

ABSTRACT

SCHNEIDER, MARGARET ELIZABETH. Whey Protein Ingredient Aggregation: A Study for Development of Beverage Applications. (Under the direction of Dr. E. Allen Foegeding.)

Addition of whey proteins to beverages is limited by aggregation facilitated by processing involving salts, pH and thermal treatment. All three factors contribute to protein destabilization; producing an unacceptable product. Two general categories of whey protein beverages exist; acidic, generally produced at $\text{pH} < 3.5$, and neutral beverages at $\text{pH} > 6.5$. At neutral pH, thermal treatment is of concern, particularly when the formula contains salts, such as calcium. Acidified whey protein beverages are limited by the development of astringency. Objectives were to develop methods to resolve obstacles encountered at both pH conditions.

Calcium reduces the electrostatic repulsion among proteins by neutralizing the negative charge and/or bridging between proteins, causing precipitation or gelation. Since little work focuses on decreasing the extent of aggregation while maintaining calcium levels, the aim was to add calcium, but prevent interactions with proteins via chelators. Native proteins require heat denaturation before the effects of calcium are observed, therefore solutions of 4% (w/v) whey protein isolate (WPI) containing 20 mM calcium chloride were heated at high temperature short time and ultra high temperature conditions. Turbidity (Nephelometric Turbidity Units, NTU) was measured as an indication of aggregation. Chelation of calcium successfully reduced turbidity. Sodium hexametaphosphate (SHMP) was the most effective chelator, requiring the lowest concentration, 10 mM, to reduce turbidity below 200 NTU

(maximum turbidity to produce a reduced turbidity beverage). Ethylenediaminetetraacetic acid (EDTA) was also effective but 20 mM was needed to create the same effect as 10 mM SHMP. Despite free calcium being below 1 mM in both solutions and both chelators at a concentration of 60 mM, solutions containing SHMP were above the 200 NTU limit, but those containing EDTA were still within the turbidity limits. This suggests that calcium chelation, indicative of free calcium levels, was not the sole explanation for this behavior. Additionally, at 60 mM, EDTA only increased ionic strength by 0.12 M, but SHMP increases ionic strength by 0.36 M, indicating that ionic strength also plays a role. Therefore it can be concluded that calcium-sequestering agents can be used to reduce turbidity, reduce calcium interactions with whey proteins, but are limited by the associated ionic strength, which destabilized whey proteins.

Astringency is associated with interactions between whey and salivary proteins at low pH. Currently, no studies were found to reduce astringency to an acceptable level. It was hypothesized that hydrolysis can reduce astringency by limiting protein-protein interactions. However, hydrolysis is known to increase bitterness. As hydrolysis increases astringency should decrease and bitterness increase. Whey protein isolate (4% w/w protein) solutions were hydrolyzed for 0, 0.33, 1, and 2 hours, and samples were analyzed using a descriptive sensory panel for bitterness and astringency. As expected, unhydrolyzed samples were the most astringent at a level of 6.5 on a 15-point scale. Astringency was reduced to 3 after 9.6 % hydrolysis was achieved and bitterness increased to 5.3. A negative correlation was seen

between bitterness and astringency with level of hydrolysis; suggesting the need to develop enzymatic processes that optimize astringency decrease and bitterness increase.

Controlling aggregation of whey proteins with other whey proteins or with saliva proteins is important to producing a high quality protein beverage. This work provides approaches that can be used to reduce aggregation and increase thermal stability and decrease astringency.

Whey Protein Ingredient Aggregation: A Study for Development of Beverage Applications

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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Food Science

Raleigh, North Carolina

2013

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ACKNOWLEDGEMENTS

First off I'd like to acknowledge, Mary Pat and Stephen Schneider, my wonderful parents who have shaped me to be the woman I am today. Thank you for the consistent support you've given me through the last 23 years. I am forever grateful to Dr. Allen Foegeding, who gave me the opportunity to be in his lab, as well as giving me challenges, patience, and guidance. Also, I appreciate Dr. Chris Daubert for being an outstanding mentor. Of course the Foegeding lab, especially Paige, without you it'd be impossible to get anything accomplished in the lab, Kelsey for teaching much of what I know about the world of whey protein, Hicran for your friendship and insightfulness, and finally Yvie, Nin, Esra, Jenny, and Quin for great discussions and laughs along the way, ultimately keeping me sane! I'm very thankful for my astringency panel, for your patience in taste my... at times not so "tasty" samples. Finally, I'd also like to thank my friends who supported me with yoga breaks, daily gifs, and laughs.

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CHAPTER1: LITERATURE REVIEW: WHEY PROTEIN INGREDIENT

AGGREGATION

1. Introduction

Whey proteins make up 20% of all bovine milk proteins, and are the globular protein fraction of milk not cleaved by chymosin or coagulated by acid (Kosaka et al., 1988; Shah, 2007). Historically, cheese whey, containing these proteins, was spread on fields as fertilizer until regulations were put in place to reduce pollution caused by the high organic matter content (Mawson, 1994; Onwulata and Huth, 2008). Since then, cheese whey has become a byproduct used for human consumption, as the starting material to produce value added high-protein ingredients. These ingredients aid in producing and stabilize food structures and impart positive health benefits such as muscle recovery, anti-cancer effects, and increased satiety (De Wit, 1990; Luhovyy et al., 2007; Onwulata and Huth, 2008).

In 2011, 2688.7 million pounds of whey and modified whey products were produced (ADPI, 2011). Additionally, functional beverages topped sales at \$22 billion in 2010. One in two adults drink these beverages, of which 59% seek these beverages as a source of calcium. Unfortunately, stability of whey proteins in beverages is extremely sensitive to calcium as, making incorporation of whey proteins and high amounts of calcium into beverages challenging, largely due to protein aggregation. In beverages, such as those designed for

quenching thirst, turbidity resulting from aggregation is not desirable since consumers prefer translucent beverages (Beucler et al., 2006). Neutral pH beverages become turbid as a result of thermal treatment, especially in the presence of calcium. If the beverage is designed to be sold refrigerated, at neutral pH, then thermal pasteurization of 63°C for 30 min, 72°C for 15 s, or 89°C for 1 s is required (CFR 2008), but in order to be shelf-stable neutral beverages are generally processed at high temperatures, such as ultra high temperature (UHT) treatment of 135°C for 1-2 sec. Shelf stable beverages can also be produced at/below pH 4.6 (acidified), with mild thermal treatment, below 100°C (21 CFR 114). Since athletic beverages, below pH 4.6, are acidified, and therefore shelf stable, minimal thermal processing is required. This and the fact that proteins are more stable at low pH allow for the production of whey protein beverages at pH 3.4-2.6 that are translucent. The combination of low heat treatment and a high net positive charge on proteins at pH < 3.4 inhibits aggregation. However, there are concerns regarding aggregation of whey proteins with saliva proteins during consumption, which causes astringency (Andrewes et al., 2011; Beecher et al., 2008; Kelly et al., 2010). Aggregation among whey proteins can result in turbid, precipitated or gelled solutions; and aggregation between whey and saliva proteins produces astringency. Therefore, understanding what causes aggregation and how to control aggregation is essential to producing high quality and desirable beverages containing whey proteins.

2. Protein Components of Whey

2.1 β -lactoglobulin

β -lactoglobulin comprises 50% of the total whey protein in bovine milk (Mulvihill and Donovan, 1987; Surroca et al., 2002). Although non-existent in human milk; β -lactoglobulin is present in bovine milk and contains all essential amino acids including large quantities of leucine (McKenzie, 1970). Leucine contributes to whey proteins' ability to increase protein metabolism aiding in exercise recovery and development of skeletal muscles for the aging population (Ha and Zemel, 2003). β -lactoglobulin consists of 162 amino acids adding up to a molecular weight of 18.367 kDa for β -lactoglobulin A and 18.281 kDa for β -lactoglobulin B (Eigel et al., 1984; Papiz et al., 1986). The structure consists of nine beta sheets and one alpha helix, with two disulfide bonds at C66-C160, C106-C119 and a free sulfhydryl at C121, which is buried in one of the beta sheets (Croguennec et al., 2003; McKenzie, 1970; Mulvihill and Donovan, 1987). At room temperature and neutral pH, the native state of β -lactoglobulin is a non-covalently bound dimer. At low pHs and non-native conditions, β -lactoglobulin exist in monomer form.

2.2 α -lactalbumin

α -lactalbumin is the second most abundant protein in bovine whey. It is a globular metalloprotein that contains one bound calcium ion and has a molecular weight of 14.175 kDa (Brew et al., 1970; Eigel et al., 1984; Mulvihill and Donovan, 1987). There are two states of α -lactalbumin; the holo- state where the calcium ion is bound and the apo- state where there is no bound calcium ion. The holo- α -lactalbumin state denatures at around 64°C while the apo-form denatures at 34-35°C (Permyakov et al., 1985). In the mixture of whey proteins found in whey protein ingredients, α -lactalbumin unfolds at temperatures much lower than β -lactoglobulin (Aparenten, 1995; Relkin et al., 1993), but remains more stable (Dalglish et al., 1997; McGuffey et al., 2005; Permyakov et al., 1985). The presumed stability is due to a low propensity of the denatured α -lactalbumin molecules to aggregate, due to lack of free thiol groups, and thus, at a macroscopic level, appear stable (i.e., no turbidity). α -lactalbumin can easily be digested and its physiological role is to assist in lactose synthesis (Mulvihill and Donovan, 1987). α -lactalbumin is high in tryptophan and aspartate and contains four disulfide bonds (6-120, 61-77, 73-91, and 28-111) (Permyakov et al., 1985).

