The Effect of Limited Enzymatic Hydrolysis and Maillard-Induced Glycation on Sodium Caseinate Allergenicity, Bioactivity, and Functionality

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Lu Liang

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Dr. Baraem Ismail

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Dedication

This thesis is dedicated to my Mom and Dad for the amazing support of all my endeavors and the unconditional love.

Abstract

With superior functional properties, especially emulsification properties, high nutritional value, and excellent physiological benefits, sodium caseinate has been widely used in the formulation of many food and beverage products. However, the major drawback of the expanded utilization of sodium caseinate is its allergenicity. Therefore, in order to prevent a decrease in the market value of casein ingredients, different protein modification techniques have been explored to reduce the allergenicity of casein. Extensive enzymatic hydrolysis (> 25% DH) can reduce the allergenicity of a protein. However, this technique is detrimental to both flavor and functionality. Limited enzymatic hydrolysis, on the other hand, can maintain the functionality, but may only reduce the immunoreactivity to a certain extent. Another protein modification technique that may reduce the allergenicity of proteins is Maillard-induced glycation. Glycation, however, should be properly controlled to prevent the formation of mutagenic compounds and brown pigments with adverse effects on functionality, digestibility, and bioactivity. It was hypothesized that combining the two mechanisms, proteolysis and Maillard glycation, under controlled and mild conditions, will have a dual effect on reducing allergenicity by altering potential epitopes, while maintaining nutritional quality, bioactivity and functionality.

Thus, the objectives of this study were twofold: (1) Determine the effect of several proteolysis conditions and Maillard-induced glycation conditions on the allergenicity of sodium caseinate. (2) Determine the effect of the dual modification approach on the bioactivity and emulsification properties of sodium caseinate.

Sodium caseinate isolate (SCI) was hydrolyzed using three enzymes separately, alcalase, neutrase and trypsin, following the pH-stat method, while maintaining limited degree of hydrolysis (DH 3.6-17 %). Different enzyme hydrolysis conditions, including enzyme to substrate ratio, incubation temperature, pH, and time were investigated. The sodium caseinate hydrolysate (SCH) with the highest emulsifying properties (emulsification capacity, stability and activity) and ACE-inhibitory activity was tested for remaining allergenicity. The same SCH was further glycated with maltodextrins at 60°C,

water activity (a_w) of 0.49, 0.63, or 0.79, and a 2:1 ratio of protein to maltodextrin over a period of 24-120 h. Sodium caseinate isolate was also glycated to determine the effect of glcation alone on modifying emulsification properties and allergenicity. Loss in free amine groups along with the formation of Amadori compounds and browning were monitored. Flouresence compound formation was monitored to determine progression of the reaction into intermediate stages before formation of brown pigments. Urea-PAGE gel was utilized to confirm the glycation formation. Select glycated samples were tested for allergenicity, emulsifying properties, and ACE-inhibitory bioactivity. ELISA was performed to determine the immunoreactivity of the samples using sera from five allergenic donors following ELISA.

Trypsin was better than Alcalase and Neutrase in producing hydrolysates that retained emulsification properties. This might be due to trypsin's narrow and unique specificity, where large molecular weight peptides with better amphiphilic properties were generated compared to those generated by Alcalase and Neutrase. Limited hydrolysis (5% DH) resulted in pronounced ACE inhibitory activity and reduced immunoreactivity (6%-45%). Differences in the reduced immunoreactivity were attributed to the sensitivity of sera used and the variation in milk-protein-specific IgE among the different sera that could react with the epitopes present in casein proteins samples and newly generated epitopes upon hydrolysis. Among all the glycation conditions studied, optimum conditions for the desired objectives were determined to be 96 h of incubation at 0.63 a_w, in a 2:1 ratio of casein protein to maltodextrin, due to a moderate Amadori compounds formation, as noted by adsorbace at 304 nm, limited % amino group blockage (9.7%), minimal progression to intermediate stages, as noted by % fluorescence intensity, and limited progression to advances stages of the Maillard reaction, as noted by minimal browning measured at 420 nm. No significant ($P < 0.05$) effect on ACE inhibitory activity, emulsification properties and immunoreactivity was observed post glycation at at 0.63 a_w. This is likely due to insufficient Amadori compounds formation and the interference from free maltodextrin and unadsorbed intact sodium caseinate, which can destabilize the emulsion through depletion flocculation.

Results from this work, for the first time, provided valuable insights into the effect of proteolysis conditions and Maillard-induced glycation conditions on the allergenicity, emulsification properties, and ACE-inhibitory activity of sodium caseinate. Combining two different protein modification techniques under controlled and mild conditions could maintain the emulsification properties and ACE-inhibitory activity, but had no dual effect on reduction of immunoreactivity. Therefore, further optimization of glycation conditions needs to be conducted for the development of hypoallergenic casein ingredient with acceptable functionality and bioactivity.

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1. Literature Review

1.1. Introduction and Objectives

Sodium caseinate is one of the most common milk protein ingredients and is utilized in various food applications due to its superior nutritional benefits and functional properties, especially solubility and emulsifying properties (Augustin et al., 2011; Munro, 2002). In addition, numerous bioactive peptides with physiological benefits are encrypted in the caseins' polypeptide chains. These peptides can be liberated through *in vivo* digestion, *in vitro* fermentation, or controlled enzymatic hydrolysis (Pihlanto-Leppälä, 2002).

However, one of the major drawbacks of the expanded utilization of sodium caseinate is its allergenicity. Cow milk protein is one of the "Big eight" food allergens, which also include peanut, soy, egg, fish, shellfish, tree nuts and wheat (FDA, 2015). These allergens are responsible for approximately 90% of allergic reactions to food (Food Allergy Research and Education, 2013). Cow milk protein allergy, especially casein, is the most prevalent allergy in infants and young children with an incidence rate of around 2-6% worldwide (Odedra, 2015). Symptoms range from mild asthma, rhinitis, and inflammation to severe anaphylactic shock and death (Kaminogawa and Totsuka, 2003). As a consequence, and despite the superior health benefits and functionality, the utilization of casein ingredients may decrease over time (Kaminogawa and Totsuka, 2003).

Several protein modification techniques have been investigated to produce hypoallergenic protein ingredients. The most studied modification is enzymatic hydrolysis. Through the specific enzymatic cleavage of peptide bonds, both linear and conformational epitopes can be destroyed (Bu et al., 2013). Linear epitopes are short amino acid sequences within the proteins' primary structure. Conformational epitopes are composed of non-linear amino acid sequences, which are brought together after a protein folds (Chen and Gao, 2012).

Limited enzymatic hydrolysis may release short peptides and polypeptides that have improved functionality, such as enhanced surface properties (Castro et al., 2015). In addition, bioactive peptides, such as angiotensin converting enzyme (ACE) inhibitory peptides, can be released upon hydrolysis. However, limited proteolysis in some cases unmasks epitopes that were originally buried within the three-dimensional native structure. Moreover, limited hydrolysis is not sufficient to ensure the cleavage of all potential epitopes. On the other hand, extensive hydrolysis can reduce protein allergenicity to a greater degree, but will result in bitter flavor and a significant loss of functionality (Høst and Halken, 2004).

Another protein modification protocol that may result in reduced protein allergenicity is Maillard-induced glycation. The initial stage of the Maillard reaction induces a covalent linkage between an amine group of a protein and a reducing carbonyl end of a carbohydrate to produce a glycated protein (the Amadori compound) (Oliver, 2011). This glycation may alter some of the epitopes' structural integrity and mask some epitopes due to the steric hindrance of the carbohydrate group, thus reducing the proteins' allergenicity (Nakamura et al., 2008; Bu et al., 2013). However, the large molecular weight carbohydrates previously used and the long glycation times employed may result in an adverse effect on the digestibility, functionality, flavor and color (Jiménez-Castaño et al., 2005a; Corzo-Martínez et al., 2010). Without proper control, the Maillard reaction will progress to advanced stages, at which point brown pigments, mutagenic compounds, and protein polymers will be formed (Oliveira et al., 2014).

It is hypothesized that combining the two mechanisms, proteolysis and Maillard-induced glycation, under controlled and mild conditions, will have a dual effect on reducing allergenicity by altering potential epitopes, while maintaining the nutritional quality, bioactivity and functionality. Therefore, the overall objective of this research was to develop a hypoallergenic sodium caseinate ingredient using a combination of enzymatic hydrolysis and Maillard-induced glycation. This work will provide new information about the combined effect of limited enzymatic hydrolysis and controlled Maillard-induced glycation on the allergenicity of casein protein. The specific objectives for this work were:

1) Determine the effect of several proteolysis conditions and Maillard-induced glycation conditions on the allergenicity of sodium caseinate.
2) Determine the effect of the dual modification approach on the bioactivity and emulsification properties of sodium caseinate.

1.2. Significance of Milk Proteins

Today, dairy products constitute an essential component of the staple diets, especially in northern Europe and North America, where milk products account for around 30% of the total dietary protein intake and about 65% of the total animal protein intake (Fox, 2003). Other than the consumption of milk products (such as cheese and yogurt), consumption of dairy products includes products containing milk proteins ingredients. The use of milk protein ingredients in various food applications has several positive ramifications including economic, health, and functional benefits.

1.2.1. Economic Benefits

The rapid growth of the protein market is boosted by consumers' increased health awareness and acceptance of functional foods and dietary supplements ("Milk casein," 2015). Dairy products are the third-largest protein source, accounting for approximately 18 percent of the total protein intake in the United States. The market is mainly composed of whey protein (whey protein concentrate, isolate, and hydrolystaes) and casein (acid casein, rennet casein, caseinates and hydrolysates) (U.S. Dairy Export Council, 2013). Approximately 1.5 million tons of whey proteins ingredients and 110,675 million tons of casein protein ingredients were exported globally in 2013, with the leading exporters being Europe and the United States (Lagrange et al., 2015). According to Technavio's Analysts, the global casein and caseinates market will continue to grow by around 3.14% over the period 2014-2019 ("Global casein," 2015).

Given the significant amount of production, the dairy protein market exerts an essential influence on the overall economy. In 2014, dairy protein contributed an estimate of $354 billion business globally, and the market is forecasted to keep increasing by more

than 10% annually over the next five years (Rockville, 2014). North America is the largest market of milk casein and caseinates, which is projected to reach approximately $2.75 billion by 2020 (“Global milk,” 2015).

The utilization of casein protein ingredients in various food applications has been positively linked with positive ramifications, ranging from health benefits to functionality. The following sections will provide a brief discussion of the associated benefits with the utilization of casein protein ingredients.

1.2.2. Health Benefits

Casein protein has long been recognized for its superior nutritional and physiological benefits. Besides a good source of energy, casein protein contains all essential amino acids for tissue synthesis and protein replacement (Haug et al., 2007). Compared to other proteins, especially plant protein, casein protein is easily digested by the human body (Gurevich, 2014). Casein protein ingredients have been applied in various food and beverage products including sports drinks and snack bars to promote weight maintenance, muscle synthesis, blood glucose control, and a boost of satiety (Giles-Smith, 2013).

1.2.2.1. Nutritional Value

Casein accounts for around 80 percent of the total milk protein and exists in a very loose and open structure that can be accessed by digestive enzymes (Sindayikengera, 2006). Because of its high digestibility and complete amino acids profile, casein’s digestibility corrected amino acid score (PDCAAS) is 1, which is the maxiumn score, similar to egg white (Gurevich, 2014). In addition, casein has a biological value of 77. Biological value measures the percentage of absorbed protein that end up in the tissue and muscle. Compared to the top score of 100, a biological score of 77 suggests that casein is utilized in body synthesis efficiently. Casein also has a protein efficiency value (PEV) of 2.7 (Gurevich, 2014), which is the highest score indicating a maximum weight gain in rats upon consumption of casein proteins.

1.2.2.2. Physiological Benefits

Casein protein has been positively linked to many physiological benefits. Compared to soy proteins, casein proteins are better in muscle recovery after resistance exercise, and promote whole body nitrogen retention (Tang et al., 2009). Casein proteins also supply a sufficient amount of amino acids precursors to maintain bone structures and prevent osteoporosis (Darling et al., 2009). In addition, casein contains several bioactive peptides (FitzGerald and Meisel, 2003). A bioactive peptide is a specific sequence of amino acids (2-20 amino acids) that exhibits physiological function (FitzGerald and Meisel, 2003). Numerous bioactive peptides from casein work in the body as regulatory compounds and exert various activities, including opioid, antithrombotic, immune-modulation and mineral utilization properties (Silva and Malcata, 2005). Other physiological benefits of casein include preventing osteoporosis, promoting cardiovascular health, increasing muscle growth, and controlling body weight.

Osteoporosis affects more than 10 million US adults aged over 50 years and lead to about $19 billion treatment cost (Huth et al., 2006; Heaney, 2009). Casein proteins are a good source of calcium and phosphorous (Manson, 1975). These minerals exist in an optimum ratio for bone formation and growth (Huth et al., 2006). Casein proteins also contain cystatin, an active component that prevents bone resorption (Toba et al., 2000; Ohashi et al., 2003).

Another physiological benefit of casein protein is promoting cardiovascular health. Hypertension is highly prevalent in the USA and affects around one-third of the adult population. Without proper control, hypertension leads to server cardiovascular diseases such as ventricular hypertrophy, smooth muscle cell hypertrophy, and coronary infarct (FitzGerald et al., 2004). Within our body, the rennin-angiotensin system plays an important role in regulating the blood pressure. When low pressure is detected, rennin activates angiotensin I from prohormone angiotensin. The angiotensin converting enzyme (ACE) then cleaves a dipeptide from the C-terminal of angiotensin I and converts it to angiotensin II, which is a vasoconstrictor, thus increasing blood pressure. Likewise, ACE also inactivates a vasodilatory peptide, bradykinin (Saito, 2008). Casein has been found to contain peptide sequences with the ACE-inhibitory activity. These peptides act as

competitive substrates for ACE, and hence limit the conversion of angiotensin I to angiotensin II (FitzGerald and Meisel, 2003). Potent ACE inhibitory peptides have been identified in casein hydrolysates (Maruyama and Suzuki, 1982; Yamamoto et al., 1994; Guo et al., 2009; Xu et al., 2014). Apart from antihypertensive activities, bioactive peptides derived from casein, especially κ-CN, inhibit platelet aggregation to counter thrombosis formation (Léonil and Mollé, 1990).

Casein proteins have been widely used in protein drinks to improve the muscle health. Casein proteins provide all essential amino acids and rich branched chained amino acids (BCCAs), such as leucine, isoleucine and valine, which are essential for the protein synthesis (Tang et al., 2009). In fact, consuming BCAAs, particularly leucine, has an anabolic effect on protein metabolism via an increased rate of protein synthesis or decreased rate of protein degradation (Louard et al., 1990; Blomstrand et al., 2006). Since they form curds and clots in the stomach instead of being emptied quickly, casein proteins are designated as "slow proteins" (Boirie et al., 1997). This unique digestive pattern results in a slow appearance of dietary amino acids in plasma after consumption, and therefore, a slightly increased protein synthesis. However, the slow protein synthesis progress is steady and prolonged within the whole body with a moderate rate of leucine oxidation, resulting in an efficient inhibition of protein breakdown and a higher leucine balance (Boirie et al., 1997).

Obesity is one of the major health problems in the United States, affecting more than 35% of women, 31% of men and 15% of children (Frenk, 2016). Obesity triggers chronic diseases such as diabetes, heart disease, cancer and joint problems (Sturm, 2002). Research has positively linked calcium to weight loss. For example, calcium rich diets such as a diet rich in casein, have been shown to accelerate weight loss for obese adults during an energy control period (Zemel, 2004). It is estimated that every 300 mg increase of calcium intake will lead to ~1 kg less body fat in children and 2.5-3.0 kg lower weight in adults (Heaney et al., 2002). In addition, casein protein contains many other active components such as bioactive peptides and high branched-chain amino acid that have a notably augmented effect of dairy calcium in promoting weight loss (Zemel, 2004).

1.3. Milk Protein Composition

1.3.1. General Composition

Cow milk protein is primarily composed of 80% casein and 20% whey protein (Fox , 2003; Swaisgood, 2003). Caseins are phosphoproteins that will precipitate from raw milk by adjusting the pH to 4.6 at 20°C or by hydrolysis with rennet enzyme at pH 7. The proteins that remain in the liquid phase are mostly whey proteins (Fox, 2003). Whey proteins are a heterogeneous mixture mainly composed of β-lactoglobulin and α-lactalbumin. Apart from casein and whey protein, numerous proteins exist in milk but in minor amounts (Farrell et al., 2004). The following sections will focus on the composition and structure of casein proteins.

1.3.2. Casein Proteins

Casein proteins constitute four subunits, α_{s1}-, α_{s2}-, β- and κ-casein (CN). Almost all casein subunits interact with each other to form casein micelles (Swaisgood, 2003). Whole casein can be further separated from whey proteins through iso-electric point precipitation, sedimentation or size-exclusion chromatography (Fox, 2003). The colloidal micelles have a varying size distribution from 500 to 3000 Angstroms (A). A micelle is composed of 93% casein (CN) with α_{s1}-: α_{s2}-: κ-: β-CN in the weight ratio of 3:1:3:1. The remaining 7% is composed , mainly of calcium, phosphate, citrate, sodium, potassium and magnesium (Wong et al., 1996).

1.3.3. Molecular Chemistry of Casein Proteins

Casein proteins are high in proline content, which precludes the formation of α-helix and β-sheet secondary structure (Slattery, 1976). Despite the non-regular structures, a significant fraction of α-helix , β-turns and β-strands were projected in casein proteins (Holt and Sawyer, 1993), based on their primary structures and various techniques such as CD spectroscopy and Raman spectroscopy (Farrell et al., 2004). The tertiary structure

was further computed using projected secondary structures and the theory of energy minimization (Whitlow and Teeter., 1985). In addition to individual casein proteins, the structure of the casein micelle has also been researched over the years via electron microscopy, small angle scattering of neutrons (SANS), and X-rays (SAXS) techniques (Holt et al., 2013). Two major structures have been hypothesized, sub-micells (Dalgleish and Corredig, 2012) and nanoclusters (Holt et al., 1998; Horne, 2006; Dalgleish, 2011). According to the nanoclusters theory, which is the most accepted theory, calcium phosphate bridges link α_{s1}-, α_{s2}-, and β-CN, with κ-CN accumulating mostly on the surface of the micelles (Kruif and Huppertz, 2012). Due to the limitations of techniques applied and the difficulty of crystallizing casein proteins, the accuracy of the predicted models is questionable and has been undergoing debate for many years (Holt et al., 2013).

Casein subunits vary in degree of phosphorylation, hydrophilicity, charge and hydrophobicity (Wong et al., 1996). α_{s1}-CN, which has a molecular weight of 23 kD and a negative net charge of 22 at pH 6.5, accounts for about 38% of the total casein (Wong et al., 1996; Fox, 2003). It has three distinct hydrophobic regions, located within the residues of 1-44, 90-113 and 132-199 (Swaisgood, 2003). In addition, α_{s1}-CN has one highly polar domain between residues 41 to 80, possessing eight phospo-seryl residues and having an approximate negative net charge of 20.6 at pH 6.6 (Wong et al., 1996). α_{s2}-CN, which has a molecular weight of 25 kD and a negative net charge of 23, comprises up to10% of the total casein and is the most hydrophilic casein containing three separated anionic clusters with a total of 10-13 phospho-seryl residues (Wong et al., 1996; Fox, 2003). In addition, two hydrophobic segments spread within the polypeptide chain, with one segment located at the C terminal at residues 162-207 and a less hydrophobic segment found between residues 90 to 120 (Fox and Mulvihill, 1982). β-CN, which has a molecular weight of 24 kD and a negative net charge of 13, accounts for 35% of the total casein and is the most hydrophobic among the different proteins (Wong et al., 1996; Farrell et al., 2004). Unlike α-CN, its hydrophilic and hydrophobic segments are concentrated in the N-terminal and C-terminal. This amphiphilic characteristic makes β-casein a very good surfactant (Fox and Mulvihill, 1982). κ-CN, which is a glycoprotein with a molecular weight of 19 kD and a negative net charge of 3, accounts for 15% of the

total casein and has similar amphiphilic structure to that of β-CN (Wong et al., 1996; Wang et al., 2013). However, the hydrophilic domain is located in the C-terminal with a negative net charge of 10 or 11 at pH 6.6, while the hydrophobic domain is in the N-terminal within residues 1-105 (Swaisgood, 2003). According to their average hydrophilicity, the correct order of these four units is α_{s2}- > α_{s1}-> κ-> β-CN (Wong et al., 1996). The varying distribution of hydrophobic and hydrophilic areas has a major effect on physiochemical properties such as the great dependence of association states on pH, ionic strength, and ion-binding (Farrell et al., 2004).

