aggregation blockers can provide ways to control aggregation.

The first objective of this research was to study the aggregation of α -lactalbumin over a pH range 3.5 to 6.0. Protein solubility and turbidity development were used to monitor aggregation. Turbidity was evaluated using

a spectrophotometer set to monitor at 400 nanometers, and a turbidimeter that measures scattering at a 90° angle from the incident light. Change in protein solubility in response to pH adjustment with 1M phosphoric acid and thermal (120 seconds in a 85°C water bath) treatment was determined by measuring dispersed protein after centrifugation at 11950 x g (18-24°C) for 60 to 90 minutes. As expected, the lowest protein solubility and highest degree of turbidity resulted near the isoelectric point of α -lactalbumin. The affect of calcium content was also evaluated. In the holo-form, α -lactalbumin was more stable to native aggregation; although no difference was seen in stability after heat treatment.

Indirect determination of the mechanism for α -lactalbumin aggregation was facilitated through the use of various compounds that had previously demonstrated aggregation-blocking or reduction abilities in different systems (ex. protein, pH, heating conditions, etc.). Blocking agents included amyloid/ β -sheet blockers (ex. thioflavin-T and quercetin), hydrophobic amino acid-interacting molecules (ex. Hydroxyl-propyl- β -cyclodextrin), and the use of proteins as blockers (ex. α_s -casein) were evaluated.

The amyloid/ β -sheet blockers were not effective in suppressing turbidity development or maintaining/increasing protein solubility. Further investigations were made to determine if a specific type of aggregate was possible. Under the conditions tested, α -lactalbumin did not form the specific aggregates

(spherulites), which helps explain why these blockers were ineffective in an α -lactalbumin protein system. The use of sodium dodecyl sulfate (SDS) was used to control aggregation by binding to exposed hydrophobic patches on the protein's surface. SDS was not effective in controlling aggregation in α -lactalbumin systems, as monitored through turbidity development and protein solubility, but was effective in systems that contained β -lactoglobulin. The difference in response may be attributed to the structural differences between these proteins.

Blocking of hydrophobic amino acid residues with hydroxypropyl- β -cyclodextrin had the most potential for success in suppressing aggregation of α -lactalbumin. However, further work is needed to determine where they are binding, strength of binding, and the effect of polar components on the core cyclodextrin molecule.

AGGREGATION OF α - LACTALBUMIN AT PH 3.5-6.0

by

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DEDICATION

To my family, teachers, and professors whom have inspired me to persevere through their guidance, enthusiasm, and encouragement; without which little is possible.

BIOGRAPHY

Paula Anne Schneider was born 04/14/1979 in Fort Wayne, IN to Stephan Paul and Nancy Ann (Bultemeier) Schneider. Paula was raised by her parents with her sibling, Jason Paul Schneider, on the family farm in Lake Township. She spent the majority of her younger years outdoors (gardening, planting trees, falling out of trees, riding bicycles, falling off bicycles, stacking straw bails, falling out of straw mows, raising chickens, getting pecked by chickens, getting stepped on and generally pushed around by hogs, stepping on rusty nails, etc), at the 'quick-clique' (getting tetanus shots and stitches), and in her grandmother's kitchen as an understudy (learned to make red-not green-jell-o, sandwich meat, fried zucchini, and a numerous varieties of cookies). Later pursuits included being a member on the academic--science, history and interdisciplinary--teams, a brief stint on the spell bowl team (not because she can spell but out of pure desperation by the coach to form a team), basketball team, track-and-field, side-kick for the men's soccer team, mat-maid for the wrestling team, 4-H, concert, jazz, and marching bands, hand-bell choir, and in numerous clubs including the political action club, national honor society, and Spanish club, in addition to briefly stint as a peer counselor.

Her interest in the biological sciences and culinary arts, spurred her brother to suggest food science as a potential major. After graduating high school, Paula commenced her studies at Purdue University, West Lafayette, IN in Smith Hall. She completed a summer internship with Central Soya, Fort

Wayne, IN, which helped her to better understand the opportunities that the food industry had to offer. Upon earning a B.S. - with distinction in 2002 (in the new food science building), Paula joined the Unilever Research and Development team in Covington, TN, where she acquired a taste for nutritional beverages and fried okra. Paula purchased a new pair of safety shoes in 2003, since her first pair disintegrated from excessive exposure to water and concentrated protein solutions. This was a turning point for her, after which she was promoted from a technologist I to II.

Later that year Paula accepted a Master's Research Assistantship with the 'fast-paced-running' laboratory of Dr. E. Allen Foegeding at North Carolina State University. Accepting this opportunity prompted a move to Raleigh, North Carolina. It also allowed for an opportunity to take a wonderful meandering journey through middle and eastern TN, exploring caves, waterways, and learning about spirit production. While in pursuit of her master's degree, she was a member of the food science club overseeing the production of many FSC newsletters and helped to guide the product development team in three different development projects; one of which placed second at the IFTSA competition. Paula was recognized for her graduate teaching assistant abilities for the undergraduate food chemistry course. She was also recognized by the NCSU food science club for her service to the club.

Paula successfully defended her M.S. thesis, Aggregation of α -lactalbumin at pH 3.5-6.0, on August 31st, 2006.

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Literature Review

α -Lactalbumin aggregation in low to intermediate pH region

Introduction

Whey is a co-product from the manufacture of cheese and casein isolation. As defined in the 21 CFR 184.1979 (58.2601), “whey is the fluid obtained by separating the coagulum from milk, cream, and/or skim milk in cheesemaking” (1). When considering fluid milk’s total protein (30-35 g/L), it is comprised of approximately 20% (5-7 g/L) whey proteins with the remaining protein in the form of casein. ‘Whey protein’ is really a category of milk proteins that are generally acid stable and heat sensitive. This category is comprised of several specific proteins. The major proteins in bovine whey include β -lactoglobulin (~45%), α -lactalbumin (~20%), and proteose-peptones (~20%). These ‘major’ and some ‘minor’ (serum albumin & immunoglobins) whey proteins are commercially available on an individual basis or as a component of a whey protein ingredient. The whey protein ingredients are available in several degrees of purity varying from a whey protein concentrate (dry product does not contain less than 25% protein; ~34-85% protein) to an isolate (90%+ protein) (2). Whey protein concentrates and isolates are manufactured using varying methods including, precipitation, filtration, electrodialysis and ion exchange. Not surprisingly, varying methods result in varying degrees of denaturation, yield, and, composition, resulting in varying functionality (2). Once discarded as waste from the manufacture of cheese, now isolated and

concentrated, whey has evolved over the last 25 years into a readily available and utilized ingredient category in countless food systems. Whey proteins have become of great economical importance, in part due to their nutritional (rich in branch chain and sulfur containing amino acids) and functional properties (2). Such functional properties include gelation, foam formation, emulsification, relatively soluble at acidic pH, and water binding to list a few (2). The functional properties of the whey protein ingredients are based upon protein composition and other factors. Based upon the functional property, relatively soluble at acidic pH, the development of sports nutritional beverages become of interest. Acidified protein solutions prepared (acidification and heating) in a manner similar to sports nutritional beverages is the focus of this investigation.

Based upon a limited market survey, nutritional beverages can be grouped by pH into two categories, those that are acidified and those that are near neutral pH. Acidified beverages range in pH from 2.8 to 3.5 whereas 'neutral' beverages have been found to range in pH from 6.5 to 6.9 (3). Sports beverages utilize a variety of proteins (ex. soy, caseinates, whey variants, milk protein concentrates). The acidified beverage category, generally was found to contain less total protein (4.5-7.5% protein) than those at neutral pH (4-10% total protein) (3). In addition to the protein content of the acidic beverage category, the mineral content was also considered an important attribute. The most abundant minerals found in the beverages included: phosphorous, sodium and potassium (3). Ionic strength, presence of divalent ions, and pH

set the chemical environment whereas thermal processing parameters contributed to the physical environment, in addition to the protein content; these inter-related parameters impact the stability of the system ((4); (5); (6); (7); (8)). The beverage environments impact the system's stability by affecting the protein structure (intramolecular interactions–folding configuration) with the potential for the occurrence of intermolecular interactions.

Sports-nutrition beverages will be generally defined as those beverages that have an acidic pH, intermediate whey protein content, contain phosphorous, sodium and potassium, and that have undergone hot-fill processing conditions. In general, hot-fill processing is used for foods/beverages with a pH below 4.6. These beverages typically are associated with acidic pH (approximate pH 3.4) (3). In the acidic pH range, the pH is below the isoelectric point (pI) of the major whey proteins (pI ~4.2-5.1). Note the isoelectric point is the pH at which the protein has a net-charge equal to zero; a net charge of zero favors self-association. The self-association is a result from the diminished charge-charge repulsion of the molecules to one another (2). Therefore, when the pH of the solution is at the extremes (relative to the given isoelectric point for the protein) self-association is not favored, due to charge-charge repulsion and a higher degree of clarity (lower opacity) results. As a positive attribute, beverage clarity would decrease as the solution pH approaches the pI of the dispersed protein. In addition to opacity, the sensory perception of astringency is also perceived as an objectionable

attribute. Astringency is positively related to low pH and the precipitation that occurs; thus the degree/intensity of perceived astringency increases as the pH of the beverage decreases (9).

The astringency could potentially be reduced by use of a different acidulant or combination of acidulants, or increasing the pH to a more alkaline state (9). However, reducing the astringency by increasing pH causes clarity to decrease (increased opacity). As noted previously, increases in pH to the proximity of the protein's isoelectric point results in loss of the protein's net charge. It is desirable to keep the pH below 4.6 to prevent requiring a more rigorous thermal processing to achieve a commercially sterile product. The balance among minimizing the overall astringency, maximizing clarity, protein content, and solubility, presents a challenge to developing high quality sports drinks.

The common constituents of sports-nutritional beverages include a protein concentration of 3 to 6% and a $\text{pH} \leq 3.5$. As noted previously, a hot-fill process is usually used in the manufacture of the beverages, although other beverages in this and similar categories could also be processed aseptically or retorted. Hot filling products, as compared to aseptic and retort processing, is less detrimental to product quality than retorting, but more so than aseptic processing. The hot fill process is used for fluid products with pH below 4.6. The product is heated to 74-83°C, transferred to finished product containers,

which are then sealed and inverted to expose the lid to the heated product. The hot product is used to commercially sterilize a clean container. Hot fill products only target vegetative microorganisms, since the pH is sufficiently acidic to ensure that pathogenic spores do not sporulate. Under the assumption of intermediary product abuse, further discussion will be focused or related to those products that are in the category of being acidic/acidified hot-filled beverages; which utilize whey proteins as their major protein source. These are designed to find a balance between astringency, aggregation, and clarity while maximizing total protein content.

Major Bovine Whey Proteins

β -Lactoglobulin

Bovine β -lactoglobulin (figures 1a and 1b) is the most abundant (globular) whey protein in cow's milk with an approximate concentration of 0.3g/100mL (~9% of total protein) (2). It was first identified and isolated in 1934 (10), consisting of a primary sequence of 162 amino acid residues. Major genetic variants include A and B, which occur at high frequency in most breeds of cows. The protein is a dimer at physiological pH and a monomer at acidic pH. Genetic variant B dimer has a molecular weight of approximately 36000 Da, where each subunit has a molecular weight of 18277 Da (11). β -Lactoglobulin structurally forms a β -barrel (generates a hydrophobic core) and is in the lipocalin family of molecule (12;13). β -lactoglobulin allows for tight

binding of small hydrophobic ligands in addition to having one free sulfhydryl group. These characteristics of β -lactoglobulin's structure contribute to the aggregation interactions both intermolecular and intramolecularly.

Composition and Structure

Native β -lactoglobulin (β -LG) contains one free sulfhydryl on cysteine-121 (13). In addition to the free sulfhydryl, there are also two disulfide bridges present between amino acid residues 66 to 160 and 106 to 119. In the approximate area of the 106 to 119 disulfide bridge, the amino acid sequence consists of a relatively large proportion of hydrophobic amino acid residues, as compared to the 66 to 160 disulfide bridge region (14). It is thought that intramolecular interchange reactions occur between free sulfhydryl 121 and disulfide bridge 106 to 119 (13). These interchange reactions occur rapidly without resulting in large regional changes (neighboring amino acid residues) (14).

β -LG is grouped into the lipocalin superfamily (15;16) with a characteristic calyx fold (17). This molecular category is a group of transport proteins that can bind non-polar/hydrophobic ligands, such as retinol (18). Bovine β -LG structurally forms a β -barrel (generating a hydrophobic core), allowing for tight binding of small hydrophobic ligands (12;13). The β -barrel is composed of antiparallel β -strands (eight total), forming the calyx (or cup-shaped cavity) fold (13;19). NMR and x-ray crystallography investigations of

dimers and monomers at pH 2 (monomeric) with low ionic strength, show that the tertiary β -barrel motif remains intact at these conditions (12). In contrast to the relatively consistent β -barrel, several loops and the terminal area appears to be in a disordered flexible state (12).

Through a complex pattern of association and aggregation, which is influenced by pH, a monomer-dimer equilibrium is present above pH 3.5 (20). At low pH (2), full protonation of the molecule is reached; resulting in a positive net charge of 21 (12). It was proposed by Fogolari et al. that the change in free energy from the dimer state (pH 7) transitions to the monomer form (pH 2) was sufficient to overcome the hydrophobic free energy, which favored the dimer structure (12). Interestingly, the monomeric protein maintains the β -barrel structure seen in the dimer at physiological pH yet there are several disordered regions leading to different surface electrostatic properties (12). In the case of β -lactoglobulin, aggregation has been proposed to occur when monomers are activated, thereby becoming reactive. There are three suggested ways for activated (reactive) monomers to associate: 1) thiol-disulfide exchange, and to a lesser extent 2) thiol-thiol oxidation, and 3) non-covalent bonding (ex. hydrophobic interactions, van der Waals, etc) (8). Also, increases in aggregate size through an additive process of monomers and dimers at pH 7.0 have been described (8). Therefore, both non-covalent and covalent interactions are critical in the formation of protein aggregates at pH 7.0 (8). It should be noted that the protein composition would determine the types of interactions involved

in aggregation process; hence it may not be as relevant for proteins that do not have a free sulfhydryl group (such as α -lactalbumin) (14).

Thermal and pH Effects

Heating (90°C for 30 or 60 min), low concentration (1% w/v) β -LG solutions at acid conditions (pH 2.5) results in not much aggregation; however, all resistance to aggregation is overcome at pH 4.5 and 6.5. (21;22). Upon heating pH 7.5 solutions, there are only slight declines in the number of free slow-reacting sulfhydryl residues. Similar results were observed when the solution's protein concentration was increased. It was concluded that the slight decrease in free slow-reacting sulfhydryl residues was less likely to drive the aggregate formation forward (6). It was deemed more probable that the aggregate formation is dependent upon the intermolecular interchange reactions (free sulfhydryl–disulfide bridge) (6). Similarly, a decrease in free sulfhydryl residues was also observed in heated pH 2.5 solutions, yet due to the lack of observed aggregate formation, it was concluded that primarily intramolecular (as opposed to intermolecular) interchange reactions were of greater significance to the resulting structure (6).

α -Lactalbumin

α -Lactalbumin (α -LA), the second most abundant protein in the whey fraction, is found at approximately 1.2-1.5g/L in fluid bovine milk (23). Unlike β -

LG, α -LA is present in human milk. As a component of human milk, it has been thought to be nutritionally more suitable for infant formulas.

α -LA is a relatively small monomer, 14,186 daltons (calculated) or 14,178 daltons (measured) and is termed a 'calcium binding' milk protein or metalloprotein (24) and structurally similar to lysozyme. It exists as a monomer in a neutral pH environment (24). α -LA's isoelectric point is 4.6 (25), yet according to Bramaud and Daufin, it has a pI range between 4.2 to 4.5 (26). Unlike β -LG, α -LA does not have free sulfhydryl groups but does possess four disulfide bridges that are located at the following amino acid positions: 6 and 120, 28 and 111, 61 and 77, and 73 and 91 (11;24).

Composition and Structure

As noted previously, α -LA is classified as a metalloprotein, since its structure is similar to this family of proteins (figures 2, 3, and 4). Structurally, the protein consists of two domains: β -domain (relatively small: 2 short sections of antiparallel β -pleated sheets, short 3_{10} -helix, & various loops) and the α -domain (relatively large: discontinuous area formed by the N- & C-terminus of the polypeptide comprised of three major α -helices and two short 3_{10} -helices); these distinct regions are separated by a cleft or space (24;27;28;29). The high affinity calcium-binding loop is located between the two domains, in the cleft separating the α -domain from the β -domain. The two domains are tethered together by two bridges. One such bridge comprises

cysteine residues 73 and 91, forming the calcium-binding loop (24). The high affinity calcium-binding site contains of three aspartic acid residues (82, 87, and 88); although a fourth aspartic acid residue is included (residue 83), which acts as a spacer with only indirect contribution to calcium binding (24;30;31). The calcium-binding site is formed by the carboxylic groups of aspartic acid, in addition to two carbonyl groups (residues 79 and 84) forming the loop between the helices in the two domains (24;29). The presence of one or two molecules of water also assists to coordinate the binding of calcium (24). The calcium binding site helps to stabilize the tertiary structure; hence the removal of calcium and water results in a less stable protein (24;26;32).

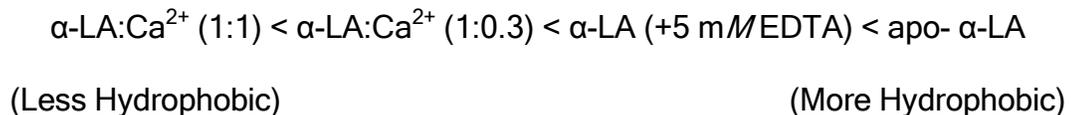
Holo verses Apo

The presence (holo-form) or absence (apo-form) of calcium in the binding loop of the protein has been found to be important in α -LA's stability to stresses; thus understanding calcium's relationship to α -LA is of importance to protein isolation and product applications. α -LA binds 1 mole of calcium per mole of protein in the holo form (calcium bound) (33). Tryptophan fluorescence and UV absorbance indicate that metal-ligand induced conformational changes are reversible (34). Dependent upon the precise conformational changes (i.e. residue exposure or coverage), this may result in conditions promoting a favorable or less favorable aggregation environment.

There is evidence that two calcium binding regions are present in α -LA: one strong binding site (K_a of $2.7 \times 10^6 \text{ M}^{-1}$) and a weaker binding region (K_a of $3.1 \times 10^4 \text{ M}^{-1}$) (34). The weaker binding region may not be specific for calcium, but perhaps other divalent metals. Some variation in the binding affinity of the loop region α -LA does exist. Bramaud and Daufin reported an apparent binding constant ranging from 10^6 - 10^{10} M^{-1} at neutral pH and room temperature (26). Regardless of the variation, the binding constant of the loop region is very strong. The binding region could also be considered a zinc binding region, since as little as 1 mM of Zn^{2+} significantly inhibits calcium binding resulting in an α -LA similar in structure to its apo-conformation (34). Kronman et al. showed that Ca^{2+} and Mn^{2+} promoted native state stabilization, whereas Zn^{2+} and possibly Tb^{3+} favored stabilization of the apo-state (34). Stabilization can be caused by intermediate concentrations of Ca^{2+} rather than abundance or absence conditions. For instance, at low concentrations of Ca^{2+} , α -LA unfolds into a stable-unfolded-intermediate tertiary state with a folded secondary structure (35). At sufficiently high Ca^{2+} concentrations, α -LA skips over the intermediately unfolded-folded structure and transitions from a native to a fully unfolded state. By undergoing this rapid and immediate transition, it is thought that this accounts for a more stable native state, as compared to the intermediate structure (35).

pH Effects

The binding of calcium to α -LA is affected by pH, which in turn results in structural changes. α -LA undergoes conformational changes when calcium is released; thus the holo versus apo-form are conformationally different (36). Calcium bound α -LA has a more compact and less hydrophobic (native-state) structure (26). Calcium begins to release from the protein at pH values less than five (26). The apo-form occurs below pH 4.0, and since hydrophobicity has been thought to increase below pH 4.5, it is probable that increases in surface hydrophobicity are coupled with calcium loss (26). Shanbhag et al. studied the calcium effect at constant pH and discovered the following relationship regarding hydrophobicity (36):



Heat Resistance

Regardless of the fact that α -LA denatures at $\sim 63^{\circ}\text{C}$, it is considered a heat resistant protein since it renatures upon cooling. Even at room

temperature near α -LA's isoelectric point, where the solubility decreases (termed acidic denatured form as opposed to the native form), unfolding is reversible (37;38). There are limitations to this classification because changes in the secondary structure can result when heated excessively. The structure of α -LA is comparable to the native state when heating to 70 and 80°C at pH 7 or 6 and cooled, but when heated at 90°C at pH 6 the resulting Fourier Transform Infra Red spectra was dissimilar to the native-state spectra (39). This dissimilar spectra from the 90°C at pH 6 samples is possibly a result from disulfide bond rearrangement (39). A more appropriate term rather than renaturation is restructuring, since after denaturation α -LA does not necessarily return to the native state (39;40).