2.3 Other Whey Proteins

The most predominate protein in whey is β -lactoglobulin, second is α -lactalbumin, and in small quantities, bovine serum albumin (BSA), lactoferrin, lactoperoxidase, immunoglobulins and other minor proteins (Mulvihill and Donovan, 1987). Bovine serum albumin is capable of binding insoluble free fatty acids, which makes them more soluble in aqueous environments (Spector et al., 1969). Lactoferrin, lactoperoxidase, and immunoglobulins are present in smaller quantities as the main contributors of antimicrobial properties. As a whole, whey proteins are high in essential amino acids, especially cysteine and branched chain amino acids (Shah, 2007). Together these individual proteins contribute overall positive health effects, but together also produce an ingredient in which protein aggregation is extremely sensitive to temperature, salts, pH and other ingredients (e.g., polyphenols) (Havea et al., 2001; Rawel et al., 2001).

2.4 Whey protein ingredients

Liquid cheese whey contains mostly water and up to 0.8 % protein, 4.7 % lactose, 0.07% lipid, and 0.25% salt (Morr and Ha, 1993). Therefore, manufacturing whey protein ingredients with high protein content requires removal of non-protein components to varying degrees. A review on whey protein processing is found at Huffman (1996) and Morr and Ha (1993).

Whey protein ingredients can be separated into categories including whey protein isolates (WPI), whey protein concentrates (WPC), and whey protein hydrolysates (WPH). Since whey protein ingredient powders may vary slightly in moisture content, the compositional requirements are often given on a dry weight basis (dw). The protein composition requirements are: WPIs have more than 90% protein; and WPCs contain 25%-90% protein (although 25%-55% protein WPCs are primarily used for animal feed, and WPC > 55% protein are used for human consumption) (Morr and Ha, 1993). Most powders contain 3-4% moisture and the remaining non-protein compounds included lactose, salts, and lipids. Lactose protects whey proteins from heat denaturation during spray drying, but remains a concern for a variety of reasons including lactose-intolerance, Maillard browning, and off flavors (Spiegel, 1999). At high concentrations, salts destabilize whey proteins against heat denaturation, especially calcium salts (Mulvihill and Kinsella, 1987). The most common forms of processing to remove non-protein compounds are microfiltration followed by ultrafiltration, or ion exchange chromatography followed by concentration. Microfiltration removes nearly all fat and most all whey proteins are recovered. Ion exchange processes are designed for selective adsorption of proteins so not all proteins are fully recovered, for example glycomacropetides are often absent in WPIs made by ion exchange (Wang and Lucey, 2003). Whey ingredients contribute nutrition, and structure to an end product, via formation of an emulsion, film, gel or foam. The quality of ingredients is often times determined by the protein content.

3. Whey Protein Functionality

Whey protein ingredient properties can contribute a variety of structures to food systems, such as edible films, foams, gels, and emulsions. It is important to fully understand the ability of whey proteins to contribute these qualities to a food system is defined as “functionality” (De Wit, 1990; Foegeding et al., 2002). Whey protein ingredients are generally sold as dry powders so the first functional necessity for production of beverages is solubility, and maintenance of solubility throughout processing. Protein solubility can be defined as the protein concentration in the liquid phase compared to the concentration in solid phase, or overall the amount of protein dispersed and retained in a supernatant after centrifugation (Pelegri and Gasparetto, 2005). Therefore, solubility is related to two physical/chemical properties. The first is the ability to disperse the protein powder in water or an aqueous solution containing other ingredients (e.g., salts, sugars, colors, and flavors). Second, once dispersed, solubility may change after thermal processing due to aggregation. Because solubility is essentially a measure of dispersing and maintaining native proteins or small aggregates, it is highly dependent on pH, thermal processing, and electrolyte content. The degree of aggregation can be observed through characteristic such as turbidity, precipitation, and gelation. These characteristics of insolubility are ultimately the results of aggregation of whey proteins; which is often preceded by denaturation, but not necessary. Before complete denaturation occurs there is an intermediate structure which maintains secondary structure but loses tertiary structure, called the molten-globule form. Although secondary structure is unchanged, the loss of tertiary structure results in exposure of different amino acids

increasing hydrophobic properties (Foegeding and Davis, 2011). The exposure of the amino acids alters functionality, and also can increase tendency to aggregate. To achieve the classical concept of a denatured structure, secondary structure must be lost without hydrolysis or break down of peptide bonds, therefore strictly maintaining primary structure (Colvin, 1964; Mulvihill and Donovan, 1987). Typically, denaturation involves the breakdown of hydrogen bonds, van der Waals, hydrophobic, and electrostatic interactions and can lead to proteins being more susceptible to aggregation (Mulvihill and Donovan, 1987; Verheul and Roefs, 1998a). Aggregation is unspecific protein-protein interactions resulting in complexes of various size (Hermansson, 1979).

Aggregation, even on the smallest level, produces complexes with higher unit mass than native individual proteins. The increase in size causes an increase in scattered light, resulting in an increase in turbidity. Aggregation usually follows an event which results in denaturation, since denaturation allows for exposure of otherwise blocked hydrophobic patches and reactive side chains such as free sulfhydryl groups. It should be noted that protein denaturation is not required for aggregation, as simply the screening of a proteins charge with salt can aggregate protein and not result in denaturation. With the exposure of side groups and hydrophobic patches, non-polar groups attract each other and eventually lead to the formation of a bi-molecular aggregates that proceed to aggregate producing a range of aggregate sizes (Prabakaran and Damodaran, 1997). Aggregates can be either soluble, forming a stable dispersion, which is called a sol (a solid dispersed in a liquid), or insoluble, which result in phase separation. The transition from one state to another is dependent on pH,

ionic strength, and heat exposure as was heavily discussed by Ako et al. (2009). While maintaining a protein concentration of 40 g/L β -lactoglobulin they propose a state diagram approach, showing the progression of different states, a sol, homogeneous gel, micro-phase separate gel and eventually a precipitate by increasing sodium concentration from 0-1 M (Ako et al., 2009). In this case and others gelation is defined as the transition from a fluid to a solid via formation of a network (Foegeding and Davis, 2011). Gelation of whey proteins requires a critical concentration of protein that will vary with conditions; for example, at least 1% protein at its isoelectric point is need to form a gel with heat treatment (Puyol et al., 2001).

4. pH

One characteristic of foods that determines quality and processing requirements is pH, defined as the negative log of the hydrogen ion activity ($-\log a_{H^+}$) and is a key regulator of microbial growth (Covington et al., 1985; Ray, 2004). Foods take on a range of pHs and can be defined based on pH. Acidified foods are those that “have a water activity (a_w) greater than 0.85 and have an equilibrium pH of 4.6 or below” and only require “[thermal processing] to an extent that is sufficient to destroy the vegetative cells of microorganisms of public health significance and those of non-health significance capable of reproducing in the food” (21 CFR 114, 2008). Although, beverages at neutral pH are desirable due to low levels of astringency, these beverages have a high propensity to aggregation, due to the required

heat treatment. Minerals added for nutritional quality often accentuate this problem.

As briefly mentioned above, protein stability in thermal-processed beverages is determined to a great degree by the beverage pH. Native proteins are stabilized by electrostatic repulsion between surface amino acids that can be positively (histidine, arginine, lysine and the N-terminal) or negatively (aspartic acid, glutamic acid and the C-terminal) charged, depending on the pH relative to the pKa of the functional group (Sillero and Ribeiro, 1989). The pH where the net charge is zero is called the isoelectric point (pI; Cannan, 1942), averaging around 5.2 for whey proteins. Consequently, solutions become clear or translucent below the pI, since proteins will have a net positive charge. Above the pI the protein will have a net negative charge. Aside from repulsive and attractive forces, extreme pHs above or below the pI, and thermal treatment facilitate unfolding of protein structure exposing hydrophobic patches (Ako et al., 2010; Pelegrine and Gasparetto, 2005), which as we now know leads to the formation of aggregates, altering functionality.

4.1 Effects of pH on individual proteins structures

β -lactoglobulin has a dynamic range of structures across the pH scale. β -lactoglobulin exists mainly in non-covalently linked dimers at neutral pH (Majhi et al., 2006; Surroca et al., 2002), but below pH 3.5 and above pH 9, β -lactoglobulin will exist in monomeric form (De Wit, 2009; Majhi et al., 2006). Around intermediate pHs such as 3.7-5.2 higher order

aggregates form. These conditions apply to β -lactoglobulin alone in solution, and may be modified in whey protein ingredients due to interactions with other proteins.

As mentioned in section 2.2, α -lactalbumin is a metalloprotein and the relative amount of holo (calcium bound) and apo (calcium free) forms depends on pH. This is significant because binding of calcium determines the stability of α -lactalbumin. At extremely low pH values, the high proton concentration creates competition with calcium for the amino acid's carboxylate oxygen within the binding site, which displaces bound calcium (Permyakov et al., 1985). This transition results in a molten globule state extremely stable, compared to fully unfolded state, against thermal denaturation, increasing denaturation temperature up (Permyakov and Berliner, 2000). The molten globule state is reversible, meaning the protein can revert back to native structure (Kuwajima, 1989).

4.2 Reduction of pH-dependant aggregation

When the combination of solution of pH and ionic strength is insufficient to prevent aggregation during heating, alternative stabilization mechanisms have been attempted. These methods involve alteration of protein structure or addition of compounds increasing repulsive forces, or reduction of attractive forces associated with protein unfolding.

In order to reduce aggregation near neutral pH, sodium lauryl sulfate can be added as was

explained by Etzel (2004). Sodium lauryl sulfate is a surfactant that binds to the proteins, imparting a stronger negative charge than the inherent negative charge, due to amino acids, increasing electrostatic repulsion.