1.3.4. Heterogeneity of Casein Proteins

Twenty different protein zones were detected following electrophoresis separation of acid casein to confirm the heterogeneity of casein (Fox and Mulvihill, 1982). This heterogeneity is in part attributed to genetic polymorphism. Genetic polymorphism refers to DNA-base pair sequence mutation on the protein gene resulting in either amino acids substitution or deletion (Swaisgood, 2003). Changes in genetic polymorphism have a great influence on its physico-chemical properties. For example, α_{s1}-CN A lacks the most hydrophobic area of the N-terminus leading to its lower hydrophobicity compared to α_{s1}-CN B, and consequently will aggregate less (Creamer et al., 1982). In addition, α_{s1}-CN A is less sensitive to calcium ions because of lower degree of phosphorylation. Consequently, the curd made from α_{s1}-CN A is much softer than α_{s1}-CN B variant (Sadler et al., 1968).

Apart from genetic polymorphism, post-translation modification also plays a critical role in the variants of casein. Unlike genetic polymorphism, post-translation involves covalent processing through either proteolytic cleavage or addition of modifying group to one or more amino acids to change the properties of the protein (Mann and Jensen, 2003). Caseins are phosphorylated at seryl residues to various degrees. Occasionally, phophorylation occurs at the threonyl residues (Swaisgood, 2003). Among the four different casein units, α_{s2}-CN may contain as high as 10-13 phosphate residues per mole, thus exhibiting the greatest variability in the extent of phosphorylation

(Brignon et al., 1977). κ-CN, on the other hand, only has one phosphorylated residue per mole, but is the only casein that is glycosylated at the threonyl residues (Mackinlay and Wake, 1965). κ-CN family is composed of one main carbohydrate-free component with one phosphate group and 6 minor components varying in the level of glycosylation and phosphorylation (Mackinlay and Wake, 1965). Addition of phosphate and /or carbohydrate groups is essential in forming hydrophilic clusters. Differences in glycosylation and phosphorylation also affect the calcium binding sensitivity of casein units. With the presence of phosphorous clusters, α_{s2}-, α_{s1}-, and β-CN units can bind significant amount of calcium ions to form aggregates. κ-CN does not bind calcium ions due to the lack of phosphorylated clusters (Fox and Mulvihill, 1982). Thus, calcium sensitivity order from high to low is α_{s2}- > α_{s1}-> β-> κ-CN, the same with that of hydrophilicity (Farrell et al., 2004). Binding of calcium plays a vital role in several cases. Firstly, the addition of calcium ions will alter the net charge of each casein unit, thus altering the original balance of electrostatic forces and hydrophobic interactions. This adjustment further affects casein units' behavior of self-association and inter-association (Swaisgood, 2003). For example, bounded calcium ions shield the net negative charges on the casein proteins' surface, leading to casein aggregation and formation of casein micelles via hydrophobic interactions (Slattery, 1976). Additionally, calcium will covalently link with phosphate group to form calcium phosphate bridges, which is essential in stabilizing the casein micelle (Wong et al., 1996). Apart from forming internal calcium- phosphate linkages to build up casein micelle structures, calcium also link with phosphate groups among paracasein peptides (formed upon enzymatic hydrolysis of κ-CN) to promote the curd formation (Cerbulis et al., 1959; Malacarne et al., 2014). A high colloidal contents of calcium was associated with better rennet coagulation properties in multiple milk samples (Jensen et al., 2012).

Another post-translation change is the formation of disulphide linkage either inter-molecularly or intra-molecularly. While α_{s1}- and β-CN do not have sulfur containing residues, both α_{s2}- and κ-CN contain two cysteine residues. Those free sulfur groups can form either inter-molecular or intra-molecular disulphide linkage (Farrell et al., 2004). Most of α_{s2}-CN molecules exist as monomers, while small amount forms

dimmers through intermolecular disulphide linkages (Rasmussen et al., 1992). κ-CN, however, could exist as 10 κ-CN molecules associated via disulphide linkages (Swaisgood, 2003). The presence of free sulfhydryl group also result in disulfide exchange with whey protein (namely β-Lg) upon heating of milk. This interchange results in aggregation in heated milk overtime (Lowe et al., 2004).

Finally, enzymatic hydrolysis makes the variants of casein subunits more complex. For example, indigenous enzymes such as plasmin can hydrolyze β-CN to produce peptides such as γ-CNs and proteose peptones. Proteose peptones mostly come from the N-terminal of β-CN, while γ-CNs mainly derive from C-terminal of β-CN (Jensen, 1995). The effect of plasmin-induced proteolysis on milk quality depends on multiple factors, such as the degree of hydrolysis and the type of dairy products. Plasmin hydrolysis promotes cheese ripening with desirable flavor and texture while it leads to undesirable gelation in pasteurized and ultrahigh-temperature milk (Ismail and Nielsen, 2010).

1.3.5. Physiochemical Properties of Casein Proteins

Casein proteins disperse in the milk system as casein micelles and are stabilized via steric hindrance or electronic repulsion. However, the stability of casein micelles is affected by many factors such as environmental pH, temperature and proteolysis. At pH 4.6, all casein proteins start to aggregate to form acid casein curds due to limited solubility (Fox, 2003). Casein curd formation is further promoted by a maximum dissolution of micellar calcium-phosphate bridges at pH 4.6.These calcium-phosphate bridges are essential for maintaining the structural integrity of casein micelles (Dalgleish and Law, 2009).

Enzymatic hydrolysis, such as chymosin proteolysis, further affects casein micelles' stability (Dalgleish and Law, 2009). Chymosin hydrolyzes κ-CN at the surface of the casein micelles and separates κ-CN into hydrophobic (para-κ-CN) and hydrophilic (glycomacropeptide) components. Losing glycomacropeptides leads to decreased

electrostatic and steric repulsion. The casein micelles start to aggregate with each other via hydrophobic interactions (Herbert et al., 1999).

On the other hand, casein is heat stable and can resist denaturation for as long as 60 min at 140°C (Fox, 2003). The thermal stability makes casein ingredients capable of surviving the pasteurization process at 62-71 °C without hurting the functionality, such as emulsifying properties ("General properties of casein," 2016).

Temperature, pH, and enzyme treatment affect the properties of the casein proteins including net charge and molecular size, which in turn will affect functionality. Casein ingredients are produced following several separation technologies that involve various environmental treatments. The following sections will describe the difference procedures used to produce different casein ingredients.

1.4. Milk Protein Ingredients

Milk proteins have long been incorporated into the human diet. Initially, milk proteins were used merely as nutrition supplements and consumed in the simple form of skim milk, whole milk or churned buttermilk (Chandan, 2011). Due to the development of analytical techniques and separation methods, milk protein has been processed into various forms of ingredients, and utilized in many food products for their unique functional properties (Kulozik, 2009). In addition, usage of milk protein ingredients is label friendly (Roginski, et al., 2003). Several milk protein ingredients are available in the market including a number of whey protein ingredients (concentrates, isolates, hydrolysates), and casein protein ingredients (renent casein, acid casein micellar casein, caseinate, hydrolysates) (Chandan, 1997). The following subsections will focus on the main casein protein ingredients.

1.4.1. Casein Protein Ingredients

Advances in protein separation technology allows not only removal of water and /or fat from milk but also the isolation of different components to produce various ingredients, that greatly enrich the choices of food industries. Of these ingredients are

caseins and caseinates ingredients. Based on the coagulation method, casein can be divided into two groups, acid casein and rennet casein. Acid separation involves dropping the pH to 4.6, which is the isoelectric point of casein. Casein proteins start to destabilize due to a loss of net charges, leading to aggregation and separation from the whey. Enzyme coagulation, on the other hand, involves hydrolysis of κ-CN by chymosin, which results in destabilization of the aggregates and coagulation of the micelles via hydrophobic interactions and calcium bridges (Fox and Mulvihill, 1982). Separated casein aggregates are subjected to additional steps including washing, removal of water and whey (Fox and Mulvihill, 1982). Since acid casein is insoluble in water, alkaline treatment is applied to form caseinates, which are easily dispersed in water. Sodium caseinate forms when using sodium hydroxide. Similarly, potassium and calcium caseinates will form when using potassium hydroxide and calcium hydroxide, respectively (Chandan, 1997).

Rennet casein is the primary ingredient used to make mozzarella cheese analogues due to its desired sensory attributes, such as bland flavor and pale color, and remarkable functionality, including firmness, stretchability, shredability, and emulsification (Aimutis, 1995; Ennis and Mulvihill, 1997; O'Sullivan and Mulvihill, 2001). Acid casein has been frequently utilized in baked products to mimic and replace wheat gluten and provide elasticity (Augustin et al., 2011). For instance, acid casein is utilized to produce gluten-free breads and cakes (Stathopoulos and O'Kennedy, 2008)

Caseinates are highly charged and exhibit high solubility and stability in solution. Sodium caseinates provide viscosity, due to a good water-holding property and protein-protein interactions, hence it is often utilized to modify the texture of food products, ranging from cutable to spreadable with a minimal fat separation (Pritchard and Kailasapathy, 2011). Sodium caseinate has also been widely used as a fining agent to reduce the astringency and improve the clarification in wine and beverages (Mulvihill and Ennis, 2003). Casein proteins remove phenolic compounds, namely tannins, by forming insoluble phenol-casein complexes, which can be removed in subsequent filtration and /or decantation steps (Patrick et al., 2009). Other applications of sodium caseinate include improving the gel firmness and reducing syneresis of yogurts (Mulvihill

and Ennis, 2003) due to their ability form gel structure (Augustin et al., 2011). When heat treatment is applied, gel firmness is further increased through the interaction between whey proteins and casein via disulfide linkages (Lucey, 2004). On the other hand, calcium caseinate forms aggregate in solutions with a turbid color and is often utilized to whiten food products such as chicken nuggets and coffee whiteners. Also, calcium caseinate has been shown to improve the gel strength in protein matrixes such as surimi (Pritchard and Kailasapathy, 2011). Calcium caseinate can enhance the polymerization and gelation of myosin, which is a major muscle protein in surimi (Yamashita et al., 1996).

Caseinate protein ingredients, especially sodium caseinates, are also famous for their excellent emulsifying, foaming and whipping properties due to their distinct hydrophilic and hydrophobic regions, and open and flexible structures, which allow them to locate and reorient on the oil-water interface easily and quickly (Dickinson, 2003b). Applications include meat products, desserts, dressings, soups, sauces, coffee whiteners, baked products, pasta, cereals, frozen dairy products, and imitation cheese (Chandan, 1997).

1.4.2. Casein Hydrolysates

Enzymatic hydrolysis of proteins results in the formation of short, intermediate chain peptides and polypeptides (FitzGerald and Meisel, 2003). A wide range of enzymes from animal, plants and microbial sources are commercially available to choose from. Due to different specificities, enzymes hydrolyze proteins in various patterns. For instance, trypsin hydrolyzes peptide bonds at lysine and arginine residues, while pancreatin has a broader specificity (Adler-Nissen, 1986). The different specificities of the enzymes used result in peptides with various physicochemical characteristics, such as hydrophobicity, net charge, and size.

The extent of enzymatic hydrolysis is often expressed and compared by calculating degree of hydrolysis (DH). DH refers to the percentage of peptide bonds cleaved and is affected by multiple factors (Panyam and Kilara, 1996). The most crucial

factors are enzyme to substrate ratio, hydrolyzing temperature, pH, and time (Adler-Nissen, 1986). A limited DH results in a mixture of small and large molecular weight peptides and intact proteins. Limited DH is often preferred to maintain the functionality and flavor of protein ingredients; however it may increase the potential allergenicity due to an increased exposure of buried epitopes (Bu et al., 2013). Extensive DH, on the other hand, results in a mixture of small molecular weight peptides and free amino acids and is detrimental to the flavor and functionality. However, extensive DH breaks down most epitopes that cause allergy within proteins and is ideal for formulating hypoallergenic products (Adler-Nissen, 1976).

Depending on the hydrolysis conditions, casein hydrolysates may have several advantages. Among the most reported advantages of casein hydrolysates are enhanced absorption efficiency by the body, enhanced bioactivity due to the release of bioactive peptides, reduced allergenicity, and enhanced functionality (Roginski et al., 2003; Luo et al., 2014; Castro et al., 2015).

1.4.2.1. Casein Hydrolysates as Nutritional Supplements

Food products have been developed to meet the special needs of patients with disorders in digestion, amino acids metabolism, and malnutrition due to chronic liver disease, cancers, and food allergy (Fischer et al., 1976; Milla et al., 1983; Silk, 1986; Heyman, 1999). Casein hydrolysates freed from phenylalanine have been provided as the dietary treatments for patients with phenylketonuria (PKU, one of the major metabolism disorders of amino acids due to a lack of hepatic enzyme that can convert phenylalanine to tyrosine). Casein hydrolysates with a high Fischer's value (ratio of branched-chain amino acids (BCAA) to aromatic amino acids (AAA) are one of the most common dietary treatments for patients with a chronic liver disease (Pedroche et al., 2004). Compared to diet treatments that are formulated with a mixture of pure amino acids, short-chain peptides are adsorbed more efficiently due to peptides' specific transport systems and an elimination of osmotic problem, caused by the high concentration of amino acids (Clemente, 2000).

1.4.2.2. Casein Hydrolysates as Hypoallergenic Formulas

Extensively hydrolyzed casein has been widely used in hypoallergenic infant formulas (Clemente, 2000; Kilara and Chandan, 2011; Borschel et al., 2013). Since dietary treatments must be tolerated by at least 90% of infants or young children with a recorded dairy protein allergy history, an extensive hydrolysis of casein is required to destroy both linear and conformational epitopes (Høst et al., 1999; Heyman, 1999). Although extensive hydrolysis based casein formulas eliminate any residue of molecular mass greater than 1500 Da, they may still trigger allergic reactions in 10% of children with the dairy protein allergy (Heyman, 1999). In addition, extensively hydrolyzed casein has unpleasant odor and taste (Pedroche et al., 2004). Partially hydrolyzed caseins are not strictly hypoallergenic due to the presence of large molecular weight peptides. However, they are useful in preventing cow milk allergies and have better taste and nutritional values (Bu et al., 2013).

1.4.2.3. Casein Hydrolysates as a Source of Bioactive Peptides

In addition to providing sufficient nutrients, caseins are rich in bioactive peptides. Bioactive peptides, which normally contains 2 to 20 amino acids, are encrypted in the primary polypeptide chain of proteins and can be released through either *in vivo* digestion, *in vitro* fermentation, or controlled enzymatic hydrolysis (FitzGerald and Meisel, 2003). The biological properties are mostly determined by the amino acid composition and sequence (Korhonen, 2009). Purification and separation steps are often applied to test the bioactivity of purified peptides (Clare and Swaisgood, 2000). Some peptide sequences are further chemically synthesized to confirm bioactivities (Clare and Swaisgood, 2000).

A lot of research has been carried out to study the bioactivity of casein hydrolysates. It is shown that casein hydrolysates are beneficial to major body systems, including the heart, bones and the digestive system. Casein bioactive peptides are also positively associated with immune defense, mood and stress control (Korhonen, 2009). In fact, most casein hydrolysates possess multiple bioactivities. This multiple bioactivities

may come from several bioactive sequences that are often protected from proteolysis due to relatively high hydrophobicity and high content of proline residues (Meisel, 2004).

Some casein bioactive peptides are potent antioxidants. They can scavenge radicals such as the superoxide anion, hydroxyl radical, and are good at inhibiting lipid oxidations and chelating metal ions (Díaz et al., 2003; Elias et al., 2006). Other casein-derived peptides have been shown to regulate several aspects of immune functions. In addition, casein bioactive peptides may possess opoid properties and participate in control of gastrointestinal functions in adults (Meisel and FitzGerald, 2000; FitzGerald and Meisel, 2003). Moreover, casein derived bioactive peptides have been found to inhibit the growth of microorganism. For instance, β-CN derived bioactive peptides were shown to successfully inhibit the growth of *Escherichia coli* DH5α (Birkemo et al., 2009). In fact, casein derived antimicrobial peptides are superior to peptides derived from other proteins since they have a wider range of targets and kill the targets much quicker (Kawasaki et al., 1992).

Antihypertensive activity is the most researched bioactivity of casein-derived bioactive peptides. Antihypertensive peptides derived from casein inhibit ACE activity (Silva and Malcata, 2005). Elevated activity of ACE results in increased blood pressure (FitzGerald and Meisel, 2003), as explained earlier. Casein-derived bioactive peptides with ACE inhibitory activity are advantageous over drug treatmens such as captopril. Drugs have potential side effects such as cough and fetal abnormalities, while bioactive peptides are natural and safe (FitzGerald and Meisel, 2003; Saito, 2008). Casein-derived bioactive peptides with ACE inhibitory activity can be released upon hydrolysis by trypsin, alcalase, and neutrase (Jiang et al., 2007). However, each produced hydrolysate differs in potency. Production of hydrolysate with antihypertensive activity is not only dependent on enzyme used, but is also dependent on hydrolysis conditions such as pH, temperature, and time (Clare and Swaisgood, 2000; Wang et al., 2011).

In addition to enhanced nutrtional and physiological benefits, casein hydrolysates have been reported to have increased functionality. Enzymatic hydrolysis using various enzymes may result in enhanced solubility, gelation, emulsificaiton, and foaming properties of casein over a wide pH range and different processing condtions (Lee et al.,

1987; Mozaffar and Haque, 1992; Ismail and Nielsen, 2010; Wang et al., 2013). Detailed discussion of preparation of casein hydrolysates with enhanced functional properties will be discussed later.

1.5. Emulsification Properties of Casein Proteins

Functionality of a protein is deteremined by a combination of extrinsic factors, such as temperature and pH, and instrinsic factors, such as the amino acids profile and protein configurations (Dickinson, 2003b). Casein proteins pertain superior functionality such as solubility, gelation, viscosity, emulsifying and foaming, with the emulsifying property being the most pronounced (Ven et al., 2001; Kennedy and Corredig, 2009; Wilde and Corredig, 2009).

Casein ingredients are among the most surface-active emulsifiers used in food manufacturing (Mohanty et al., 1988). The distinct amphiphilic property of the amino acid distribution provides casein proteins with a strong tendency to adsorb at the oil-water interface during emulsion formation (Mohanty et al., 1988). Additionally, casein proteins contain a high content of proline residues, which are secondary structure breakers, and are deficient in cysteine residues, which could potentially be involved in disulphide linkages. Accordingly, casein proteins have highly flexible structures allowing them to quickly spread and reorient at the interface (Dickinson, 1989; Dalgleish, 1997). These adsorbed caseins interact with each other on the interface to form an entangled monolayer around dispersed droplets (Dickinson, 2001). Resulting casein layers of fine droplets provide long term stability to an emulsion through a combination of steric hindrance and electrostatic repulsion to prevent flocculation, coalescence, and creaming (Dickinson, 1999).

As previously mentioned, different processing methods result in various forms of casein ingredients with different properties. Using sodium caseinate, in particular, as the only emulsifying agent results in an emulsion with good resistance to coalescence (Dickinson, 1997; Surh et al., 2006). Emulsions formed using sodium caseinate are thermally stable, since heat does not induce major conformational change to the casein

molecular structure (Surh et al., 2006). In fact, processing has minimal impact on the emulsification properties of sodium caseinate (Mohanty et al., 1988). However, heating sodium caseinate extensively at ≥ 120 °C will alter its emulsification properties (Dickinson, 2003). Compared to other casein ingredients, sodium caseinate proteins are the most soluble and can fully cover the water-oil interfaces (Mohanty et al., 1988).