In addition to the native and nonnative folding patterns, α -LA forms a fleetingly-stable conformational state known as the molten globule (41;42). The molten globule state is an intermediary form between the native and non-native 3-D folding pattern (41;42). The folding between native/functional protein and a non-native folded state, not classically functional, is important in understanding folding versus function. The folding or refolding process can result in a native monomeric protein or an aggregate system. Griko et al. studied α -LA's folding at neutral pH (with calcium) and discovered that at high temperatures there existed cooperative unfolding with increases in both enthalpy and heat capacity (43). A similar experiment conducted at low pH, resulted in a two stage unfolding process; the first stage was characterized as being cooperative with distinct heat absorption and, since the expected heat capacity for complete

unfolding was not reached, a second stage was suggested (31). The second stage of unfolding did not have the sharp absorption of heat (i.e. small enthalpy); thus it was unclear if this stage was a cooperative or gradual process (31). It was also unclear on the relative molecular location where these stages of unfolding occurred in α -LA. Assuming that unfolding occurred similarly to equine lysozyme, the first stage of unfolding (cooperative process) was assumed to take place in the β -sheet domain whereas the second stage was occurring in the α -helical domain of α -LA (43;44). Interactions between the two domains help to understand the folding process. The domain to domain interactions were studied by varying number of disulfide bonds coupled with calorimetry and circular dichroism at a range of temperatures and various solvent conditions (31). It was determined that even with only two stabilizing disulfide bonds, α -LA's calcium binding β -sheet domain remained intact, yet the α -helix rich domain lost its native conformation, making it appear that β -sheet domain does not require the other domain for proper folding and calcium binding (31). It was also suggested that bound calcium is critical in determining protein stability and resistance to further unfolding.

Whey Protein Aggregates

Polymerized whey proteins or whey protein polymers refer to soluble aggregates of whey proteins that are formed during heating and would otherwise form a gel, based upon heating temperature and protein

concentration, but do not since the salt concentration is low (5). Polymerization of whey protein isolate or β -lactoglobulin can be induced by heating at pH 7 (low ionic strength) at temperatures above the denaturation temperature and concentrations below their critical gel concentration (45). Heating β -LG combined with α -LA yields coaggregates, whereas pure α -LA solution under the same conditions do not form aggregates (46;47). It seems that β -LG is an initiator or more sensitive to aggregate formation than α -LA. This observation coupled with the fact that increases in salt concentration can help to reduce the denaturation rate, but increase the aggregation rate, may help in producing a more stable protein beverage (5;48). Increasing stability could be arrived at through reductions in or eliminating β -LG and optimizing the salt concentration to achieve fewer total and smaller soluble aggregates that resist settling.

Potential Aggregation Minimizing or Blocking Agents

As discussed earlier, protein aggregation can lead to detrimental quality effects in a beverage system; such quality changes that could be experienced include precipitation, mouth feel alterations, diminished clarity, and phase separation. Protein aggregation is enhanced when in the vicinity of the isoelectric point of the protein and with the addition of salts (change in ionic strength of the solution). The ideal beverage would be a high protein drink that is low in astringency and with no visible aggregation. One way to reduce beverage astringency is to increase the solution's pH, from the more acidic range closer to neutrality, but this causes an increase in aggregation.

Therefore, this approach for reducing astringency would only be successful if the quality decrease, resulting from aggregation, was controlled to an acceptable level.

Using this thought process; a literature search was conducted for information on possible aggregation controlling compounds to generate a more stable beverage through reduction of total aggregation. Aggregation rate could be slowed, reduced or perhaps initially a more favorable aggregate size and/or shape could be formed (i.e. one that remains in suspension over time).

Aggregate Blocking or Disassembling

Blocking Highly Organized Structure (amyloid fibrils) Formation or Organization

Increasing attention has been devoted to characterizing protein aggregates associated with pathogenesis of neurodegenerative diseases. The presence of aggregates, specifically amyloid fibrils, which characteristically have a high degree of β -sheet structure, are associated with a variety of diseases (49). The binding of thioflavin-T, Congo Red, and 8-anilino-1-naphthalenesulfonate (ANS) is used in the assessment of amyloid fibrils. As a member of the flavin family, thioflavin-T (cationic benzothiazole dye) (50) would be more likely than Congo Red or ANS to find a similar structure that is already a food grade compound (figure 5b). Thioflavin-T has substantial binding, as

indicated by fluorescence, when more ordered protein aggregates have formed.

Attenuated total reflectance Fourier transform infra-red (ATR-FTIR) spectroscopy has been used to help characterize the various stages of aggregation. A shift from peaks characteristic of α -helical or loops/mobile tails to an increased intensity in the region indicating β -sheet structure, indicates that the overall process to form amyloid protofibrils occurs more rapidly than native unfolding (51). Extensive unfolding of the native structure is not required in the initial steps of aggregation leading to amyloid protofibril formation. Once the protein system has been seeded with protein-aggregates (intermediate oligomers), the remainder of the monomeric proteins partially unfold and aggregate much more rapidly than the pure monomeric system (51). These characterization studies suggest that β -amyloid protofibril structures are formed in the early stages of the aggregation process.

Thioflavin-T is amphiphilic in nature with both polar and hydrophobic regions. The amphiphilic nature lends to the formation of micelles (3 nanometers in diameter as determined by atomic force microscopy) when exceeding the critical micelle concentration (52). The micelles have been suggested to have the hydrophobic dimethylaminophenyl group making up the core whereas the positively charged benzothiazole nitrogen is left exposed to the solvent. The critical micellar concentration was determined to be $4.0 \pm 0.5 \mu\text{M}$ (52). The fluorescence assay used to monitor fibrils requires a thioflavin-T concentration of approximately $10\text{-}20 \mu\text{M}$, which means that thioflavin-T is

predominately in a micellar form when binding to amyloid fibrils (52). When thioflavin-T concentrations are greater than 10 μM , the molecules are associated into micelles of a constant size (52). These findings are important when considering appropriate concentrations that bind well to protein aggregates. Solution pH is also important because when the pH is below 3.0 the micelles become disrupted resulting in decreased binding (52;53).

The dependence upon pH suggests that electrostatic interactions play a role in the binding of thioflavin-T (positively charged). Atomic force microscopy and fluorescence output have shown that the charged state of thioflavin-T is important for micelle formation, involving both ionic and hydrophobic interactions, which ultimately effects the degree of binding to the amyloid fibrils (52).

The precise binding location of thioflavin-T is not clearly established. One model proposes that thioflavin-T binds as a micelle to β -sheet structure (27) (figure 5a and 5b), while another model suggests thioflavin-T binding to β -sheet structure as a single molecule (figure 6). This later model has thioflavin-T bind along and amongst the radially extended β -sheets from the core of a low-pH formed spherulite-shaped protein aggregate (52;54), which implies hydrophobic interactions because the arms are composed of β -sheets and ionic interactions could act to destabilize the structure. Spherulites formed using bovine insulin and bovine β -lactoglobulin range in size from 10 to 150 μm (54). This type of binding interaction has been challenged since thioflavin-T binding to nucleic acids can only be based upon charged interactions, due to their

structural nature; hence the dye is not specific to β -sheet structure. The charged interactions (positive dye to negative nucleic acid or protein) are lost when the pH is lowered (52;53;54). This specifically challenges the model of a single molecule of thioflavin-T binding to a spherulite. The single molecules of dye binding to the spherulites occur through ionic interactions, as opposed to those proposed by the thioflavin-T micelle binding model. The dye-micelle binding model involves both ionic and hydrophobic interactions (52); although the later seems improbable since the hydrophobic portion of thioflavin-T is buried forming the core of the micelle. In defense of the spherulite-binding model, an older study under acidic pH conditions showed specific binding of thioflavin-T to amyloid sheets interactions (53).

These previous observations are critical to consider when understanding how thioflavin-T binds to protein aggregates. It is unclear which binding mechanism predominates, whether it binds as a micelle through charge-charge interactions with amino acid side groups in addition to some hydrophobic interactions or as an individual molecule to the secondary structure (β -sheet) of a spherulite. If the spherulite structure is not present, then single molecules of thioflavin-T would bind less; although if the concentration of the thioflavin-T exceeded the CMC, then thioflavin-T would bind more favorably to elongated fibrils of β -sheets since the micelle size would not be conducive to binding to the closely packed spherulite structure.

As illustrated using *Sulfolobus solfataricus*'s 101 residue acylphosphatase, the binding of thioflavin-T to β -structured aggregates

occurred faster than the destabilization of the native state (potentially that intermediately folded proteins aggregated) (57). This suggests that the first aggregation step promotes the generation of the resulting amyloid structures. It can be thought that the final amyloid structures are derived from the reorganization of non-amyloid aggregates of the initial step (57).

Naturally occurring polyphenols in food systems, such as flavonoids (e.g. flavanone and isoflavones), have been shown to specifically inhibit aggregates self-assembling into ordered structures (specifically β -fibrils) (55). Flavonoids have abilities to scavenge radicals, after which they are transformed into highly stable flavonoid radicals, which do not further react (similar to antioxidants) (56). Flavonols and flavones, including the flavonol quercetin, occur ubiquitously in plant foods (56). The average intake of the flavonol quercetin is 16 mg/day for individuals in the Netherlands (56).

It has been suggested that a free C-3 hydroxyl group in flavonols is key to the inhibition of β -fibril formation in purified β -amyloid peptides (figure 7) (57). In addition, the loss of a hydroxyl group from the C-5 position on quercetin caused a significant increase in the inhibitory response (57). Quercetin, at the levels demonstrating inhibitory activity (50% inhibition of β -amyloid fibril formation at 2.4 $\mu\text{g/mL}$), also exhibited no cytotoxicity. This is beneficial to know when determining whether or not it could be added to foods and at what levels. In general, based upon consumption data from the Netherlands and United States of America, the low levels required to inhibit fibril formation would be acceptable for supplementation into a beverage/food formulation.

A recent study by Porat et al. also looked at structural mechanisms to explain why some of the small polyphenol molecules are such remarkable inhibitors to fibril formations, regardless of the oxidative conditions (58). It has been suggested that wine derived polyphenols (including quercetin) could prevent Alzheimer development not only through their ability to scavenge reactive oxygen, but also through direct interactions with the fibril deposits in the brain (55). Based upon the structures of polyphenols that inhibit aggregation, they all are composed of two phenolic rings with two to six atom linkage and at least three hydroxyl groups bonded to the aromatic rings (58). It has been suggested that the phenol rings are stacked in such a way as to optimize their interactions with amyloidogenic aromatic residues. This is important to consider when identifying compounds that could function to inhibit aggregation/fibril formation and also to understand the molecular mechanism driving the inhibition. Even though this category of polyphenols are characterized as being protein aggregation inhibitors, more specifically they are good inhibitors of the elongation phase of the fibril assembly and/or the association into large oligomer. They do not have any effect to the initiation nucleation phase (58).

Blocking Hydrophobic Groups

Interactions between proteins and surfactants are of great importance to numerous industries such as the personal care and food. The intermolecular

interactions can be described as possibly having ionic and/or hydrophobic characters. One such example of a surfactant is sodium dodecyl sulfate (SDS) (figure 8). It is an anionic surfactant (charged-hydrophilic sulfate head), composed of a twelve carbon hydrophobic tail (trans conformation).

Binding of SDS to protein can occur either through specific or non-specific interactions. Specific binding has been demonstrated in β -lactoglobulin, but this type of binding interaction is unlikely to occur in α -LA due to the structural differences (59). α -LA lacks the hydrophobic calyx cavity present in bovine β -lactoglobulin. The calyx cavity allows for hydrophobic molecules, like SDS and palmitate (16 carbons in length) to bind with great affinity (59). In contrast, weak hydrophobic binding of the alkyl chain of SDS to localized-exposed hydrophobic areas on the protein could be a way to increase the net negative charge of the protein (60). The increase in the charged character results in a repulsive affect, which minimizes aggregation and increases stability (61). Both hydrophobic and electrostatic interactions occur between SDS micelles and proteins (61). Interestingly, it was pointed out that the SDS micelles and the protein particles of ovalbumin ($\sim 44,300$ Da/molecule) were of similar size, which was important to be able to negate a depletion potential that forms with colloids of different sizes. The depletion potential is a result from osmotic pressure that pushes particles together (interparticle attraction) (62). This may be a contributing factor to solution stability when considering a smaller protein like α -LA.

Aoki and coworkers characterized the interactions between SDS and ovalbumin at acidic (pH \sim 4.2) conditions (60;63;64;65). The mixing ratio of SDS to ovalbumin was critical, as noted by the quantity of white precipitate that formed. Precipitation was observed when SDS and protein were mixed below the isoelectric point of the protein (serum albumin pI=4.7), but precipitation was not detected when SDS was added to the protein solution above the protein's pI (60). By increasing the relative concentration of SDS, the white precipitate decreased to where no precipitate was present when SDS exceeded 45:55 mixing ratio of protein (ovalbumin) to SDS (60;61).

The effect of SDS on heat stability of whey protein isolate solutions at pH 4.0-5.5 has been investigated using SDS (66). In solutions containing 25g/L whey protein isolate, and 100g/L sucrose, the addition of 16g/L SDS caused a marked decrease in heat induced turbidity. Another family of molecules, cyclodextrins, has been used to increase protein stability. The three types of cyclodextrins that are the most industrially useful are α -, β -, and γ -cyclodextrins, which are composed of six, seven and eight glucose units, respectively (figure 9) (67). The arrangement of the glucose units forms a ring structure that has a hydrophobic core (center) and a surface with varying degrees of hydrophilicity dependent upon the type and degree of substitution on the glucose units. These compounds are used in air fresheners and in dressings for wounds as an odor entrapment system. Irie and Uekama (1997) found that cyclodextrins are non-toxic and result in little irritation to the skin,

mucosae, as well as to the cornea of the eye (68). The non-toxicity allows for their use in the pharmaceutical industry. In pharmaceuticals, cyclodextrins are used to increase the shelf-life of therapeutic proteins (ex. growth hormones) by inhibiting or decreasing the rate of protein aggregation (67;69;70) and increasing the solubility of small molecules (71). Sigurjónsdóttir et al. found using salmon calcitonin (sCT), a water-soluble polypeptide hormone, that cyclodextrins at 5% (w/v) or less are effective in maintaining chemical stability. Two derivatives of cyclodextrin, 2-hydroxypropyl- β -cyclodextrin (HP β CD) and randomly methylated β -cyclodextrin (RM β CD), successfully inhibited aggregation and provided increased physical stability in the sCT system (69). Similarly, HP β CD inhibits aggregation (pH \sim 7.4) of ovine growth hormone, interleukin-2, and insulin (72). Under conditions where human growth hormone (hGH, \sim 20 μ M) readily aggregates (pH 2.5 in 1M NaCl), various β -cyclodextrin (β CD) derivatives at 25 to 50 mM concentrations are able to quell the formation of the amorphous aggregates (67). The α and γ -CDs were less effective at avoiding the formation of aggregates (67). The difference between α and γ -CDs and the β CD derivatives could stem from the size of the available hydrophobic core; where the α CDs is too small, the γ CDs too large, and the β CD is just the right 'size' of hydrophobic character.

Cyclodextrin's are unique with a water soluble exterior and a hydrophobic core (71;73;74;75). Aachmann et al. found that substantial surface/solvent exposure of a protein's aromatic residues was required for

binding (capping) with β -cyclodextrin (67;71). The capping of hydrophobic moieties by the cyclodextrin molecules can lead to increased protein solubility. The increased protein solubility is imparted not only from the decrease in the proteins hydrophobicity through capping, but also the residual hydrophilic covering-layer provided from the exterior of the cyclodextrin molecule (67).

Cyclodextrins require high concentrations, as compared to protein, to be effective, since one molecule of cyclodextrin is able to bind to only one amino acid residue. It also appears that of the three commonly used cyclodextrins (α -, β -, and γ -), only β CD has the appropriate cavity diameter to allow for a benzene ring to fit into the glucose ring structure of cyclodextrin (67). The cyclodextrin caps on the hydrophobic amino acid residues would reduce the likelihood that these sticky patches would interact with one another to form aggregates.

Blocking with Proteins

Bovine caseins are molecules that could be useful in suppressing protein aggregation. α_s -caseins are largely composed of hydrophobic β -strands and sheets that are connected to a short hydrophilic segment (76). Due in part from the large hydrophobic character of α_s -caseins, they been proposed to act as a non-reactive/physical molecular-barrier to deter the interaction of reactive (heat-denatured) proteins that would aggregate primarily through hydrophobic interactions with each other (77). Matsudomi et al. used ovotransferrin as their model protein to evaluate aggregation development, since this is the most heat-

labile egg white protein (77). A 50% suppression of insoluble aggregates was achieved at a 1:<0.2 (w/w) ratio of ovotransferrin/ α _s-casein (composition of α _{s1} or α _{s2} was not specified). Total suppression of aggregation occurred at a 1:0.6 (w/w) ratio of ovotransferrin/ α _s-casein; although the suppression was greatly hindered by the presence of NaCl (~20 mM) at pH 7 (77). It was proposed that the hydrophobic surfaces exposed during heat-denaturation are bound with α _s-casein, and that further complexation is decreased due to repulsive electrostatic forces from the α _s-casein phosphoserine residues (77). Recently, the ability of α _{s1}/ β -casein and micellar casein to stabilize whey protein solutions from heat-induced aggregation/precipitation has been investigated (78). The addition of α _{s1}/ β -casein to whey proteins helped to inhibit aggregation of denatured whey proteins; whereas the addition of micellar casein controlled aggregation, yet was implemented in accelerating the denaturation process (78). It is noteworthy to add that both of these studies that used α _s-casein's to control aggregation were in solutions of low protein concentrations (77;78). At these low concentrations aggregation is generally not a significant challenge to overcome, in comparison to systems are at higher protein concentrations.

Characterization of Protein Aggregation

Protein Solubility

The change in protein solubility, whether comparing various ionic strengths, heat treatments or combinations, is critical. Solubility has been

evaluated based on centrifugation for a range of g-forces and time, then measuring soluble (supernatant) and insoluble (pellet) protein (6). For example, Shimada et al. first homogenized solutions followed by centrifugation at 20000 x g for 15 minutes, after which protein solubility was calculated as a percent of protein content in the supernatant versus total protein content. Throughout the literature investigators have used an assortment of both g-forces and time to report solubility of various protein systems. The most ideal centrifugation parameters would be those which simulate a beverage system's shelf-life. The correlation between centrifugation parameters and shelf-life of a protein beverage has not been published in the literature.

Turbidity Measurements

Turbidity can be measured using turbidimeters or spectrophotometers. Turbidity measurements using spectrophotometers and turbidity meters are non-destructive, rapid, inexpensive, and require small sample sizes. The use of turbidimeters has been approved for an official evaluation of water quality.

A schematic of the internal workings of a spectrophotometer is depicted in figure 10a. The light from a tungsten lamp emits polychromatic or white light. The emitted light is then separated into individual wavelengths by a prism, so that a specific wavelength can be selected by the aperture. The light that enters the sample chamber where it is either scattered in multiple directions, transmitted, and/or absorbed. The transmitted light is detected by a photocell and the percent of absorbance or transmittance can be provided.

Optical density can be monitored at various wavelengths from 400-700 nanometers. Four hundred nanometers is often used for protein solutions, since this wavelength is sensitive to particulate size while not being absorbed.

A turbidimeter is depicted in figure 10b. Particles scatter light waves in a multitude of directions, but a turbidimeter evaluates only that which is at a 90° angle between its photo-detector and incident light beam. The type of particle can effect the measurement. A negative bias, where the measurement observed is lower than actual turbidity, occurs when the particle is not physically dense (79). The Orbeco-Hellige turbidimeter uses a tungsten-filament lamp that is associated with a large band width of wavelengths, which is beneficial for evaluating a wide range of particle sizes. Interferences contribute to a greater extent when evaluating samples with high turbidity (79). A disadvantage occurs with pigmentation, since light could be absorbed, decreasing the amount of scattered light to the detector. Molecular movement is the source for the instability of turbidity. This can be minimized by controlled temperatures and time frame of evaluation.