An alternative approach is to stabilize proteins against pH dependant unfolding. When whey proteins are pre-heated and internally cross-linked using transglutaminase, they are more stable to unfolding and therefore more stable against pH induced aggregation (Lorenzen, 2007; Tanimoto and Kinsella, 1988). Cross-linking occurs by transglutaminase covalently linking the γ -carboxamide group of glutamine and the ϵ -amino group of lysine (Tanimoto and Kinsella, 1988). At neutral pH, 10% cross-linked β -lactoglobulin held at 95°C for 30 min failed to form gels, results indicated that intramolecular cross-linking prevented unfolding (Tanimoto and Kinsella, 1988).

Yet another approach is to covalently link specific compounds to amino acids to alter the physical/chemical properties of the protein. Glycosylation, covalently attaching a carbohydrate to a protein, can be done through the hydroxyl groups on serine and threonine or with the ϵ -amino group of lysine (Lis and Sharon, 1993). The latter approach is the most common as it can be done via the initial phase of the Maillard reaction (Monnier and Cerami, 1983). Wang and Ismail (2012) glycosylated whey proteins through the Maillard reaction with dextran, primarily at the ϵ -amino group lysine, but also at the imidazole group of histidine and indole group of tryptophan. Glycosylated whey proteins are more soluble than unglycosylated proteins around pH 4.5 and 5 (Wang and Ismail, 2012). The increase in

stability at pH 5 with partially glycosylated whey proteins is thought to be due to the increase in negative charge, creating greater electrostatic repulsion (Wang and Ismail, 2012). Two concerns with this method are increased browning and reduced digestibility. Browning can be reduced through extreme control of the Maillard reaction. Digestibility was found to increase according to Wang at Ismail (2012), although others have found glycosylation to reduced digestibility (Erbersdobler et al., 1981). The increase in digestibility was suggested to be due to the partial unfolding that occurs during glycosylation (Wang and Ismail, 2012).

5. Thermal Treatment

The degree of unfolding and the time for aggregation to occur is dependent on thermal treatment. At temperatures between 40-50 °C, and pHs above the isoelectric point, proteins typically increase in solubility until a denaturation temperature is achieved (Pelegrine and Gasparetto, 2005). Changes in structure lead to exposure of hydrophobic groups and other transformations that promote aggregation. These hydrophobic areas reduce solubility in water and individual proteins are therefore attracted to each other leading to aggregation. Individual proteins behave differently having different denaturation temperatures and also interact uniquely in combination with other proteins and ingredients (McGuffey et al., 2005; Mulvihill and Donovan, 1987).

5.1 Heating of pure β -lactoglobulin

β -lactoglobulin behavior is of great concern since it is the most predominate protein in whey protein ingredients and shows a high degree of aggregation when denatured. From a food-functionality perspective it should be emphasized that stability of a protein in a food systems is associated to the interactions among proteins caused by heat rather than a loss of tertiary and secondary structure, per se. It is recognized that β -lactoglobulin denatures around 70 °C at pH 7 (De Wit and Swinkels, 1980; Relkin and Mulvihill, 1996). At temperatures starting around 40°C, reversible structural changes occur to produce an intermediate molten-globule state that is partially unfolded (Kuwajima, 1989). The structure further unfolds with increasing temperature (Palazolo et al., 2000). At 40 °C β -lactoglobulin transitions to mostly in monomer form at neutral pH. Above 40 °C this monomer state is more open to interactions with other proteins and small molecular weight molecules (De Wit and Swinkels, 1980; Majhi et al., 2006). Increasing the temperature to 60 °C initiates structural changes in, which the tertiary structure unfolds and exposes hydrophobic groups (De Wit and Swinkels, 1980; Relkin and Mulvihill, 1996). As temperature approaches 70°C, more structural changes occur and irreversible aggregates are formed due to the exposure of hydrophobic patches and inter-molecular sulfhydryl-disulfide exchange (Iametti et al., 1998; Majhi et al., 2006; Verheul and Roefs, 1998a). As temperature increases, dimers, trimers, and eventually polymers are formed (Figure 1).

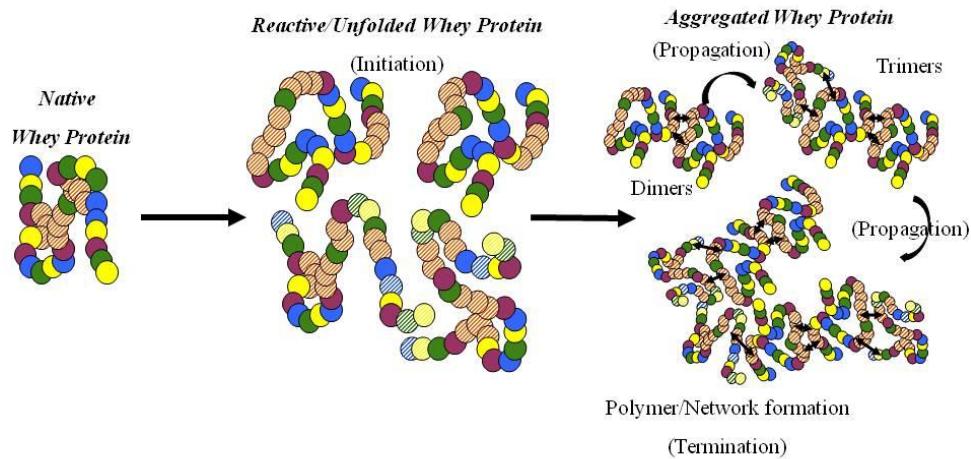


Figure 1.1 Whey protein aggregation

Polymers will eventually be produced, if two conditions are met: 1) a temperature of 75°C and 2) all monomeric proteins have been incorporated into either dimers or trimers (De Wit, 2009; Surroca et al., 2002). The mechanism of polymer formation involves the oxidation of sulfhydryls and sulfhydryl-disulfide exchange. Progressing above 85°C and up to 105 °C, hydrophobic interactions become less common, and instead disulfide aggregation dominates (De Wit, 2009).

The rate and degree of aggregate formation is also pH dependant. With increasing pH above the isoelectric point, denaturation temperature decreases, the rate of aggregation increases, and aggregates also become increasingly irreversible (De Wit and Swinkels, 1980; Relkin and Mulvihill, 1996). Inversely, denaturation temperature increases when pH is below the isoelectric point (De Wit and Swinkels, 1980; Verheul and Roefs, 1998b), indicated by a shift of β -lactoglobulin denaturation temperature of 70°C at pH 6.7 (De Wit and Swinkels,

1980) to 85°C when at pH 3 (Relkin and Mulvihill, 1996). Typically thiols do not interact until thermal treatment is introduced to the system (Hoffmann and van Mil, 1999), but in some cases pH will affect disulfide interactions (Surroca et al, 2002). At pHs close to or above the pKa of cystine, 9-9.5, sulfhydryl groups are more reactive (McGuffey and Foegeding, 2001), and therefore will be involved in aggregation.

5.2 Heating of pure α -lactalbumin

α -lactalbumin denatures at 64 °C, above this temperature denaturation begins to occur, creating a molten globule state (Dagleish et al., 1997). Aggregation of denatured α -lactalbumin progresses at a much slower rate than denatured β -lactoglobulin (McGuffey et al., 2005), since α -lactalbumins lack free thiol groups (Dagleish et al., 1997). Additionally, denatured α -lactalbumin is less prone to aggregation than β -lactoglobulin and has the ability to successfully re-nature upon cooling (De la Fuente et al., 2002). Above 85 °C, disulfides break down exposing free thiol groups, allowing irreversible aggregation and rearrangement of disulfide bonds (Chaplin and Lyster, 2009; Hong and Creamer, 2002; McGuffey et al., 2005). Once the molten globule state is exceeded 85°C, disulfides begin to rearrange creating a non-native form of α -lactalbumin and molecules begin to interact with neighboring α -lactalbumins in the molten globule state. Pure α -Lactalbumin is less prone to aggregate with itself in pure solution compared to β -lactoglobulin. In the presence of β -lactoglobulin α -Lactalbumin becomes more likely to form aggregates with itself and β -lactoglobulin (Havea et al., 2001; Hong and Creamer, 2002; McGuffey et al., 2005).

5.3 Heating mixtures of whey proteins

Aggregation of whey protein at 75°C begins with the formation of reactive β -lactoglobulin monomers and unfolding of α -lactalbumin molecules. Interactions start off as reversible non-covalent bonds including electrostatic, hydrophobic, and van der Waals interactions. As the interactions progress with increasing temperature and time, α -lactalbumin continues to interact in this fashion, while β -lactoglobulin begins to also interact via thiol/disulfide exchanges. Initially, α -lactalbumin does not participate in this exchange since it has no free thiols, therefore α -lactalbumin is more stable against the thiol/disulfide exchange than β -lactoglobulin. In a mixed solution, α -lactalbumin is more susceptible to disulfide exchange and after an initial lag phase will undergo thiol/disulfide exchange (Havea et al., 2001). As the ratio of β -lactoglobulin/ α -lactalbumin in a formulation increases, the thermal aggregation increases, and the rate of α -lactalbumin aggregation increases in comparison to its pure state, but the rate of β -lactoglobulin remains the same as its pure state (Dalglish et al., 1997; McGuffey et al., 2005).

More comprehensive investigations have examined the mechanisms of interactions in whey protein solutions heated at 60-100°C. At these higher temperatures β -Lactoglobulin dimers dissociate into reactive monomers leading to several stages, in which free thiols react with undenatured β -lactoglobulin or unfolded α -lactalbumin molecules. This results in a mixed population of homo (β -lactoglobulin alone) or hetero (β -lactoglobulin and α -lactalbumin) dimers (Bauer, 1998).