Several environmental factors influence the formation and stability of an emulsion. At pH 4.6, for example, casein-stabilized emulsion starts to aggregate and precipitate due to casein proteins' minimal solubility (Swaisgood, 2003). The decreased hydration of casein proteins at pH near the isoelectric point is detrimental to emulsification properties due to insufficient proteins to fully cover the water-oil interface (Chandan, 1997). Extrem pH (basic and acidic) resulted in increased surface hydrophobicity of the protein due to inducing partial unfolding, therefore, affecting the protein surface activity and emulsification properties significantly (Jiang et al., 2009). For example, emulsification stability and activity of soy protein isolate were markedly improved by adjusting the pH to 1.5 to 3.5 and 10.0 to 12.0 (Jiang et al., 2009). Similary, both the emulsifiaciton capacity and stability of casein-stabilized emulsion increased dramatically as pH shifted to extrem alkaline pH (pH 11) (Chobert et al., 1988).

Emulsification properties can further be influenced by the protein concentration (Mulvihill and Murphy, 1991; Srinivasan et al., 1996; Dickinson and Golding, 1997a; Dickinson and Golding, 1997b). High concentration of sodium caseinate (>2%, w/v) forms unstable emulsion, which is more prone to creaming, than emulsions made with a lower amount of sodium caseinate. The instability of emulsion might result from depletion flocculation, induced by a considerable amount of unadsorbed casein proteins in solution (Dickinson and Golding, 1997a).

Calcium ions can also affect the emulsion properties. The emulsifying capacity of a 0.25% w/v sodium caseinate solution significantly increased after adding $CaCl_2$ up to 20 mM (Mohanty et al., 1988). The emulsifying capacity might be enhanced due to extensive casein aggregation via calcium bridging (Mulvihill and Murphy, 1991).

Casein proteins are generally composed of several sub-unit proteins, which are a_{s1}-, a_{s2}-, β- and κ-CN. They compete with each other for adsorption onto the interface

during emulsion formation (Dalgleish, 1997). For instance, β-casein has better adsorbtion than the other caseins in sodium caseinate emulsions (Srinivasan et al., 1996). Due to the increased demand for novel dairy ingredients, individual caseins have been isolated for various emulsification applications. For example, β-CN is the most surface active casein due to its distinctive amphiphilic structure and flexible backbone (Dickinson, 2001). Compared to β-CN, α_{s1}-CN is less surface active due to a more random distribution of amino acids distribution (Srinivasan et al., 1996). Being more surface active, β-CN adsorb more than α_{s1}-CN in emulsions stabilized by a mixture of β- and α_{s1}-CN (Dickinson et al., 1988). Moreover, α-CN has high surface charge, and thus the emulsion is more vulnerable to flocculation in the presence of electrolytes such as NaCl or $CaCl_2$ (Dickinson et al., 1997a; Dickinson et al., 1997b; Dickinson, 1999). While α-CN adsorbs at the interface in the configuration of a loop, forming a thinner layer, β-CN protrudes a long hydrophilic tail into the aqueous region and forms a thicker layer around the droplets. A thicker film promotes stability to the emulsion (Dickinson et al., 1997a; Dickinson et al., 1997b). On the other hand, κ-CN does not have strong tendency to adsorb at the interface (Sánchez and Patino, 2005). However, emulsions formed by κ-CN are more viscous than those formed by either α-CN or β-CN, since κ-CN can form inter-molecular disulphide linkages (Mulvihill and Murphy, 1991). Using a combination of α_s-/κ- CN results in reduced surface reactivity and emulsification capacity, yet promote increased emulsion stability compared to emulsions formed using β-CN (Murphy and Fox, 1991).

Despite the superior emulsification properties of existing casein ingredients, there is still room for further improvement (Kilara and Chandan, 2011). Modification of casein properties may enhance not only emulsification capacity, but also emulsification stability.

1.6. Casein Protein Modification to Enhance Functionality

Protein functionality can be improved through different types of modifications including chemical reactions, thermal treatment, genetic engineering, enzymatic hydrolysis, and Maillard-induced glycation (Damodaran, 1997). Each approach has

limitations and advantages. Thermal treatment is often applied to improve the texture (heat-induced gelation), solubility, flavor, digestibility and microbiological safety (Davis and Williams, 1998). However, it can also induce protein polymerization, which is detrimental to the emulsifying properties (Raikos, 2010). Chemical modification (oxidation, acetylation, carboxymethylation, de-amination, and others) affects functionality (solubility, emulsification properties, gelation, etc) through the substitution or addition of compounds to the protein molecules by covalent linkages (Richardson, 1985; Modler, 1985). However, chemical modification reduces proteins' nutritional value, by the blockage of essential amino acids such as lysine, or the formation of covalent linkages that cannot be digested by enzymes (Puigserver et al., 1979a; Puigserver et al., 1979b). Additionally, the generation of new hybrid proteins and residues of chemicals create food safety concerns among customers (Haque and Mozaffar, 1992). Genetic engineering, on the other hand, modifies the primary structure of the proteins through altering the coding system of a cloned gene to produce mutant proteins with increased functionality (Nakai, 1996). However, genetic modification is costly and time-consuming, which limits its applications (Clark, 1996). The most researched protein modification is enzymatic hydrolysis, and the most recently studied approach is Maillard-induced glycation. The following sections will focus, therefore, on enzymatic hydrolysis and Maillard-induced glycation.

1.6.1. Effect of Hydrolysis

Modifications of proteins via enzymatic hydrolysis have several advantages including specificity, minor side effects, wide availability of enzymes, and high efficiencies (Kester and Richardson, 1984). Enzymatic hydrolysis results in a decreased molecular weight and increased overall ionization, and exposure of concealed hydrophobic groups (Panyam and Kilara, 1996). Based on the degree of hydrolysis and the characteristics of the peptides generated, hydrolysates can be utilized to produce hypoallergenic infant formula, nutritional diets, bioactive peptides, and ingredients with enhanced functionality (Doucet et al., 2003).

1.6.1.1. Factors Affecting Enzymatic Hydrolysis

Enzymatic hydrolysis and resulted hydrolysates are influenced by many factors. Primary factors include substrate concentration, enzyme to substrate ratio, pH, temperature, and time of incubation (Adler-Nissen, 1986). Other factors, such as enzyme specificity, denaturation status of the proteins, and the presence of inhibitory substances, also affect hydrolysis pattern and extent (Panyam and Kilara, 1996). The higher the concentration of enzymes, the larger the DH. The same is true for the substrate when enzyme is more than ample (Gonzàlez-Tello et al., 1994). The DH, however, does not increase linearly with hydrolysis time (Gonzàlez-Tello et al., 1994). In fact, the reaction progresses extensively in the initial stage and then slows down gradually afterwards as the substrate gets depleted (Mahmoud et al., 1992). For instance, porcine pancreatin hydrolyzed acid casein rapidly during the initial 10% of the hydrolysis time, resulting in 48% DH (Mahmoud et al., 1992). Temperature and pH are important factors since different enzymes have different optimum pH and temperatures (Adler-Nissen, 1986). Extreme pH and temperature are undesirable since the enzyme might denature, resulting in a reduced enzyme activity (Adler-Nissen, 1986; Agboola and Dalgleish, 1996b).

Enzyme specificity matters since it determines both the number and location of peptide linkages to be hydrolyzed (Panyam and Kilara, 1996; Doucet et al., 2003). For example, alcalase has high specificity to aromatic (Phe, Trp, and Tyr), acidic (Glu), sulfur-containing (Met), aliphatic (Leu and Ala), hydroxyl (Ser), and basic (Lys) residues (Doucet et al., 2003), while trypsin is limited to lysine and arginine residues (Agboola and Dalgleish, 1996a). Therfore, under similar hydrolysis conditions, alcalase might produce a hydrolysate with higher DH than that produced using trypsin.

The protein hydrolysis patterns, namely the characteristics (molecular weight, sequence, hydrophobicity, etc.) of the peptides generated, are also affected by the amino acid profile and the three dimensional structure of the protein. For example, although trypsin has high preference to lysine and arginine residues, major components of casein get degraded at different rates, with α_{s1}- and α_{s2}-CN the fastest, followed by β-CN and κ-CN (Agboola and Dalgleish, 1996a). This can be explained partially by the different contents of lysine and arginine in individual caseins, where α_{s1}-, α_{s2}-, β-, and κ-

CN contains 30, 20, 15, and 14 lysine and arginine residues, respectively. In addition, β-CN molecues tend to associate with each other through hydrophobic interactions, and κ-CN can form polymers through S-S linkages. The aggregates make it harder for trypsin to access the lysine and arginine residues (Agboola and Dalgleish, 1996a). On the other hand, open structure proteins such as caseins are more rapidly and easily hydrolyzed compared to compact globular proteins such as whey proteins (Guo et al., 1995).

1.6.1.2. Effect of Degree of Hydrolysis on the Functional Properties of Sodium Caseinate

The extent of hydrolysis is often quantitatively monitored by determining the DH, which is defined as the percentage of hydrolyzed peptide bonds (Panyam and Kilara, 1996). The DH has significant effects on the functional properties of a protein. Limited and controlled DH may maintain or enhance functionality (Panyam and Kilara, 1996). One good example of enhanced functionality is protein solubility. Casein and caseinate have very limited solubility at pH close to their pI (pH 4.6) due to reduced electrostatic repulsion. This limited solubility hinders the utilization of casein ingredients in acidic products such as sports drinks (Panyam and Kilara, 1996). Upon enzymatic hydrolysis, peptides with increased ionization groups are released, enhancing the solubility close to the pI (Panyam and Kilara, 1996). In fact, a higher DH results in a higher degree of ionization and short chain peptides that are relatively more stable and soluble (Quaglia and Orban, 1987). However, extensive hydrolysis often leads to undesirable bitterness due to the accumulation of low-molecular-weight peptides containing hydrophobic amino acids (Panyam and Kilara, 1996). Casein hydrolysates have often been found to be associated with bitterness due to the presence of high content of hydrophobic amino acid residues, especially proline (FitzGerald and O'cuinn, 2006).

The relationship between emulsification properties and hydrolysis is much more complicated and conflicted due to different enzyme/substrate (E/S) combinations and hydrolysis conditions, namely pH and temperature (Ven et al., 2001). Casein hydrolysates produced upon hydrolysis by trypsin under mild hydrolysis conditions (pH 8, 37°C, E/S from 0.5 to 1%) had a DH ranging from 4.3 to 9.9 (Chobert et al., 1988).

These hydrolysates demonstrated enhanced emulsification activity compared to unmodified casein. The improved emulsification activity was pH dependent, with best activity at pH above 5 and below 4. Similarly, acid casein hydrolyzed by immobilized Rhozyme-41 lead to a 3.2-fold increase in the emulsifying activity when the DH was controlled to less than 5% (Haque and Mozaffar, 1992). On the other hand, emulsification activity of casein protein decreased progressively with an increased DH (greater than 22%) (Mahmoud et al., 1992). A two-fold reduction in emulsification activity was observed when the DH reached 58 % (Mahmoud et al., 1992). A limited DH maintains the molecular structure of the major casein components, releasing only a limited amount of small molecular weight peptides (Haque and Mozaffar, 1992; Mahmoud et al., 1992). A limited degree of hydrolysis further improves the emulsion formation since the peptides generated have less secondary structure and are more flexible, allowing them to adsorb more readily at the interface and consequently form smaller droplet size (O'Regan and Mulvihill, 2010). On the other hand, a high DH generates a wide distribution of molecular weight peptides, some of which have significantly reduced amphiphilicity, which is essential for good emulsification properties (Haque and Mozaffar, 1992; Mahmoud et al., 1992).

The main drawbacks of enzymatic hydrolysis are bitterness and decreased functionalities associated with extreme DH conditions (Panyam and Kilara, 1996). In addition, pure enzymes are often costly with a limited life-time (Haque and Mozaffar, 1992). Moreover, heating or adjusting the pH is often applied post hydrolysis to inactivate the enzymes. These treatments might induce protein polymerization, which is detrimental to the functionality (Mahmoud et al., 1992). Hence, new techniques are needed to overcome these limitations.

1.6.2. Effect of Maillard-Induced Glycation

The Maillard reaction, a non-enzymatic browning reaction, occurs in heated foods containing proteins and reducing sugars, and significantly affects the aroma and color of the final products (Ames, 1990). Some good examples are roasted coffee and cocoa

beans, baked products, toasted cereals, and cooked meat (Martins et al., 2000). Compared to other chemical reactions, the Maillard reaction is a natural occurring process and is accelerated by heating without additional chemicals (Oliver, 2011; Zhuang and Sun, 2013; Oliveira et al., 2014).

1.6.2.1. The Maillard Reaction

The first scheme of the Maillard reaction pathway was described by Hodge in 1953. However, the Maillard reaction is still complicated and not fully understood (Oliver, 2011). Generally, there are three stages involved- initial, intermediate and advanced stages. In the initial stage, a covalent linkage is formed between carbonyl groups of carbohydrates and free amine groups of proteins or peptides (Liu et al., 2012). In most cases, the most reactive groups are ε-amino group of the lysine residues. The α-amino groups of terminal amino acids, the imidazole groups of histidine, the indole group of tryptophan, and the guanidino group of arginine residues, also react with carbonyl groups but to a lesser extent (Liu et al., 2012). The resulting condensation produces Schiff bases, which further rearrange form Amadori products (Martins et al., 2000). In the intermediate stage, the Amadori products under specific condition may further degrade to form aldehydes and a-aminoketones via strecker degradation (Martins et al., 2000). The advanced stage involves a wide range of reactions through various pathways, such as cyclizations, dehydrations, and retroaldolizations. These reactions generate new compounds that are poorly understood. When the reaction approaches the final stage, brown colored polymers, known as melanoidin, are formed (Martins et al., 2000). Melanoidins lead to a decreased digestibility and reduced protein solubility. Off-flavor compounds and mutagenic compound may also be formed (Corzo-Martínez et al., 2010). Additionally, extensive Maillard reaction results in lysine and other essential amino acids blockage, leading to reduced biological value (Oliver et al., 2006). On the other hand, limiting the Maillard reaction to the initial stage might result in several benefits as will be discussed in the following sections.

1.6.2.2. Factors Affecting the Reaction Rate

The Maillard reaction is hard to control since it is affected by many variables in a system. Among them, water activity (a_w), temperature, pH, amino: carbonyl ratio, amino acid composition, and properties of the reducing carbohydrate (mono-, di-, oligo-, and polysaccharides) are the most crucial factors (Liu et al., 2012).

Water activity has a major influence on the rate of the Maillard reaction. At low a_w (<0.5), the reactants can only have limited mobility despite their high concentration. On the other hand, high a_w (>0.8) increase the mobility but greatly dilute the reactants' concentration (Ames, 1990). Since water is the by-product of Maillard reaction, it significantly hinders the initial Amadori rearrangement product formation (Boekel, 2001). The Maillard reaction often precedes at maximum rate at intermediate a_w values (0.5-0.8) (Baltes, 1982).

Incubation temperature and time contribute further to the rate of the Maillard reaction. To initiate the Maillard reaction, activation energy between 10 and 160 kJ/mol is normally required. Therefore, heating is necessary to provide this energy (Oliveira et al., 2014). The higher the heating temperature, the higher the reaction rate. A longer heating time promotes a greater extent of glycation (Jiménez-Castaño et al., 2005b; Oliver, 2011; Liu et al., 2012; Oliveira et al., 2014). However, high reaction rate is best to be avoided to limit the formation of undesired reaction products and protein aggregates. (Dickinson and Semenova, 1992).

The pH of the environment also plays a key role in the propagation of the reaction. The pH affects the degree of ionization of the functional side chains. The Maillard reaction progresses faster at a higher pH, where sugars exist in an open form and amino groups are unprotonated, both of which are in the highly reactive forms. An alkaline pH promotes Schiff base formation due to its nucleophilic nature. Schiff base formation is the rate-determining step of the Maillard reaction. Therefore, the more Schiff bases are formed, the faster the Maillard reaction rate is (Ge and Lee, 1997).

In addition to environmental factors, the characteristics of intrinsic reactants have a pronounced effect on the reaction rate. Simpler sugars have a higher reactivity with amino groups since they exist more as available acyclic forms (Corzo-Martínez et al.,

2010). For example, riboses react more quickly than hexoses, while hexoses are more efficient than disaccharides (Ames, 1990). Larger carbohydrates such as polysaccharides are less reactive due to their bulky carbohydrate chains, which prevent carbonyl groups from accessing available amino groups (Kato, 2002). The reaction rate is further limited by the fact that a polysaccharide only has one reactive carbonyl group at its end (Oliveira et al., 2014). Maltodextrins are saccharides with a broad range of molecular weight, ranging between 1,000 to 155,000 g mol^{-1} (Chronakis, 1998; Avaltroni et al., 2004). Maltodextrins are often classified based on the dextrose equivalent value (DE), with DE often between 3 and 20 (Dokic et al., 2004; Matsuura et al., 2015). DE is determined by comparing the reducing powder of certain maltodextrins with glucose, while assuming the glucose's reducing power to be 100 (Chronakis, 1998). The higher the DE value, the shorter the maltodextrin chain is, and hence the more reactive it is (Sun et al., 2010). Besides the nature of carbohydrates, the carbohydrate to protein ratio also affects the Maillard rate. If the carbohydrate concentration is too low, the Maillard reaction will be restricted due to limited carbonyl availability (Oliveira et al., 2014).

Protein amino acid composition and molecular structure also affect the rate of the reaction. Proteins with open and flexible structures have more exposed lysine residues than compact and globular protein, therefore they are more reactive. α-CN, an open structure protein, was glycated with four galactomannan molecules only after 24 hours of incubation at a relative humidity of 79% and 60°C (Kato et al., 1992), while lysozme, a globular protein, had only two galactomannan molecules conjugated after 2 weeks of incubation under the same incubation conditions (Nakamura et al., 1992). A moderate degree of protein denaturation promotes the progression of the reaction due to more exposed amine groups (Seidler and Yeargans, 2002). Enzymatic hydrolysis is another way to modify the Maillard reaction progression. Peptides are more reactive than the intact protein due to decreased molecular weight (increased mobility), increased free amino groups, and enhanced accessibility to the amino groups (Oliver, 2011).

Control of the Maillard condition to the initial stage, in order to prevent introducing mutagenic and brown colored compounds formation, has been achieved by

several researchers (Ames, 1990; Kato et al., 1992; Martins et al., 2000; Kato, 2002; Oliveira et al., 2014). The benefit of controlled Maillard glycation is summarized below.

1.6.2.3. Initial Stage of the Maillard Reaction

The initial stage of the Maillard reaction is illustrated in Fig. 1. The Amadori compound formed is a glycated protein (Seo et al., 2012). The glycated protein can be stable to changes in pH, ionic strength, and temperature, if glycated with polysaccharide that provide steric hindrance (Ge and Lee, 1997; O'Regan and Mulvihill, 2009). The Maillard reaction can be stopped at the initial stages by changing the environmental conditions (Ge and Lee, 1997).

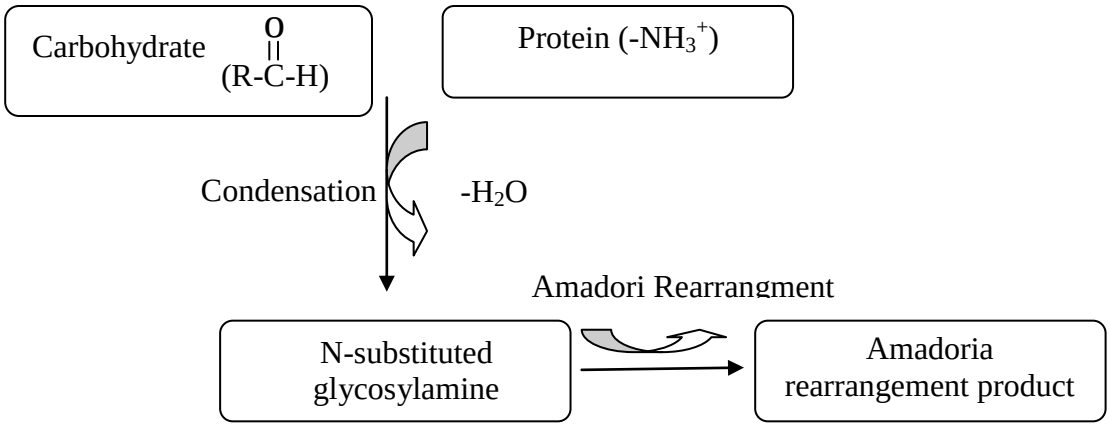

Figure 1. Initial Stage of Maillard Reaction Scheme adapted from Martins et al. (2000).