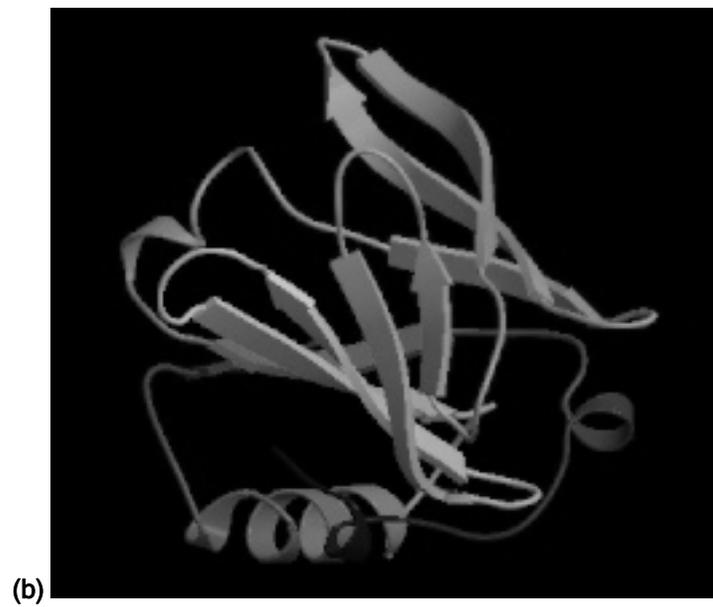
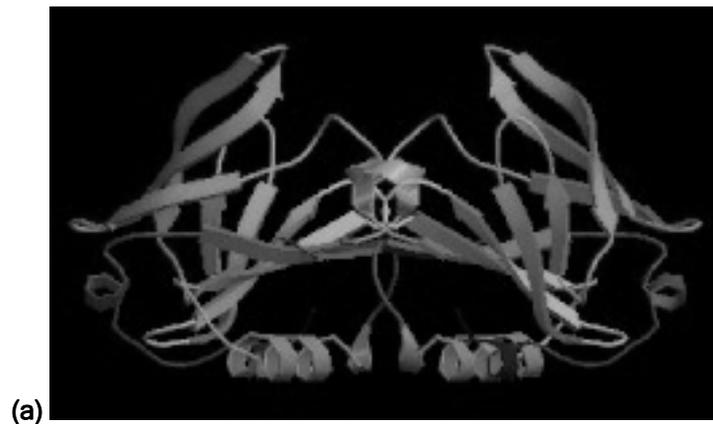


Figure 1. (a) Bovine β -Lactoglobulin dimer (isoforms A & B), from a high resolution crystal structure using X-ray diffraction (1.95 Å), (b) asymmetric unit From the RCSB Protein Data Bank, 1UZ2, 1.90 Å resolution (27)

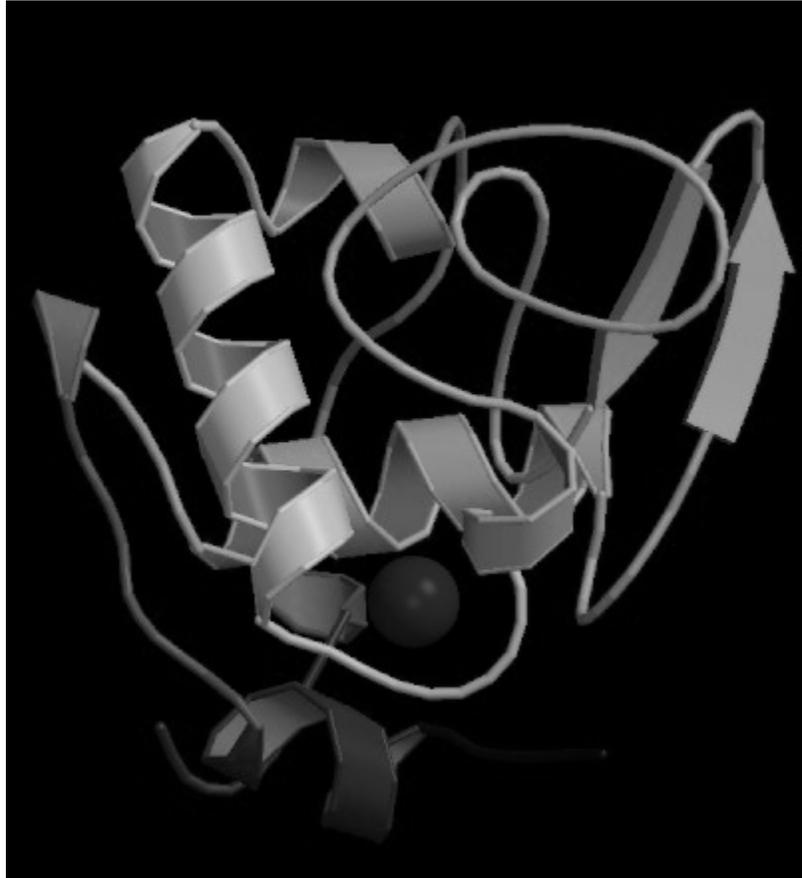


Figure 2. Native asymmetric unit of guinea pig α -lactalbumin with bound calcium. From the RCSB Protein Data Bank, 1HFX, 1.90 Å resolution (27)

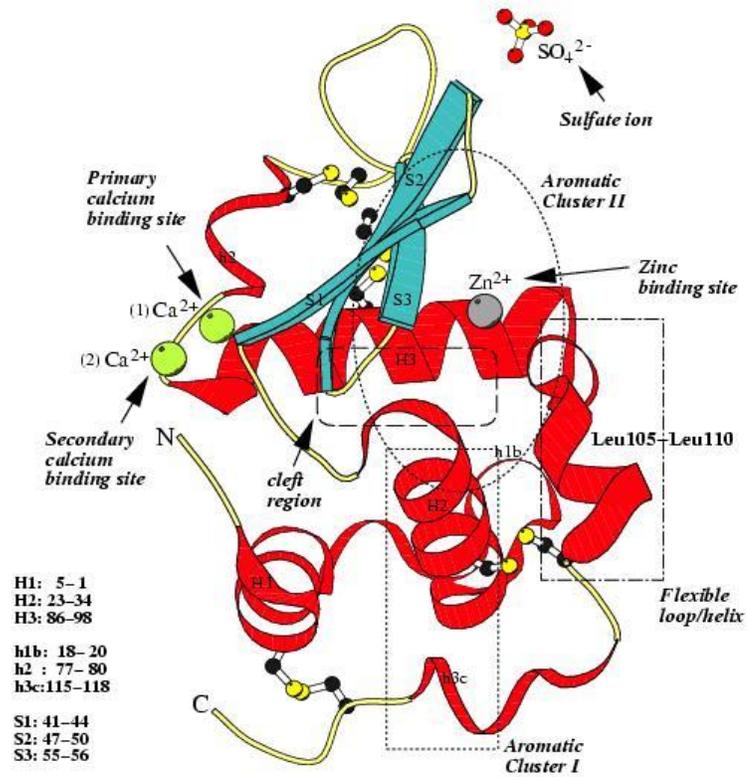


Figure 3. α -Lactalbumin monomer with metal binding sites identified. Structure of α -LA and the functional regions of the molecule showing the location of metal ions identified (80)

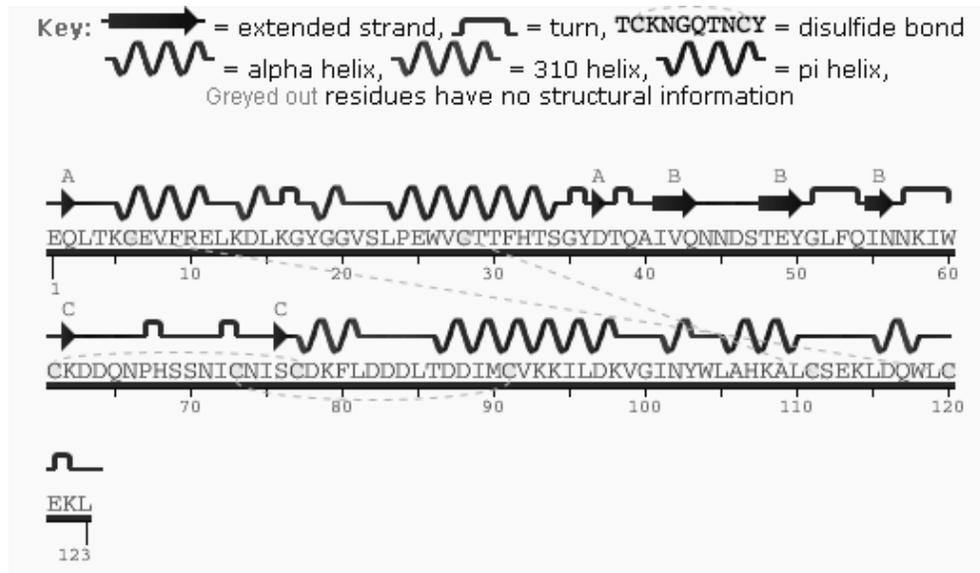


Figure 4. Amino acid sequence and secondary structure of α -lactalbumin (A chain). RCSB PDB 1F6R. Source: www.rcsb.org/pdb/explore/sequence.do?dssp=on&domainMethod=SCOP&disulphides=on&customDisplay=on&chainID=A&domainID=d1f6ra_&component=chain

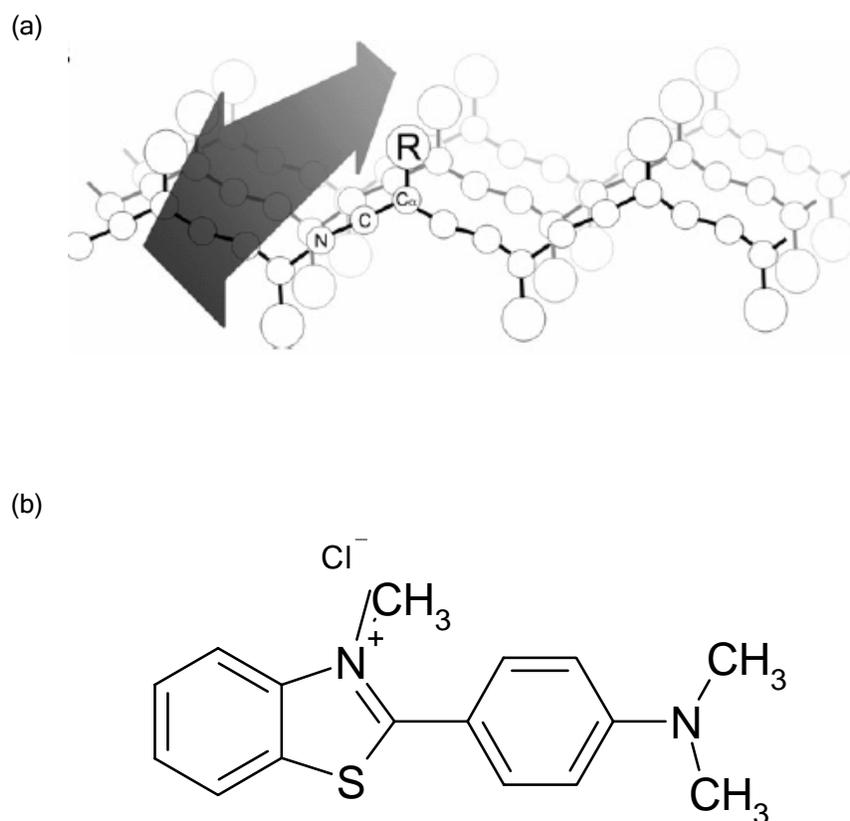


Figure 5. a) β -sheet schematic, proposed structure of a section of a radially extended that make up a spherulite (54;81) with the atoms of the backbone represented by the small circles (N, C, and C_{α}) and the side chain (R) for each residue (hydrogen bonds have been omitted). This double arrow represents the proposed binding off thioflavin-T for the amyloid/ β -sheet, where the molecule's long axis runs parallel to the long axis of the arrow. (b) The structure of thioflavin-T, generated with ISIS/Draw.

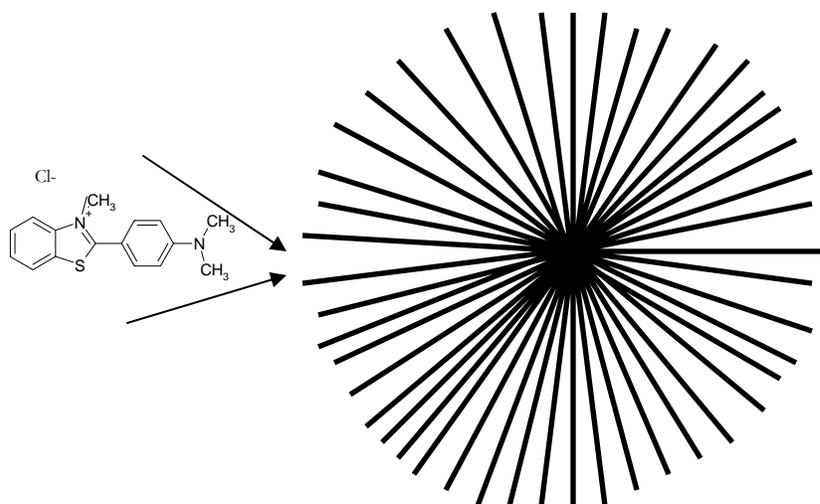


Figure 6. Spherulite schematic that depicts radially associated amyloid fibrils (straight black lines), which originates from a disordered core (dark area). As shown with arrows, thioflavin-T binds in between the radially extended amyloid fibrils (54).

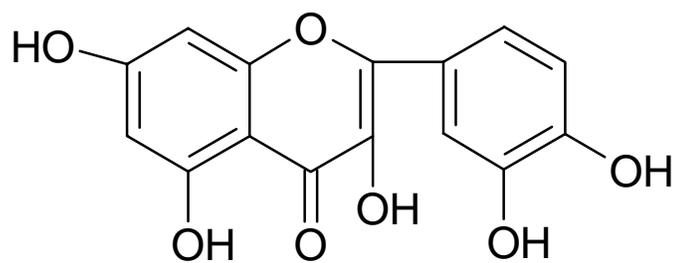


Figure 7. Two-dimensional representation of a quercetin molecule generated using ISIS/Draw.

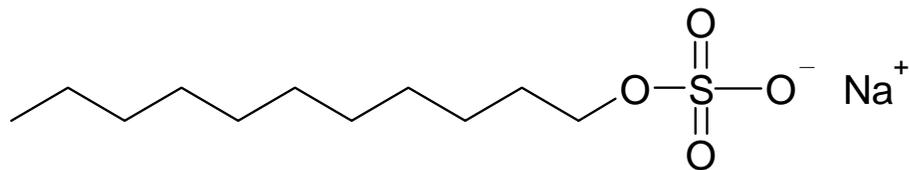


Figure 8. Sodium dodecyl sulfate (SDS), generated with ISIS/Draw.

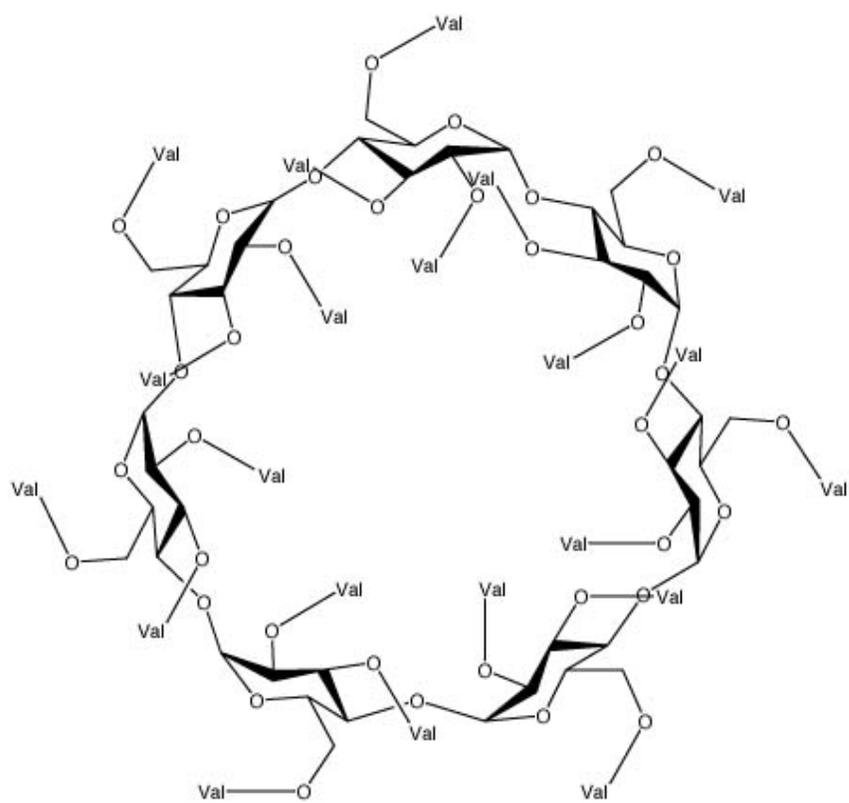


Figure 9. Hydroxy-propyl-β-cyclodextrin

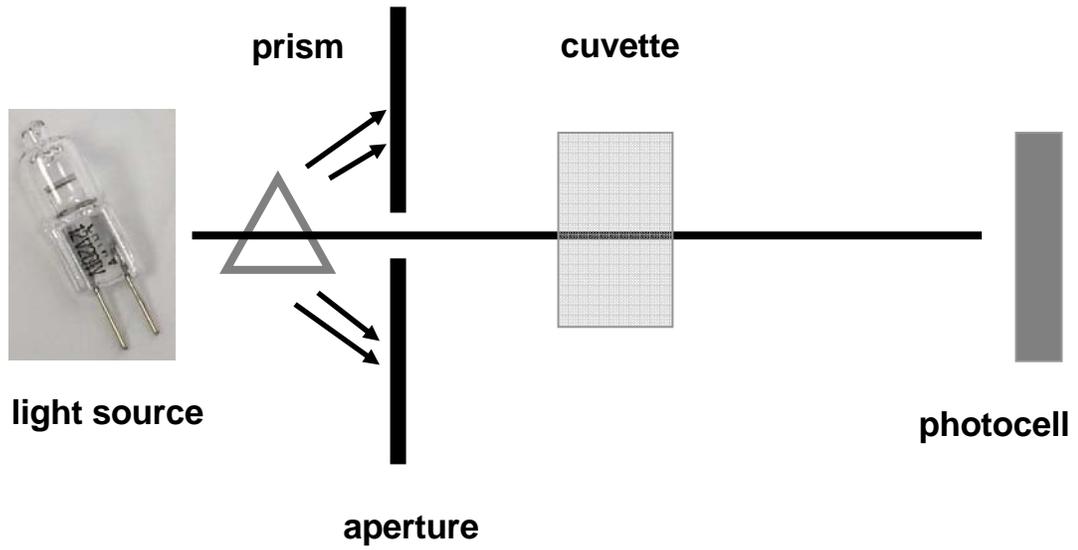


Figure 10a. Schematic of the internal workings of a spectrophotometer.

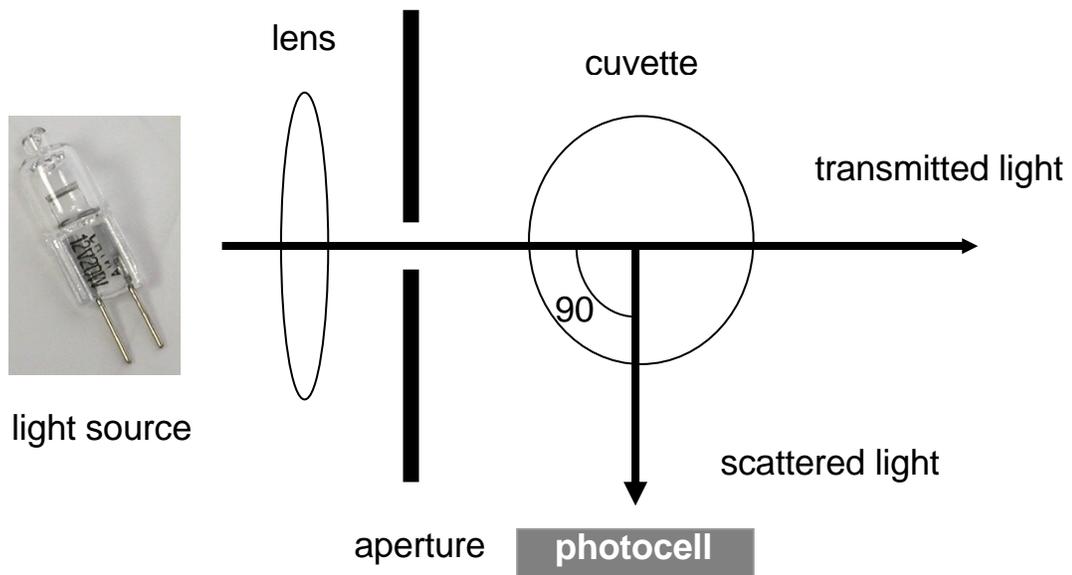


Figure 10b. Schematic of the internal workings of a turbidity meter.

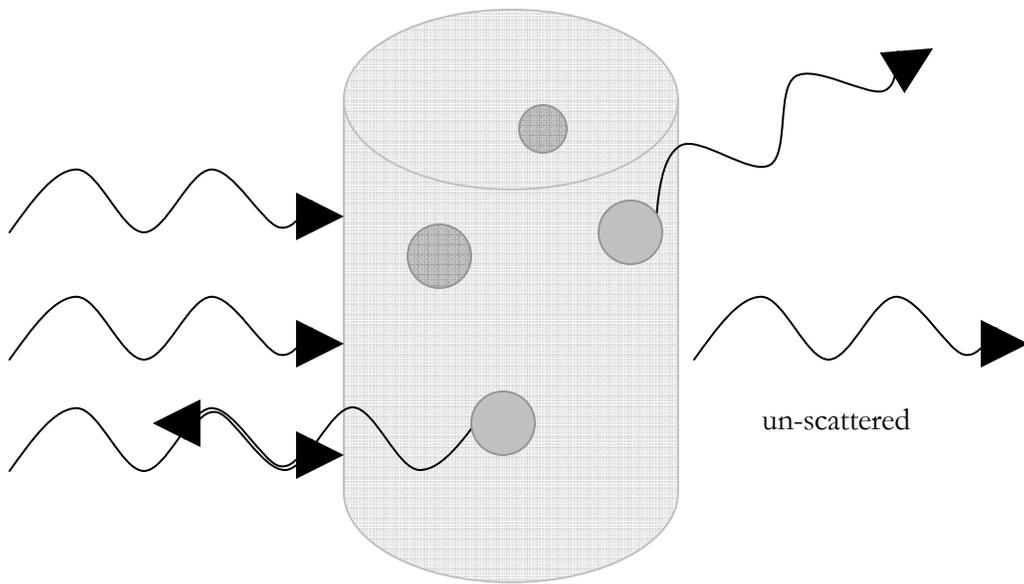


Figure 11. Depiction of light being scattered when encountering particulates and the transmission of light of un-scattered light.