Without β -lactoglobulin and other proteins such as BSA, α -lactalbumin would not form irreversible dimers (de la Fuente, 2002, Hines and Foegeding, 1993). In the presence of other proteins, α -lactalbumin will form dimers through disulfide bonding, but primarily α -lactalbumin interacts through hydrophobic interactions unless temperature approach 100°C (Chaplin and Lyster, 2009; Hong and Creamer, 2002). As heating time increases, aggregation progresses from unfolding of monomers to formation to dimer, trimer, tetramer, oligomers, and finally polymers. For example after 15 minutes of thermal treatment at 63 °C at pH 6.7, trimers, form and reach a maximum after an hour, at which point oligomers, >300 kDa, are formed (De la Fuente et al., 2002). A general model for aggregation can be seen in Figure 1. This model is similar to polymerization models introduced by others such as Roefs and de Kruif (1994) and Prabakaran and Damodaran (1997) for β -lactoglobulin, which propose three main stages; initiation, propagation, and termination. In Roefs' model *initiation* involves multiple reversible reactions, but truly begins with a first order reaction involving a pseudo-reversible development of reactive sulfhydryls. *Propagation* occurs between a reactive, denatured, monomer/intermediate and an undenatured monomer. At any point only one sulfhydryl, per monomer, is available to interact, therefore aggregates form linear polymers that can be defined as flexible rods composed of semi-spherical monomers. Propagation will continue until *termination* is reached when all reactive intermediate have reacted with one another, recall this process is limited by the availability of one sulfhydryl per intermediate (Roefs and Kruif, 1994).

5.4 Improving Thermal Instability

As mentioned in section 1.2, thermal stability in beverages is achieved when aggregates remain dispersed, at a desired level of opacity and viscosity, for a desired length of time. In other words, it is a form of operational stability defined by physical properties associated with beverage quality. Therefore, the solution to producing a stable beverage is to control aggregation under thermal processing conditions (time and temperature) required to achieve product safety.

Individual protein concentration effects- The presence of multiple proteins in solution can alter stability. Examples include, but are not limited to, the increase in susceptibility of α -lactalbumin in the presence of β -lactoglobulin, as discussed in section 1.4.2, or the concentration effect of BSA which begins forming disulfide bound aggregates before β -lactoglobulin or α -lactalbumin (Havea et al., 2001). The amount of individual proteins in a whey protein ingredient is one way to alter stability to manipulate stability (Wang and Lucey, 2003). This can be done by a combination of increasing protein structural stability (e.g., increased denaturation temperature) and decreasing aggregation propensity.

Co-solute effects - One approach is to add co-solutes that minimize denaturation, aggregation or both. For example addition of sorbitol and glycerol improve heat stability in 10% β -lactoglobulin solutions by altering denaturation temperature: 75°C without co-solute, 82.2°C with sorbitol, and 76.8°C with glycerol (Chanasattru et al., 2007).

Glycosylation - As noted earlier, Wang et al. (2010) showed glycosylation of whey proteins to increase heat stability, as well as pH stability. Gelation was reduced near the isoelectric point and clarity maintained. This is most likely due to the increase in steric stabilization, which is where the surface has almost linear extensions of solvent-loving molecules extending from the surface. When the linear molecules from two proteins overlap, the local increase in concentration causes solvent to move in to dilute the local concentration, preventing aggregation (Hooper and Schweizer, 2005; Napper, 1977). Glycosylation also increased denaturation temperature of proteins by 15-18°C.

Formation of stable aggregates - The creation of soluble aggregates through treatment of 7% WPI (90°C for 10min, pH 6.8) was also found to increase heat stability (Ryan et al., 2012). The nano-scale particles formed are thought to be less prone to secondary interactions because they have are stabilized by greater electrostatic repulsion with less hydrophobic attractions (Ryan et al., 2012). The combination of preheating followed by cross-linking via transglutaminase, stabilized whey proteins around pH 5 (Lorenzen, 2007). At pH 5 whey proteins typically are not stable since this is near the average pI, but the combine thermal/crosslinking treatment prevents unfolding, reducing exposure of free thiol groups and therefore aggregation (Lorenzen, 2007).

Recently, Zhang and Zhong (2010) also developed a unique method to increase heat stability by forming nano-scale whey protein particles. This was accomplished by first forming water (whey protein solution) in oil emulsions, followed by heating to form particles, and

recovering and drying the particles. It was hypothesized that the heat treatment creates irreversible disulfide bonds that prevent unfolding and aggregation upon further heat treatment (Zhang and Zhong, 2010).

6. Calcium

Some salts in small concentrations can have a stabilizing effect on whey protein, but the levels required for the majority of nutritional applications result in destabilization of proteins (Mulvihill and Kinsella, 1988; Zhu and Damondaran, 1994; Varunsatian et al., 1983).

Calcium presents a multifactor problem because 1) it is inherent in whey so the amount present in a whey protein ingredient is controlled by the ingredient process, 2) it is a divalent cation that is very effective in promoting aggregation, and 3) it is desirable from a nutritional standpoint. The presence of calcium in whey protein solutions induces aggregation and decreases heat stabilization. Particularly above pH 6, calcium decreases denaturation temperature by 2-4 °C (Varunsatian et al., 1983) and increases aggregation (Towend and Gyuricsek, 1974) .

The mechanism by which calcium acts on whey protein is debated and several pathways of aggregation have been proposed. Calcium has been proposed to: 1) form bridges between negatively charged β -lactoglobulin monomers, 2) shield charges of β -lactoglobulin, or 3) interact at specific binding sites and promote or stabilize structures that favor aggregation (Kinsella et al., 1989; Ju Kilara, 1998; Simon et al., 2002). Mechanisms of screening charge

and site specific binding were observed by Simons et al. (2002) who used succinylation and methylation of whey protein to show that crosslinking was not likely. Although at low calcium concentration (5mM CaCl₂) Mulvihill and Kinsella, (1998) observed crosslinking of proteins, but charge screening to be the dominant mechanism at concentrations above 5mM CaCl₂.

6.1 β -lactoglobulin

Calcium induces aggregation of all whey proteins, but has the greatest effect on β -lactoglobulin (Varunsatian et al., 1983). When approaching the study of calcium interactions with proteins, both structure stability and aggregation rate have been studied. It should be noted that, for increased protein content, structure stability is increased in the presences of calcium (Varunsatian et al., 1983). This indicates that to a degree calcium has a protective effect on the native structure of β -lactoglobulin. After a sufficient amount of calcium is added to the system (Sherwin and Foegeding (1997) suggested 3.33-23.3 CaCl₂ (mM)/protein (%)) destabilization occurs through internal binding of calcium to native β -lactoglobulin altering and reinforcing the structure (Petit et al, 2011). Calcium also catalyzes both the unfolding and aggregation of β -lactoglobulin (Li et al. 1994). Arrhenius plots of β -lactoglobulin suggest a reaction rate order of 1.5, with two mechanisms of destabilization occurring at different temperatures. Structural breakdown occurs up to 80°C, after which aggregation is increased and disulfides form (Petit et al, 2011).

6.2 α -lactalbumin

Unlike β -lactoglobulin, α -lactalbumin actually requires calcium for structural stability (Bernal and Jelen, 1985). The four disulfide bonds in α -lactalbumin are dependent on the presence of a bound calcium. Removing calcium and forming the apo state allows for a random mixing of disulfide bonds (Rao and Brew, 1989). This can be seen as the biological activity of α -lactalbumin is lost when the calcium is removed and not available for refolding of the protein (Kuwajima, 1989). Calcium is thought to reduce charge repulsion in the bend of the binding site, which supports appropriate cysteine pairing. With removal of calcium, the bend will not form, leaving an open structure that is more prone to aggregation (Rao and Brew, 1989).

6.3 All Whey Protein

Since α -lactalbumin and β -lactoglobulin are stabilized with small amounts of calcium; it would be suspected that small amounts of calcium would stabilize whey protein ingredients in the same manor, which is found to be true. Work done by Varunsatian et al. (1983) showed that increasing ionic strength with calcium between 0.03-0.05 stabilized whey protein, through reduction in aggregation and denaturation of 1% WPC. The effect was seen with other salts, but stabilization was greatest with calcium. Greater amounts of calcium, at ionic strengths above 0.05, destabilized the proteins. The binding/interaction between

calcium and proteins increases as pH increases to more alkaline pHs (Varunsatian et al, 1983). Calcium destabilization for whey protein increases with increasing severity of heat treatment (Ju and Kilara, 1997). Aggregation was not linearly related to calcium concentration but instead dependant on the ratio of calcium to protein (Sherwin and Foegeding, 1997; Ju and Kilara, 1997). As briefly mentioned in section 6.1 Sherwin and Foegeding (1997) observed a maximum aggregation rate between 3.33 and 23.3 CaCl₂ (mM)/protein (%), and excess calcium did not increase aggregation.

It was found that a certain ratio of calcium to whey protein results in negative effects by Ju and Kilara (1997). Initially a constant calcium concentration was added to 5, 15, and 20% whey protein solutions and only 5% whey protein solutions resulted in increase turbidity, but when studies were done according to protein to calcium ratio, turbidity effects were seen.

6.4 Reducing Calcium Thermal Instability Effects

Resolving calcium induced aggregation proves an immense challenge. Regardless of the process used to produce a whey protein ingredient, it is inevitable that a small amount of calcium remains in the dried ingredient. Industry also must cater to the desire for nutritional beverages that contain both whey protein and calcium. One solution to the problem is to bind the calcium so that it is unavailable to interact with whey protein, but available to the human body. Chelators such as ethylenediaminetetraacetic acid (EDTA) and sodium citrate are the

most commonly explored sequestering agents for beverage applications (Deshpande et al., 1995). Utilization of chelators for prevention of calcium-associated protein aggregation has only been used to study mechanism of calcium in protein gelation (Keowmaneechai and McClements, 2002; Kuhn and Foegeding, 1991a). EDTA is an effective chelator of calcium and reduced gel network formation and aggregation caused by the presence of calcium (Keowmaneechai and McClements, 2002; Kuhn and Foegeding, 1991a).