Glycated proteins formed during the initial stage of the Maillard reaction may have enhanced functional properties (Kato et al., 1992; Flanagan and FitzGerald, 2003; Seo et al., 2012). Enhanced solubility has been reported for various protein-carbohydrate conjugates such as whey protein-dextran, soy protein- acacia gum, β-lactoglobulin-galactose, rice dregs glutelin- κ-carrageenan, peanut protein isolate- dextran, and casein-maltodextrin (Katayama et al., 2002; Jiménez-Castaño et al., 2007; Medrano et al., 2009; Li and Yao, 2009; Qi et al., 2009; Liu et al., 2012; Markman and Livney, 2012; Wang

and Ismail, 2012; Du et al., 2013). Also glycated proteins may have enhanced emulsification (Dickinson and Euston, 1991; Dickinson and Semenova, 1992; Darewicz and Dziuba, 2001; Diftis and Kiosseoglou, 2004; Oliver et al., 2006; O'Regan and Mulvihill, 2009; Liu et al., 2012) and foaming properties (Wilde and Clark, 1996; Dalgleish, 1997; Medrano et al., 2009; Oliveira et al., 2014). Effect of Maillard-induced glycation of casein protein on their emulsification properties will be discussed below in more details.

1.6.2.4. Effect of the Maillard-Induced glycation on the Emulsification Properties of Casein Ingredients

Maillard-induced glycation of sodium caseinate was shown to increase the emulsification stability and activity compared to intact protein in a number of studies. For instance, α_s -CN-dextran and casein-galactomannan conjugates incubated at 60°C and humidity of 79% for 24 h resulted in 1.5 times higher emulsification activity and 10 times higher emulsion stability than the non-glycated controls (Kato et al., 1992). Sodium caseinate hydrolysates glycated with maltodextrin (>~12 kDa) also exhibited higher emulsification stability than sodium caseinate stabilized emulsion (O'Regan and Mulvihill, 2009).

Emulsions formed with glycated casein have better properties than emulsions formed with intact casein proteins due to increased activity and stability to changes in pH and temperatures (Oliveira et al., 2014). For example, heating a casein-dextran conjugate formed emulsion at 100 °C for 5 minutes resulted in a limited change without significant aggregation of polymers (Kato et al., 1992). This increased thermal stability makes glycated casein a better ingredient that resists the pasteurization processing (Kato et al., 1992). Sodium caseinate-maltodextrin emulsions specifically, are colorless and stable at lower pH conditions, especially near the iso-electric point of casein (pI=4.6) (Shepherd et al., 2000). Glycated casein also has enhanced emulsifying capacity (Kato et al., 1992; Shepherd et al., 2000; Cardoso et al., 2010). For instance, the emulsifying capacity of β-

CN was increased by glycating with dextran with molecular weight ranging from 5 to 62 kDa (Mu et al., 2006).

The enhanced emulsification activity, stability, and capacity of glycated caseins could be attributed to several reasons. Glycated caseins are more surface active than unmodified polysaccharide and casein proteins, therefore they adsorb to the interface more quickly and form films around oil droplets more efficiently at a lower concentration (Oliveira et al., 2014). In addition, covalent linking of long chain carbohydrate generates long range steric and electronic repulsion between the surfaces of the emulsion droplets, which prevent oil droplet coalescence during heating (Akhtar and Dickinson, 2007; Dickinson, 2009; O'Regan and Mulvihill, 2013). Emulsification stability is further improved by colloidal stability provided by the polysaccharides through the thickening and gelling behavior of the aqueous phase (Ru et al., 2009).

Despite the superiority in improving the functionality of casein proteins, Maillard reaction should be properly controlled to avoid negative bioloigcal effects, such as generating toxic and antinutritional compounds (Echavarrías et al., 2012), producing off-flavor (Kokkinidou and Peterson., 2014), decreaing protein solubility (Xia et al., 2015), shortening shelflife (Oliver et al., 2006), and resulting in undesirable browning color (O'Regan & Mulvihill, 2010).

1.7. Casein as a Food Allergen

A food allergy refers to specific and reproducible immunoglobulin (IgE) mediated type I hypertensive reactions that is intrigued by immune response to inhalation, ingestion, or skin contact with allergens, namely proteins (Hill et al., 1986; Boyce et al., 2010). Dairy proteins belong to the "Big Eight" allergens list (Peanuts, soybeans, fish, crustacea, milk, eggs, tree nuts, and wheat) responsible for approximately 90% of all IgE-mediated food allergies (FDA, 2015). Dairy protein allergy affects around 0.3 to 7.5 % of children in the USA, and an estimate of 15% of allergic children remain allergic to dairy proteins in adulthood (Bahna and Gandhi, 1983; Branum and Lukacs, 2008; Jackson et al., 2013). Although all dairy protein components can be allergenic, even at trace

amounts (Isolauri, 1997), casein proteins (namely α_{s1}-CN and β-CN), as well as whey proteins (β-lactoglobulin and α-lactalbumin), are considered the dominant dairy allergens (Kaminogawa and Totsuka, 2003; Monaci et al., 2006; Bu et al., 2013). In a study conducted by Goldman (1963) using symptom-provoking challenge tests, as high as 60% of the dairy protein allergeic patients reacted to casein fractions. Similarly, Bernard et al. (1998) reported about 85% of sera obtained from patients allergic to dairy proteins had positive casein specific IgE meditated reaction using immunoblotting methods.

1.7.1. Mechanisms Involved in Protein Allergy

Allergy develops in two phases, allergen sensitization and the allergic reaction. During the initial exposure to antigens, antigens are recognized by antigen presenting cells (APC), which place the processed antigens on the surface and induce immature CD4+ T cells to differentiate into allergen-specific $CD4^{+}T_H2$ helper cells. $CD4^{+}T_H2$ helper cells secrete TH2 cytokines (such as interleukin-4 (IL-4) and IL-13), which promote the maturity of B-cells. Maturated B-cells produce IgE antibodies, which are bound by the mast cells and basophils with their high-affinity receptor for IgE (FcεRI) on the cells' surface. At this point, the sensitization process is accomplished. No allergic clinical symptoms occur during the sensitization phase. However, an allergic reaction will occur during a second encounter with the antigen, where the IgE antibodies of the antigen crosslink with FcεRI and lead to a degranulation of mast cells and basophils. Numerous mediators, such as histamine, leukotreines and cytokines, will be released into the blood-stream and tissues to trigger the onset of the clinical symptoms of allergic reactions immediately or hours later (Larché et al., 2006).

Casein specific IgE bindings are mostly associated with α_{s1}-CN and β-CN (Kaminogawa and Totsuka, 2003). Despite the fact that each individual component can trigger an allergic response, studies have shown that cross-sensitization mechanism also provoke allergic reactions. In other words, IgE antibodies react with different caseins interchangeably through some common or similar epitopes (Isolauri, 1997). This cross-reactivity might result from homologeous clusters of phosphors in the primary amino acid

sequence of caseins, which are both immunogenic and resistant to enzyme digestion (Meisel and Frister, 1989).

1.7.2. Allergic Symptoms and Epitopes

Dairy protein allergy symptoms could occur in the gastrointestinal tract, resulting in vomiting, recurrent diarrhea, and excessive colic abdominal pain. Alleric reaction to dairy protein can also occur in the respiratory tract resulting in rhinitis, wheezing, chronic cough, and pulmonary infiltrate. Addtionally, allergic reactions to dairy proteins can occur in the cutaneous areas resulting in atopic dermatitis, atopic eczema, and angioedema. Serious anaphylactic shock could also occur, leading to death. Dairy protein allergy could occur immediately after the consumption of milk products or can appear gradually (Roginski et al., 2003).

Antibodies react stereospecifically with regions on a protein antigen, known as epitopes. Epitopes exist in two forms- linear and conformational epitopes. Linear epitopes refer to linear amino acids sequences within a polypeptide chain. Conformational epitopes are composed of amino acids located in different regions of the polypeptide chains and are brought together through polypeptide chain folding, thus possessing certain degree of conformational structures (Davis and Williams, 1998). To identify linear epitopes on individual caseins, numerous overlapping decapeptides were synthesized on cellulose derived membranes to cover the entire length of the casein (Chatchatee et al., 2001). Enzyme-linked immunosorbent assay (ELISA) testing was then utilized to determine potential allergenicity for each of these peptides, using sera from children allergic to milk. Six major segments (f 17-36), (f 69-78), (f 109-120) , (173-194), (f 123-132), (f 159-174) as well as two minor IgE-binding regions (f 39-48), (f 82-102) were identified on α_{S1}-CN (Chatchatee et al., 2001). Similarly, six major IgE binding segments (f 1-16), (f 45-54), (f 57-66), (f 83-92), (f 107-120), and (f 135-144) and three minor IgE binding segments (f 149-164), (f 167-178) and (f 173-184) were identified on β-CN (Chatchatee et al., 2001). On κ-casein, eight major IgE binding

regions (f 1-14), (f 23-24), (f 55-68), (f 79-92), (f 107-120), (f 135-144), (f 149-160), (f 169-184), (f 183-208) were identified (Chatchatee et al., 2001).

Conformational epitopes, on the other hand, are more difficult to identify than linear epitopes since they cannot be isolated from protein molecules completely (Harrer et al., 2010). Till now, only a limited number of comformational epitopes have been identified through techniques such as animal testing, X-ray crystallography and panning of random peptide libraries (Chen and Gao, 2012). Database for the conformational epitopes are set up to help develop predictive methods in computational immunology (Chen and Gao, 2012).

Linear epitopes are more involved in persistent allergy than conformation epitopes, which are assumed to result in transient allergies. This is mainly because digestions will break down most conformational epitopes while linear epitopes are preserved and can penetrate the gastrointestinal systems (Chatchatee et al., 2001). The epitopes recognizing pattern differs within patients. One example is among younger patients, who are more likely to outgrow dairy protein allergy, and older patients with persistent dairy allergy. Two epitopes on β-CN and six on κ-CN were only recognized by older group of patients (Chatchatee et al., 2001). Children with persistent dairy protein allergy have a higher level of IgE specific to linear epitopes than conformational eptiopes in α- and β-CN, consistently (Vila et al., 2001).

1.8. Casein Protein Modification to Reduce Casein Allergenicity

Casein allergenicity limits the expanded utilization of casein protein ingredients, therefore it is of great importance to find ways to reduce or eliminate caein allergenicity. Several methods have already been developed to modify casein proteins to reduce allerginicity including thermal treatment, genetic modification, enzymatic hydrolysis, and Maillard-induced glycation.

Heating proteins leads to unfolding of the proteins' compact and globular structure, which may destroy conformational epitopes (Heyman, 1999). However, heating may increase allergenicity as well by exposing potential epitopes that are originally

buried within the three-dimensional structure of proteins (Heyman, 1999). However, thermal treatment has little influence on the antigenicity of casein since they already have open structures with little secondary and tertiary structure to unfold (Kaminogawa and Totsuka, 2003).

Genetic modification is another method that can be applied to produce novel hypoallergenic protein ingredients through amino acids substitution technique. Proteins' allergenicity can be reduced by substituting the amino acids residues that are critical for the epitope formation (Totsuka et al., 1990). However, the utilization of genetically engineered proteins in food applications is chanllenged by decreased functionality (Ogawa et al., 2000) and consumers' acceptance of genetically modified crops (Riascos et al., 2009).

Enzymatic hydrolysis is the most researche hypoallgenic approach and Maillard-induced glycation is a newly emerging technology. Accordingly, the following sections will discuss the effect of enzymatic hydrolysis and Maillard-induced glycation on reducing casein allergenicity.

1.8.1. Enzymatic Hydrolysis

Through enzymatic cleavage of peptide bonds, several linear epitopes could be destroyed (Bu et al., 2013). Conformational epitopes can be cleaved as well due to protein unfolding and a change in the three-dimensional structure of proteins (Bu et al., 2013). For instance, allergenicity of acid casein was reduced significantly upon hydrolysis by porcine and pancreatin, which resulted in DH of 20% and 70%, respectively (Mahmoud et al., 1992). Casein hydrolysates with a DH of 23.2% produced by alcalase via a two-step memberane system removed approximate 98% of allergenicity (Lin et al., 1997). However, enzymatic hydrolysis could increase the allergenicity through exposing buried epitopes due to protein unfolding (Bu et al., 2013).

The residual allergenicity is affected by many factors. One of them is the extent of hydrolysis. Partial hydrolysis of a protein reduces the number of large molecular weight peptides or intact proteins. However, partially hydrolyzed proteins can't be claimed as

hypoallergenic since they still contain some intact proteins or large peptides (Lee, 1992; Agamy, 2007). Extensively hydrolyzed proteins, on the other hand, have high DH (>25%) with little residual peptides that are larger than 1,500 Da (Heyman, 1999; Nielsen, 2009). Extensively hydrolyzed caseins have been used in infant formulas intended for infants with dairy protein allergies (Clemente, 2000). Although extensively hydrolyzed proteins are hypoallergenic, they suffer from flavor challenges, mainly bitterness (Bu et al., 2013). In addition, extensively hydrolyzed proteins cannot be used for all allergic patients. About 10 percent of children with dairy protein allergy develop allergic responses to these hydrolysates (Boissieu et al., 1997). In fact, no matter how small the peptide is, if the enzyme does not hydrolyze the epitopes, an allergic reaction can still be triggered (Wal, 2001). For these individuals, only free amino acid based formulas could be used (Rosendal and Barkholt, 2000). Due to the diverse degrees of sensitivity among patients and the hydrolysis methods utilized, there is no agreement on the molecular weight cutoff that can be considered safe (Bu et al., 2013).

Despite the DH and peptides' molecular weight, remaining allergenicity is also affected by the enzyme specificity. β-CN, hydrolyzed by trypsin to result in either moderate or excessive DH, had up to 80% reduction in allergenicity (Heddleson et al., 1997). However, only a slight reduction of allergenicity was found in extensively hydrolyzed sodium caseinate using alcalase, pepsin, and lactozyme sequentially (Wroblewska et al., 2007).

On the other hand, various hydrolysis approaches affects functionality of the proteins. Extensive hydrolysis leads to not only flavor challenge but also reduced functionality, making the ingredient undesirable in the food industry. For instance, after acid casein was hydrolyzed by alcalase to produce a hydrolysate with DH of 58%, more than twofold's reduction of emulsification activity was observed for these hydrolysates (Mahmoud et al., 1992). Also, a siginificant reduction of solubility and a generation of strong bitter taste was observed for sodium caseinate hydrolysates (DH >10%) produced by Corolase (Vegarud and Langsrud, 1989).

1.8.2. Maillard-Induced Glycation

Maillard-induced glycation may have the potential to modify protein allergenicities. Several studies have shown that glycation can reduce dairy protein allerginicity since both B-cell and T-cell epitopes are shielded and blocked by the conjugates (Kobayashi et al., 2001; Nakamura et al., 2008). For instance, soy protein-galactomannan conjugates successfully removed the major allergic band s-34 kDa in soy proteins, while maintaining good solubility and emulsification properties (Babiker et al., 1998). Fag e 1 (protein extracted from buckwheat) was conjugated with food-grade polysaccharides, arabinogalactan or xyloglucan, using dry heating methods at 60°C under 65% relative humidity. A significant reduction of allergenicity (up to 98.8%) was observed. It was assumed that the carbohydrates conjugated with the protein shielded exposed epitopes. In addition, heating at 60°C induced protein aggregation, which further blocked some exposed epitopes (Nakamura et al., 2008).

On the other hand, glycation was also reported to be associated with enhanced protein allerginicity. For example, milk proteins often form complexes with free lactose through Maillard reaction during pasteurization. Lactose was found in β-lactoglobulin extracted from heated milk, and the allergenicity was found to be 100 times higher than the unheated β-lactoglobulin in milk (Bleumink and Young, 1968). It was presumed that conjugation lead to protein structural changes. The change included partial exposure of regions buried originally in the core of the protein, therefore new epitopes were exposed (Nakamura et al., 2008; Li et al., 2013). Also, new epitopes were identified on β-LG–glucosamine and β-LG–chitopentaose conjugateds (Nakamura et al., 2008; Li et al., 2013).

The effect of glycation on residual allergenicity can be affected by several reaction parameters. Changing glycation conditions including the weight ratio of protein to sugar, temperature and time of incubation, and length of the reducing carbohydrate influences the degree of glycation and consequently the residual allergenicity. For instance, the antigenicity of conjugates of β-Lg and α-La with glucose was dramatically reduced through optimizing the glycation conditions using response surface methodology. An optimum of 90% of reduction in antigenicity was observed (Bu et al.,

2009). The larger the molecular size of carbohydrate and the higher amount of carbohydrates attached, the lower the residual allergenicity (Bu et al., 2013). For instance, β-Lg glycated with larger molecular weight carboxymethyl dextran lead to more significant reduction of the allergnicity up to 71% reduction (Kobayashi et al., 2001). β-Lg-carboxymethyl dextran conjugates with a higher amount of dextran attached showed a higher reduction of allegenicity (Hattori et al., 2000). However, the Maillard-reaction must be propertly controlled to prevent it progressing into the advanced stages, where hazardous compounds are formed with reduced digestibility and undesirable browning. For example, advanced glycation endproducts that are formed in foods can be easily adsorbed into the bloodstream, leading to protein crosslinking, which has a positive correlation with Type I and Type 2 Diabetes (Berg et al., 1998; Aso, 2000). Moreover, long incubation times (< 2 weeks) are often employed to achieve glycation, which is not industry feasible (Oliver et al., 2006).

Most of the reported glycation as a technique to reduce allergenicity focused on whey protein and soy protein. Limited research was done on the effect of glycation on the allergenicity of casein proteins. Due to the structural difference (open v.s. globular), glycation may reduce caseins' allergenicity more readily. Therefore, it is of great interest to explore the allergenicity of glycated casein.

1.9. Conclusions

Incidences of allergy to casein in the United States are continuing to grow, limiting the potential utilization of casein ingredients in food applications. Therefore, it is of great importance to develop hypoallergenic casein ingredients that have acceptable functionality and quality. Currently, several methods have been utilized to produce hypoallergenic casein ingredients. However, extensive conditions are often required, leading to inferior functionality, nutritional attributes, and overall quality. Limited enzymatic hydrolysis can enhance functionality and liberate bioactive peptides. However, this might reduce allergeicity only to a certain degree or even increase allergenicity. Similarly, limited glycation could also improve functionality and reduce allergenicity to a

certain degree through steric hindrance. Combining these two mechanisms, enzymatic hydrolysis and Maillard-induced glycation under controlled and mild conditions, may have a dual effect on reducing allergenicity while maintaining or enhancing functionality and bioactivity.