β -lactoglobulin were used as electrophoresis standards. BCATM protein assay kit was used from Pierce.

Protein solution preparation. α -Lactalbumin powder was slowly mixed with deionized water and hydrated with ~85% of the total deionized water required. The solutions were stirred using a magnetic stir plate at ambient temperatures (23°C +/-2°C) and stirred for 2 hour after all of the required protein powder had been added into the deionized water. The prepared solutions contained 3, 4.6, and 5.4% w/v protein. If solutions were not intended for use within one hour after the 2 hour hydration time, then they were sealed and stored (1°C +/-2°C). Refrigerated solutions were brought back to room temperature in 1 hour on a low stir setting. Stock solutions were centrifuged 11950 x g (21°C +/-3°C) for 60 minutes. After which the pH was adjusted accordingly (1M H₃PO₄ or 1M HCl or 1N NaOH) and brought up to volume. Note, at least three different solution per pH were generated and evaluated. Adjusted solutions were centrifuged 11950 x g (21°C +/-3°C) for 90 minutes. The solution's pH was checked and adjusted if needed; also samples were collected for analysis (figure 1).

Heating. Solutions were transferred into borosilicate glass culture tubes 16 x 150mm, capped with aluminum foil and heated for 120 seconds at 85°C in a water bath (Precision microprocessor controlled 280 series). Samples were quenched with ice to room temperature. The solution's pH was checked and adjusted if needed; also samples were collected for chemical and physical analysis. Heated solutions were centrifuged 11950 x g (21°C +/-3°C) for 60

minutes. After which the solution's pH was checked and adjusted if needed, also samples were collected for chemical and physical analysis (figure 1).

Protein Assay. BCA™ protein assay kit was used from Pierce to determine protein content in samples at various analysis points.

Turbidity Evaluation. Optical density development was monitored at 400 nanometers in a Shimadzu 160U spectrophotometer. An Orbeco-Hellige Digital, Direct-Reading Turbidimeter (model 965-10A) was used to obtain readings in Nephelometric Turbidity Units (NTUs) over three ranges: 0-20.00, 0-200.0, and 0-1000, with a test resolution of 0.01, 0.1, and 1 NTU, respectively. The sample cell used an optically matched glass vial with screw cap, 28 mm OD x 61 mm high.

Results and Discussion

Minimal changes in protein solubility and solution turbidity were observed from pH 3.5 to 6.0 (figures 2, 3, and 4a-c). Protein solubility was reduced, while turbidity increased, especially between pH 4.5 and 5.0, with gelation occurring upon heating at pH 4.0. Protein solubility decreased similarly across concentrations (figure 2). Interestingly, even when protein content had been significantly reduced, the optical density [high-low value equates respectively to high (cloudy)-low (clear) turbidity] after final centrifugation remained high. It appeared that a small amount of soluble aggregates were able to greatly increase turbidity.

Gelation occurred at pH 4.0 in the thermally treated samples. Hence these samples were unable to be evaluated. Protein solubility and turbidity are

markedly affected by pH and thermal treatments, respectively solubility decreases and turbidity increasing when nearing the protein's pI. Solution pH greatly affects the protein solubility at the specified processing conditions. Of the small quantity of protein remaining in the solution after pH adjustment, heat treatment, and centrifugation, there seemed to be a large portion of soluble aggregates (an aggregate that remains suspended after centrifugation) remaining (figures 3 and 4a-c). Acidification using HCl was compared to H₃PO₄, which resulted in similar effects regarding protein solubility (data not included).

The α -lactalbumin had intermediate calcium content (0.05%) between holo, fully calcium loaded, and apo, devoid of calcium. α -Lactalbumin binds 1 mole of calcium for every mole of protein (26;33) and based on this 1:1 molar ratio, calculations for the addition of calcium were determined. Based upon the previous findings that concentration did affect the fraction of soluble protein, a dilute protein system was prepared to study the impact of calcium concentration on the aggregation of α -lactalbumin. Apo- α -lactalbumin preparations resulted in the largest degree of turbidity, followed by the commercially prepared (provided by Davisco) α -lactalbumin preparations, with the holo- α -lactalbumin preparations resulting in the least degree of turbidity (figure 5). When calcium was added to the commercially prepared (provided by Davisco) α -lactalbumin solutions, via CaCl₂, the turbidity decreased. This could be a result of the processing conditions between that of commercially prepared (provided by Davisco) and that which was purchased from Sigma. An additional study was

conducted to determine the affect of calcium content in the commercially prepared α -lactalbumin (intermediate verses holo) before and after heating (figures 6 and 7). Visually, upon acidification, the solutions that had intermediate calcium content (no added CaCl_2) aggregated immediately upon addition of the phosphoric acid and remained aggregated and turbid. The holo commercially prepared α -lactalbumin aggregated immediately upon addition of acid; although large aggregates did not appear and the coloration change from a yellow to opaque disappeared quickly. As both solutions approached pH 4.5, there was no visually perceivable difference. The holo- α -lactalbumin solution's maintained protein solubility after the acidification and heating (figures 1 and 6); whereas the intermediate calcium content α -lactalbumin solution's maintained c.a. 80% protein solubility after acidification and heating (figure 6). The protein solubility dropped to a level comparable to the intermediate calcium content solutions after centrifugation; hence the holo-solutions were not stable. It appears that holo- α -lactalbumin solution's developed significantly less turbidity in native conformation at pH 4.5 but this does not alter aggregation and loss of solubility after heating (figure 7). The benefit diminished to a point where the depressed turbidity became comparable to the intermediate calcium concentration α -lactalbumin solutions.

Conclusion

The effects of acidification and heating resulted in a marked decrease in protein solubility and increase in optical density around the iso-electric point with little effect at the extreme pH evaluated (pH 3 and 6). Using the previously stated conditions, gelation occurred during the heat treatment of samples at pH 4.0. Although gelation is unacceptable for beverage applications it could be exploited to evaluate the effects of potential aggregation blocking agents. The protein solubility was independent of the protein concentrations evaluated and of the acidulant type (hydrochloric and phosphoric acid), since these different solutions reacted in a similar fashion (percent solubility). Interestingly, the relatively small quantity of protein that remained in solution after the pH adjustment and heat treatment at pH 4.5 & 5.0 characteristically resulted in large degree of soluble aggregates.

Calcium concentration was an important to consider when evaluating aggregation of α -lactalbumin. Holo (fully loaded) α -lactalbumin preparations resulted in less pH-associated aggregation of the native state than apo preparations and the degree of difference in α -lactalbumin types was more apparent around α -lactalbumin's iso-electric point. Upon heating and centrifugation, the protein solubility of the holo- α -lactalbumin became comparable to the intermediate calcium content. Hence the benefit (lower

turbidity) of having α -lactalbumin in a holo state (by addition of calcium chloride) was lost upon heating.

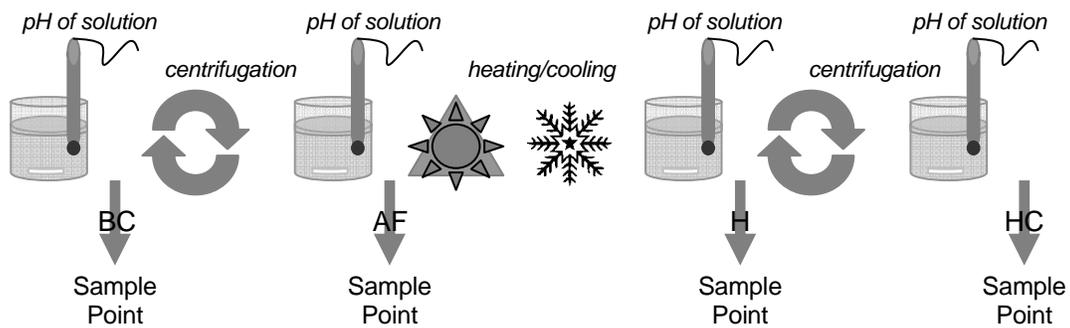


Figure 1. Process flow diagram for sample preparation and collection. In order from left to right: pH adjustment of protein solution, sample-BC, centrifugation, pH check, sample-AF, heating, cooling, pH check, sample-H, centrifugation, pH check, and sample-HC. Readings were taken: BC-before centrifugation, AF-after centrifugation, H-after heating, and HC-after heating and a second centrifugation.

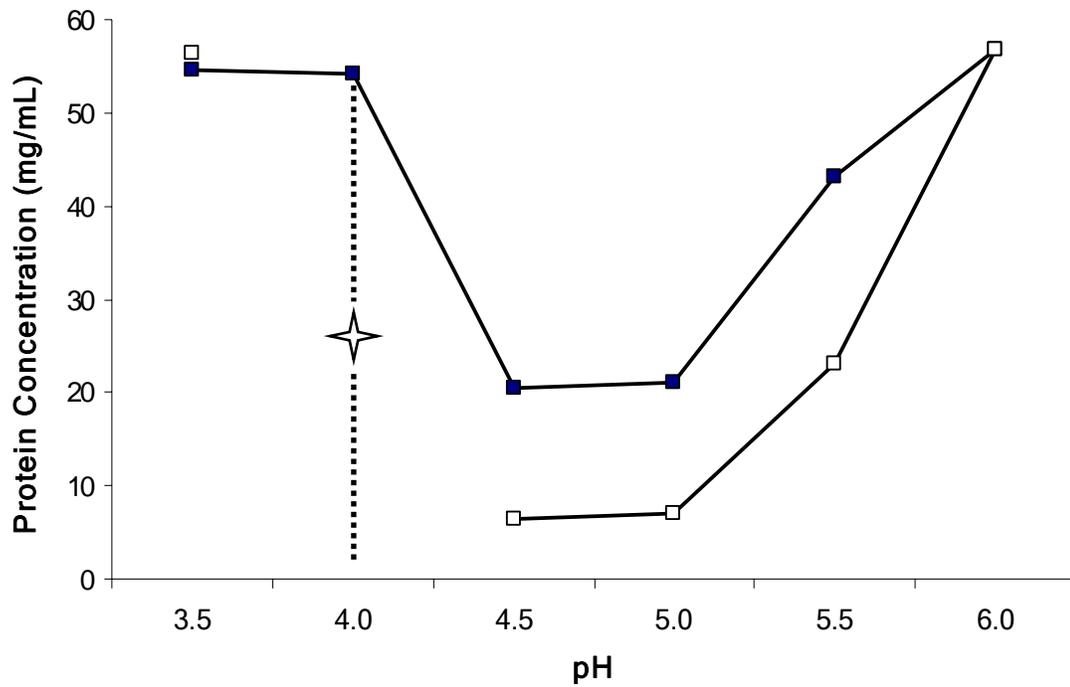


Figure 2. Effect of pH (3.5-6.0) and heating (85°C for 120s) on protein solubility of 56 mg/mL α -lactalbumin solutions. Protein concentration was determined after pH adjustment and centrifugation-AF (■) and after heating and centrifugation-HC (□). Heated sample gelled and protein content was not determined (✦).

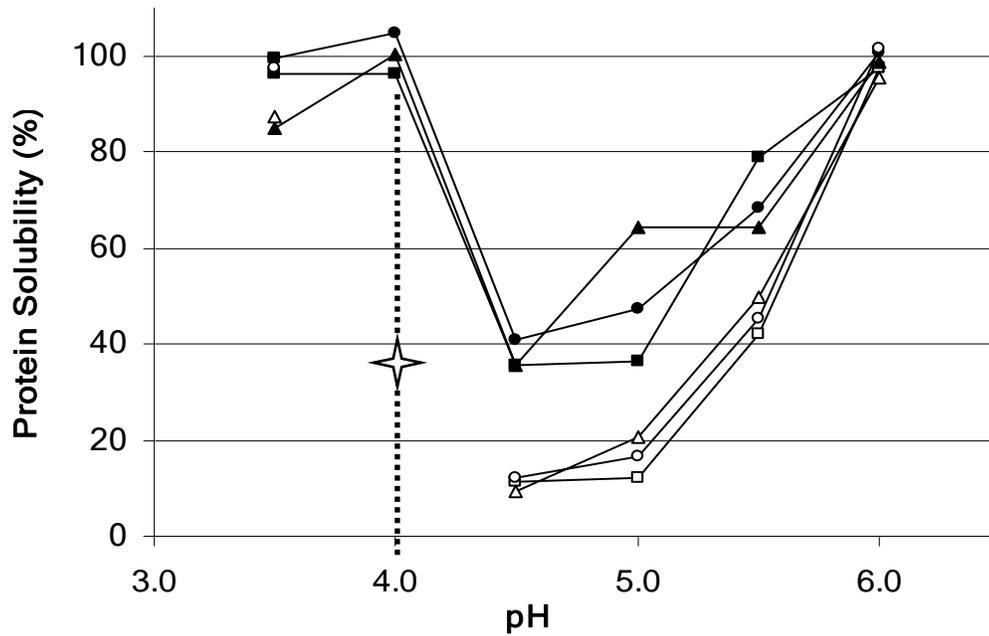


Figure 3. Effect of pH (3.5-6.0) and heating (85°C for 120s) on protein solubility of 30, 46, and 56 mg/mL α -lactalbumin solutions. Protein concentration was determined after pH adjustment and centrifugation-AF (●-30, ▲-46, and ■-56 mg/mL) and after heating and centrifugation-H (○-30, △-46, and □-56 mg/mL). pH 4.0 heated sample gelled and protein content was not determined (↔).

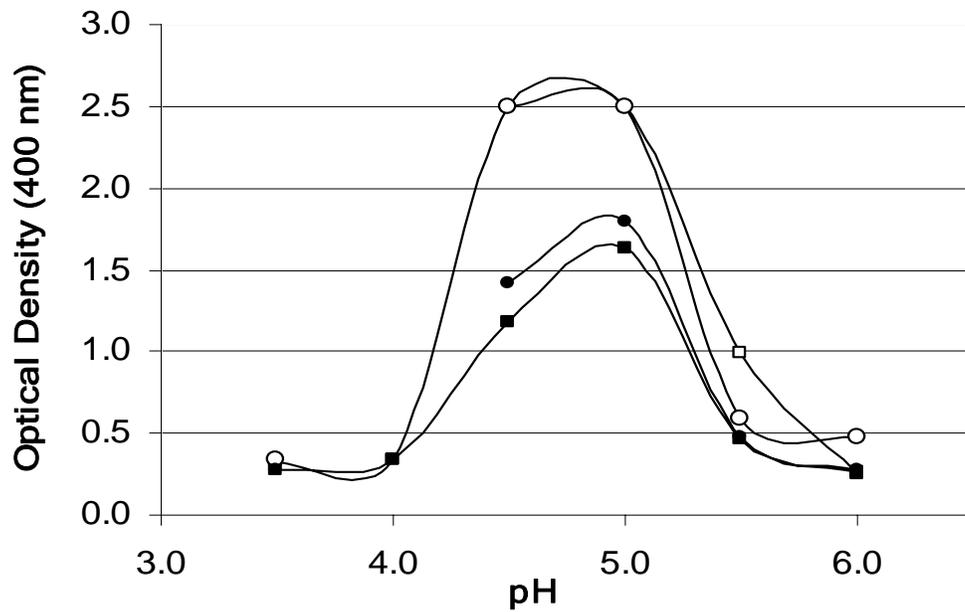


Figure 4a. Effect of pH (3.5-6.0) and heating (85°C for 120s) on protein solution's optical density at 3.0% w/v α -lactalbumin. Optical density was measured after pH adjustment-BC (□), after centrifugation-AF (■), after heating-H (○), and after heating and centrifugation-HC (●).

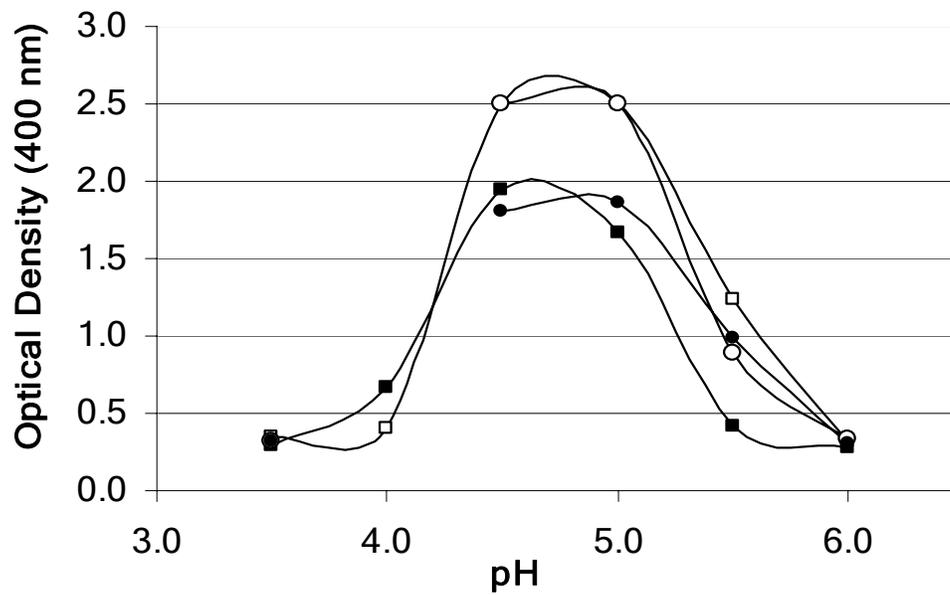


Figure 4b. Effect of pH (3.5-6.0) and heating (85°C for 120s) on protein solution's optical density at 4.6% w/v α -lactalbumin. Optical density was measured after pH adjustment-BC (□), after centrifugation-AF (■), after heating-H (○), and after heating and centrifugation-HC (●).

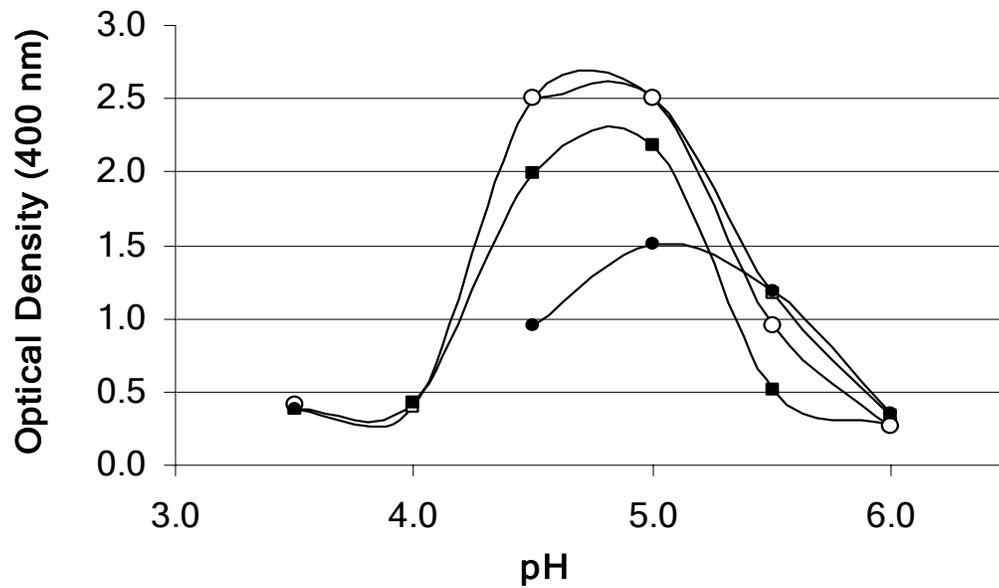


Figure 4c. Effect of pH (3.5-6.0) and heating (85°C for 120s) on protein solution's optical density at 5.6% w/v α -lactalbumin. Optical density was measured after pH adjustment-BC (□), after centrifugation-AF (■), after heating-H (○), and after heating and centrifugation-HC (●).