7. Astringency

As mentioned previously, one of the challenges in making palatable, clear/translucent whey protein-containing beverages is the level of astringency when pH is ≤ 3.5 (Beecher et al. 2008). Astringency is characterized as a sensation of dryness of oral surfaces and/or puckering/tightening of the mucosa (Lee and Lawless, 1991, Bate-Smith, 1954). It can also be understood as the sensation of roughness when the tongue is moved laterally, due to friction (Smith and Noble, 1998). Astringency is thought to be due to interactions between compounds and saliva proteins and/or the mucosa surface. This is supported by Guinard et al. (1986), who gave evidence that upon exposure of consecutive astringency samples, de-lubrication occurred, but lubrication did increase when given recovery time. Astringency can be seen in other foods such as wine, green tea, and fruits. This astringency is associated with precipitation of saliva proteins with polyphenol compounds.

Beecher et al. (2008) proposed that astringency of whey proteins is due to the interaction between positively charged whey proteins and negatively charged saliva proteins. This was supported by increased turbidity of saliva and whey protein mixtures at low pH (Beecher et al. 2008). Ye et al. (2012) also proposed that astringency is associated with interactions of salivary proteins and positively charged whey proteins. This was reinforced by Kelly et al. (2010) and Ye et al. (2012) by showing that lactoferrin is astringent at pH 7. Lactoferrin has an isoelectric point of 8.7 so it is positively charged at pH 7 and therefore upholds the concept of electrostatic interactions between beverage proteins and saliva proteins causing astringency (McKenzie, 1970).

It has long been established that proline rich proteins (PRP) cause astringency of polyphenols (Baxter et al., 1997; Lu and Bennick, 1998; Luck et al., 1994). In particular to polyphenol-based astringency, proline rich saliva proteins are said to act as sticky patches attracting the polyphenols resulting in aggregation (Luck et al., 1994).

Building on the observation that whey proteins are astringent at pH values where the net charge is positive, a general associative polymer-polymer interaction is proposed to be involved. This hypothesis coincides with the behavior of chitosan, a positively charged polysaccharide at pH 7.0, being very astringent (Rodriguez et al., 2003),

Astringency is difficult to study since imitating the oral cavity is complicated due to individual differences in saliva proteins and saliva flow (Kelly et al. 2010). Groups of people

with higher flow rates have slower onset of perceived astringency (Kallithraka et al., 2001). A fully agreed upon mechanism(s) of astringency remains elusive, in part because it can be caused by a variety of compounds, may not be one sensation, and involves interactions with oral surfaces that are difficult to investigate. Often, astringency assessed by a sensory panel is correlated with *in-vitro* tests, to allow for more rapid screening. One such test is measuring the increase of turbidity when an astringent compound is mixed with salivary proteins (Horne, 2002, Monteleone, 2004). This test is based on the assumption that, turbidity indicates protein aggregation, since increases in turbidity increase in mean particle size and therefore astringency.

7.1 Minimizing Astringency

To our knowledge, there are no studies showing how protein-based astringency can be significantly reduced at pH 3.4. Some studies attempt to imitate solutions that are successful in reducing polyphenol astringency, and prove to be fruitless. One example is increasing viscosity, since Smith and Noble (1998) found increasing viscosity to 1-9 mPas to reduce astringency of tannins. Unfortunately, this was found to be unsuccessful for whey proteins (Beecher et al., 2008), but it should be noted that the viscosity range is specific to beverages and greater viscosities may reduce astringency of protein solutions.

8. Conclusion

Whey proteins are functionally diverse ingredients; therefore mechanisms of stabilization in beverage applications are extremely complex. However, the ability to reduce heat-induced aggregation by chelation of calcium and decrease astringency by decreasing whey protein-saliva proteins interactions are plausible approaches that will be explored in this thesis.

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CHAPTER 2: INCREASING WHEY PROTEIN HEAT STABILITY THROUGH CALCIUM CHELATION

1. Introduction

Whey protein ingredients contain various amounts of non-protein components, including calcium salts. The functional properties of whey proteins, such as foaming, gelation, and solubility, are affected by the presence of these salts (Ju and Kilara, 1998; Kuhn and Foegeding, 1991b; Varunsatian et al., 1983; Zhu and Damodaran, 1994). The desire to have protein and salts combined for nutritional purposes is a challenge to utilization of whey protein in high protein beverages. Comparing calcium, magnesium, and sodium salts, calcium causes the greatest decrease in denaturation temperature, i.e. greatest reduction in stability (Varunsatian et al., 1983). To ensure product safety and extend shelf life, beverages require a thermal treatment during processing. The time/temperature combination used dependent on if the product is designed to be refrigerated or shelf stable. Heating causes protein denaturation (unfolding); and the denatured structure is more prone to aggregation. Salts decrease electrostatic stabilization and thereby increase aggregation. Aggregation, which is diffusion controlled and time dependent, can take place only after protein unfolding takes place, which is very rapid and determines the potential for intermolecular interactions. Work by Petit (2011) suggests that calcium increases the rate of aggregation, having less of an effect on the rate of unfolding of β -lactoglobulin. Interactions which facilitate aggregation include 1) calcium bridging of adjacent negatively charged whey proteins, 2) charge

screening effects, 3) and site-specific binding (Kinsella et al. 1989, Ju and Kilara 1998, Simon et al. 2002). Work by Xiong (1992) and Simons et al. (2002) validates the charge screening mechanism, but not crosslinking. If calcium crosslinking was the main mode, less calcium would be required to bind proteins with greater affinity for each other, but such was not the case (Simons et al., 2002; Xiong, 1992). The third possible association between calcium and whey proteins is site specific interacts. These interactions take place at free carboxylic groups on aspartic and glutamic acid (Brew et al., 1970).

The role of calcium in aggregation of whey proteins has been investigated from the perspective of characterizing the aggregation mechanism and properties of the aggregates. The goal of this study is to maintain calcium in beverages but prevent it from facilitating protein aggregation. The simplest way to reduce the effect of calcium on aggregation is to simply not have calcium in the system. However, there is a need to have calcium present in beverages, say for nutritional purposes, but at the same time diminish aggregation.

In other applications, such as protein gelation, chelators have been used to control the availability of calcium. These studies typically use EDTA and sodium citrate, common chelating agents utilized for beverage applications (Deshpande et al., 1994). Other chelating agents, such as ethylene glycol tetraacetic acid (EGTA) and sodium tripolyphosphate (TPP) have also been investigated. For instance, the addition of 10 to 50 mM EDTA reduces the hardness and strength of whey protein gels (Smith and Rose, 1994). In other work EDTA and citrate were shown to reduce aggregation, resulting in weaker gels (Keowmaneechai and

McClements, 2002; Tsuge and Koyama, 2007). Of the two chelators EDTA was more effective at chelating calcium than citric acid, since it binds calcium at a much faster rate (Keowmaneechai and McClements, 2002). Regardless of compound, the reduction of gel formation is possibly explained by a lower degree of aggregation when calcium is chelated, and unavailable to the protein.

Chelators inhibit aggregation by binding the calcium, but also increase ionic strength which favors aggregation. The level of ionic strength determines, in part, the degree of electrostatic repulsion. As ionic strength increases, proteins solutions will become less stable to heat and transition to more turbid solutions and eventually precipitation will occur (Ako et al., 2009).

In the absence of other ions, calcium will cause a concentration-dependent increase in the of denatured protein rate of aggregation. Therefore, under fixed heating temperature/time conditions, there will be an increase in the extent of aggregation in the presence of calcium. It is proposed that adding a chelator will decrease free calcium and alter the aggregation rate. If the mechanism is ionic strength driven, then successful chelators must act by decreasing ionic strength. If it is calcium specific, then it will not follow a simple ionic strength trend. Regardless of ion type, once a maximum ionic strength is achieved there will be no benefit of chelating calcium.

2. Methods

2.1 Materials

All chemicals used were reagent grade. Ethylenediminetetraacetic acid (EDTA) was obtained from Fischer Scientific (Fair Lawn, NJ, USA), calcium chloride from The British Drug Houses (West Chester, PA, USA), and sodium hexametaphosphate (SHMP) from Sigma-Adrich (St. Louis, MO, USA). BiPro, Whey protein isolate (WPI) was gifted from Davisco Foods International (Le Sueur, MN, USA). Three batches used contained 93.7%/750ppm, 94.4%/579ppm, 92.9%/605ppm protein/calcium content. Protein and calcium content was determined by inductively coupled plasma spectroscopy nitrogen analysis, and inductively coupled plasma emission spectroscopy respectively.

2.2 Solution Preparation

Stock solutions of 10% w/v protein were prepared by mixing in deionized water ($>17\text{M}\Omega$) and stirring overnight. Stock solutions of 100mM EDTA and 100mM SHMP were also prepared. Working solutions were made by mixing stock solutions and deionized water to produce final concentrations of 4 % w/v protein and a range of chelator and calcium

concentrations. A study comparing 5, 6 and 7 % w/v protein content was also conducted. The pH of all solutions were adjusted to 7 using 1 N HCl/NaOH

2.3 Thermal Treatment

Two thermal treatments were used to cover requirements for refrigerated or room temperature beverage sales. Conditions for high temperature short time (HTST) were heating samples in a water bath at 70°C for 165 sec; and ultra high temperature (UHT) treatment was attained by heating samples in an oil bath at 143 °C for 60 sec. It should be noted these conditions were validated by assuring the internal temperature of the tubes reached necessary temperature. After heating, samples were immediately put on ice for 2 minutes, and cooled overnight at 4°C.