2. Materials and Methods

2.1. Materials

Sodium caseinate 90 (ML492314, 96.6% protein) was kindly provided by Milk Specialties (Eden Prairie, MN, USA). The food grade Alcalase 2.4L (2.4 AU-A/g) and Neutrase (0.8 AU/g) were kindly provided by Novozymes (Denmark). Maltodextrin (MD 01956) was kindly provided by Cargill (Wayzata, MN, USA). Pronase from Streptomyces griseus (10165921001, 7.0 U/mg) was purchased from Roche Diagnostics (Indianapolis, IN, USA). Porcine pancreas trypsin 1X-S (T0303, 13,000-20,000 BAEE units/mg protein), rabbit lung angiotensin converting enzyme (A6778, 2.0 units/mg), N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (F7131), streptavidin alkaline phosphatase (S2890), p-nitrophenol phosphate (N7653), o-phthaldialdehyde (OPA) (79760), L-lysine stand ard (56871), quinine sulfate (22640), sodium dodecyl sulfate (SDS)(L4390), phosphate buffered saline (P5368, pH=7.4) , tris buffered saline (T6664, pH=8.0), urea for electrophoresis (U6504), tween® 20 (p1379), potassium bromide (76444), α-CN (C6780, ≥70% αs-casein basis), β-CN (C6905, BioUltra, ≥98%), and κ-CN (C0406, ≥70%,) were purchased from Sigma Aldrich (St. Louis, MO, USA). All casein standards are derived from cow milk proteins. Bicinchoninic acid (BCA) protein assay kit (23237), Pierce™ glycoprotein staining kit (PI-24562), and snakeskin™ dialysis tubing (3.5kDa MWCO) (38035) were purchased from Thermo Scientific (Waltham, MA, USA). Tris/Glycine running buffer (161-0734), ammonium persulfate (161-0700), 40% acrylamide/Bis solution (161-0148, 37.5:1, 2.6% C), N, N, N', N'-tetra methylethylenedimine (TEMED) (161-0800), criterion empty Cassettes (#3459902, 18 well, 1.0mm thick), and Coomassie Brilliant Blue G-250(1610406) were purchased from BioRad (Hercules, CA, USA). Sudan Red 7B (191630050), 2-Mercaptoethanol (BP176100), tris base (BP1525), and potassium iodide (7681-11-0) were purchased from Fisher Scientific (Waltham, MA, USA). Pure corn oil (Mazola corn oil) was purchased from grocery stores. Biotinylated mouse anti-human IgE (ab99807) was purchased from Abcam (Cambridge, England). Goat anti-human IgE (16-10-04) was purchased from Kirkland and Perry Laboratories

(Gaithersburg, Maryland, USA). Human serum samples from milk allergic patients were kindly provided by Jaffe Food Allergy Institute (NY, USA). Polystyrene microplates specific for ELISA (Costar # 3370), UV (Costar #3635), and fluorescence (Costar #3916) were purchased from Corning (Tewksbury, MA, USA). All other chemical grade reagents were purchased from Fisher Scientific or Sigma-Aldrich.

2.2. Experimental Design

Sodium caseinate Isolate (SCI) was hydrolyzed by three different enzymes (alcalase, neutrase and trypsin) at various hydrolysis conditions (Table1). Sodium caseinate hydrolysates (SCH) were tested for their emulsifying properties (namely the capacity and stability), ACE-inhibitory activity, and allergenicity. The hydrolysate with the highest emulsifying activity, ACE-inhibitory activity, and the lowest allergenicity was selected and glycated with maltodextrins at different glycation conditions. Samples collected at the optimum glycation condition, with the highest degree of glycation and lowest degree of browning formation, were tested for allergenicity, emulsifying properties, and ACE-inhibitory bioactivity.

2.3. Preparation of Sodium Caseinate Hydrolysates (SCH)

Sodium caseinate isolate (SCI) was subjected to enzymatic hydrolysis by alcalase, neutrase and trypsin separately for 45 min under different pH, temperature and enzyme/substrate ratio (E/S) conditions as listed in Table 1. Preliminary testing was performed to determine hydrolysis condtions that would result in a degree of hydrolysis (DH) less than 8%. Limited DH was targeted to avoid production of excess bitter peptides. Alcalase, neturase and trypsin were chosen since they have been reported to produce different protein hydrolysates with reduced allergenicity. Walter et al. (2014) found a significant reduction of immunoreactivity (up to 58%) in soy protein hydrolysates produced by alcalase using response surface methodology. Herranz et al. (2013) found that Neutrase is a promising enzyme to obtain hypoallergenic hydrolysates from β-CN. Neturase and trypsin have been found to reduce the allergenicity of whey

proteins as well (Bu et al., 2013). Trypsin specifically was chosen to retain the functionality of casein proteins. For instance, Chobert et al. (1988) found that trypsin hydrolyzed casein had higher emulsifying activity than that of casein. Haque and Mozaffar (1992) found that trypsin hydrolyzed acid casein had significantly improved emulsion stability. The DH was controlled and monitored using a DL22 titrator (Mettler Toldeo, Columbus, OH) following the pH-stat method (Adler-Nissens, 1986). Briefly, 150 mL of 5% protein solution (w/v) was prepared in distilled deionized water (DDW) in triplicates. Prior to adding the enzymes, the pH value was adjusted to the specific starting value using 0.2 M NaOH. The solution was then pre-heated to the desired temperature in a water bath, followed by the addition of the enzyme. Example of enzyme volume calculations can be found in Appendix A. During hydrolysis, the pH was adjusted back and kept constant by titrating 0.2 N NaOH every 5 min. Once the hydrolysis was completed, hydrolysates were immediately boiled for 5 min to inactivate the enzymes. Samples were lyophilized and stored at -20˚C for future testing. The DH was calculated following the formula reported by Adler-Nissens (1986) (**Eq.1**). Example calculations can be found in Appendix B.

$$DH = \frac{h}{h_{tot}} \times 100 = \frac{B \times N_b}{MP \times \alpha \times h_{tot}} \qquad \textbf{Eq.1}$$

Where:

B is the volume of NaOH (mL)

N_b is the normality of the base

MP is the mass of the protein

h is the number of peptide bonds of casein being cleaved

h_{tot} is the total number of peptide bonds specific for casein

α refers to the degree of dissociation of the α-NH_2 groups (Adler-Nissen, 1986).

Table 1. Enzymatic hydrolysis conditions of sodium caseinate using different enzymes.

Enzymes	Hydrolysis Conditions		
	pH	Temperature (°C)	E/S[a] ratio (mL/g)
Alcalase[b]	7.5	55	0.2:100
	7.5	55	0.1:100
	7.5	55	0.04:100
Neutrase[c]	6.5	50	0.2:100
	6.5	50	0.067:100
Trypsin[d]	7.0	37	1.37:100
	7.0	37	0.8:100

[a] E/S ratio: enzyme to substrate ratio ; [b] 0.216 U/mL; [c] 0.072 U/mL; [d] 0.216 U/mL.

2.4. Analysis of Protein Content

The protein content of SCI and the hydrolysates was determined following the AOAC Official Method Dumas nitrogen combustion method (Method 968.06, AOAC International, 1998) using a nitrogen analyzer (LECO® TruSpecTM, St. Joseph, MI, USA). For all casein protein sample types, a nitrogen conversion factor of 6.38 was utilized. The protein content of SCI was 91%, and protein contents of the hydrolysates ranged from 83% to 92%.

2.5. Urea Gel Electrophoresis (Urea-PAGE)

Protein patterns of the different protein samples (SCI, SCH, partially glycated sodium caseinate hydrolysates (PGSCH) and partially glycated sodium caseinate isolates (PGSCI) were monitored following the urea gel electrophoresis method as outlined by Andrews (1983). A 10% resolving gel solution was prepared (2.4 mL of 40% acrylamide / bis, 7 mL of resolving buffer (pH 8.9), 0.6 mL DDW, 10 uL TEMED and 80 uL of 10% ammonium persulfate (APS) (0.1 g dissolved in 1 mL of DDW). A stacking gel solution was also prepared (1 mL of 40% acrylamide / bis, 9 mL of stacking buffer (pH 7.6), 10 uL TEMED, and 50 uL of 10% APS). The resolving and stacking gel solutions were polymerized separately, in the 18-well gel cassette for 1 h at room temperature.

Each sample was fully dissolved in DDW (25 mg protein /mL DDW) and diluted in sample buffer (pH 8.9) with the volume ratio of 1:25 to obtain a final protein

concentration of 1mg protein /mL sampling buffer. Samples were boiled for 5 minutes before loading onto the gels. Aliquots of samples (20 μL) and pre-stained caseinate stand ards (20 μL) were loaded into the wells. Gels were electrophoresised at 200 V for approximately 1.5 h and then were stained with commassie blue staining solution (45% w/v methanol, 10% glacial acetic acid v/v, 45% DDW v/v, and 3g/L Coomassie Brilliant Blue G-250) for detection of the protein distribution, and with glycoprotein detection kit for the detection of glycoproteins. Glycoprotein staining was carried out on a second set of gels using a Pierce™ Glycoprotein Staining Kit following manufacturer's instructions. Visualization of the gel was done using a Molecular Imager Gel DoxTM XR system (Bio-Rad Laboratories) with the gel positioned over a fluorescent back-lit platform for optimal resolution.

2.6. Emulsification Capacity (EC)

Emulsification capacity was determined following the method outlined by Mohanty et al. (1988) and Rickert et al. (2004), with modifications. Briefly, a protein sample solution (10 mL of a 1% protein solution, w/v) was prepared, in triplicate, in DDW and adjusted to pH 7.0 with 0.1N HCl or NaOH. Corn oil colored with Sudan Red (4ug/mL) was continuously titrated into the sample solution constantly at 4mL/min using a homogenizer (IKA® RW 20 Digital, IKA Works, Inc., NC, US) with the blade (R 1342 propeller stirrer, 4-bladed) rotating at 860-870 rpm. Oil titration was stopped when a sudden phase inversion was observed. The weight of oil added was calculated by multiplying the total volume of oil titrated by the oil density. Emulsification capacity was then calculated as g oil /g protein. EC at pH 7, 4, and 5.3 were further compared to determine effect trypsin hydrolysis on the EC of casein hydrolysates at acidic conditions.

2.7. Emulsification Stability (ES) and Activity (EA)

Emulsification stability (ES) and activity (EA) were determined following the methods outlined by Tang et al. (2003) and Chove et al. (2001), with modifications. Protein solutions (1% protein, w/v), in triplicate, were prepared in DDW. An aliquot (6

mL) was added into 2 mL soybean oil and homogenized using a homogenizer (Power Gen 700, Fisher Scientific, Pittsburg, PA, US) set at 10,000 rpm for 1 min. After homogenization, 50 µL of the emulsion (pipetted from the lower half of the emulsion) was diluted in 5 mL of 0.1% SDS to achieve a 0.01% protein solution (w/v), vortexed for 5 sec, and adsorbance was measured at 500 nm using a spectrophotometer (Beckman DU 640 B, Beckman Coulter, Inc., CA, US). The initial adsorbance reading at time zero (A_0) was recorded as the EA. After 10 min, another 50 uL of the emulsion were diluted into 5 mL of 0.1% SDS and adsorbance was recorded as A_{10}. Emulsification stability was determined using **Eq. 2**. ES and EA at pH 7, 4, and 5.3 are further compared to determine effect trypsin hydrolysis on the ES and EA of casein hydrolysates at acidic conditions.

$$ES = \frac{A_0}{A_0 - A_{10}} \times 10 \qquad \textbf{Eq. 2}$$

Where

A_0 is the adsorbance at t = 0 min

A_{10} is the adsorbance at t =10 min

2.8. Controlled Maillard-Induced Glycation of SCI and SCH

Both SCI (91% protein) and SCH (trypsin, pH=7, 37˚C, E/S=0.06:100, and 91% protein) were subjected to Maillard-induced glycation with pre-dialyzed maltodextrin (DE of 4.7) under controlled conditions. SCH made from dialyzed SCI was only used when incubation was conducted at 0.63 a_w. SCI was glycated to determine the effect of glycation alone on modifying functionality and allergenicity of casein proteins. A protein sample was mixed, in triplicate, with maltodextrin in a 2:1 ratio (w/w), dissolved in phosphate buffer (0.01 M, pH 7), and lyophilized. The mixture was then incubated in desiccators at 0.49, 0.63, and 0.79 water activity (a_w), 60°C for 24 to 120 h. Different salt slurries, sodium bromide, potassium iodide, and potassium bromide were placed in the bottom of desiccators to control the water activity at 0.49, 0.63, and 0.79 a_w, respectively (Shepherd et al., 2000; O'Regan and Mulvihill, 2009; O'Regan and Mulvihill, 2013). Lyophilized samples were spread in a thin layer in petri-dishes and placed in the

incubator. As controls, SCI and SCH without maltodextrin were incubated under the same conditions. Samples were removed after several intervals of time (every 24 h) and stored at -20°C for further testing. Maltodextrin (3-10 kDa) was chosen instead of small sugars with reducing power, to control the reaction rate and prevent fast progression into advanced stages (Jiménez-Castaño et al., 2007). The protein-to-maltodextrin ratio was chosen to reduce the extent of free and unreacted maltodextrin. Unreacted maltodextrin remaining in the sample after incubation is not desired as it may promote further Maillard reaction during storage, and may negatively affect functionality. The temperature of incubation was maintained at 60°C, which is a moderate temperature to induce Maillard reaction, yet it is not high enough to result in a high and uncontrolled rate of the reaction.

2.9. Estimation of Maillard Glycation Extent

To estimate the extent of glycation that occurred over the course of the 24-120 h of incubation, and to aid in selecting an optimum incubation condition for the production of glycated SCH for further study, a variety of analyses were carried out, including estimation of Amadori compounds and browning, estimation of amino group loss, estimation of fluorescent Maillard compounds, and visualization of protein/peptide molecular weight distribution following Urea-PAGE gel.

2.9.1. Monitoring of Amadori Compound Formation and Browning

The difference UV (DUV) absorption spectroscopy method (Wang and Ismail, 2012) was utilized to monitor Amadori compound formation and browning. Incubated protein samples and controls (incubated SCH and SCI with and without maltodextrin), as well as non-incubated SCH and SCI, were prepared in DDW to obtain 0.2% protein solutions (w/v). Protein solutions were further centrifuged at 13, 800 x g for 10 min. The adsorbance of the supernatant (200 µL) was measured at 280 nm to correct for protein content. Amadori compound formation was monitored by measuring the DUV absorption at 304 nm for both samples and controls. As an indicator of the propagation of the Maillard reaction to advanced stages, browning was monitored by measuring DUV

absorption at 420 nm for both samples and controls. The change in Amadori compound formation and browning over time was compared amog SCH with or without maltodextran and non-incubated controls.

2.9.2. Loss of Free Amino Groups

The loss of free amino groups was determined through a modified O-Phthaladehyde method reported by Goodno et al. (1981) and Rao and Labuza (2012). In triplicate, 0.01 g of protein samples (PGSCI, PGSCH, non-incubated SCI, and non-incubated SCH) were diluted in 1 mL 1% SDS solution and centrifuged at 18,894 x g for 10 min. Supernatants were further diluted four times in 1% SDS. L-lysine (0-200 μg/mL) stand ards were prepared in 1% SDS (w/v) for the generation of a stand ard curve. 1% SDS (w/v) served as the blank. The OPA reagent was prepared by dissolving 80 mg of OPA (dissolved in 2 mL 200 proof ethanol), 3.81g sodium tetraborate, 200 uL of β-mercaptoethanol (BME), and 0.5 g SDS in DDW to make up a volume of 100 mL. OPA reagent was prepared fresh and kept in the dark. An aliquot (50 uL) of each of the stand ards, sample, and blank was loaded into polystyrene microplates specific for UV-readings followed by 200 μL of OPA reagent. Plates were immediately transferred into the microplate reader (Biotek, Winooski, VT, USA), and were incubated at room temperature for 2 min prior to reading the adsorbance at 340 nm. For each sample, the free amino group concentration (mg/mL) was adjusted for the protein content (mg/mL) to obtain % free amino groups per protein content, which was obtained as % lysine using the standard curve. The protein content in the prepared sample solutions was determined using the BCA kit following manufacturer's instructions. Remaining free amino group content over time was determined using Eq. 3. Sample calculation can be cound in Appendix C (Fig. 15 and 16).

$$\% \text{ Remaining Free Amino Groups} = 100 - 100 \times \frac{\% \text{ Lysine}_0 - \% \text{Lysine}_{\text{ stored}}}{\% \text{ Lysine}_0} \quad \textbf{Eq. 3}$$

Where

$\% \text{ Lysine}_0$ is the value for non-incubated SCI or SCH

$\% \text{ Lysine}_{\text{ stored}}$ is the value for PGSCH, PGSCI from different intervals of incubation time.

2.9.3. Determination of Fluorescent Compounds

Fluorescent compounds formation was determined following the method outlined by Rao and Labuza (2012), with modifications. Briefly, SCI and SCH incubated with or without maltodextrin, and non-incubated controls were dissolved, in triplicate, in DDW to make 1.0% protein solutions. An aliquot of 0.18 mL fresh pronase solution (20 U/mL in 50 mM Tris-HCl, pH 7.2) was added to each sample, and the mixture was incubated on a carousel mixer for 50 min at room temperature to ensure complete digestion. After digestion, the samples were centrifuged at 15, 682 x g for 15 min, and the supernatant was further diluted 1:1 (v/v) with phosphate buffered saline (PBS) solution (20 mM phosphate buffer, 15 mM NaCl, pH 7.0). Aliquots (200 µL) of each sample were placed into the wells of a black and opaque microplate. The fluorescence was measured at the excitation of 360 nm and emission of 460 nm. Fresh 5 ppm quinine sulfate solution, made by diluting quinine sulfate sulfuric acid solution (100 µg/mL quinine sulfate in 50 mM sulfuric acid) with PBS was used to correct the readings. Readings were corrected for protein content using the BCA assay kit, following the manufacturer's instructions. The fluorescence intensity (FI) of samples was determined by first correcting for the protein content of the sample and then by subtracting the FI values of their respective non-incubated controls, which served as blanks. The FI values were further corrected to the FI of 5 ppm quinine sulfate (**Eq. 4**). Sample calculation can be found in Appendix D.

$$\%\frac{\text{FI}}{\text{g protein}} = \frac{\left(\frac{\text{Emi}_{\text{sample}}}{\text{g protein}} - \frac{\text{Emi}_{\text{control}}}{\text{g protein}}\right)}{\text{Emi}_{\text{5ppm quinine sulfate}}} \qquad \textbf{Eq. 4}$$

Where

Emi = emission reading at 460 nm (band width 30 nm)

2.10. Measurement of Angiotensin-Converting Enzyme (ACE) Inhibitory Activity

The ACE inhibitory activity of SCH and glycated samples was measured, in triplicate, following the assay outlined by Otte et al. (2007) and Shalaby et al. (2006), and modified by Margatan et al. (2013), using furanacryloyl-L-phenylalanylglycylglycine (FA-PGG) as the ACE substrate and sodium borate buffer (0.1 M sodium tetraborate with 0.3 M sodium chloride, pH 8.3 at 37°C). The ACE was dissolved in cold DDW to reach a final concentration of 0.288 units/mL. The FA-PGG was diluted in 0.1 M sodium borate buffer to the concentration of 0.88 mM. Aliquots of both ACE and FA-PGG were stored at -20°C for further utilization. Samples (20 mg/mL) were prepared freshly in DDW, centrifuged at 15, 682 x g for 10 min, and filtered through 0.45 µm syringe filters. The filtered supernatant was further diluted to the concentration of 1, 1.75, 2.5, 5, 7.5, 10.0, 12.5, 15.0 and 17.5 mg solids/mL. Protein concentrations of the supernatant were determined using the Thermo ScientificTM PierceTM BCA assay kit, following manufacturer instructions.The reaction mixture including protein samples, ACE (0.288 units/mL of DDW), and FAPGG (0.88 mM prepared in 0.1M sodium borate buffer) was preheated to 37˚C before being added to a 96-well plate (Table 2). The adsorbance was read at 340 nm every 30 sec for 30 min in the microplate reader (BioTek Synergy HT, BioTek Instruments, Winooski, VT, USA) at 37°C. The ACE inhibitory activity was expressed as the slope of the decrease in adsorbance at 340 nm taken from 10-25 min of incubation. The IC_{50} values (protein concentrations that inhibit ACE by 50% in comparison to the control) were determined by plotting % ACE inhibition by protein concentration. The % ACE inhibition was calculated using **Eq. 5** and **Eq. 6**. The IC_{50} value was deduced from the plot of % of ACE activity reduction vs. the protein

concentration for each sample using the equation of the line (**Eq. 7**). Sample calculations can be found in Appendix E.

$$\text{Conc. of protein in the well (mg/mL)} = \frac{\text{Sample concentration (mg/mL)} \times 10\mu\text{L}}{170\mu\text{L}} \quad \textbf{Eq. 5}$$

$$\%\text{ACE Inhibition} = \frac{\text{slope}_{\text{control}} - \text{slope}_{\text{inhibitor}}}{\text{slope}_{\text{control}}} \times 100\% \quad \textbf{Eq. 6}$$

$$\text{IC}_{50} = y = mx + b \quad \textbf{Eq. 7}$$

Table 2. ACE activity assay mixtures

	Na-borate buffer[a] (uL)	**FA-PGG[b] (uL)**	**Sample[c] (uL)**	**ACE[d] (uL)**
Control blank	170	—	—	—
Control	10	150	—	10
Sample blank	160	—	10	—
Sample	—	150	10	10

[a] Sodium tetraborate buffer: 0.1 M borate, 0.3 M chlorine ion, pH 8.3; [b] 0.88 mM FA-PGG in Na-borate buffer; [c] Diluted concentrations of SCH; [d] 0.288 units/mL ACE in DDW.