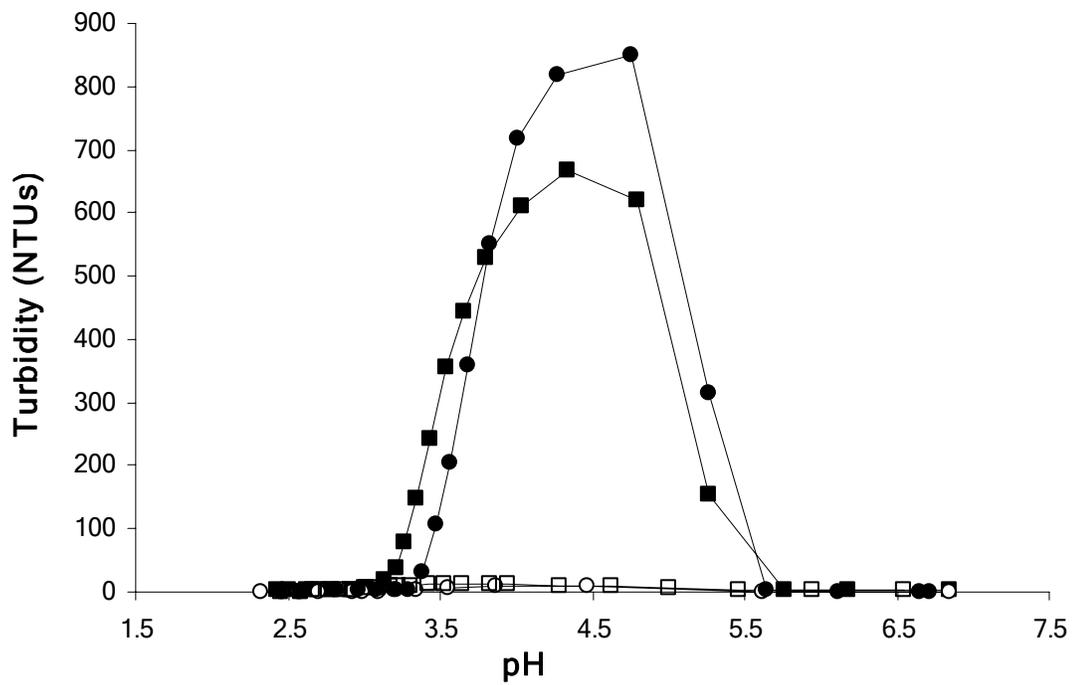


Figure 5. Turbidity development of various degrees of calcium content of unheated α -lactalbumin solutions (0.25% w/v protein) over a range of pHs. Apo- α -lactalbumin (Sigma) (●), intermediate- α -lactalbumin (Davisco) (■), holo- α -lactalbumin (Sigma) (○), and holo- α -lactalbumin (Davisco) (□).

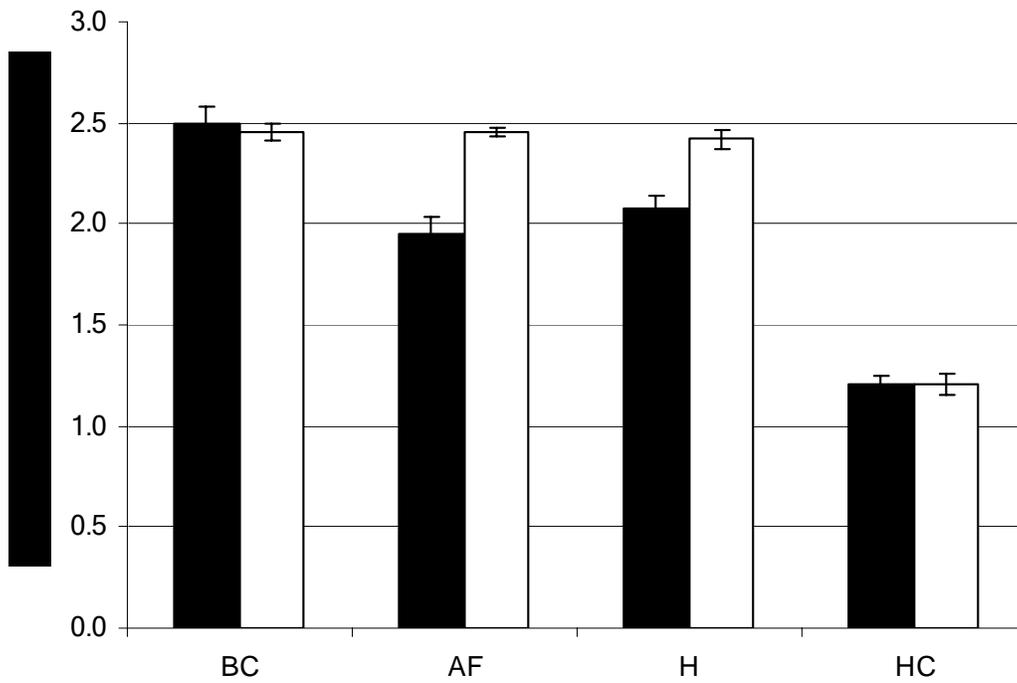


Figure 6. Protein concentration comparing calcium content of unheated and heated α -lactalbumin solutions (0.25% w/v protein) at pH 4.5. Intermediate- α -lactalbumin (Davisco) (■) and holo- α -lactalbumin (Davisco) (□).

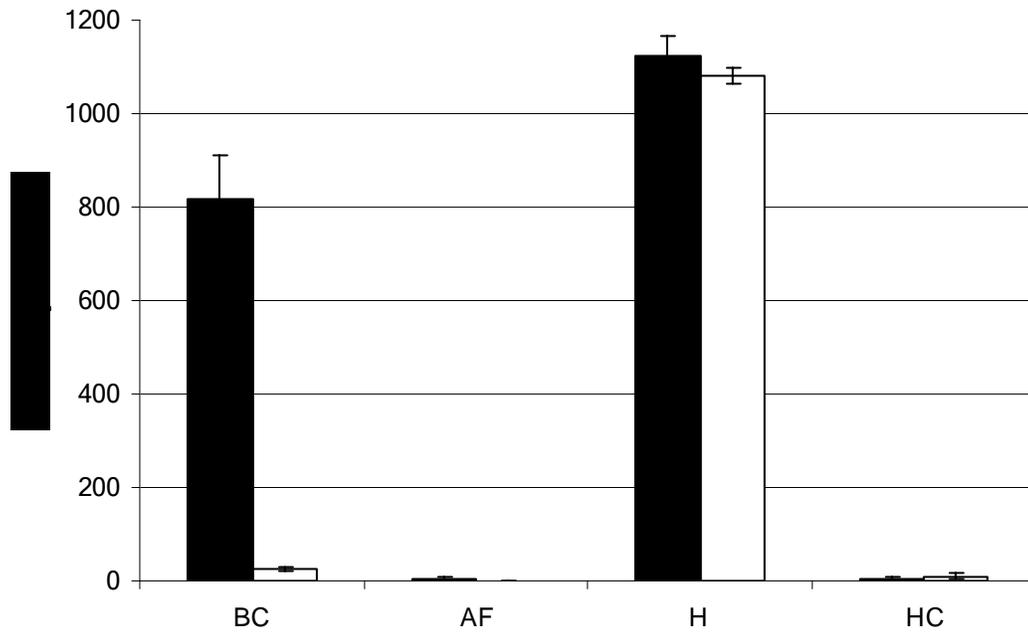


Figure 7. Turbidity development comparing calcium content of unheated and heated α -lactalbumin solutions (0.25% w/v protein) at pH 4.5. Intermediate- α -lactalbumin (Davisco) (■) and holo- α -lactalbumin (Davisco) (□).

CHAPTER II, Part A . BLOCKING OR DISASSEMBLING β -STRUCTURES OF α -LACTALBUMIN AT PH 4.5 WITH THIOFLAVIN-T

Introduction

As determined in the pervious chapter, differences in pH as well as heating, has an affect to the resulting turbidity and protein solubility. Thioflavin-T, a cationic benzothiazole dye (50) was selected for investigation to minimize the affects of pH and heating. It has been shown to bind to cross β -structures (β -sheets that run perpendicular to fibril), characteristic structure in amyloidogenic proteins (83) and thus could bind to unfolding/unfolded α -lactalbumin, pending that sufficient cross β -structures form. Thioflavin-T is an amphiphilic molecule with both polar and hydrophobic regions. The amphiphilic nature lends to the formation of micelles (3 nanometers in diameter) when exceeding the critical micelle concentration of $4.0 \pm 0.5 \mu\text{M}$ (52). Solution pH is important since values below 3.0 results in disrupted micelles, leading to decreased binding (52;53).

The binding of thioflavin-T could act to decrease the aggregation of the non-native α -lactalbumin at acidic conditions (yet, above pH 3.0). Protein aggregates are thought to be stabilized by inter and intramolecular hydrophobic interactions. The cross β -structures are localized areas of hydrophobic interactions, which would suggest that molecules that favor binding to cross β -structures could potentially act to inhibit (ex. sterically) further interaction of the hydrophobic dense areas. Both dilute and more concentrated solutions of

ovalbumin have been shown to readily bind thioflavin-T, which resulted in suppression of heat-induced aggregation (83).

In this study, thioflavin-T was studied as an inhibitor to aggregation of α -lactalbumin samples at pH 4.0 and 6.0. Visual observation was used to determine the effectiveness of thioflavin-T on α -lactalbumin aggregation before and after heating.

Materials and Methods

Materials. Thioflavin-T (molecular weight 318.87), a dry yellow powder, was purchased from Sigma-Aldrich (St. Louis, MO). See chapter I for additional materials information.

Solution preparation. Thioflavin-T was dispersed into deionized water at room temperature, pH was adjusted accordingly (1N HCl or 1N NaOH) and then was brought to volume to yield an 8 mM solution. The α -lactalbumin stock solutions were mixed at room temperature for ~2hr, after which the pH was adjusted accordingly (1N HCl or 1N NaOH) and then brought up to volume to yield an 8 mM α -lactalbumin solution.

The 8 mM stock solutions of α -lactalbumin, thioflavin-T and water were transferred into test tubes in the following order: water, α -lactalbumin, and thioflavin-T. Solution ratios of thioflavin-T: α -lactalbumin consisted of the following: 1:1, 1:4, 0:4, and 4:0. The solutions were vortexed, capped and heated in a water bath for 10 and 20 minutes at 82°C in a water bath (Precision

microprocessor controlled 280 series). After heating, the solutions were quenched with ice/ice-water to room temperature was obtained.

Results and Discussion

The study was conducted at pH 4.0 and 6.0. In the previous study gel-formation occurred at pH 4.0, so gel-properties could potentially be measured to monitor aggregation. As determined in the pervious study, aggregation was not detectable at pH 6.0, so by conducting this study at this pH it could be monitored if aggregation was induced by addition of thioflavin-T to protein. Observations before heating at pH 6.0 and 4.0 were similar in that only localized aggregation was notable when thioflavin-T was added (1:1 and 1:4), but after further mixing the aggregates disappeared (Table 1). Potentially, if a low level of aggregates were present, the thioflavin-T may be able to disassociate the aggregates that were present. The α -lactalbumin solutions at pH 6.0 with thioflavin-T resulted in visible particulates after heating for 10 and 20 minutes at 82°C, whereas the protein or thioflavin-T only solutions were void of visible particulates. After heating the pH 4.0 solutions all solutions gelled with entrapped bubbles. It was noteworthy that the resulting gels were free of visible particulates.

Thioflavin-T binding has been shown to decrease heat-induced aggregation of ovalbumin at neutral pH conditions (83). The binding between ovalbumin and thioflavin-T is more specific than general hydrophobic interactions, which could explain why similar inhibition was not detected with α -lactalbumin. If cross β -structures (specific for amyloid formation) are not

formed during heating, as with lysozyme (similar to α -lactalbumin structure) and ovotransferrin, then binding and consequently inhibition does not occur (83). Lysozyme is structurally homologous to α -lactalbumin, which could explain why thioflavin-T was not affected in the α -lactalbumin system (34;44). The cross β -structures must be present for binding to occur, so that further extension of these structures can be minimized. Thioflavin-T, through visual observations, did not reduce the turbidity in thioflavin-T- α -lactalbumin solutions; it was concluded that the number or sheer presence of cross β -structures may not be sufficient for binding to occur between the protein and thioflavin-T.

Conclusion

The effect of thioflavin-T on the heat-induced aggregation of α -lactalbumin was investigated in pH 4.0 and 6.0 solutions at 8 mM protein concentration. It was shown that thioflavin-T did not have an effect on aggregation at these stated conditions. The structures to support thioflavin-T binding may not be present or present in sufficient quantities hence the blocking of these structures can not occur. Other types of aggregates may be formed that can not be controlled by the presence of thioflavin-T.

Table 1. Observations of thioflavin-T: α -lactalbumin solutions at pH 4.0 and 6.0, before and after heating (82°C for 10 and 20 min)

Ratio	Solution pH (22°C +/- 2°C)	Observations	
		Before Heating	After Heating
1 to 1	4.0	solution transparent, no visible aggregates	gelled, no visible aggregates, entrapped bubbles
	6.0	localized aggregation throughout test tube	gross aggregation (soft-opaque)-concentrated at the top-middle of test tube, semi-continuous
1 to 4	4.0	solution transparent, no visible aggregates	gelled, no visible aggregates, entrapped bubbles
	6.0	localized aggregation (as 1:1)	slightly lighter yellow, no visible aggregates
0 to 4	4.0	solution transparent, no visible aggregates	gelled, no visible aggregates, entrapped bubbles
	6.0	no aggregates visible	opaque no visible aggregates
4 to 0	4.0	solution transparent, no visible aggregates	gelled, no visible aggregates, entrapped bubbles
	6.0	transparent-yellow solution, no aggregates	transparent yellow, no visible aggregates

CHAPTER II, Part B BLOCKING OR DISASSEMBLING β -STRUCTURES OF α -LACTALBUMIN AT PH 4.5 WITH QUERCETIN

Introduction

After evaluating a range of pH in the context of α -lactalbumin aggregation, in chapter I, pH 4.5 was selected for the evaluation of potential aggregation blockers. This pH is also the highest pH still acceptable for hot fill processing. In chapter II part A, it was established that thioflavin-T was not effective at controlling aggregation of α -lactalbumin. In theory thioflavin-T binds to peptide regions rich in cross β -structures, amyloid type structures. Since it is still unclear if thioflavin-T binds in a specific or non-specific way, another amyloid binding type of molecule was selected for evaluation. Additional insight into the aggregative process of α -lactalbumin was sought to evaluate this second type of amyloid binding molecule.

Naturally occurring polyphenols in food systems, such as flavonoids (e.g. flavanone and isoflavones), have been shown to inhibit aggregates self-assembling into ordered structures (specifically β -fibrils) (55). Flavonols and flavones, including the flavonol quercetin, occur ubiquitously in plant based foods (56). Flavonoids have abilities to scavenge radicals, after which they are transformed into highly stable flavonoid free radicals, which do not further react (similar to antioxidants) (56) and also through direct interaction with fibril deposits (55). Structurally polyphenols found to inhibit aggregation are composed of two phenolic rings with two to six atom linkage and at least three hydroxyl groups bonded to the aromatic rings (58). It has been suggested that

the phenol rings are stacked in such a way as to optimize their interactions with amyloidogenic aromatic residues.

The objective of this study was to determine if quercetin could block aggregation of α -lactalbumin at pH 4.5. Solubility and turbidity were monitored for native-aggregation and after thermal denaturation.

Materials and Methods

Materials. See chapter I for additional materials information. Quercetin dehydrate was purchased from Sigma-Aldrich (St. Louis, MO) and +95% purity.

Protein solution preparation. See chapter I for additional solution preparation information. After protein powders had mixed for at least 30 minutes, quercetin (0.03 g/100mL) was slowly added and mixed for 30 additional minutes. After the addition and 30 minutes of mixing the quercetin-protein solution, the pH was adjusted to 4.5 with 1M H₃PO₄ or 1N NaOH and brought to volume (5.6% +/-1.7 w/v protein). Adjusted solution were either centrifuged {centrifuged 11950 x g (21°C +/-3°C) for 90 minutes to remove insoluble material} and heated or just heated (figure 1).

Heating. See chapter I for heating protocol.

Protein assay. Protein concentration was determined using the biuret assay (84).

Turbidity evaluation. See chapter I for turbidity evaluation information.

Results and Discussion

Quercetin-protein solutions were acidified to pH 4.5 in preparation for heating. The solutions, after acidification, were either centrifuged or not

centrifuged before heating. The solutions that were heated directly after acidification resulted in decreased turbidity development, as monitored by a turbidimeter (figure 2a). Although the decrease in turbidity was not detected, using a spectrophotometer (figure 2b) some samples required diluting prior to evaluation; which may have introduced error. Samples evaluated using a turbidimeter did not require diluting, since this instrument has a large working range. The addition of quercetin, with regard to measured turbidity was negligible, except for the evaluation point in figure 2a, after heating. This decrease in relative turbidity was not detected at 400 nanometers (figure 2b); therefore either the sample dilution required for detection at 400 nanometers or the inherent detection of scattered light could be attributed to the discontinuity in the monitored turbidity. The turbidimeter only detects light that is scattered at a 90° angle from the light-path generated by the light source. Protein solubility was not affected by the addition of quercetin (figure 3). Both the negligible affect to turbidity development and protein solubility indicate that the addition of quercetin did not decrease or enhance aggregation of α -lactalbumin. The aggregates that did form in the quercetin- α -lactalbumin system did not behave differently than those aggregates formed in a solution containing only α -lactalbumin. Keeping this in mind, it would seem that thioflavin-T may not be binding to aggregates in a specific way, but could be binding in a more general way, as is the quercetin, to these regions rich in β -strands or to the more organized structures of spherulites.

Conclusion

The addition of quercetin to α -lactalbumin solutions and then acidified to pH 4.5 with subsequent heating (either with centrifugation or not) did not effectively block aggregation of α -lactalbumin in terms of protein solubility and turbidity development.

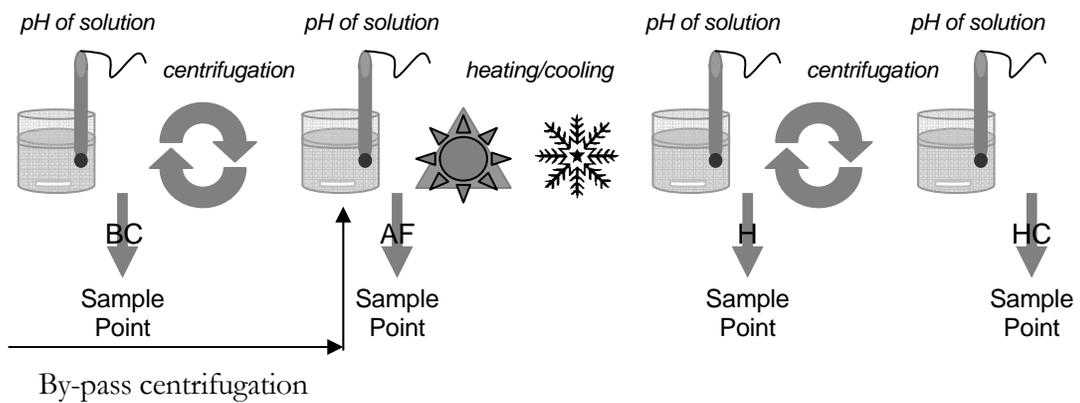


Figure 1. Process flow diagram for sample preparation and collection. In order from left to right: pH adjustment of protein solution, sample-BC, centrifugation, pH check, sample-AF, heating, cooling, pH check, sample-H, centrifugation, pH check, and sample-HC. Readings were taken: BC-before centrifugation, AF-after centrifugation, H-after heating, and HC-after heating and a second centrifugation. Note, some samples were acidified and by-passed the initial centrifugation step to proceed directly to heating.

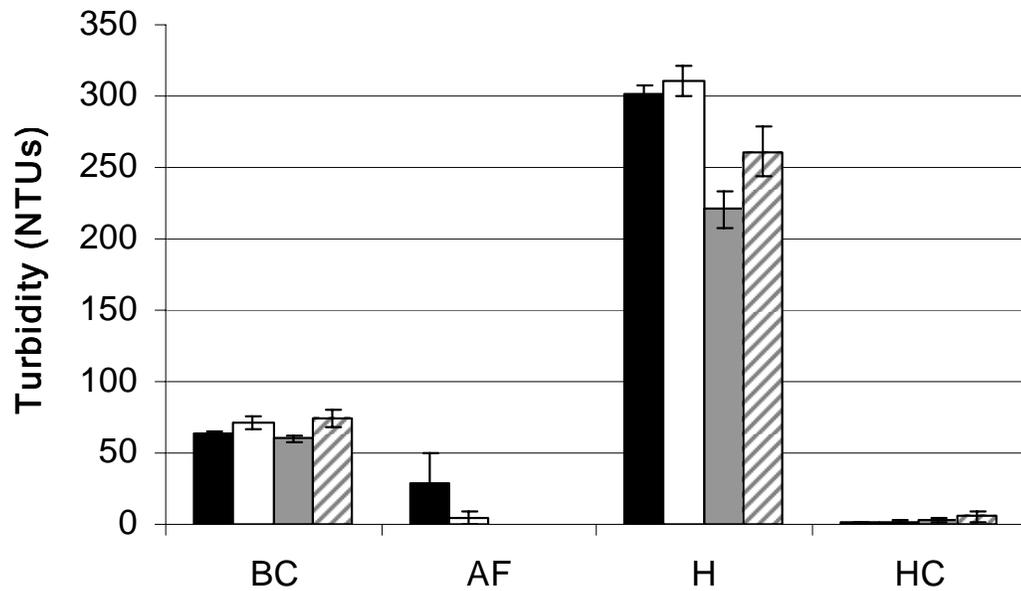


Figure 2a. Turbidity development (NTUs) of quercetin- α -lactalbumin (0.03% quercetin and 5.6% \pm 1.7 w/v protein) solutions at pH 4.5 and after heating at 85° for 120 seconds. Quercetin and protein with centrifugation before heating (■), protein only with centrifugation before heating (□), quercetin and protein without centrifugation before heating (▒), and protein only without centrifugation before heating (▨). BC-after pH adjustment, before 90 minutes centrifugation, AF-after pH adjustment, after 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation, see figure 1.