2.4 Turbidity

The turbidity of the solutions was measured one the following thermal treatment using a Hach 2100AN turbidimeter (Hach, Loveland, CO). The turbidimeter measures scattered light by measuring light scattered at 90° between the light source and photodetector. The turbidity was measured in 12 mm internal diameter tubes in which the solutions were heated; allowing for measuring the turbidity of fluid or solid (gelled) samples. Turbidity values are reported in

nephelometric turbidity units (NTU). All samples were equilibrated to room temperature before measuring. A value of <200 NTU was set as the cut off for maximum acceptable turbidity.

2.5 Free Calcium

Free calcium was measured using an Accumet® ion selective calcium probe. Ionic strength adjustment solution was acquired from Fischer Scientific (Fair Lawn, NJ, USA) and was added to each sample at a concentration of 2% v/v to account for any other ion interactions.

3. Results

The objective was to evaluate a range of chelating compounds regarding their ability to bind calcium and reduce protein aggregation in heated whey protein solutions. To begin, sodium citrate, EDTA, fish gelatin hydrolysate, SHMP and medical chelators, those used for treatment of metal toxicity, were evaluated for potential to reduce heat induced aggregation. Heated samples containing 20 mM CaCl₂ SHMP, sodium citrate or EDTA, had reduced turbidity in comparison to controls containing no chelators (data not shown). Medical chelators, lactobionic acid and D-penicillamine, either increased turbidity significantly or gelled solutions. EDTA and sodium citrate are sequestering agents which are well known for use in

the food industry (Deshpande et al., 1994). Although both EDTA and sodium citrate were found to bind calcium at 1:1 ratio by isothermal titration calorimetry, EDTA binds calcium at a faster rate (Keowmaneechai and McClements, 2002). Therefore, SHMP and EDTA were chosen for further analysis based on their ability to sequester calcium and reduce turbidity.

3.1 Chelators

The goal of the first experiment was to determine the effect of chelators alone, without added calcium. A range of concentrations of EDTA or SHMP were added to 4% w/v WPI solutions and heated. At lower concentrations the chelators were effective under HTST heated solutions, but they began to exceed 200 NTUs between 80-100 mM added EDTA, and as low as 20-40 mM for added SHMP (Figure 2.1); both of these chelators concentrations are around an ionic strength of 0.8 M. Ignoring the innate 0.57-0.75 mM calcium of WPI ingredients used. The low level of calcium was not enough to increase aggregation so there was no reduction in turbidity for either chelator. This treatment revealed the maximum amount of chelator that can be added to WPI before excessive turbidity (turbidity >200 NTU) occurred. Chelators combined with whey, sans calcium, favors aggregation with increasing concentration; this could be due the increasing ionic strength associated with increasing chelator concentration (Figure 2.1). Beyond an ionic strength of 0.8 M the system appears to be over loaded and destabilized. Trends of EDTA and SHMP appear slightly different from each other, but this could be since the same ionic strengths were not measured for each chelator or because of different interactions between each chelator and protein.

3.2 Efficacy of EDTA and SHMP

The amount of chelator needed to inhibit aggregation of a fixed amount of calcium was investigated. In the presence of 20 mM CaCl₂, varying amounts of chelator were added to 4% w/v protein solutions and heated. Control samples containing 20 mM CaCl₂ and 4% w/v protein gelled after exposure to HTST and UHT heat treatments. A critical chelator concentration range was observed (Table 2.1). Below 20 mM EDTA was ineffective, but turbidity remained lower than the control at concentration, up to 60 mM added EDTA. The minimum concentration of SHMP to see a significant drop in turbidity was 10 mM, and turbidity remained below 200 NTUs up to 55 mM. For both HTST and UHT treatments, SHMP was more effective at reducing calcium-associated aggregation at chelator concentrations below 60 mM. In HTST treated samples, above 60 mM added SHMP, stability began to decrease in comparison to EDTA. Contrary to results with no added calcium, SHMP solutions with as little as 40 mM added SHMP were beyond 200 NTU. This suggests alternate interactions are occurring, possibly complexes between SHMP and calcium are what prevent aggregation by reducing net ionic strength on whey proteins, but at higher concentrations contribute to destabilization.

As discussed for Figure 2.1 reduction in chelator efficacy at higher concentrations is most likely due to increased ionic strength, since whey proteins are stabilized by electrostatic repulsion. Additionally, free calcium data suggests there is a critical amount of free calcium, below which turbidity will be reduced. There was 1.4 mM free calcium with the addition of

10 mM of EDTA, but only 0.09 mM free calcium with the addition of 10 mM SHMP (Table 2.2). Free calcium results follow turbidity trends, suggesting the initial turbidity reduction is due to binding of calcium. The positive effect of EDTA and SHMP is proposed to be due to calcium no longer being available to screen charge, maintaining electrostatic stabilization and reducing formation of insoluble aggregates that cause turbidity.

3.3 SHMP vs Calcium Concentrations

A range of CaCl_2 and SHMP concentrations were evaluated (Table 2.3). Results again support not just calcium chelation, but also ionic strength and chelator interactions with whey protein contributing to stability. In protein solutions containing 10 mM CaCl_2 , just 10 mM SHMP was sufficient to reduce turbidity below 200 NTUs, but 30 mM SHMP and above resulted in high opacity or gelation. As calcium concentration increased the turbidity also increased in all samples, suggesting a critical SHMP to calcium ratio might be required to counter act calcium effects.

3.4 Sodium Hexametaphosphate Efficacy vs Protein Concentrations

The overall goal is to incorporate as much protein into beverages as possible, while maintaining stability. Therefore, stability of protein solutions containing 5, 6, and 7% w/v whey protein were investigated to determine the maximum protein concentration that could

be stabilize by SHMP (Table 2.4). Addition of 10 mM SHMP reduced turbidity at all three protein concentrations. Interestingly there was no trend seen between protein concentration turbidity at 10 mM added SHMP 4 % w/v WPI. 6% w/v WPI solution had the highest turbidity, and turbidity decreased at 7% w/v WPI. This suggests that after a certain amount of protein is added and any addition to the system might be having a minimal effect, due to crowding of the system. The crowding will reduce interactions between any added compounds and whey protein.

4. Discussion

4.1 Chelators

Chelators alone have the ability to decrease electrostatic stabilization by adding ionic strength. This resulted in a significant level of aggregation producing turbidity above 200 NTU at concentrations above 40 mM for SHMP and 80 mM for EDTA, equating to an ionic strength of 0.8. Addition of chelator to the whey protein-calcium samples prevented gelation that occurred in the control samples containing no chelator (Figure 2.1). The lowering of turbidity along with free calcium data suggested chelation of calcium has a vastly different effect than the calcium or chelator alone on proteins. It was shown that SHMP was more effective at lower concentration, possibly due to its ability to bind calcium at a 3:1 ratio (De Kort et al., 2009) and EDTA typically binds calcium at a 1:1 ratio (Keowmaneechai and

McClements, 2002). Therefore, it would be expected that 20 mM of EDTA would be required to bind 20 mM of CaCl₂, but only ~6.6 mM SHMP would be required to bind the same amount. Beyond this concentration chelator would be in excess contributing to ionic strength, assuming chelators bind to available calcium. Free calcium results indicate SHMP indeed is more effective at binding calcium at lower levels.

Above 60 mM SHMP, stability was lost, likely due to increased ionic strength. A similar phenomenon was also observed by Mizuno and Lucey (2007) when adding SHMP to milk protein concentrate, which contains calcium. There was a shift in the type of calcium complexes formed, between low ($\leq 0.1\%$) and high ($\geq 0.5\%$) SHMP concentrations. In this study, a shift away from soluble calcium phosphate complexes was hypothesized at low concentrations. This could also be the case for WPI and SHMP systems, where different calcium-SHMP complexes are formed at higher concentrations. Since, in the current work trends were seen between free calcium, and ionic strength, but data is still parabolic suggesting other interaction could be taking place. Further investigations of protein concentrations effects also suggest this hypothesis (see next section).

4.2 Sodium Hexametaphosphate Efficacy at Higher Protein Concentrations

Protein concentration was seen to have a non-linear effect on aggregation. Protein-calcium interactions appear to peak in a particular calcium-protein range suggesting a ratio effect, which was also found by Sherwin and Foegeding (1997). Sherwin and Foegeding (1997)

observed a maximum rate of aggregation between 3.33 to 23.3 CaCl₂ (mM)/protein(%). Similar trends might be concluded in current work, but cannot be directly compared since rate of aggregation was not measured and the true amount of available calcium to protein ratio is not known given the presence of chelators.

5. Conclusion

Calcium can be chelated using SHMP or EDTA to increase thermal stability of whey protein beverages. Whey protein solutions containing 4% protein and 20mM CaCl₂, which normally gel upon exposure to heat, were stabilized to liquid form and brought below 200 NTUs using 20-60mM EDTA or 10-55mM SHMP. The most effective chelator was SHMP which only required 10 mM to reduce turbidity below 200 NTU for solutions containing 20 mM CaCl₂. Trends in ionic strength and free calcium indicated calcium specific interactions as well as ionic strength to be the mechanism for reduced turbidity.