2.11. Patient Recruitment for Determining Allergenicity

All sera and clinical laboratory services for this work were provided by Jaffe Food Allergy Institute (NY, USA). A total of 6 participants with milk protein specific IgE levels of 3.5 kU/L or greater were chosen for participation in this project (Table 3). A specific IgE level of 3.5 kU/L or greater is considered a high level according to the manufacturer of the ImmunoCap assay that was used to determine milk protein specific IgE concentrations.

Table 3. Milk protein specific IgE levels of different sera used.

Sera from Participant	IgE Milk Protein
1 (#1387)[a]	33.50
2 (# 824)	>100.00
3 (# 855)	>100.00
4 (#1010)	>100.00
5 (#1095)	>100.00

[a] Patient serum number.

2.12. Enzyme Linked Immunosorbant Assay (ELISA)

An indirect ELISA was used to assess the immunoreactivity of SCI, SCH, PGSCI, and PGSCH using sera from milk protein sensitive donors, as outlined by Brandon and Friedman (2002), with modifications. The 96-well microtiter plates were coated with 100 μL antigen (5 μg protein/mL phosphate buffered saline (PBS), pH 7.4, either SCI, SCH, PGSCI, or PGSCH) overnight at 4°C. Before moving onto the next step, plates were washed 4 times with 300 μL of PBST (PBS + 0.05% w/v Tween 20) using an automatic plate washer (Stat Fax, Orlando, Florida). Aliquots (200 uL) of (3% w/v) bovine serum albumin (BSA) + 0.05% w/v Tween 20 were added to each well to block the plate for 1 h at room temperature. After blocking and washing, diluted serum (1:20-1:400 using PBST) was added to the plate (100 μL/well), and the plate was incubated for 1 h at room temperature. After attaching a layer of primary antibody to the plate, aliquots (100 μL) of diluted secondary antibody, biotinylated anti-human goat IgE, (1:250 in TBST, tris buffered saline + 0.01% w/v Tween 20) was added to each well. The plate was further incubated for 1 hour at room temperature. This step was followed by adding diluted streptavidin alkaline phosphatase (SAP) (1:500 in TBS+ 0.1% w/v Tween 20) to the plate (100 μL/well) and incubating the plate for 30 min at room temperature. Lastly, 100 uL of p-nitrophenol phosphate, substrate to the SAP enzyme, was added to each well, and the plate was incubated for 30 min at room temperature. Adsorbance was then read at 405 nm using a microplate reader (Biotek). Apart from samples, blank was also set up by adding all reagents except the antigen. To preclude the background noise, such as the non-specific binding of the secondary antibodies and false positive reading from negative

serum, the secondary antibody control and negative control were also incorporated into each plate (Table 4). The % reduction in allergenicity of the different samples was determined in **Eq.8**. Sample calculation can be found in Appendix F.

$$\%\ \text{Reduction in allergenicity} = 100 \times \frac{\text{Corrected ABS}_{SCI} - \text{Corrected ABS}_{SCH}}{\text{Corrected ABS}_{SCI}} \qquad \textbf{Eq.8}$$

Where ABS is the adsorbance at 405 nm

Corrected ABS_{SCI} is equivalent to ($ABS_{SCI\ positive\ sera} - ABS_{2nd\ antibody}$)

Corrected ABS_{SCH} is equivalent to ($ABS_{SCH\ positive\ sera} - ABS_{2nd\ antibody}$)

Corrected ABS_{PGSCH} is equivalent to ($ABS_{PGSCH\ positive\ sera} - ABS_{2nd\ antibody}$)

Corrected ABS_{PGSCI} is equivalent to ($ABS_{PGSCI\ positive\ sera} - ABS_{2nd\ antibody}$)

Table 4. Reaction mixtures for ELISA

Assay	**Antigen[a] (µL)**	**BSA[b] (µL)**	**Pos Serum[c] (µL)**	**Neg Serum[d] (µL)**	**2nd Antibody[e] (µL)**
Positive	100	200	100	--	100
Blank	--	200	100	--	100
2nd Antibody	100	200	--	--	100
Negative	100	200	--	100	100

[a] SCI, SCH, PGSCI or PGSCH (5ug/mL); [b] Bovine Serum Albumin (3% in 0.05% Tween20); [c] Sera from individuals with casein specific IgE > 3.5kU/L; [d] Serum from an individual with no medical history of milk protein allergy; [e] Biotinylated Anti-Human Goat Secondary Antibody.

2.13. Statistical Analysis

Analysis of variance (ANOVA) was carried out using R software. Significant differences among the respective means were determined when a factor effect was found to be significant ($P \leq 0.05$) using the Tukey-Kramer multiple means comparison test. ANOVA tables can be found in Appendix G.

3. Results and Discussion

3.1. Extent of Hydrolysis and Protein Profiling

The extent of catalytic action of the different proteases was determined by comparing the DH values. The DH (3.6-16.7%) varied considerably among the different hydrolysates produced using the different enzymes and hydrolysis conditions, and was significantly higher in samples that had a higher enzyme to substrate ratio (Table 5). According to Nielsen (2009), extensive hydrolysis usually generates peptides with DH values greater than 25%. Therefore, the extent of enzymatic hydrolysis performed in this study can be considered limited to moderate.

Table 5. Percent DH and emulsification properties of sodium caseinate isolate (SCI) and hydrolysates (SCH) produced using different enzymes under various conditions, with hydrolysis time held constant (45 min)

Protein Sample	**DH[A] % ± SE[B]**	**EC[C] ± SE**	**EA[D] ± SE**	**ES[E] ± SE**
SCI	0^{f*}	1860 ± 10^{a}	0.110 ± 0.000^{f}	15.17 ± 0.17^{f}
SCH –Neutrase (50 °C, e/s = 0.067:100, pH=6.5)	5.20 ± 0.27^{d}	501 ± 13^{d}	0.140 ± 0.005^{de}	16.97 ± 0.59^{cd}
SCH –Neutrase (50 °C, e/s = 0.2:100, pH= 6.5)	16.7 ± 0.2^{a}	301 ± 13^{f}	0.220 ± 0.007^{a}	19.91 ± 0.24^{b}
SCH –Alcalase (55 °C, e/s = 0.04:100, pH =7.5)	3.60 ± 0.20^{e}	368 ± 19^{e}	0.120 ± 0.006^{e}	16.03 ± 0.91^{ef}
SCH –Alcalase (55 °C, e/s = 0.1:100, pH=7.5)	7.00 ± 0.20^{c}	289 ± 13^{f}	0.120 ± 0.002^{e}	16.31 ± 0.60^{de}
SCH –Alcalase (55 °C, e/s = 0.2:100, pH=7.5)	10.1 ± 0.1^{b}	249 ± 27^{g}	0.140 ± 0.004^{d}	21.58 ± 0.31^{a}
SCH-Trypsin (37°C, e/s=1.37:100, pH=7)	7.20 ± 0.10^{c}	706 ± 20^{c}	0.170 ± 0.002^{c}	17.75 ± 0.33^{c}
SCH-Trypsin (37°C, e/s=0.8:100, pH=7)	5.00 ± 0.10^{d}	1070 ± 13^{b}	0.190 ± 0.022^{b}	20.13 ± 0.94^{b}

[A] Degree of Hydrolysis; [B] Standard Error; [C] Emulsification Capacity; [D] Emulsification Activity; [E] Emulsification Stability ; [*] Means followed by the same lower case letter are not statistically different according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

The protein/peptide profile of casein was further visualized using urea-polyacrylamide gel electrophoresis (PAGE) (Fig. 2). Although SDS-PAGE is a powerful tool in protein separation, it is insufficient to separate individual casein proteins (α-, β-, and κ-CN) due to their similar molecular weight (20-25 kDa) and strong interactions (O'Mahony and McSweeney, 2002). Therefore, urea was added to both samples and gel formulas to prevent casein aggregation and increase the separation resolution of individual caseins based on size/charge ratios (Fox, 2003; Recio and López-Fandiño, 2009).

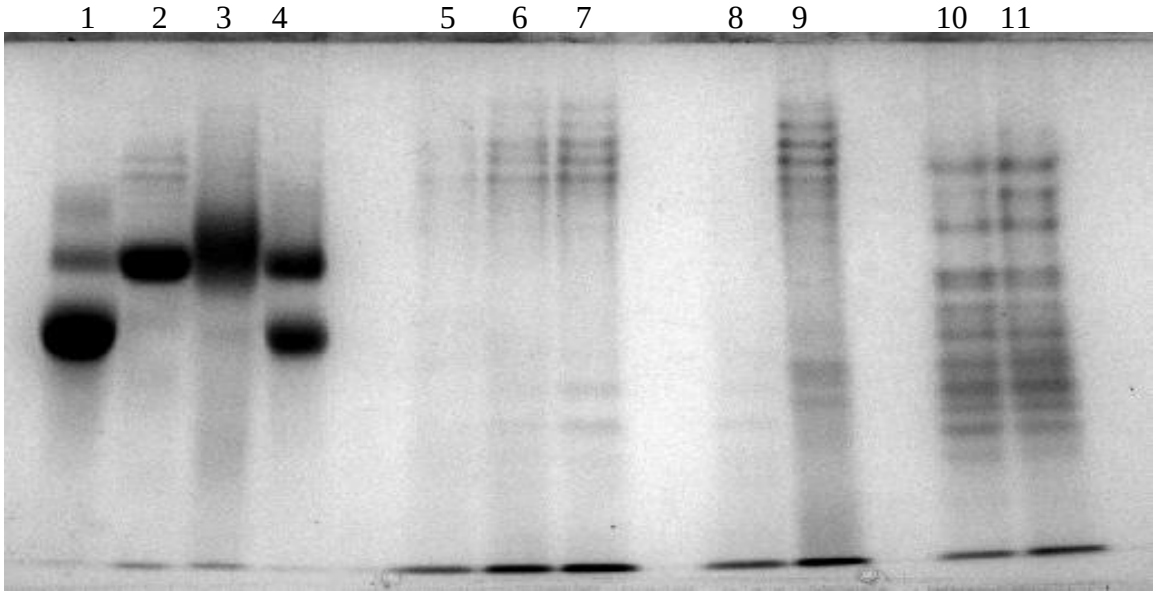

Figure 2. UREA-PAGE visualization of the hydrolysis pattern of prepared sodium caseinate isolate (SCI), sodium caseinate hydrolysate (SCH) produced using different enzymes under various conditions. The amount of protein loaded in each lane was 20 μg. Lane 1: α-caseinate standard; 2: β- caseinate standard; 3: κ-caseinate standard; 4 : SCI; 5: SCH with DH of 10.1 %, produced by alcalase at pH=7.5, 55 °C, 45 min, e/s=0.2:100; 6: SCH with DH of 7.0 %, produced by alacalase at pH=7.5, 55 °C, 45 min, e/s=0.1:100; 7: SCH with DH of 3.6 %, produced by alcalase at pH=7.5, 55 °C, 45 min, e/s=0.04:100; 8: SCH with DH of 16.7 %, produced by neutrase at pH=6.5, 50 °C, 45 min, e/s=0.2:100; 9: SCH with DH of 5.2 %, produced by neutrase at pH=6.5, 50 °C, 45 min, e/s=0.067:100; 10: SCH with DH of 7.2 %, produced by trypsin at pH=7, 37°C, 45 min, e/s=1.37:100; 11: SCH with DH of 5.0 %, produced by trypsin at pH=7, 37°C, 45 min, e/s=0.8:100.

After hydrolyzing SCI, using alcalase, neutrase, or trypsin, under various conditions, major protein bands faded or disappeared, and new peptide bands were visualized, indicating hydrolysis of the major caseins. In accordance with the DH results (Table 5), increasing the enzyme to substrate ratio significantly decreased the intensity of the protein bands in all SCHs (Fig. 2), indicating more protein and peptides were broken down and shifted to small molecular weight peptides. Compared to the other two enzymes, trypsin hydrolysis resulted in moderate DH (Table 5) and the SCH displayed highest band intensities (lane 10-11, Fig. 2), indicating a higher content of peptides with moderate molecular weight. Neutrase generated yet another hydrolysis pattern and a unique distribution of peptide in relation to DH. Alcalase, on the other hand, resulted in the production of lowest intensity of peptide bands (lane 5-7, Fig. 2), even at similar DH to that of trypsin-produced hydrolysates (Table 5), suggesting the production of more short chain peptides. Similar result was reported by other researchers. Adamson and Reynolds (1996) found casein phosphopeptides produced by alcalase were relative short to that produced by trypsin.

The different molecular weight distribution of peptides among the different hydrolysates maybe is due to enzyme specificity. Alcalase is an alkaline protease produced from Bacillus licheniformis (Hyun and Shin, 2000), and it cleaves peptide bonds at the carboxyl groups of hydrophobic, hydroxyl (Ser), acidic (Glu), and basic (Lys) amino acid residues (Doucet et al., 2003). On the other hand, neutrase is a metallo protease (Hyun and Shin, 2000), which hydrolyzes proteins less efficiently due to limited cleavage at the carboxyl groups of phenylalanine and leucine residues (Adler-Nissen, 1986). Simlarly, trypsin is specific to carboxyl groups that are only contributed by positively charged arginine and lysine residues (Olsen et al., 2004). Due to narrower cleavage specificity, trypsin hydrolyzed the casein molecules into larger peptides (Carreira et al., 2004). According to Qi et al. (2003), tryptic hydrolysis of whole casein (5%-30% of DH) resulted in predominate amount of polypeptides with limited amount of oligopeptides. The polypeptides were difficult to be further hydrolyzed by trypsin due to limited amount of available peptide that can be susceptable to trypsin hydrolysis (Qi et al., 2003). On the other hand, alcalase had broader specificity and could break up

peptides even between adjacent aromatic amino acid residues (Kim et al., 2007). Therefore, alcalase would further hydrolyze large peptides into smaller chains, leading to fainter bands (lane 5-7, Fig. 2).

3.2. Effect of Enzymatic Hydrolysis on Emulsification Properties

3.2.1 Effect of Enzymatic Hydrolysis on Emulsification Capacity (EC)

Emulsification capacity was evaluated at pH 7 for SCI and the different SCH samples (Table 5). Intact SCI exhibited high EC value. The high EC of SCI is attributed to casein's superior amphiphilic and flexible structure, which allow them to adsorb at the water-oil interface quickly and easily (Arai et al., 1988). Intact SCI was also reported to have high EC by other researchers but at a lower value. Mohanty et al. (1988) reported that sodium caseinate could emulsify 268 g oil/g protein at pH of 7. The inconsistent result of EC might be due to different test settings utilized such as equipment, protein concentration and the type of blender and oil. In fact, EC is a property of the whole emulsion system rather than just the protein (Tornberg and Hermansson, 1977).

After enzymatic hydrolysis, EC of all SCHs decreased significantly (Table 5). When comparing hydrolysates produced by the same enzyme, reduction of EC was greater in the hydrolysate that had higher DH. Trypsin-produced hydrolysates retained the highest EC, with more than half of the EC being retained at 5.0% DH compared to SCI. Alcalase-produced SCH, even at 3.6% DH, lost more than five-fold of the EC. The drastic loss of EC is mostly attributed to elevated levels of short-chain peptides generated during enzymatic hydrolysis as discussed in the earlier section. Short-chain casein hydrolysates had lower molecular flexibilities, determined as the ratio of α-helix to β-structure, than the original casein protein (Wang et al., 2013), which limited their ability to unfold and reorient at the water-oil interface, thus reducing the interfacial tension less efficiently (Kato et al., 1995). In fact, a minimum peptide length of 20 residues is often required to provide good emulsification properties for casein proteins (Lee et al., 1987). Apart from molecular weight, having good amphiphilicity of peptides is also of critical

importance to the emulsification properties (Lee et al., 1987; Chobert et al., 1988; Gauthier et al., 1993). The data suggested that trypsin-hydrolzyed peptides were more amphiphilic than neutrase- and alcalase-generated peptides, which allowed them to be adsorbed at the interface.

It should be noted that conflicting observations related to the effect of hydrolytic modification on the EC of casein have been reported. Haque and Mozaffar (1992) found that controlled trypsin-produced casein hydrolysates (2% DH) improved the oil-holding capacity by more than 600%, while Agboola and Dalgleish (1996a) and Chobert et al. (1988) determined superior EC in native casein, rather than trypsin-digested hydrolysates. The ambiguous relationship might result from different DH of casein hydrolysates investigated and various methods utilized to measure the emulsification properties. In addition, factors such as the inefficiency for blender to mix very viscous emulsions, and operators' subjective determination of the amount of oil required for phase inversion can further lead to great variation in final results (Pearce and Kinsella, 1978). Therefore, a standardized manner should be adopted to characterize all hydrolysis products with the same testing method for a more accurate comparison.

3.2.2. Effect of Enzymatic Hydrolysis on Emulsification Stability (ES) and Activity (EA)

Apart from EC, emulsification properties were also determined for ES and EA. EA refers to the maximum interfacial area formed by the unit weight of a protein and is related to how quickly the protein forms film. ES, on the other hand, describes the stability of an emulsion during a certain period of time at a given temperature and gravitational field (Panyam and Kilara, 1996).

Overall hydrolysis under the conditions tested resulted in increased EA and ES. Trypsin-hydrolyzed samples had higher EA and ES than alcalase and neutrase-hydrolyzed samples at similar DH (Table 5). This was in agreement with Chobert et al. (1988) who found increased EA for SCH produced by trypsin under mild hydrolysis conditions (pH 8, 37°C, E/S of 0.5%, 1.0% and 1.0% for 5-, 17-, and 110-min, with DH of 4.3%, 8.0% and 9.9%, respectively). Mietsch et al. (1989) found that casein

hydrolysates made by alcalase and neutrase (DH <10%) did not significantly improve the emulsification stability, but had improved emulsification activity. The beneficial effect of limited enzymatic hydrolysis on emulsification activity and stability might be due to the exposure of hydrophobic groups and the increased number of ionization groups, which promotes a better balance between hydrophobicity and hydrophilicity. Therefore, enzymatic hydrolysis affects the amphiphilic characteristics of casein proteins and the efficiency of peptides adsorbing at the oil-water interface (Lee et al., 1987; Chobert et al., 1988; Gauthier et al., 1993). For instance, cutting off the C-terminal peptide (f 146-199) of α_{s1}-CN via a protease made the modified fragment more surface-active with its typical amphipilic nature (Shimizu et al., 1983). Depending on different enzyme specificities, various peptides were released that differed in amphiphlicity. This was substantiated by Gauthier et al. (1993) who compared the emulsification activity of whey protein hydrolyzed by trypsin and chymotrypsin and found that tryptic hydrolyzates were more surface active than the chymotyrptic hydrolyzates. The data have suggested that trypsin-hydrolyzed peptides were more amphiphilic than neutrase- and alcalase-generated peptides, which allowed them to be adsorbed at the interface more efficiently.

EA is also affected by the molecular weight of the peptide. Peptides should not be so small that they lose the capacity to interact with both aqueous and nonaqueous phases (Turgeon et al., 1992). A certain chain length is required to have a greater probability of having both hydrophilic and hydrophobic moieties on the same unit (Agboola and Dalgleish, 1996a). According to Kilara and Panyam (2003) and Enser et al. (1990), peptides exhibit emulsifying activity only when they possess some helical confirmation and have sufficient residues to support at least three turns of the helix. The inferior EA of alcalase generated peptides might be due to the presence of a higher amount of short-chain peptides (Fig. 2).