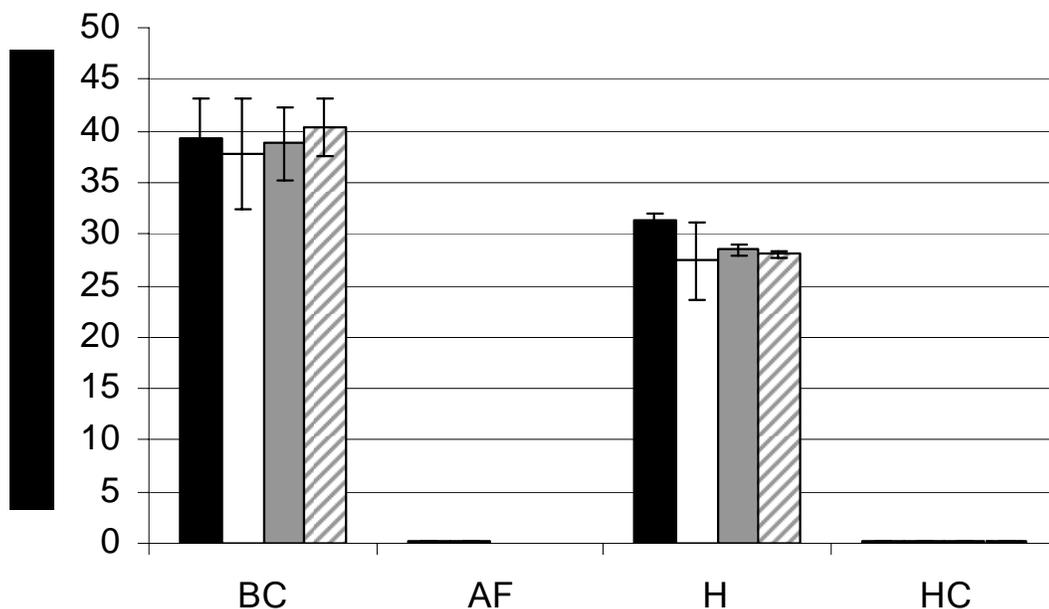


Figure 2b. Turbidity development (at 400 nanometers) of quercetin- α -lactalbumin (0.03% quercetin and 56 mg/mL \pm 17 mg/mL protein) solutions at pH 4.5 and after heating at 85° for 120 seconds. Quercetin and protein with centrifugation before heating (■), protein only with centrifugation before heating (□), quercetin and protein without centrifugation before heating (▣), and protein only without centrifugation before heating (▤). BC-after pH adjustment, before 90 minutes centrifugation, AF-after pH adjustment, after 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation, see figure 1.

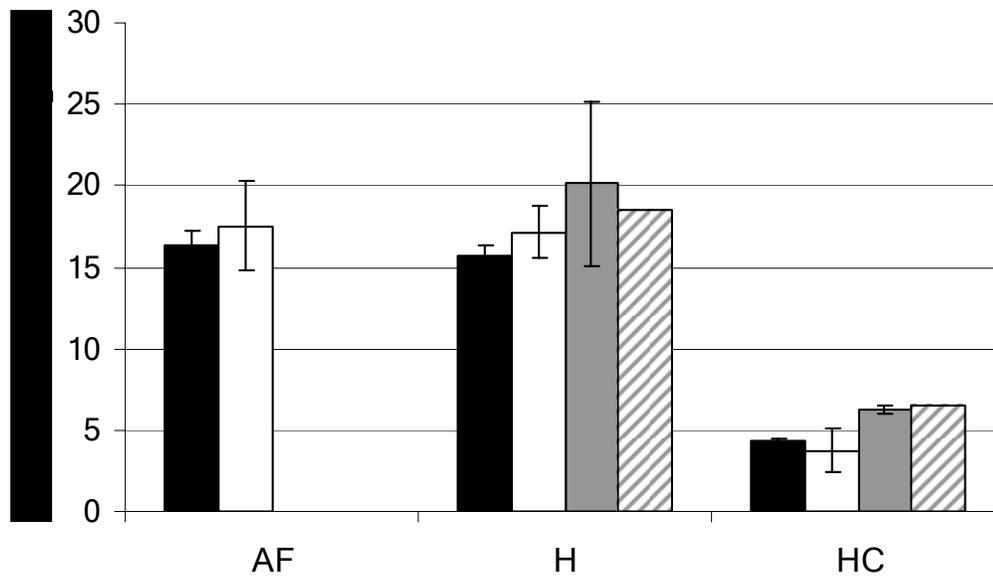


Figure 3. Protein solubility of quercetin- α -lactalbumin (0.03% quercetin and 5.6% \pm 1.7 w/v protein) solutions at pH 4.5 and after heating at 85° for 120 seconds. Quercetin and protein with centrifugation before heating (■), protein only with centrifugation before heating (□), quercetin and protein without centrifugation before heating (■), and protein only without centrifugation before heating (▨). BC-after pH adjustment, before 90 minutes centrifugation, AF-after pH adjustment, after 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation, see figure 1.

CHAPTER II, Part C. DETECTION OF SPHERULITE FORMATION IN α -LACTALBUMIN OR β -LACTOGLOBULIN SOLUTIONS

Introduction

As discussed previously, the addition of thioflavin-T did not block the aggregation of α -lactalbumin. Even though the precise binding of thioflavin-T to β -amyloid rich structures is not well established, two models for binding have been proposed. The two proposed binding models for thioflavin-T consist of micelles of thioflavin-T binding to the β -sheet structure (27), while another model suggests that thioflavin-T binds as a single molecule to a spherulite-shaped protein (52;54).

To better understand why thioflavin-T was not effective in blocking α -lactalbumin aggregation, a survey of spherulite formation using both β -lactoglobulin and α -lactalbumin was conducted using polarized light. Polarized light has been used to categorize the aggregates formed in other protein systems (54). A Maltese cross is observed under polarized light when spherulites are present, due to their inherent highly organized radially extended regular β -sheet structures.

Materials and Methods

Materials. See chapter I for additional materials information. Commercial α -lactalbumin, and β -lactoglobulin acknowledgments (Davigo Food International, Le Seur, MN), contained 92.83% and 93.45% protein, respectively, which was determined on a wet basis ($N \times 6.38$) (82) based on micro-Kjeldahl nitrogen analysis.

Methods. The method of Krebs et al. was used to form spherulites. In brief, samples containing c.a. 2 mM protein in solution was adjusted to pH 2.0, 3.0, 4.0, or 4.5. Loosely sealed glass-disposable vials containing the protein solutions were incubated at 65°C for 24 hours in a water bath. α -Lactalbumin samples were incubated for an additional 2 hours and 30 minutes, after observing that spherulites had not formed. After incubation, the samples were cooled to room temperature and evaluated under polarized light at both 10 and 20 x objective magnification using a Nikon Optiphot-2.

Electrophoresis. A non-heated α -lactalbumin sample was analyzed by non-dissociating (native) polyacrylamide gel electrophoresis (PAGE) using a BioRad mini Protean II apparatus. Samples were made to yield 2 ug/uL stock solution and mixed with native sample buffer (Bio-Rad Laboratories, Hercules, CA) in a 1:1 (1:4) relationship; thus yielding a loaded protein concentration of 1 ug/uL (0.5 ug/uL). Coomassie blue R250 staining solution was used to stain the protein and destained with destaining solution (Bio-Rad Laboratories, Hercules, CA).

Results and Discussion

After incubation at pH 2.0, the β -lactoglobulin sample vials had small specs of material adhering to the interior walls of the vials. There were also particulates suspended free in solution. This was not observed in α -lactalbumin samples that were incubated at pH 2.0 (recommended by protocol), 3.0, 4.0 or 4.5. The pH 4.0 and 4.5 samples showed increasing levels of turbidity, due potentially to non-spherulite aggregation.

As seen in figure 1a and 1b, β -lactoglobulin samples had detectable spherulites at pH 2.0. Krebs et al. presented similar images (54). Spherulite formation was not detected at pH 2.0, 3.0, 4.0, and 4.5 in α -lactalbumin samples when incubated for 24 hours at 65°C; although after c.a. 26 hours and 30 minutes the pH 2.0 and 3.0 samples of α -lactalbumin did contain a very small quantity of particulate matter when compared to the pH 2.0 β -lactoglobulin samples. α -Lactalbumin samples had just one or two spherulites detected. Those spherulites detected in the α -lactalbumin are most likely due to the small amount of contaminating β -lactoglobulin. Native-polyacrylamide gel electrophoresis indicated a small band that was at the same position as the band associated with the β -lactoglobulin sample. The observation that α -lactalbumin did not form spherulites under similar conditions was recently supported by Bolder, et al.(85). The lack of detectable spherulite formation could help to explain why amyloid binding molecules were not effective in reducing aggregation.

Conclusion

α -Lactalbumin did not form spherulites; whereas β -lactoglobulin readily formed spherulites. These findings correlate well with recently (2006) published findings, even though a different solution preparation protocol was followed. Knowing that α -lactalbumin is not likely to form spherulite-aggregate structures helps to better explain why certain types of potential aggregation blocking compounds do and do not work, specifically thioflavin-T.

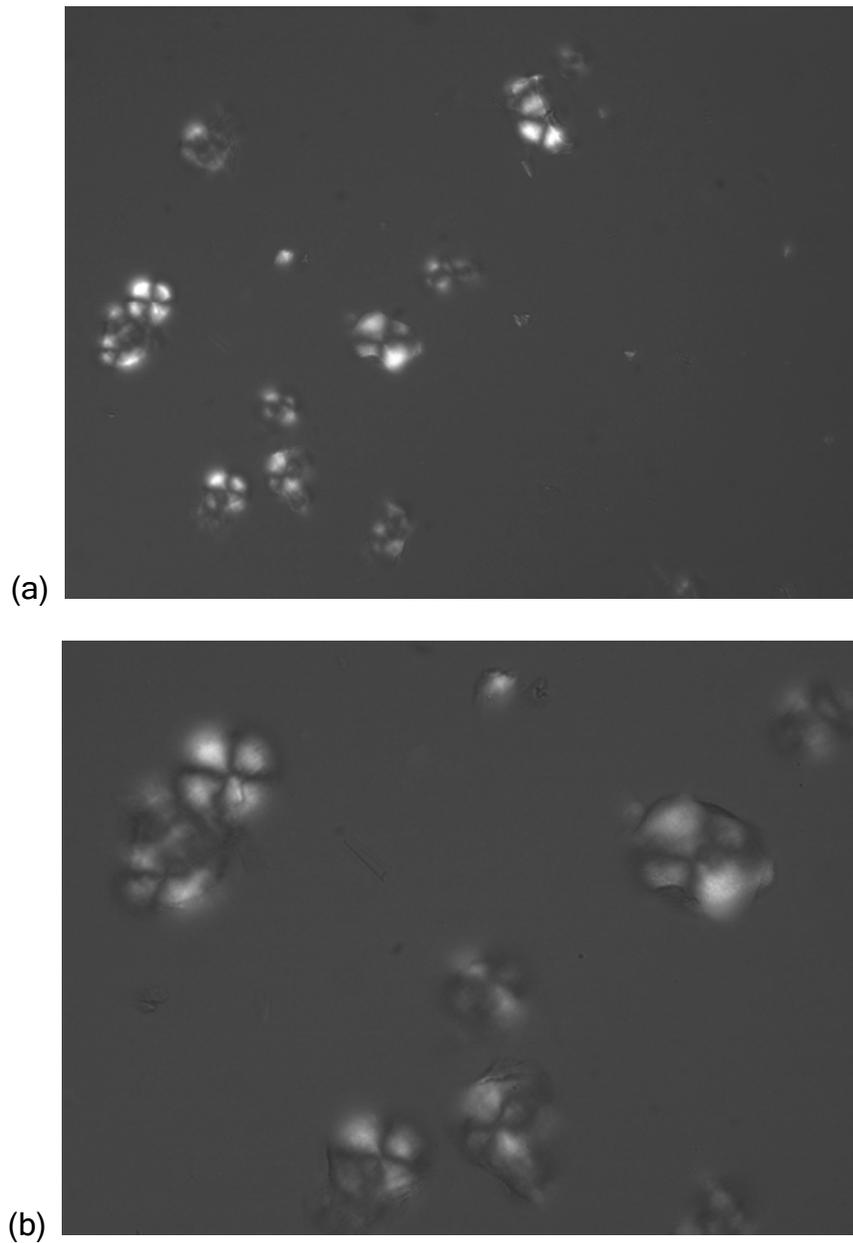


Figure 1. β -lactoglobulin spherulites. The polarized light spherulite images were taken at (a) 10 x and (b) 20 x magnification using a Nikon Optiphot-2 light microscope.

CHAPTER III. EXPOSED HYDROPHOBIC PATCHES OF α -LACTALBUMIN AT PH 4.5 WITH SDS AND/OR SUCROSE

Introduction

Sodium dodecyl sulfate (SDS) has been shown to block aggregation of proteins (66). It's attributes of being an anionic surfactant (charged-hydrophilic sulfate head), with a hydrophobic tail, allows for possible binding and aggregation blocking to hydrophobic patches (sticky-patches) on proteins. Once binding has occurred the protein's sticky-patches are less accessible and will have a resulting charge imparted from the SDS charged head group. This charge allows for charge to charge repulsion, further minimizing the opportunity for protein aggregation to occur. Specifically with whey protein isolate, SDS-sucrose mixtures at pH 4.0-5.5 decrease heat induced turbidity (66).

The addition and concentration of SDS are critical in the preparation of protein solutions to be able to fully incorporate the SDS into the hydrated protein solutions. Precipitation was observed when SDS and protein were mixed below the isoelectric point of the protein (serum albumin $pI=4.7$), but precipitation was not detected when SDS was added to the protein solution above the protein's pI (60). By increasing the relative concentration of SDS, the white precipitate decreases to where no precipitate was present when SDS exceeded 45:55 mixing ratio of protein (ovalbumin) to SDS (60;61).

The objective of this study was to determine if sodium dodecyl sulfate (SDS) and/or sucrose can be used to control the aggregation (turbidity) and solubility of α -lactalbumin.

Materials and Methods

Materials. Commercial α -lactalbumin, β -lactoglobulin, and BioPro whey protein isolate, acknowledgement (Davisco Food International, Le Seur, MN), contained 92.83%, 93.45%, and 91.19% protein, respectively. The protein content was determined on a wet basis ($N \times 6.38$) (82) based on micro-Kjeldahl nitrogen analysis. All salts and chemicals used were analytical grade. Crystallin sucrose was Pharmacopeia (EP)/British Pharmacopeia (BP)/National Formulary (NF) grade. See chapter I for additional materials information.

Protein solution preparation. See chapter I for additional solution preparation information. Powdered SDS was added, yielding 1.6% w/v SDS, to the protein and protein-sucrose solutions 30 minutes into the warm-up stir phase to prevent precipitation. After the SDS dissolved, the pH was adjusted to 4.5 with 1M H_3PO_4 or 1N NaOH and the solution brought to volume. Solutions were centrifuged at 11950 x g ($21^\circ C \pm 3$) for 90 minutes to remove insoluble material (figure 1).

Protein solution preparation for one step centrifugation study. Preparation was the same as previously stated for the protein solution preparation except for the following: Stock solutions of SDS were prepared and adjusted to c.a. pH 4.0.

The SDS stock solution was 16% SDS, of which 10 mL were delivered for each required 100 mL total sample solution (1.6% w/v SDS). The SDS stock solution was added slowly to a continuously stirred, pH 4.5 protein solution. Acid addition was required after each aliquot of the SDS solution to maintain a protein-SDS solution pH range of 3.8-4.8 until all of the required SDS had been added.

Heating. See chapter I for heating protocol and figure 1.

Protein Assay. The Biuret protein assay was used (84).

Turbidity Evaluation. See chapter II, part B. for additional information.

Results and Discussion

Sodium dodecyl sulfate (SDS), in theory, binds to exposed hydrophobic patches on the proteins, which increase upon the addition of acid or by heating (60). SDS's 12-carbon hydrophobic tail interacts with these exposed patches which results in an induced surface charge from the sulfate head group (61). SDS increased precipitate of the protein upon the addition of H_3PO_4 to adjust pH. The precipitation had a dense-compact rounded and/or oblong shape. The precipitation made it difficult to quantify the protein since the precipitant was difficult to transfer from the volumetric flask and SDS-protein complex would not go back into solution upon vortexing. Inaccurate protein concentrations were found in protein solutions containing the SDS (figure 2). When SDS was added after acidification and centrifugation, protein quantification was more reliable, since it did not readily form a precipitate (figure 3). Significant precipitation occurred in the α -LA-SDS solution when the

pH was lowered to 4.5. This resulted in a small fraction of protein remaining soluble after centrifugation. The turbidity that was detected after acidification was similar, although the control protein solutions were less particulate (less clumpy) and more homogeneous. After centrifugation the turbidity was similar, regardless of the protein content. The pellets from the solutions containing SDS were rubbery and dislodged as a tight mass; whereas the control solutions had tight pellets that were not rubbery and did not dislodge as a tight mass.

After heating, both control (α -LA only) and α -LA-SDS solution's developed turbidity. The protein only control drastically developed turbidity, higher than that which was developed through acidification (figures 4). The addition of SDS can not be attributed to developing less turbidity, since these solutions contained significantly less protein; thus less substrate was available for thermal denaturation to contribute to light scattering.

When SDS was added after acidification and centrifugation, the protein quantification difficulty no longer existed (figure 3). Based upon protein solubility and turbidity development, there was no benefit to add SDS (figures 3 and 5). Upon heating, the α -LA-SDS solutions again had large quantities of precipitant that would not re-dissolve. This accounted for the low level of turbidity after heating (figure 5, treatment H), because the aggregated protein precipitated. After the final centrifugation step, little protein was remaining in either treatment (figure 3).

In other studies it has been shown that binding of small hydrophobic molecules (ex. fatty acids) to β -LG occurs, primarily through hydrophobic and

hydrogen bonds, and is further enhanced with increasing hydrophobic chain length or overall hydrophobicity (59). Limited and weak binding of flavor compounds occurs with α -LA (86). This observation of limited binding through hydrophobic interactions and hydrogen bonds may also explain the ineffectiveness of SDS. SDS was shown to decrease aggregation in heated WPI solutions, where the major protein is β -LG, which has a binding site for hydrophobic molecules (86). The specificity of SDS to β -LG is depicted in figure 6, through lower development of turbidity (relative to protein concentration), when compared to the α -LA-SDS solutions. The bovine β -LG specificity for hydrophobic compounds has been attributed to the hydrophobic calyx cavity. The calyx cavity allows for hydrophobic molecules, like SDS and palmitate to bind with great affinity (59). Upon binding of the alkyl chain of SDS through hydrophobic interactions a net negative charge results (60). The increase in the charged character results in a repulsive affect, which minimizes aggregation and increases stability, as measured by turbidity development (61). However, as shown in figure 6, it does not increase solubility.

The addition of sucrose was also evaluated both with SDS-protein solution and protein only solutions. The addition of sucrose to these solutions did not change turbidity or solubility when compared to without sucrose solutions. This data is not presented here.

Conclusion

SDS is an ineffective means to stabilizing α -lactalbumin at pH 4.5. It enhances precipitation upon acidification to pH 4.5, forms unstable soluble

aggregates during heating that are removed during centrifugation, and upon refrigeration or setting out at room temperature will precipitate readily. Sucrose did not significantly affect the system in either a positive or negative way. There does seem to be specific binding of SDS to β -lactoglobulin that does not occur with α -lactalbumin potentially due to structural differences.