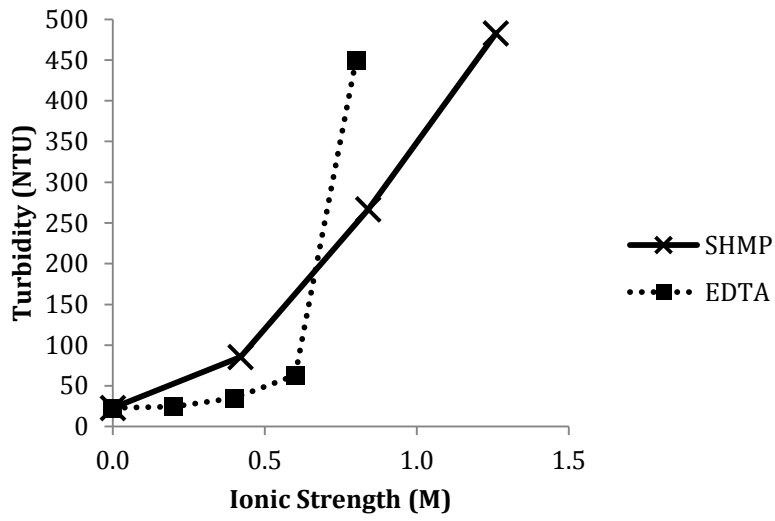


Figure 2.1. Turbidity of 4% w/v protein WPI solutions containing various amount of SHMP and EDTA heated at HTST.

Table 2.1. Turbidity of heat treated 4% WPI solutions containing 20 mM CaCl₂ and a range of EDTA and SHMP concentrations.

Added Chelator (mM)	EDTA		SHMP	
	Turbidity (NTU)		Turbidity (NTU)	
	HTST	UHT	HTST	UHT
0 (no CaCl ₂)	23 ± 3 ^c	62 ± 17 ^e	23 ± 3 ^c	62 ± 17 ^e
0 (with CaCl ₂)	Gel	Gel	Gel	Gel
5	Gel	Gel	268 ± 21 ^a	454 ± 180 ^f
10	Gel	Gel	67 ± 2 ^e	222 ± 51 ^a
15	5700 ± 103 ^g	243 ± 53 ^a	28 ± 3 ^c	77 ± 28 ^e
20	29 ± 2 ^c	252 ± 32 ^a	26 ± 2 ^c	166 ± 26 ^h
25	30 ± 2 ^c	690 ± 28 ^f	29 ± 3 ^c	164 ± 59 ^h
30	31 ± 2 ^c	Liquid	33 ± 4 ^c	Gel
35	39 ± 4 ^c	Liquid	37 ± 7 ^c	Gel
40	44 ± 5 ^{cd}	Gel	55 ± 7 ^{ed}	Gel
45	45 ± 5 ^d	Gel	75 ± 11 ^e	Gel
50	55 ± 7 ^{ed}	Gel	75 ± 7 ^e	Gel
55	57 ± 4 ^{ed}	Gel	74 ± 11 ^e	Gel
60	69 ± 6 ^e	Gel	273 ± 15 ^a	Gel

Grey indicates turbidity in within acceptable turbidity, 200 NTU range. Different letters indicate significant different between values.

Table 2.2. Free calcium in solutions with addition of chelator to 20 mM CaCl₂.

Added Chelator	<i>Free Calcium (mM)</i>	
	EDTA	SHMP
<i>10</i>	1.4 ^a	0.09 ^b
<i>20</i>	<0.01	<0.01
<i>30</i>	<0.01	<0.01
<i>40</i>	<0.01	<0.01
<i>50</i>	<0.01	<0.01

Different letters indicate significant different between values.

Table 2.3. Turbidity of a range of calcium and SHMP concentration in 4% WPI solutions, exposed to HTST treatment.

SHMP (mM)	0 mM CaCl₂	10 mM CaCl₂	15 mM CaCl₂	20 mM CaCl₂
0	23 ± 3 ^a	Solid	Solid	Solid
10	44 ± 5 ^{ab}	23 ± 2 ^a	34 ± 1 ^b	45 ± 3 ^b
20	50 ± 13 ^b	39 ± 11 ^b	49 ± 5 ^b	56 ± 2 ^b
30	184 ± 21 ^{cd}	57 ± 16 ^b	94 ± 18 ^{cd}	111 ± 29 ^{cd}
40	117 ± 77 ^c	223 ± 122 ^d	278 ± 162 ^d	405 ± 28 ^f
50	438 ± 25 ^f	455 ± 263 ^f	918 ± 204 ^g	1209 ± 321 ^g
60	938 ± 130 ^g	983 ± 69 ^g	Solid	Solid
70	Solid	Solid	Solid	Solid
80	Solid	Solid	Solid	Solid
90	Solid	Solid	Solid	Solid
100	Solid	Solid	Solid	Solid

Grey indicates turbidity in within acceptable turbidity, 200 NTU range. Different letters indicate significant different between values.

Table 2.4. Effects of protein concentration on turbidity of solutions containing 20 mM CaCl₂.

SHMP (mM)	Turbidity (NTU)		
	5 % WPI	6% WPI	7% WPI
0	Solid	Solid	Solid
5	Solid	Solid	Solid
10	128±78 ^a	193±52 ^a	160±51 ^a
15	108±37 ^a	169±28 ^b	Solid
20	164±47 ^a	210±69 ^a	445±269(Solid) ^b

Grey indicates turbidity with in 200 NTU range, defined as translucent. Different letters indicate significant difference within rows

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CHAPTER 3: HYDROLYSIS OF WHEY PROTEINS AS A WAY TO DECREASE ASTRINGENCY: PROOF OF CONCEPT

1. Introduction

Whey protein beverages have become popular among individuals seeking specific health effects, such as a protein source for muscle recovery after strenuous exercise, but also among the general populations seeking a more healthy diet. Studies show translucent beverages are perceived to quench thirst more efficiently than opaque beverages (Beucler et al., 2006). This is a challenge with protein-containing beverages where aggregation decreases clarity. To most effectively achieve translucent beverages with whey protein, a pH below 3.5 is generally used (Andrewes et al., 2011; Beecher et al., 2008; Kelly et al., 2010). This is because at pH 3.5 and below the whey proteins have a high net positive charge that resists aggregation facilitated by the presence of salts and thermal processing. The caveat is that at this pH whey proteins are astringent (Beecher et al., 2008).

It is currently not fully resolved whether astringency is a taste sensation (Breslin et al., 1993) or a tactile mouth feel (Schwarz and Hofmann, 2007) because of the many sensations that go into the cognitive representation of astringency. However, the tactile sensation is more commonly accepted, as seen in the American Society for Tasting and Materials (ASTM, 2004) definition as the combined oral sensations resulting from “shrinking, drawing, or puckering of the epithelium”. Astringency was first applied to the sensation accompanying

the consumption of polyphenols, but as mentioned, is also experienced when consuming acidic whey protein beverages.

The mechanism(s) of astringency are un-resolved and there are an array of different astringent compounds; with possibly different modes of astringency. The most explored mechanism is for polyphenol-associated astringency. The prevailing proposal is that astringent compounds bind with salivary proteins and by some mechanism cause astringency (Horne, 2002; Payne et al., 2009). There is debate on the mechanism of protein-based astringency. Acids alone causes astringency (Sowalsky and Noble, 1998) and this has led some to suggest simply that the acidity of whey protein solutions cause astringency (Lee and Vickers, 2008). An alternate mechanism independent of pH, involving interactions between negatively charged saliva proteins, and positively charged whey proteins is suspected. Whey proteins are positively charged below pH 5.2 as well as lactoferrin at pH 7. Work involving whey proteins, below pH 5.1, and lactoferrin, pH 6.8, found both to be astringency supporting interaction dependent on charge interactions between proteins and saliva (Streicher, 2010). Kelly et al. (2010) also found astringency reaches a maximum at around 4% protein and any additional acid, required to achieve desired pH in higher protein concentrations, was associated with increased sourness but not astringency. Instead it was suggested that astringency was dependant on a complex process involving buffering capacity of solutions, initial pH, and saliva interaction (Kelly et al., 2010). Furthermore, there is considerable support for a mechanism based on whey protein-salivary protein interactions, as seen with β -lactoglobulin forming aggregate with salivary mucins (Vardhanabhuti et al.,

2010). Beecher et al. (2008) observed a decrease in astringency of whey protein from pH 3.4 to 2.6, likely due to the reduction of negative charge associated with saliva inhibiting whey protein-saliva interactions.

At low pH, whey proteins are heat stable but, according to manufacturers, increased astringency with decreased pH is the major issue limiting their uses (Lemieux and Simard, 1994). Astringency is known as a defect in dairy products, but as with all defects, there is an acceptable level (Lemieux and Simard, 1994). Therefore, the aim is to reduce astringency to an acceptable level. It was thought that increasing viscosity, which is effective with astringency polyphenols, could be a solution. But increasing viscosity from 1.6 to 7.7 mPa had no effect on perceived protein astringency (Beecher et al., 2008), this does not exclude the possible inhibition of astringency at viscosities greater than 7.7 mPa. No studies have successfully reduced astringency at low pH, and due to the growth of functional beverages, a better understanding of astringency is required to produce better products.

If astringency is based on protein-protein interactions associated with their polymer properties, then reducing the size of protein polymers may be a way to reduce astringency. Enzymatic hydrolysis of proteins is a common unit operation used to modify protein functionality (Panyam, 1996; Schmidt et al., 1984; Siso, 1996), but it has not been explored as a solution to astringency. However, one limitation to food applications of hydrolysates is an increased level of bitterness (Ney, 1979; Adler-Nissen, 1976; Springett and Saxby, 1993)

Our hypothesis is that hydrolysis of whey proteins will decrease astringency at pH 3.5 but also increase bitterness. If this is proven true, then it indicates that enzymes and hydrolysis conditions can be developed that have the combined goal of 1) decreased astringency and 2) the lowest increase in bitterness. The goal of the current study was to investigate the process of hydrolysis at several time points to determine the relationship between astringency and bitterness.