The improved ES of peptides might result from their different mechanism of adsorption and emulsification than intact caseins. While caseins form an adsorbed layer by extending and spreading at the surface, casein peptides aggregate with each other at the interface and form a more densely packed and thick film, confirmed by electron microscopy observation (Lee et al., 1987). A thicker interface gave a more viscous

emulsion (less free flowing) than one with a thinner interface, therefore, making the emulsion more stable (Haque, 1993). With the exception of trypsin-produced hydrolysates, a higher DH was associated with an increased ES. Although larger peptides generally provide higher ES than smaller peptides (Panyam and Kilara, 1996), the increased ES at higher DH in this study might result from a higher degree of peptide-peptide association at the water-oil interface. In addition, some synergistic effect might have existed from the presence of peptides having different properties, which increased the adsorption sites of peptides by interacting with each other at the surface (Lee, et al., 1987). One good example was β-CN (f l93-209) and hydrophilic glycomacropeptide. The interaction between these two peptides resulted in an amphipathic complex with an increased EC and thicker film (Lee, et al., 1987). However, it should be noted that all hydrolysis conditions were controlled to a limited degree (<25%, Table 5). Extensive hydrolysis, on the other hand, is detrimental to emulsification properties due to the increased amount of free amino acids (Panyam and Kilara, 1996). The superior ES of trypsin-hydrolyzed samples might result from the different peptide generated. Trypsin-generated peptides has positively charged residues (lysine and arginine) at the C-terminals (Baker and Sali, 2001), which might have increased the emulsification stability through electrostatic repulsion.

3.3. Effect of pH on Emulsification Properties of Sodium Caseinate Hydrolysates

Casein proteins were found to have inferior emulsification properties at pH close to their isoelectric point (pH 4.6), which significantly limits the utilization of casein protein ingredients in acidic food products (Chandan, 1997; Chandan and Kilara, 2011). Inferior emulsification properties at pH close to the isoelectric point were attributed to flocculation due to reduced protein solubility (Chobert et al., 1988; Dickinson, 2003b). Therefore, it was of great interest to investigate whether enzymatic hydrolysis will have a positive effect on emulsification properties at pH conditions that are acidic or close to the original pI of the protein. Accordingly, the emulsification properties of SCI, and trypsin-produced SCH at pH 4, 5.3 and 7 were measured and compared (Table 6).

Trypsin produced hydrolysates (DH of 5%) had significantly higher EC compared to that of intact SCI at pH of 4.0 and 5.3 (Table 6). The enhancement in EC could be attributed to an increased solubility under these conditions. According to Chobert et al. (1988), the solubility of SCH (trypsin, pH of 8, 37°C, and E/S from 0.5%, 1% and 1% for 5-, 17-, and 110-min) was increased particularly in the pI range, with 45%, 65%, and 70%, respectively, higher than control casein, due to an increased amount of ionizable groups upon hydrolysis of peptide bonds (Panyam and Kilara, 1996). Higher solubility of SCH might facilitate migration, adsorption and spreading at oil/water interfaces at the interface of protein, therefore forming the film more efficiently (Chobert et al., 1988).

Table 6. Effect of pH on emulsification properties of sodium caseinate isolate (SCI) and hydrolystates (SCH) produced by trypsin at pH=7, 37°C, and e/s=0.8:100 for 45 min

Protein Sample	SCI			SCH		
	EC[B] ± SE[A]	EA[C] ± SE	ES[D] ± SE	EC[B] ± SE	EA[C] ± SE	ES[D] ± SE
pH 7	1860 ± 10[a*]	0.105 ± 0.004[b]	15.1 ± 0.2[b]	1071 ± 11[a]	0.232 ± 0.004[a]	22.1 ± 3.9[a]
pH 5.3	448 ± 9[b]	0.133 ± 0.006[a]	16.5 ± 0.9[a]	665.5 ± 21.4[c]	0.027 ± 0.002[b]	11.5 ± 0.4[b]
pH 4	382 ± 13[c]	0.072 ± 0.002[c]	14.7 ± 0.1[b]	772.5 ± 48.5[b]	0.054 ± 0.016[b]	11.5 ± 0.5[b]

[A] Standard Error; [B] Emulsification Capacity; [C] Emulsification Activity; [D] Emulsification Stability;
[*] Means followed by the same lower case letter are not statistically different according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

However, ES and EA of SCH were significantly lower than that of SCI at pH 4 and 5.3 (Table 6). This observation is in agreement with Chobert et al. (1988) as well, where significant reduction of EA and ES of trypsin-derived SCH (DH of 4.3%, 8.0%, and 9.9%) at pH 3.5-5.5 were reported based on the turbidity measurement. The turbidity absorbance of an emulsion is a function of both particle size and concentration, and the change in turbidity indicates instability of the emulsion (Reddy and Fogler, 1981).

Despite higher solubility of SCH than SCI at acidic conditions, the actual concentration of peptide at the interface might be low. Therefore, the film formed by SCH at acidic conditions was thinner and unstable due to limited protein-protein aggregation on the interface. According to Mohanty et al. (1988), the potential barrier for protein to adsorb at the water-oil interface was enhanced as pH approached the iso-

electric point, which reduced the surface area covered by protein films. Therefore, proteins formed less densely packed films with reduced stability. In addition, pH might change the amphiphlic property of SCH more easily than SCI via the exposed ionizable groups (Lee et al, 1987). One example is β-CN (f1-25), which contains 7 glutamine, 4 phospho-serum, and 2 arginine residues. At pH 3, the dissociation of glutamine residues was suppressed, which makes the N-terminal portion of β-CN (f1-25) less hydrophilic and changed the amphiphilic properties of β-CN (f1-25) (Lee et al., 1987). It was likely that when pH was adjusted near the isoelectric point, trypsin-generated SCH were not amphiphilic enough, which resulted in reduced EA than that of SCI. While adjusting pH to the acidic condition significantly improved the EC of SCH, but was detrimental to both ES and EA.

Combining the emulsification results of this study, trypsin was more efficient in producing SCH with good emulsification properties than the other two enzymes tested. Among all hydrolysates generated, SCH (trypsin, 5% DH) retained the maximum emulsification properties. Choices must be carefully considered when using such a product under different conditions, keeping in mind an acceptable balance between EC and ES.

3.4. Effect of Different Enzymatic Hydrolysis Conditions on the ACE Inhibitory Activity of the Produced Caseinate Hydrolysates

Limited enzymatic hydrolysis releases peptides with not only modified functionality but also bioactivity. In this study, SCH produced by different enzymes under various hydrolysis conditions were compared for the angiotensin converting enzyme inhibitory activity due to its critical role in regulating blood pressure and preventing hypertension (FitzGerald and Meisel, 2003).

The ACE-inhibitory activity of SCH produced using alcalase, neutrase and trypsin that resulted in similar DHs, was determined in order to assess differences in bioactivity resulting from the use of different enzymes. The differences were assessed by determining the IC_{50} values, the protein concentration required to inhibit ACE activity by

50%. A lower IC_{50} value corresponds to higher ACE inhibitory activity because less protein is required to cause a 50% inhibition. The ACE-inhibitory activity was assessed for SCH samples that had the lowest DH. In most cases, moderate hydrolysis is enough to achieve the maximum release of bioactive peptides. For instance, Xu et al. (2014) found the casein hydrolysates prepared by neutrase showed the highest ACE-inibitory activity at DH of 13% with the IC_{50} value being 0.04 mg/mL. In another work by Zhao and Li (2009), where casein hydrolysates were produced by alcalase and to a DH of 11.2% exhibited IC_{50} value of 0.047 mg/mL. In this study, SCHs with the least DH were investigated due to the need to maintain highest emulsification properties. The SCH generated had a prominent ACE-inhibitory activity (Table 7). The IC_{50} values of the three SCH samples tested did not vary much and fell within the range of reported IC_{50} values—0.028-1.067 mg protein/mL for casein-derived peptides (Pihlanto-Leppälä et al., 1998). Since there was no major differences in the activity of the hydrolysates, SCH produced using trypsin was selected for further testing given its significantly better emulsification properties.

Table 7. IC_{50} value (mg protein / mL) and the degree of hydrolysis (DH) of sodium caseinate hydrolysates (SCH) produced using different enzymes

SCH ± SE[A]	**IC_{50}[B] (mg/mL) ± SE**	**%DH± SE**
SCH-Trypsin (37°C, e/s=0.8:100, 45 min, pH=7)	0.223 ± 0.004[a*]	5.0 ± 0.1[a]
SCH –Neutrase (50 °C, e/s = 0.067:100, 45 min, pH 6.5)	0.222 ± 0.008[a]	5.3 ± 0.3[a]
SCH –Alcalase (55 °C, e/s = 0.04:100, 45 min, pH 7.5)	0.192 ± 0.003[b]	3.6 ± 0. 2[b]

[A] Standard Error; [B] IC_{50}: Protein concentration leading to 50% inhibition of ACE activity; [*] Means followed by the same lower case letter are not statistically different according to the Tukey-Kramer multiplmeans comparison test ($P \leq 0.05$).

3.5. Effect of Enzymatic Hydrolysis on Immunoreactivity of Sodium Caseinate

Comparing SCH produced under all conditions, trypsin-hydrolyzed sodium caseinate (5% DH) maintained the highest emulsification properties and angiotensin converting enzyme inhibitory activity, and therefore it was of great interest to investigate its remaining allergenicity. To investigate the effect of enzymatic hydrolysis on modifying the allergenicity of sodium caseinate, the immunoreactivity of SCH produced by trypsin (DH of 5%) was quantitatively assessed following ELISA, using sera from five allergenic donors for varying milk protein specific IgE levels (Table 3). Overall, significant reductions in immunoreactivity, ranging from 4.49% to 44.49%, were observed for SCH relative to SCI, indicating that trypsin hydrolysis reduced the immunoreactivity of sodium caseinate to some degree (Table 8). The differences of reductions in immunoreactivity might result from the sensitivity of the sera used and the variation in milk-protein-specific IgE among the different sera that could react with the epitopes present in the casein protein samples. In addition, enzymatic hydrolysis can generate additional conformational epitopes when proteins unfold and rearrange, and may expose epitopes that were originally buried within the original protein structures (Bu et al., 2013). IgE from some patients' sera may not.

Table 8. Reduction in Immunoreactivity of sodium caseinate hydrolysates (SCH), produced by trypsin at 37°C, e/s=0.8:100, 45 min, pH=7 with DH of 5.0 % relative to sodium caseinate isolate using sera from five participants.

Serum	% Reduction in immunoreactivity of SCH compared to SCI ± SE[A]
1	$24 \pm 3^{b*}$
2	45 ± 5^{a}
3	18 ± 3^{c}
4	25 ± 4^{b}
5	6.3 ± 2.5^{d}

[A] Standard Error; [*] Means followed by the same lower case letter are not statistically different according to the Tukey-Kramer multiplmeans comparison test ($P \leq 0.05$).

Several studies found that enzymatic hydrolysis can be an effective tool to modify allergenicity of proteins. For instance, casein hydrolysates with a DH of 23% produced

by alcalase via a two-step membrane system reduced allergenicity by 98% (Lin et al., 1997). Through specific enzymatic cleavage of the peptide bonds, both linear and conformational epitopes can be destroyed, therefore reducing the immunoreactivity of the protein (Bu et al., 2013). In this study, DH was limited. Therefore, the reduction in immunoreactivity was not high enough to claim a hypoallergenic product (% reduction in allergenicity ≥ 90%). Additional modification to SCH produced in this study is needed for further reduction in immunoreactivity.

3.6. Extent of Maillard-Induced Glycation of Sodium Caseinate Hydrolysates Incubated with Maltodextrin

Glycation has been found effective for enhancing emulsification properties (Shepherd et al., 2000; O'Regan and Mulvihill, 2009; Oliveira et al., 2014) and reducing allergenicity of dairy proteins (Kobayashi et al., 2001; Nakamura et al., 2008). Additionally, according to the recent work by Lasky (2015), limited and controlled glycation did not affect the ACE- inhibitory activity of whey protein hydrolysate. Therefore, it was of great importance to control the Maillard reaction to a limited degree to maximize the emulsification properties, further reduce the allergenicity, while maintaining the bioactivity of SCH.

3.6.1. Amadori Compounds and Melanoidins Formation

Maillard glycation was first induced at 0.49 a_w and 60°C. Although incubation temperatures usually range from 40-80°C, 60 °C is the most common across several previous studies (Oliveira et al., 2014). The choice of 0.49 a_w was based on the work of Wang and Ismail (2012) who found that the Maillard reaction between whey protein isolate and dextran (10 kDa) can be successfully limited to initial stages by adjusting the a_w to 0.49, right below the reaction's optimum a_w values (0.5-0.8). However, minor differences in the adsorbance at 304 nm of SCH incubated with and without maltodextrin were observed even after 120 h of incubation (Fig. 18, Appendix H), indicating that glycation and hence Amadori compound formation was not initiated under these

conditions. It is possible that 0.49 a_w was too low to promote good mobility between casein and maltodextrin, due to the different protein structures among casein and whey.

Incubation at 0.79 a_w was investigated, keeping other parameters (temperature, incubation time, and protein to maltodextrin ratio) constant. Several reported studies have successfully induced glycation between casein proteins and carbohydrates at 0.79 a_w and 60 °C (Regan & Mulvihill, 2009; Shepherd et al., 2000; Regan & Mulvihill, 2013; Kato et al., 1992). A significant difference in the adsorbance at 304 nm of SCH incubated with and without maltodextrin confirmed that glycation did occur (Fig. 3). The significant difference in adsorbance between 0 h (un-incubated) and 24 h incubated SCH with maltodextrin indicated that Maillard-induced glycation was initiated within the first 24 h of incubation.

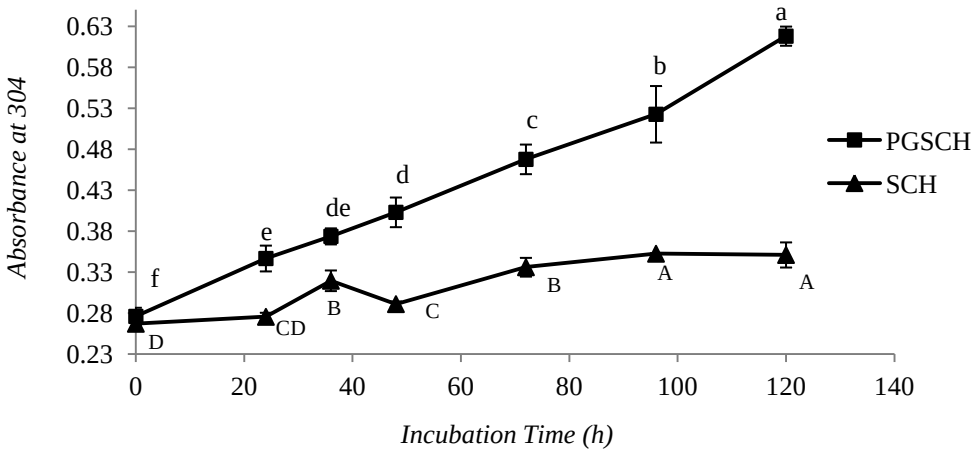

Figure 3. Amadori compound formation in samples of sodium protein hydrolysate (SCH) incubated with maltodextan and control SCH (trypsin at pH=7, 37°C, 45 min, e/s=0.8:100 with DH of 5.0 %) incubated without maltodextran at 60˚C, 0.79 a_w for 24 to 120 h, as determined determined by UV-Visible difference spectroscopy at 304 nm. PGSCH referred to glycated hydrolysate. Error bars represent standard errors (n=3). Different lower case and upper case letters above and below the shape indicate significant differences between different time points of samples according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

Formation of Amadori compounds continued to occur as incubation time increased up to120 h, as indicated by the significant increase in adsorbance over time.

This observation was in agreement with Kato et al. (1992) who found that Maillard reaction occurred in casein-polysaccharide (60-90 KD) mixtures incubated in a dry state at 79% relative humidity and 60°C for 24 h.

However, visual browning and caking were observed in powders after only 24 h of incubation, indicating that the Maillard reaction progressed too fast into the undesirable advanced stages. Both browning and caking increased as incubation time progressed. To further confirm the formation of brown-colored melanoidins in the final stages of the Maillard reaction, adsorbance at 420 nm was measured. The progression of the Maillard reaction into the advanced stages is often monitored by measuring adsorbance at 420 nm (Martins et al., 2000; Jiménez-Castaño et al., 2005b; Corzo-Martinez et al., 2008; Wang and Ismail 2012). Absorbance values indicated that browning significantly increased over the 120 h incubation period (Fig.19, Appendix I).

It should be noted that Amadori compound formation and browning were also observed in the incubated controls (SCH incubated without maltodextrin). This observation was likely due to the presence of lactose in the SCH (~3.5% lactose, according to manufacturer's specifications), which could participate in the Maillard reaction with the casein proteins, leading to the formation of Amadori compounds and browning.

In order to better control the Maillard reaction rate, SCI was dialyzed prior to incubation to remove residual lactose and a_w was reduced to limit the mobility of proteins and maltodextrins (Ames, 1990). Accordingly, samples were incubated at 0.63 a_w, keeping other parameters (temperature, incubation time, and protein to maltodextrin ratio) constant. Difference in adsorbance at 304 nm of SCH incubated with and without maltodextrin was also observed after 24 h of incubation, indicating that glycation did occur (Fig. 4). However, the extent of glycation was reduced compared to that at 0.79 a_w (Fig. 3). The formation of Amadori compounds continued to occur as incubation time increased up to 120 h, as indicated by a significant increase in the adsorbance over time. A plateau was observed after 48 h, suggesting equilibrium between new Amadori compound formation and loss of Amadori compounds, as a result of conversion to intermediate and advanced glycation end-products. The extent of browning monitored at

420 nm was lower than what was observed at 0.79 a_w (Fig. 20, Appendix I). Only a slight increase of adsorbance at 304 nm was also noted in the control sample (SCH incubated without maltodextrin) between 24 and 120 hours (Fig. 4), suggesting that dialysis successfully removed most of the lactose from SCI. This observation was in agreement with Shepherd et al. (2000), who found that dialysis of casein significantly reduced the browning formation of incubated casein-maltodextrin at 60°C and 79% a_w for 96 h.

Amadori compounds also formed in SCI incubated with maltodextrin (Fig. 5 and 6). SCI was glycated to determine the effect of glycation alone on modifying functionality and allergenicity of casein proteins. However, a higher UV-Visible difference at 304 of glycated SCH-maltodextrin was observed than that of glycated SCI-maltodextrin, indicating a higher degree of glycation in incubated SCH. This observation is attributed to more exposed free amino groups in SCH due to breakage of peptide bonds, which increased the reactivity of SCH with maltodextrin.