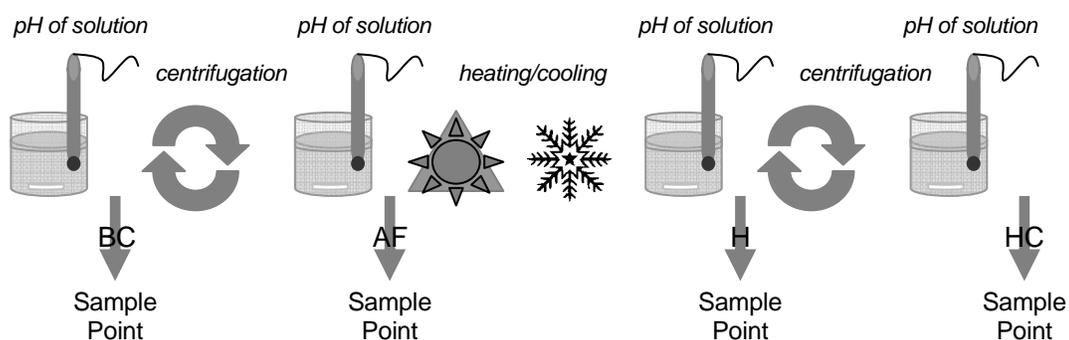


Figure 1. Process flow diagram for sample preparation and collection. In order from left to right: pH adjustment of protein solution, sample-BC, centrifugation, pH check, sample-AF, heating, cooling, pH check, sample-H, centrifugation, pH check, and sample-HC. Readings were taken: BC-before centrifugation, AF-after centrifugation, H-after heating, and HC-after heating and a second centrifugation.

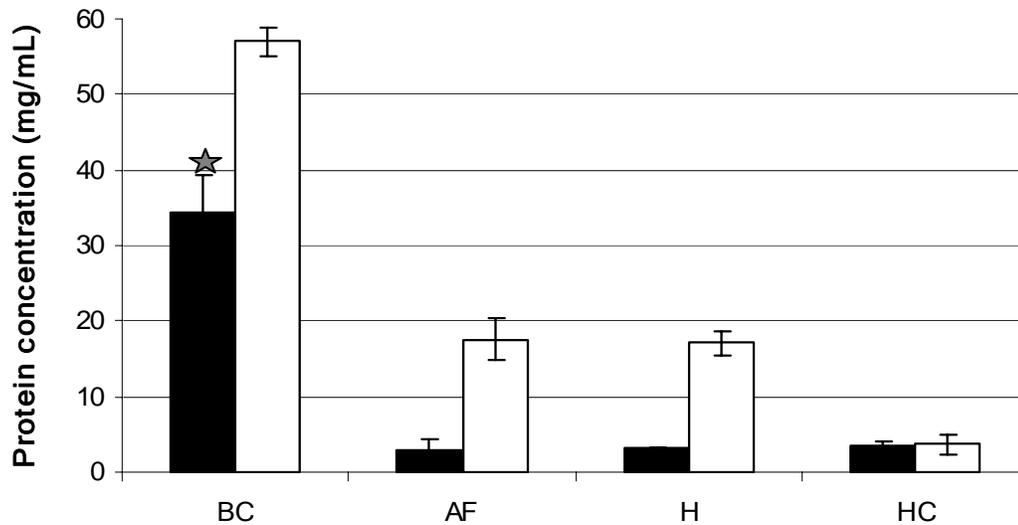


Figure 2. Influence of sodium dodecyl sulfate with regard to protein solubility at pH 4.5. α -lactalbumin-SDS (1.6% w/v SDS) solution (■). α -lactalbumin only solution (□). BC-after pH adjustment, before 90 minutes centrifugation, AF-after pH adjustment, after 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation. Protein concentration is inaccurate due to SDS (★).

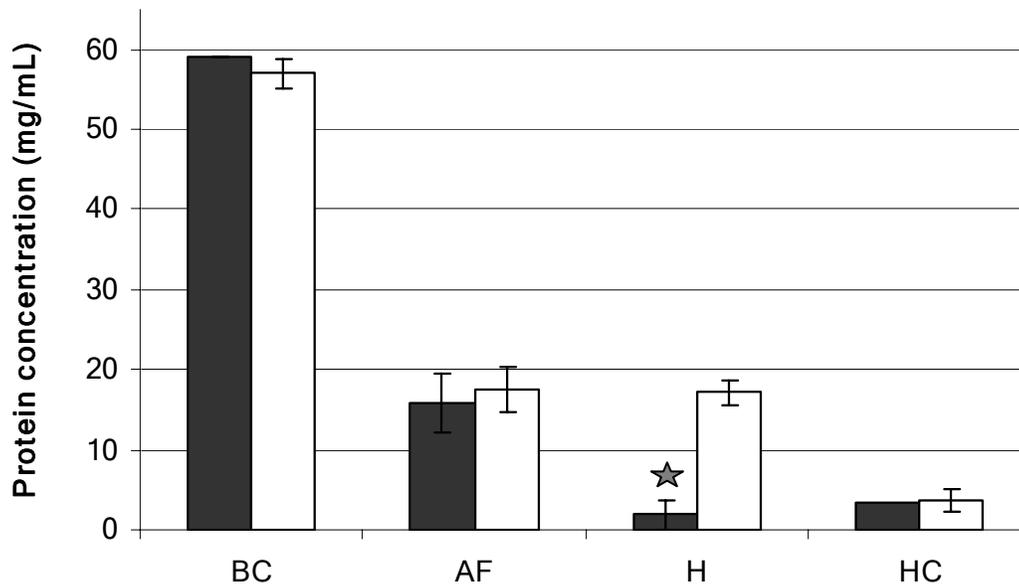


Figure 3. Influence of sodium dodecyl sulfate on protein solubility when added after adjusted to pH 4.5 and centrifuged. α -lactalbumin-SDS (1.6% w/v SDS) solution (■). α -lactalbumin only solution (□). BC-after pH adjustment, before 90 minutes centrifugation, AF-after pH adjustment, after 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation. Protein concentration is inaccurate due to SDS (★).

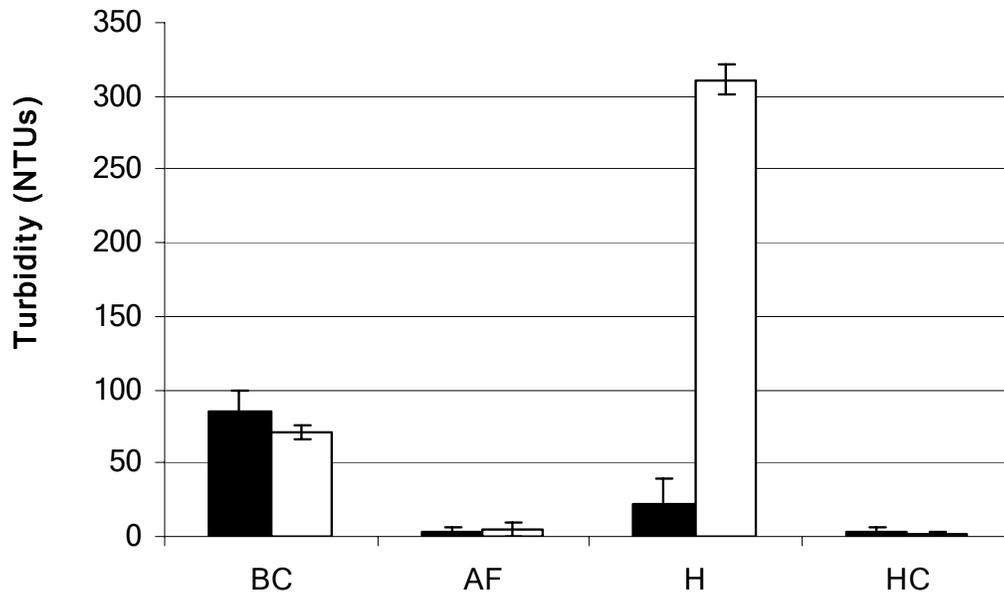


Figure 4. Influence of sodium dodecyl sulfate on turbidity development at pH 4.5. α -lactalbumin-SDS (1.6% w/v SDS) solution (■). α -lactalbumin only solution (□). BC-after pH adjustment, before 90 minutes centrifugation, AF-after pH adjustment, after 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation.

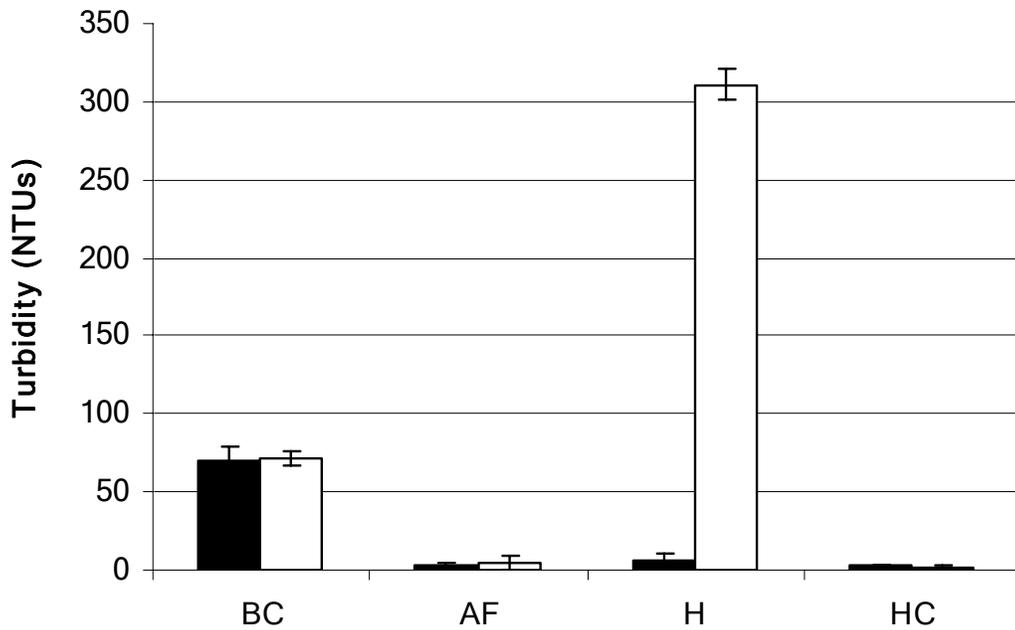


Figure 5. Influence of sodium dodecyl sulfate on turbidity development when added after adjusted to pH 4.5 and centrifuged. α -lactalbumin-SDS (1.6% w/v SDS) solution (■). α -lactalbumin only solution (□). BC-after pH adjustment, before 90 minutes centrifugation, AF-after pH adjustment, after 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation.

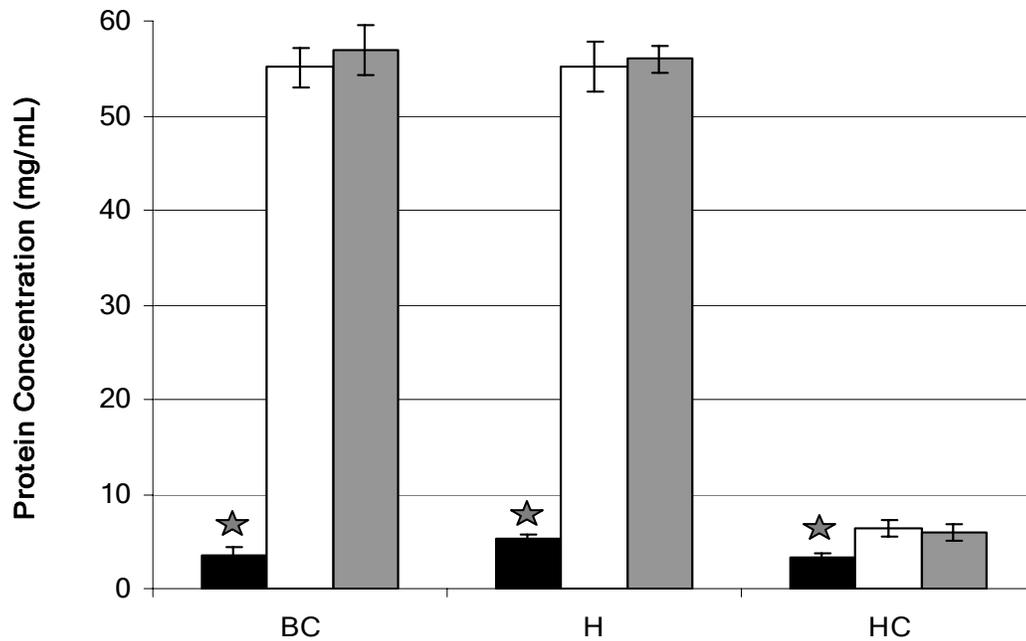


Figure 6. Influence of sodium dodecyl sulfate on protein solubility at pH 4.5, before and after heating. α -lactalbumin-SDS (1.6% w/v) solution (■). β -lactoglobulin-SDS (1.6% w/v) solution (□). Whey protein isolate-SDS (1.6% w/v SDS) solution (▣). BC-after pH adjustment, before 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation. Protein concentrations are inaccurate due to SDS (★).

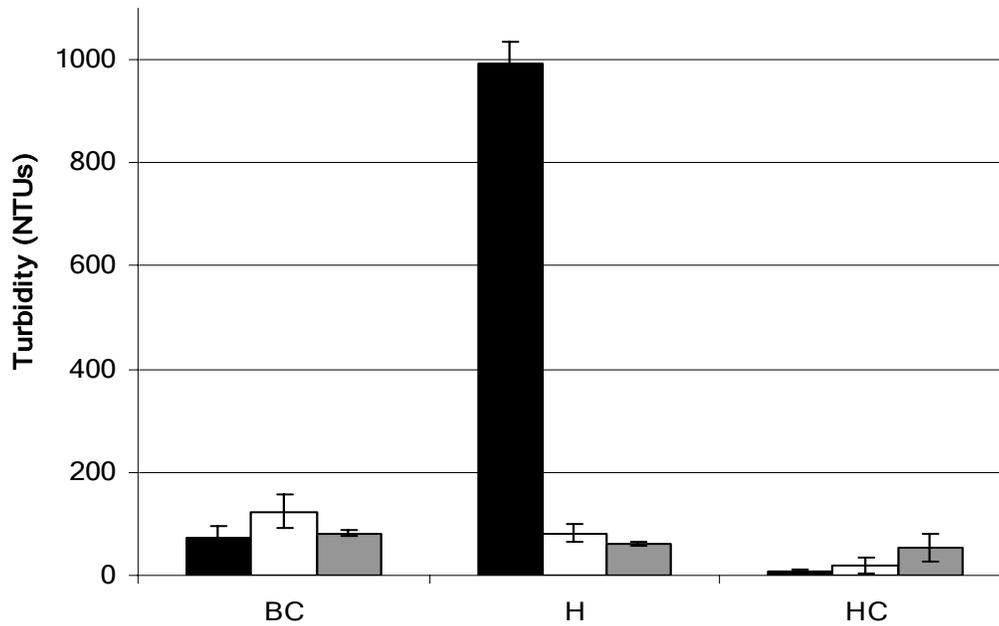


Figure 7. Influence of Influence of sodium dodecyl sulfate on protein solubility at pH 4.5, before and after heating. α-lactalbumin-SDS (1.6% w/v SDS) solution (■). β-lactoglobulin-SDS (1.6% w/v) solution (□). Whey protein isolate-SDS (1.6% w/v SDS) solution (▒). BC-after pH adjustment, before 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation.

CHAPTER I AGGRIGATION OF α -LACTALBUMIN FROM PH 3.5-6.0 AS DETERMINED BY PROTEIN SOLUBILITY AND TURBIDITY DEVELOPMENT

Introduction

Recently, protein beverages, formulated with whey proteins, have been increasing in popularity. These beverages are consumed because they contain a high concentration of branched chain amino acids, which have been linked to increasing and maintaining muscle mass. Generally, these whey protein (isolates and concentrates) containing beverages have $\text{pH} \leq 3.5$ to maintain clarity and stability and are hot fill processed. As a consequence to the low pH, the final beverage is stable. However, the beverage may be considered less desirable due to astringency (parched sensation). Formulation with α -lactalbumin may result in a more stable beverage at higher pHs with reduced levels of astringency, since it is the least reactive whey protein. Although using a hot fill process limits the range of acceptable pH, since the product (except for tomato products) must have a $\text{pH} \leq 4.5$.

Materials and Methods

Materials. Commercial α -lactalbumin, acknowledgements (Davisco Food International, Le Seur, MN), contained 92.83% protein on a wet basis (N x 6.38) (82) based on micro-Kheldahl nitrogen analysis. The α -lactalbumin contained 0.044% phosphorous, 0.048% potassium, 0.05% calcium, 0.0038% magnesium, 2.04% sulfur, and 0.6127% sodium (9190 mg/15 grams powder) as determined by inductively coupled plasma. All salts and chemicals used were analytical grade. Sigma Chemicals (Saint Louis, MO) α -lactalbumin and

CHAPTER IV. BLOCKING HYDROPHOBIC RESIDUES OF α -LACTALBUMIN AT PH 4.5 WITH HYDROXY-PROPYL- β -CYCLODEXTRIN

Introduction

SDS, decreased turbidity development in solutions composed of whey protein isolate and β -lactoglobulin, but generated precipitant and cloudiness when α -lactalbumin was the protein source for the solution. SDS is effective when it has access to bind proteins that have large hydrophobic regions that are prone to aggregation. Specifically, α -lactalbumin does not have the calyx binding site or the large regions of β -strands that β -lactoglobulin has inherent to its structure. Regardless, α -lactalbumin still undergoes aggregation most likely through hydrophobic interactions; thus if SDS is ineffective then another hydrophobic compound that interacts in a more general way may be effective.

One such possible way to minimize aggregation is to use hydroxyl-propyl- β -cyclodextrin (HP β CD). Its structure, a ring of seven propylated glucose molecules, allows for the formation of a hydrophobic pocket inside the ring and a more hydrophilic character on the outside of the ring (69;70;73;87). HP β CD has been shown to interact with exposed hydrophobic amino acid residues, acting as a molecular blocking agent for protein to protein hydrophobic interactions that are associated with aggregate formation (69;70;73;87). Another benefit for HP β CD use as an anti-aggregator is that it has been approved for use in drug formulations for parenteral drug administration (67); hence it would likely be approved for use in beverages.

The objective of this study was to determine if HP β CD can block aggregation (turbidity) and maintain solubility of α -lactalbumin at pH 4.5. The HP β CD, in theory, binds to the unfolded molecule due to the increased number of exposed hydrophobic amino acid residues (primarily aromatic amino acid residues, ex. phenylalanine) making up the main chain of α -lactalbumin (69;87).

Materials and Methods

Materials. See chapter I for additional materials information. All salts and chemicals used were analytical grade. Sorbitol, powder, was food grade.

Protein solution preparation. See chapter I for additional solution preparation information. After protein powders were mixed for at least 30 minutes, HP β CD or sorbitol was added and mixed for the time remaining. After the HP β CD or sorbitol dissolved, the pH was adjusted to 4.5 with 1M H₃PO₄ or 1N NaOH and brought to volume. Adjusted and heated solutions were centrifuged [11950 x g (18-24°C) for 90 minutes] to remove insoluble protein.

Heating. See chapter I for protocol.

Protein Assay. Biuret protein assay was used (84).

Turbidity Evaluation. See chapter II, part B for additional information.

Results and Discussion

HP β CD was evaluated at two different ratios to α -lactalbumin. The higher concentration of HP β CD had a more pronounced effect on protein solubility and turbidity development. HP β CD was most effective in decreasing turbidity development of the unheated only solutions (figures 1, 2a, and 2b, treatment BC). In contrast, there was no effect on turbidity development of

thermally denatured α -lactalbumin-HP β CD (figure 1, 2a, and 2b, treatment H). There was a slight increase in protein solubility after heating (figures 3 and 4, treatment HC). This is consistent, since the general use for cyclodextrins is to increase peptide solubility (increase stability) in pharmaceuticals in non-thermally treated suspensions or those where minimal heating is administered (c.a. 55°C/130°F) (69). Cooper (1992) found that increasing the concentration of cyclodextrins (specifically α -CD in this study) in a globular protein system actually decreased the thermal stability of the proteins. It was proposed once cyclodextrins bind to exposed hydrophobic residues that a change in the equilibrium is fostered; hence destabilized the native state and favoring an unfolded state (73). Simply, that cyclodextrins bind more strongly to the unfolded state than native state proteins, so they actually act in destabilizing the native state. Differential scanning calorimetry showed a decrease in the mean unfolding temperature when cyclodextrins were added to the protein system and that increasing the concentration of cyclodextrins reduced the thermal stability in such a way that it was consistent with how non-covalent binding would affect the system (73). Regardless of the fact that cyclodextrins favor unfolding, they do sequester the hydrophobic amino acid residues, so they are not available to participate in the aggregation process. The type of cyclodextrin, in conjunction with the peptide sequence, is critical in its ability to effect solubility and decrease aggregation in heated and non-heated conditions (69;71).

Potential anti-aggregation enhancement of this system could come from use of a variety of cyclodextrins at once, to make a tailored cyclodextrin cocktail specific for the amino acid residues in the protein. HP β CD is highly effective at binding aromatic residues; whereas α -cyclodextrins are effective at binding aliphatic amino acids as well as protonated aspartic and glutamic acids which would be present at lower pHs (67). α -Cyclodextrins are composed of a ring of six glucose units which make an ideal fit for linear aliphatic amino acid residues (ex. isoleucine) (67).

Sorbitol was used as a control for HP β CD. It was selected due to its non-reactive properties. There was no discernable difference in turbidity development or protein solubility when compared to protein-only solutions (figures 5 and 6). This is consistent to what Otzen et al. found when using glucose in place of a blend of cyclodextrins (67). By using this type of control, it is evident that the lower turbidity values in the cyclodextrin containing protein solutions could not be attributed to solvent effects alone, but requires the unique property (hydrophobic cavity) that cyclodextrins offer.