2. Methods

2.1 Materials

Commercial whey protein isolate (WPI) was a gift from Davisco Foods International Inc. (Le Sueur, MN, USA), and contained 94.4 % protein, measured by inductively coupled plasma spectroscopy nitrogen analysis. Phosphoric acid (85% \, food grade) was obtained from Fischer Scientific (Fair Lawn, NJ, USA). Protease ProteAX was acquired from Amano Enzyme Inc. (PAX, Nagoya, Japan). Sucrose was purchased at the grocery store; food grade citric acid and caffeine were acquired from Sigma-Aldrich (St. Louis, MO, USA). Alum was also purchased from the grocery store (McCormick, Hunt Valley, MD, USA).

Carboxymethyl cellulose type 7 MF was acquired from Hercules-Aqualon (Wilmington, DE, USA).

2.2 Solution Preparation

Stock solution of 4% (w/w) protein was prepared and adjusted to pH 7.5 using 0.5 M NaOH. Solutions were then heated to 50°C, in a water bath, in preparation for hydrolysis, and enzymes were added at 84 u PAX/g of protein. Once 2 L of protein solution reached 50°C, protease was added and mixed. Hydrolysis was monitored over 2 hr incubation at 50 °C, with 500mL samples taken at hrs 0, 0.33, 1, and 2 and pH immediately adjusted to 3.4 to inactivate enzyme. To guarantee the inactivation of enzyme and imitate beverage thermal treatment, solutions were heated at 85 °C for 15 min in a water bath.

2.3 Degree of Hydrolysis

The degree of hydrolysis (DH %) was determined through trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen, 1979) and calculated using equation 1,

$$DH\% = 100 \left(\frac{AN_2 - AN_1}{N_{pb}} \right) \quad \text{Eqn. 1}$$

where AN_2 is the amino nitrogen content of the hydrolysate, AN_1 is the amino nitrogen content of the substrate before hydrolysis, and N_{pb} was 123.3 (Spellman et al., 2003), which

was the nitrogen content of the peptide bonds in whey protein. The amount of primary amines was determined at 340 nm using 0-2mM L-leucine as a standard curve.

2.4 Turbidity

The turbidity of solutions was measured using a Hach 2100AN turbidimeter (Hach, Loveland, CO). All samples were equilibrated to room temperature before measuring.

2.5 Descriptive Analysis

A panel of 10 females ranging from age 21-39 were trained to evaluate sweet, sour, bitter, and astringency using the Spectrum 15-point intensity scale (Meilgaard et al., 1999).

Panelists were calibrated for at least 20 hrs to evaluate astringency and bitterness. For panel training, sucrose was used as a sweet reference, citric acid as sour references, caffeine as bitter references, and alum for astringency references. Prior to sample evaluation panelist were calibrated with astringency standards, 2 (0.02% alum), 5 (0.05% alum), and 8, (0.08% alum) and bitter standards, 2(0.05% caffeine), 5 (0.125% caffeine), 10 (0.25% caffeine). For sample assessment, solutions were swished in panelists' mouths for 5 s, expectorated, and evaluated for astringency up to 30 s following expectoration. Between each sample, panelists

rinsed with water, took a bite of cracker, rinsed with 0.55% CMC, sipped water, and waited 2 min before tasting the next sample. No more than four samples were evaluated at one time to prevent panelist fatigue.

2.6 Statistics

Statistical analysis was performed using SAS version 9.2 (SAS Institute Inc, Cary, NC). Analysis of Variance (ANOVA) with t-test to test significance at $p \leq 0.05$ significance level was used to determine differences among treatments.

3. Results

3.1 Effect of degree of hydrolysis on astringency and bitterness

Varying degrees of hydrolysis were achieved to determine the effect on astringency of whey protein isolate. Samples were hydrolyzed for 0-2 hours resulting in varying degrees of hydrolysis 0, 4.6, 7, and 9.6 %. A trained descriptive analysis panel rated astringency and bitterness. As the degree of hydrolysis increased, astringency decreased and bitterness increased (Table 3.1). With hydrolysis, astringency was reduced from 6.5 (un-hydrolyzed) to 3 at 9.6% hydrolysis. As expected, bitterness increased with hydrolysis from 0.8 to 5.3. The

increase in bitterness is expected as the degree of hydrolysis increases the amount of bitter peptides. Bitterness is associated with small peptides, < 6 kDa, which can bind to bitter receptors (Ney, 1979). This highly negative correlation between bitterness and astringency indicates hydrolysis could be a potential method for controlling astringency and bitterness of whey protein solutions.

3.2 Turbidity

As discussed in Chapter 2, turbidity should be kept to a minimum to meet consumer needs.

Therefore turbidity was analyzed to determine potential for beverage applications.

Hydrolysis and/or the thermal treatment associated with the process, caused an increase in protein aggregation, as indicated by an increase in turbidity, turbidity remained below 100 NTU (Figure 3.2). Interestingly, the increase in turbidity peaked at one hour and decreased after two hours, but never returned to the original turbidity (Figure 2.2).

4. Discussion

4.1 Astringency and Bitterness

Our hypothesis was proven in that hydrolysis decreased astringency. This observation supports the protein-based astringency model describing astringency related to the polymer-polymer interactions caused at pH 3.5 and not the pH or acidity alone. All solutions were adjusted to pH 3.4. This adjustment required additional phosphoric acid, due to the increased buffering capacity of the hydrolysates, but astringency was inverse correlated with added acid so acid alone, clearly is not the cause of astringency. This would seem to disprove Lee and Vickers (2008) who state the added acid is what causes astringency.

Results not only prove that astringency can be control with hydrolysis, but also might begin to illuminate a more specific mechanism for astringency. Polyphenol astringency is reduced upon ripening of course fruit, due to polymer type properties in which polyphenols aggregate with proteins creating insoluble compounds. The insolubility removes the ability of polyphenols to react with salivary proteins and this is what it thought to cause the reduction in astringency (Ozawa et al., 1987). This can be viewed as a way of effectively removing the astringent compound. Inverse polymer effects may explain hydrolysis and the associated reduction in astringency. It is plausible that the smaller peptides have reduced astringency because they are less reactive with saliva proteins. Results suggest that whole proteins, or close in size to whole, are what induces astringency, since breakdown decreases astringency.

This does not preclude interactions with saliva proteins. Although these smaller peptides may interact with saliva, they may not be large enough to precipitate proteins, which is thought to be the cause of astringency.

4.2 Turbidity

As discussed in Chapter 2, turbidity is an important factor in determining beverage acceptance/liking by consumer. All samples remained below 100 NTU indicating they were close to the goal of below 40 NTU for a clear beverage (Rittmanic, 2006). Since only the unhydrolyzed samples maintained a “clear” appearance, future work on exact cause of aggregation would be important. Turbidity increase suggests an increase in protein-protein interaction, possibly due to exposed hydrophobics. Turbidity trends do not coincide with astringency, since it would be expected that these compounds be more astringent as more proteins are precipitated. But this again supports a protein size effect on aggregation and precipitation of saliva proteins, which is thought to cause astringency.

5. *Conclusion*

Astringency was effectively reduced through hydrolysis of whey protein. With increasing degree of hydrolysis, a greater reduction in astringency was observed. However, coinciding increases in bitterness indicated that hydrolysis needs to be precisely controlled to reduce

astringency with minimal increase in bitterness. This work provides a model that can help better understand the mechanism of whey protein astringency at low pH. It is argued whether the mechanism of astringency is purely pH dependant or more complex involving protein-saliva interaction and solution condition. Since all solutions were tested at pH 3.4, it can be suggested that astringency is affected by the buffering capacity of the protein solution and effect of structural changes induced by hydrolysis. Further work is needed to characterize the relationship between degree of hydrolysis and interactions with saliva proteins. Also other enzymes should be analyzed for ways to minimize bitterness.

Table 3.1. Astringency and bitterness associated with degree of hydrolysis

Degree of Hydrolysis (%)	Astringency	Bitterness
<i>0</i>	6.5±1.3 ^a	0.8±1.1 ^a
<i>4.6</i>	5.5±1.1 ^b	2.2±1.2 ^b
<i>7.1</i>	4.7±0.6 ^c	3.9±0.9 ^c
<i>9.6</i>	3.0±0.7 ^d	5.3±1.9 ^d

Different letters in the same column indicate significant difference at $p \leq 0.03$

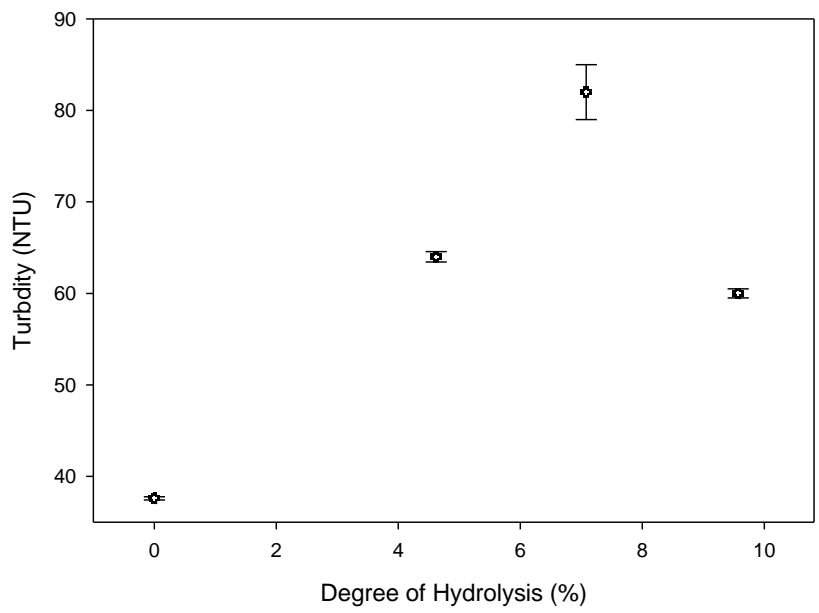


Figure 3. 2. Turbidity of hydrolysates.

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