Conclusion

HP β CD was effective at controlling the aggregation of native proteins but not heat-denatured. There is potential to explore other types of cyclodextrins and possibly for the generation of a custom blend of different cyclodextrins to maximize the effect for turbidity development.

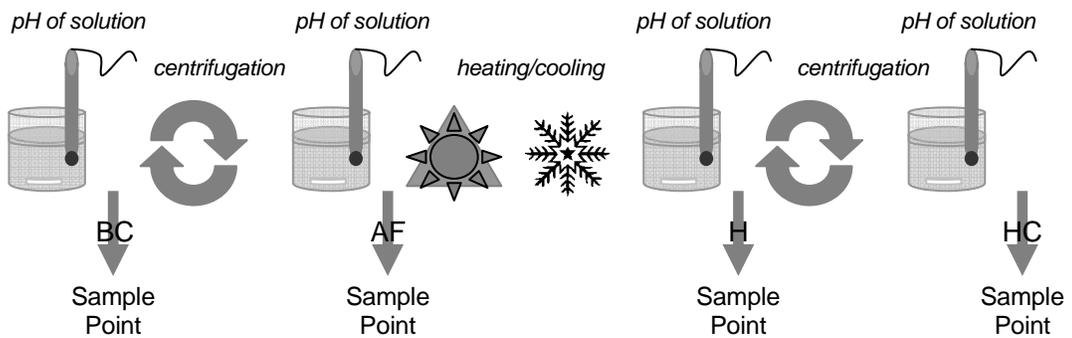


Figure 1. Process flow diagram for sample preparation and collection. In order from left to right: pH adjustment of protein solution, sample-BC, centrifugation, pH check, sample-AF, heating, cooling, pH check, sample-H, centrifugation, pH check, and sample-HC. Readings were taken: BC-before centrifugation, AF-after centrifugation, H-after heating, and HC-after heating and a second centrifugation.

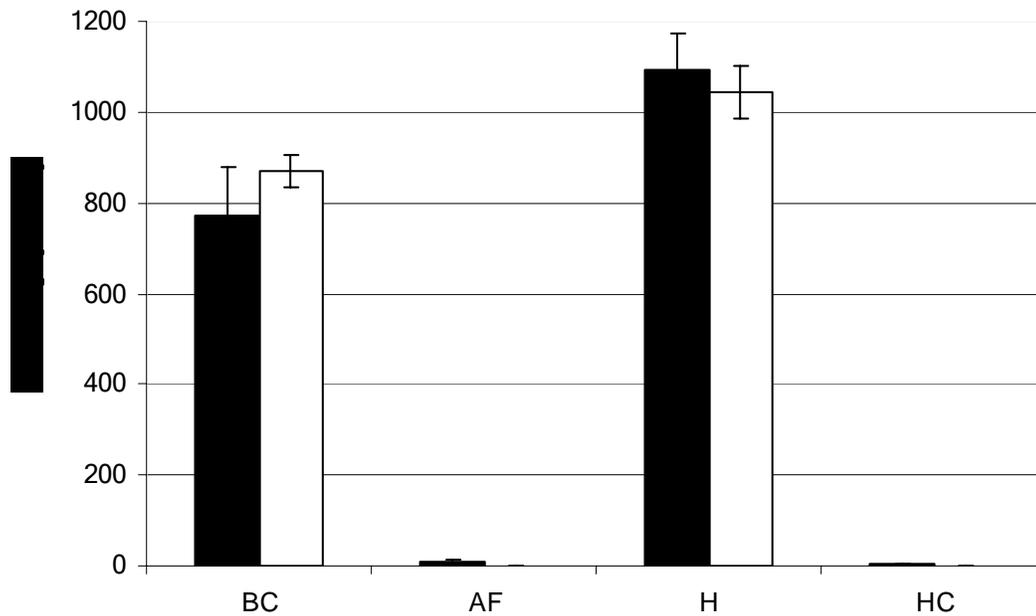


Figure 2a. Influence of HPβCD on turbidity development at pH 4.5 in solutions containing a 1:100 molar ratio of protein to HPβCD. α-Lactalbumin (0.35% w/v)--HPβCD (2.47% w/v) (■) and α-lactalbumin (0.35% w/v) only solution (□); representing three replications. BC-after pH adjustment, before 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation.

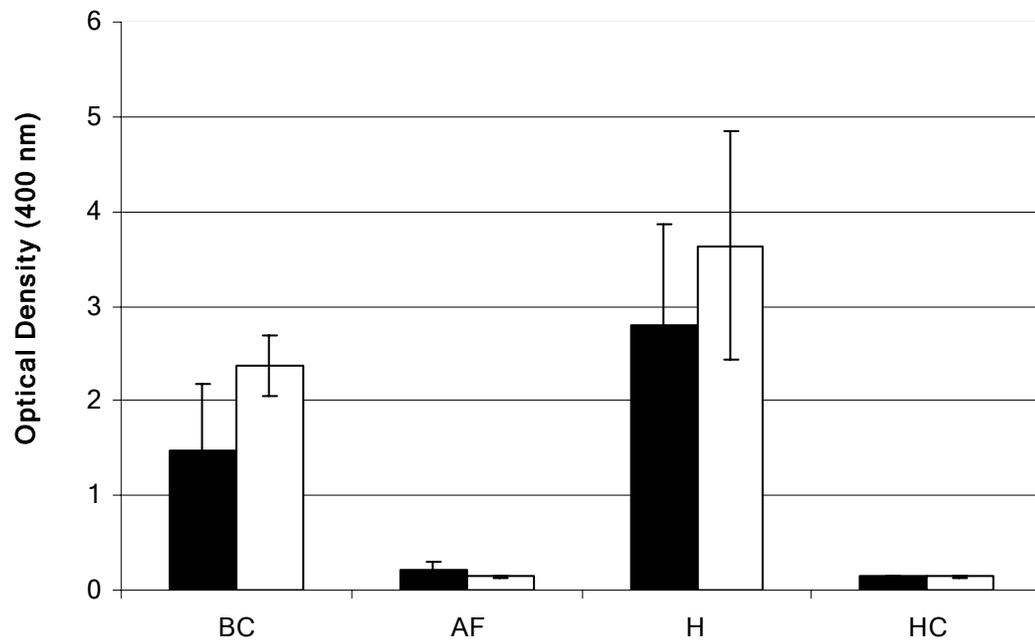


Figure 2b. Influence of HPβCD on turbidity development at 400nm at pH 4.5 in solutions containing a 1:100 molar ratio of protein to HPβCD. α-Lactalbumin (0.35% w/v)-HPβCD (2.47% w/v) (■) and α-lactalbumin (0.35% w/v) only solution (□); representing three replications. BC-after pH adjustment, before 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation.

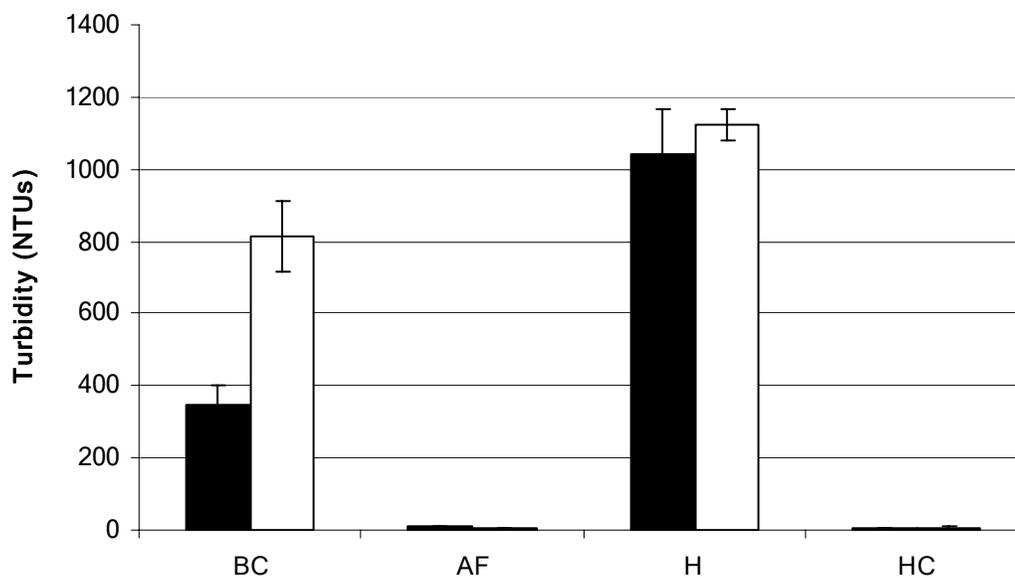


Figure 3a. Influence of HPβCD to turbidity development at pH 4.5 in solutions containing a 1:205 molar ratio of protein to HPβCD. α-Lactalbumin (0.2% w/v)--HPβCD (2.47% w/v) (■) and α-lactalbumin (0.2% w/v) only solution (□); representing three replications. BC-after pH adjustment, before 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation.

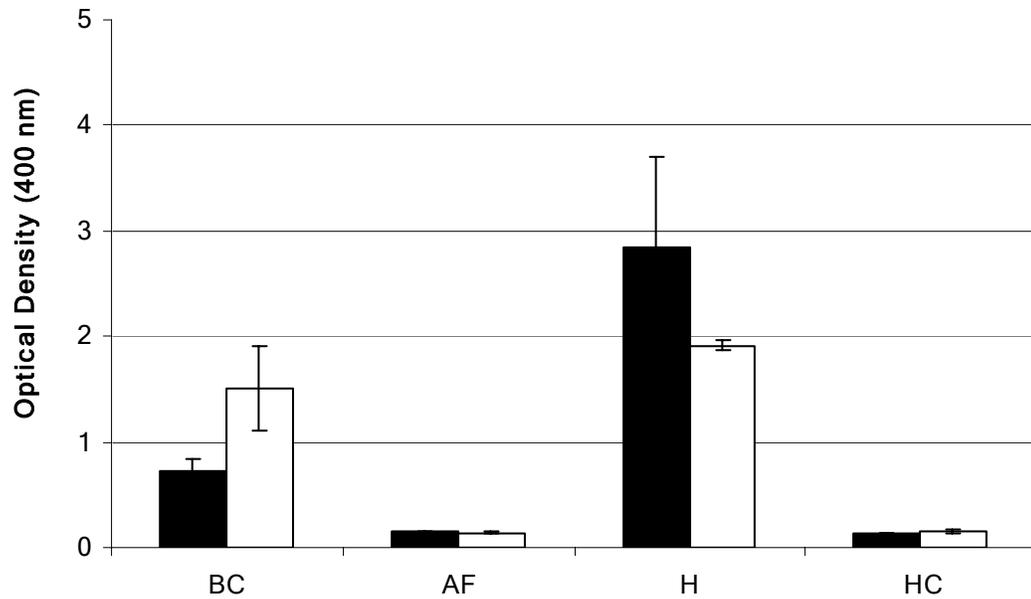


Figure 3b. Influence of HPβCD to turbidity development at 400nm at pH 4.5 in solutions containing a 1:205 molar ratio of protein to HPβCD. α-Lactalbumin (0.2% w/v)-HPβCD (2.47% w/v) (■) and α-lactalbumin (0.2% w/v) only solution (□); representing three replications. BC-after pH adjustment, before 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation.

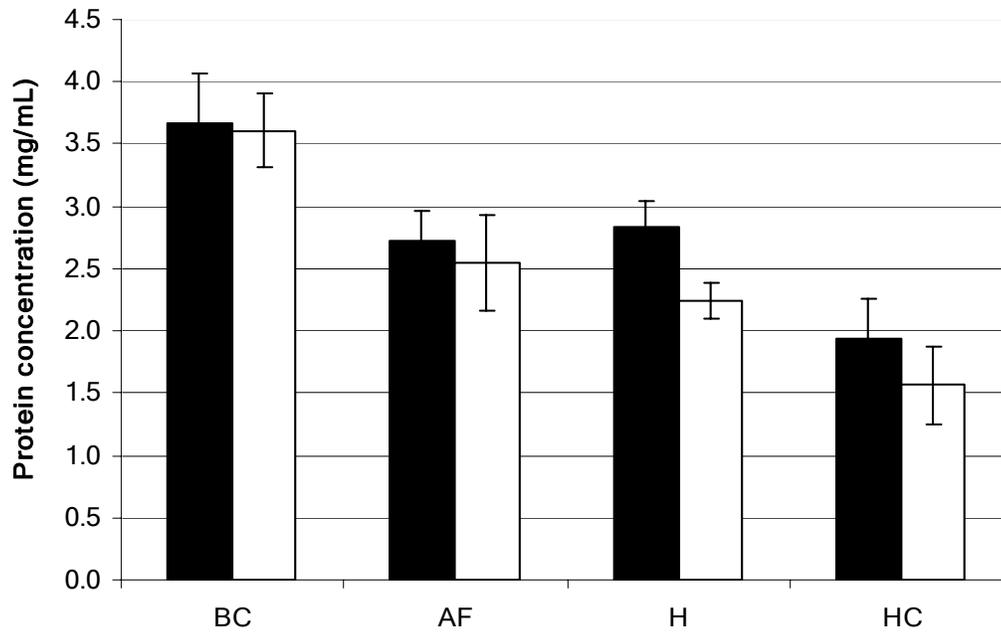


Figure 4. Influence of HPβCD to protein solubility at pH 4.5 in solutions containing a 1:100 molar ratio of protein to HPβCD. α-Lactalbumin (0.35% w/v)--HPβCD (2.47% w/v) (■) and α-lactalbumin (0.35% w/v) only solution (□); representing three replications. BC-after pH adjustment, before 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation.

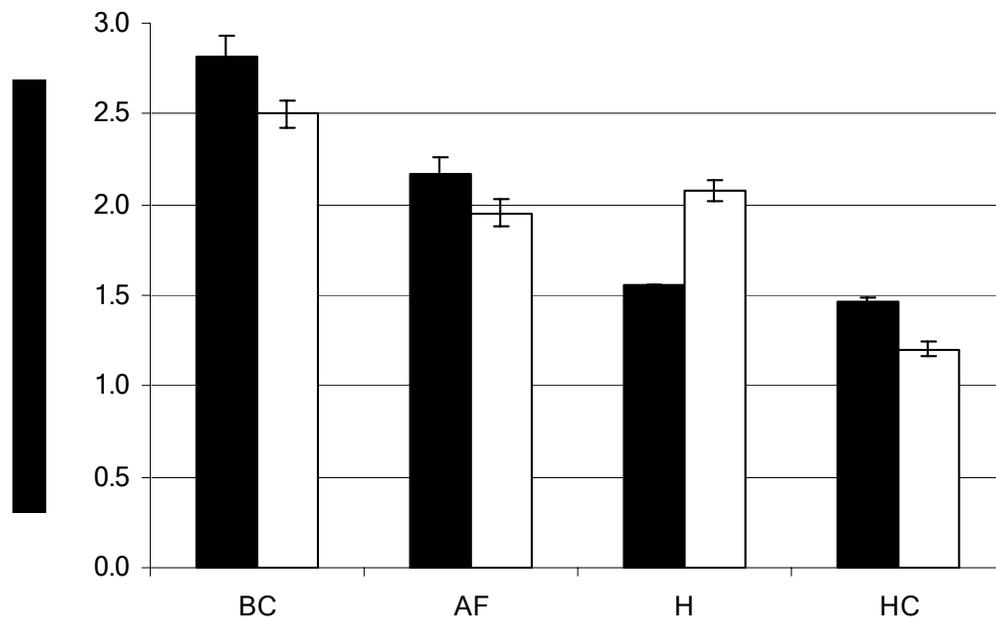


Figure 5. Influence of HPβCD to protein solubility at pH 4.5 in solutions containing a 1:205 molar ratio of protein to HPβCD. α-Lactalbumin (0.25% w/v)--HPβCD (2.47% w/v) (■) and α-lactalbumin (0.25% w/v) only solution (□); representing three replications. BC-after pH adjustment, before 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation.

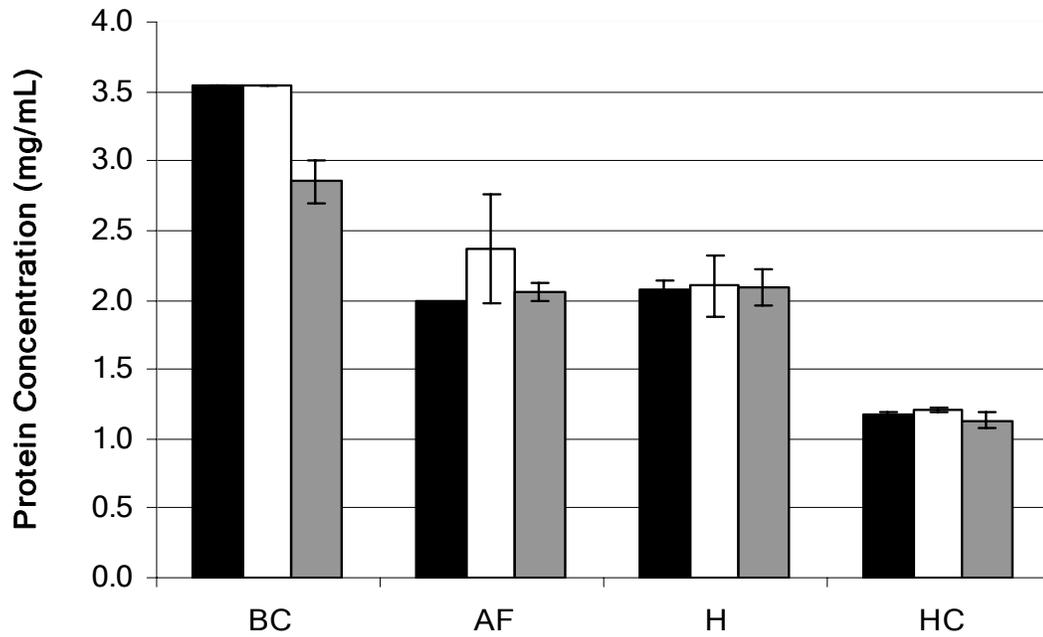


Figure 6. Influence of sorbitol to protein solubility at pH 4.5, centrifuged and heated. 1:205 molar ratio of protein to sorbitol. α -Lactalbumin (0.25 grams) only (■) and α -lactalbumin (0.25% w/v)–sorbitol (added after initial 'AF' centrifugation) (□), α -lactalbumin (0.25% w/v)–sorbitol (□); representing three replications. BC-after pH adjustment, before 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation.

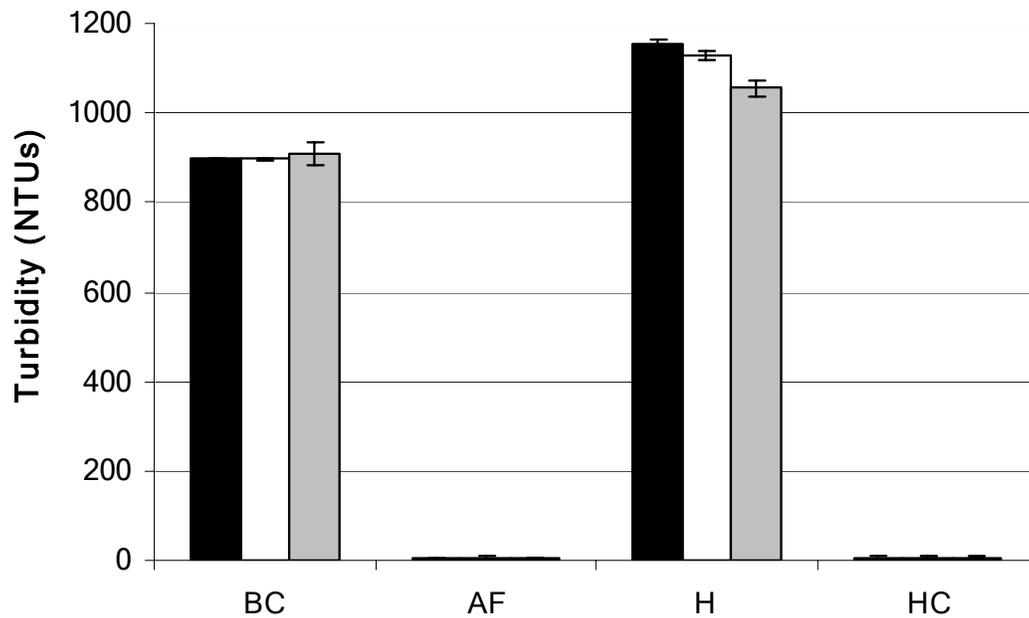


Figure 7. Influence of sorbitol to turbidity development at pH 4.5, centrifuged and heated. 1:205 molar ratio of protein to sorbitol. α -Lactalbumin (0.25% w/v) only (■) and α -lactalbumin (0.25% w/v)-sorbitol (added after initial 'AF' centrifugation) (□), α -lactalbumin (0.25% w/v)-sorbitol (▣); representing three replications. BC-after pH adjustment, before 90 minutes centrifugation, H-heating, and HC-after heating and 60 minutes centrifugation.

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