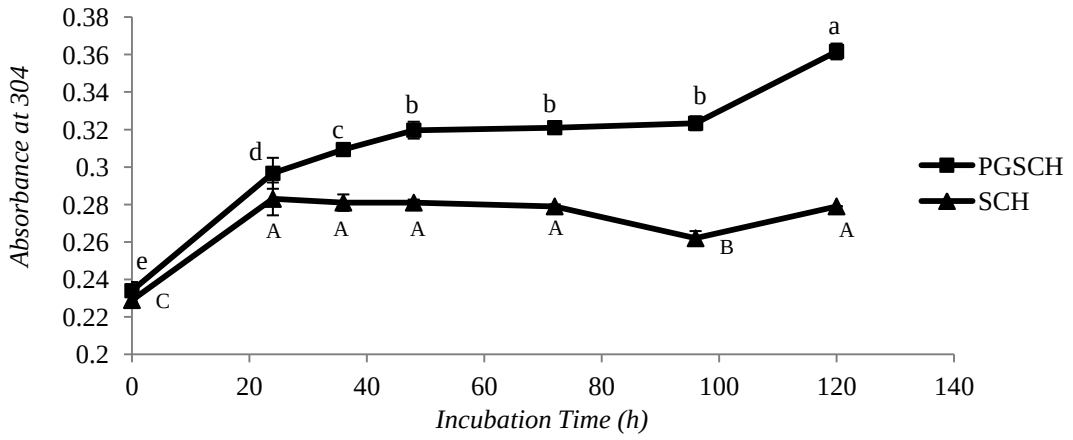

Figure 4. Amadori compound formation in samples of sodium protein hydrolysate (SCH) incubated with maltodextan and control SCH (trypsin at pH=7, 37°C, 45 min, e/s=0.8:100 with DH of 5.0 %) incubated without maltodextran at 60˚C, 0.63 a_w for 24 to 120 h, as determined by UV-Visible difference spectroscopy at 304 nm. PGSCH referred to glycated hydrolysate. Error bars represent standard errors (n=3). Different lower case and upper case letters above and below the shape indicate significant differences between different time points for samples according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

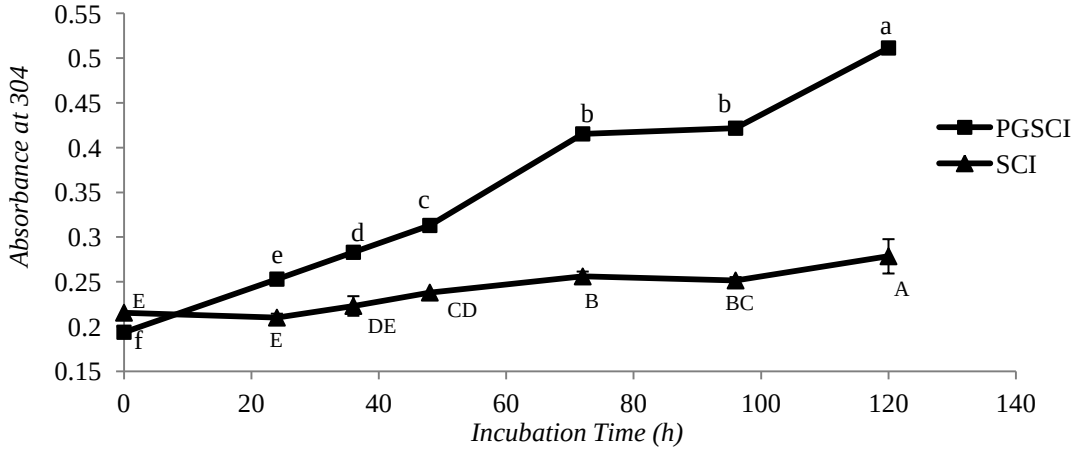

Figure 5. Amadori compound formation in samples of sodium protein isolate (SCI) incubated with maltodextan and control SCI incubated without maltodextran at 60˚C, 0.79 a_w for 24 to 120 h, as determined by UV-Visible difference spectroscopy at 304 nm. PGSCI referred to glycated sodium caseinate isolate. Error bars represent standard errors (n=3). Different lower case and upper case letters above and below the shape indicate significant differences between different time points for samples according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

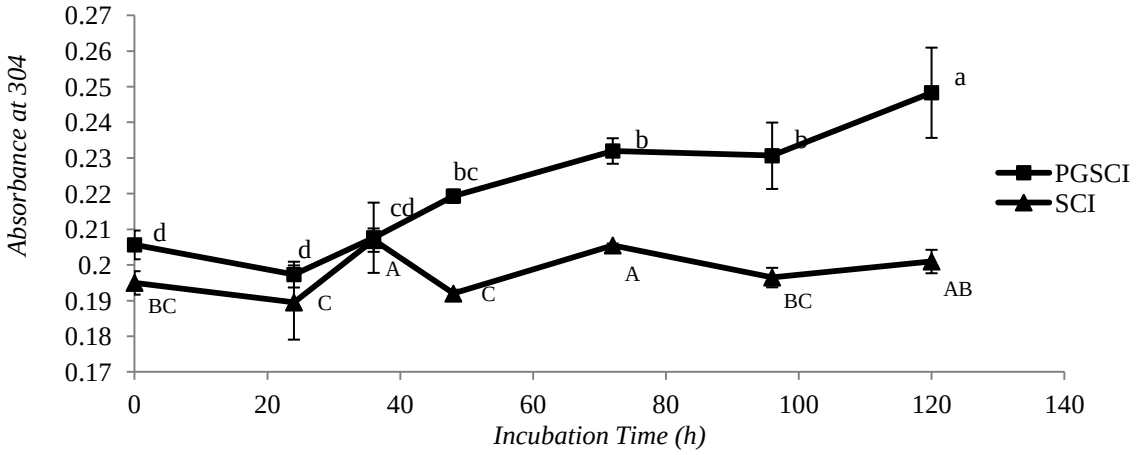

Figure 6. Amadori compound formation in samples of sodium protein isolate (SCI) incubated with maltodextan and control SCI incubated without maltodextran at 60˚C, 0.63 a_w for 24 to 120 h, as determined by UV-Visible difference spectroscopy at 304 nm. PGSCI referred to glycated sodium caseinate isolate. Error bars represent standard errors (n=3). Different lower case and upper case letters above and below the shape indicate significant differences between different time points for samples according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

3.6.2. Loss of Free Amino Groups

The Maillard reaction induces a covalent linkage between a reducing sugar and a free amino group (mainly the ε-amino group of lysine, and occasionally α-amino groups of terminal amino acids), so the percent of amine blockage can be used to indicate the extent of the Maillard-induced glycation (Ames 1990; Wang and Ismail 2012). The percent of free amino group loss was extensive at 0.79 a_w. After 24 h of incubation, a significant decrease in free amine groups (11.7%) was noted for SCH, confirming that the Maillard-glycation was initiated within the first 24 h with maltodextrin (Fig. 7). Maillard-induced glycation continued thereafter, as the % reduction in free amine groups continued to increase reaching to 28.6% after 120 h of incubation. These results are in accordance with the UV- Visible difference spectroscopy data discussed earlier, which

also indicated that the Maillard-glycation was initiated within the first 24 h of incubation and continued thereafter.

At 0.63 a_w, a slight decrease in free amine groups (1.1%) was noted for SCH after 24 h of incubation (Fig. 8). Decrease in free amine groups reached 14% after 120 h of incubation. Similar to UV-Visible difference spectroscopy data, less reduction in % free amino groups was observed compared to what was seen at 0.79 a_w, confirming that glycation at 0.63 a_w occurred at lower rate.

A higher % loss of free amino groups was observed in SCH than in SCI (Fig. 9 and 1), which was also in agreement with UV-VIS difference spectroscopy data suggesting a higher degree of glycation in SCH than in SCI, under both conditions. Combining both UV-Visible difference and OPA results, glycation at 0.63 a_w was chosen over 0.79 a_w for further investigation, due to a slower reaction rate and minimum bowning formation.

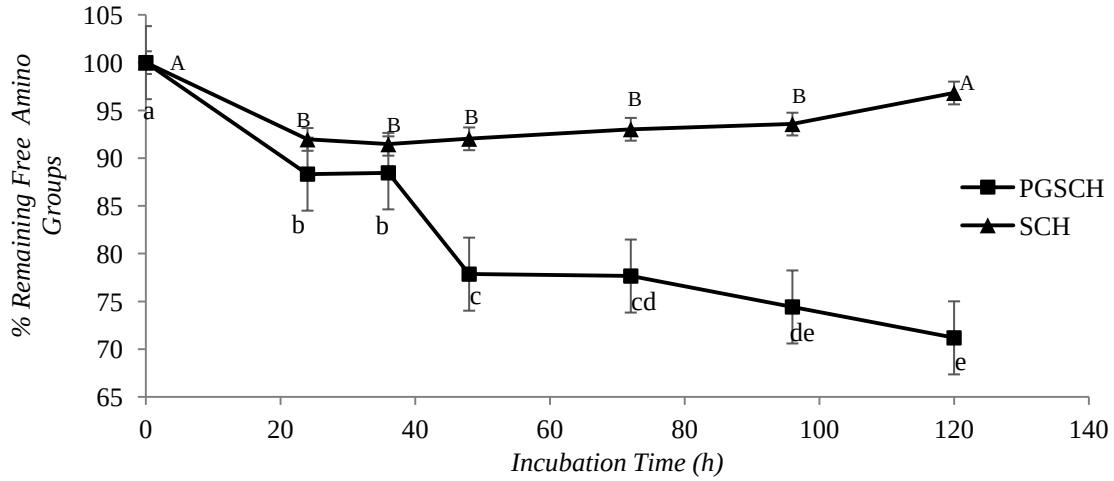

Figure 7. % remaining free amino groups for sodium caseinate hydrolysates (SCH) trypsin at pH=7, 37°C, 45 min, e/s=0.8:100 with DH of 5.0 % and incubated with maltodextran at 60˚C, 0.79 a_w for 24 to 120 h, as determined by OPA method. PGSCH referred to glycated hydrolysate. Error bars represents the standard errors (n=3). Different lowercase and upper case letters indicate significant differences among samples incubated at different time points according to Tukey-Kraemer multiple means comparison ($P \leq 0.05$).

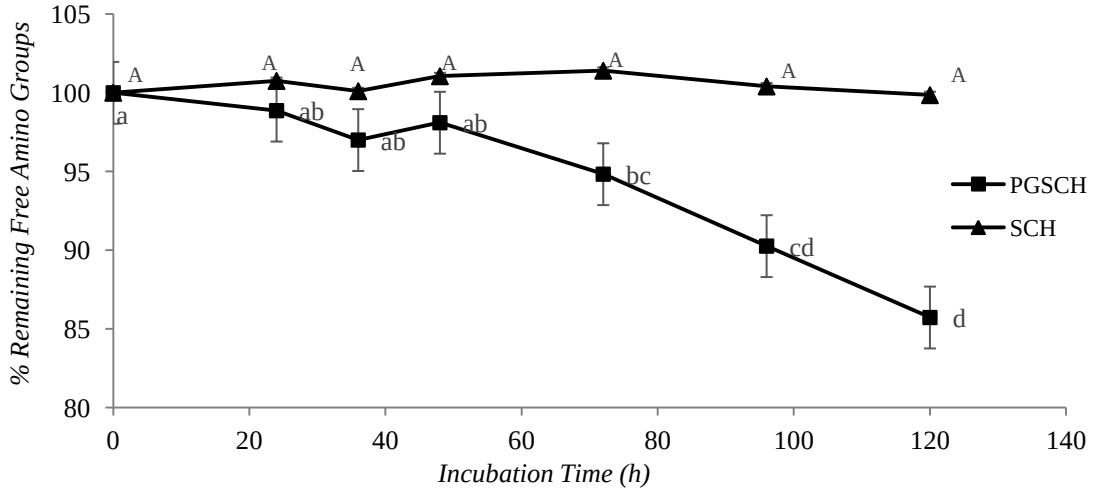

Figure 8. % remaining free amino groups for sodium caseinate hydrolysates (SCH) produced by trypsin at pH=7, 37°C, 45min, e/s=0.8:100 with DH of 5.0 % and incubated with maltodextran at 60˚C, 0.63 a_w for 24 to 120 h, as determined by OPA method. PGSCH referred to glycated hydrolysate. Error bars represents the standard errors (n=3). Different lowercase and upper case letters indicate significant differences among samples incubated at different time points according to Tukey-Kraemer multiple means comparison ($P \leq 0.05$).

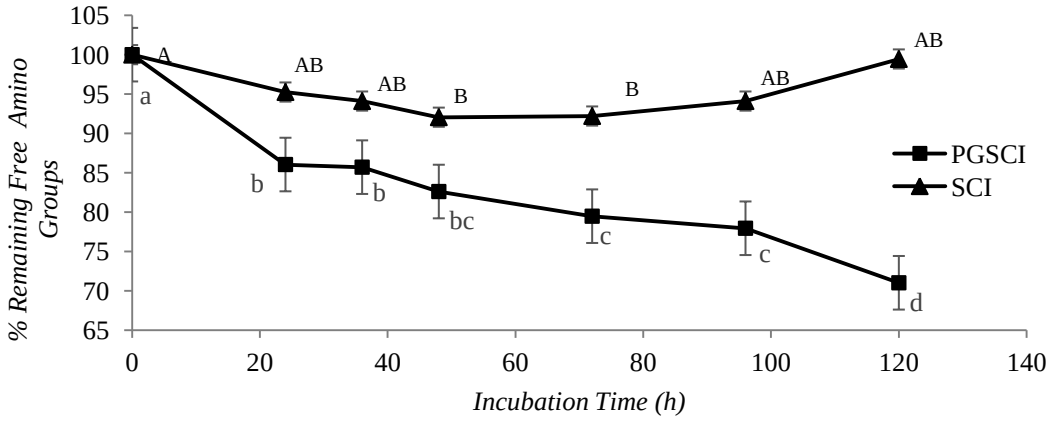

Figure 9. % remaining free amino groups for sodium caseinate isolate (SCI) and incubated with maltodextran at 60˚C, 0.79 a_w for 24 to 120 h, as determined by OPA method. PGSCI referred to glycated sodium caseinate isolate. Error bars represents the standard errors (n=3). Different lowercase and upper case letters indicate significant differences among samples incubated at different time points according to Tukey-Kraemer multiple means comparison ($P \leq 0.05$).

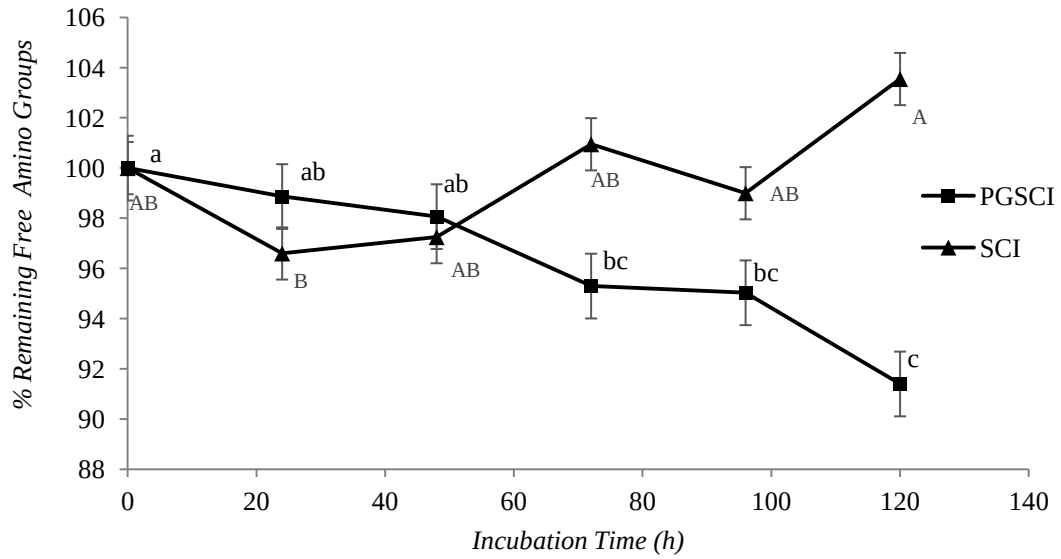

Figure 10. % remaining free amino groups for sodium caseinate isolate (SCI) and incubated with maltodextran at 60˚C, 0.63 a_w for 24 to 120 h, as determined by OPA method. PGSCI referred to glycated sodium caseinate isolate. Error bars represents the standard errors (n=3). Different lowercase and upper case letters indicate significant differences among samples incubated at different time points according to Tukey-Kraemer multiple means comparison ($P \leq 0.05$).

3.6.3. Fluorescence Compound Formation

Amadori compounds, formed in the initial stage, further rearrange and degrade through reactions, such as dehydration and fission, to form fluorescent colorless intermediates before the development of brown pigments (Bastos et al., 2012). Therefore, fluorescence compounds formation was assessed in the samples incubated at 0.63 a_w to determine the propagation of the Maillard reaction into secondary stages before the advanced stages, where visual browning can be observed (Burton et al., 1963).

The % fluorescence intensity of SCH incubated with maltodextrin significantly increased over time (Fig. 11), indicating fluorescence compound formation, and hence a propagation to the intermediate stages. The % fluorescence intensity increased more rapidly (steeper slope) between 96 and120 h of incubation. The control SCH incubated without maltodextrin also exhibited some fluorescence activity over time, indicating the

occurrence of the Maillard reaction. This is attributed to the presence of residual lactose that remained after dialysis. Although fluorescent compounds might also originate from lipid oxidation (Castilho et al., 1994; Hidalgo and Zamora, 2007), casein proteins only had approximately 0.9% of lipid, according to the manufacturer's specification. Therefore, the extent of fluorescent compound formation was mainly attributed to the Maillard reaction. Compared to a control, fluorescence formation in PGSCH was minimal, indicating that the reaction was controlled and did not excessively progress to intermediate stages.

Fluoresence compounds also formed in SCI incubated with and without maltodextrin (Fig. 17, Appendix D). However, higher % fluorescence intensity was observed in SCH and PGSCH than that of SCI and PGSCI, indicating Maillard reaction progressd into the intermediate and advanced stages more readily in SCH and PGSCH than in SCI and PGSCI. This observation could be attributed to higher amount of free amine groups in SCH compared to SCI.

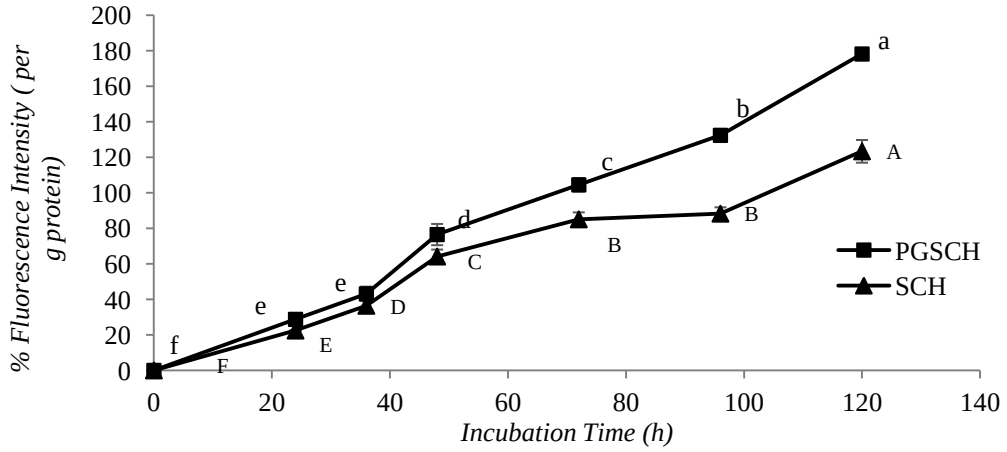

Figure 11. Fluorescent compound formation for sodium caseinate hydrolysates (SCH) incubated with maltodextran and control SCH, produced by trypsin at 37ºC, e/s=0.8:100, 45 minutes, pH=7 with the DH of 5.0 %, incubated without maltodextran (n) at 60°C for 0-120 h at a_w of 0.63 as determined by fluorescent intensity quantification. Error bars represent standard errors (n=3). Different uppercase and lowercase letters above or below the shapes indicate significant differences between different time points according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

3.6.4. Visualization of Glycated Proteins and Peptides Using UREA-PAGE

Urea–PAGE was performed to confirm conjugates formation, since the molecular weights of casein peptides change after covalently binding with maltodextrin. A noticeable reduction in band intensity of the different casein peptides was observed for SCH incubated with maltodextrin compared to non-incubated SCH (Fig. 12). To further confirm the presence of glycated peptides, another gel was run and stained using glycoprotein staining (Fig. 13). As incubation time increased, a longitudinal smearing appeared at the upper layer of the gel, which was absent in non-incubated SCH. The smearing was attributed to the heterogeneous molecular weight distribution of glycated proteins and peptides. The intensity of smearing increased as incubation progressed, supporting previous conclusions that the Maillard-glycation was initiated within the first 24 h of incubation, and continued to progress over time. High intensity bands were observed at the border between the stacking gel and the resolving gel of all SCHs glycated with maltodextrin. The intensity increased considerably as the incubation time increased, indicating the formation of high molecular mass protein-maltodextrin conjugates, which were too large to penetrate the separating gel. Presence of large conjugates could be attributed to having multiple maltodextrin molecules linked to a single peptide/protein at several possible glycation sites. The appearance of large molecular weight bands and the smearing were also reported by other researcher studying glycated casein proteins (Kato et al., 1995; Shepherd et al., 2000; O'Regan and Mulvihill, 2009; Oliveira et al., 2014).

After considering results from UV-Visible difference spectroscopy, loss of free amino groups, fluorescence intensity, and urea-PAGE visualization of glycated peptides, the sample incubated for 96h period at 0.63 a_w and 60°C was selected for further analysis, since this time point resulted in sufficient Amadori compound formation, as noted by adsorbance at 304 nm, limited % amino group blockage (9.7 %), minimal progression to intermediate stages, as noted by % fluorescence intensity, and limited progression to advanced stages of the Maillard reaction, as noted by adsorbance at 420 nm.

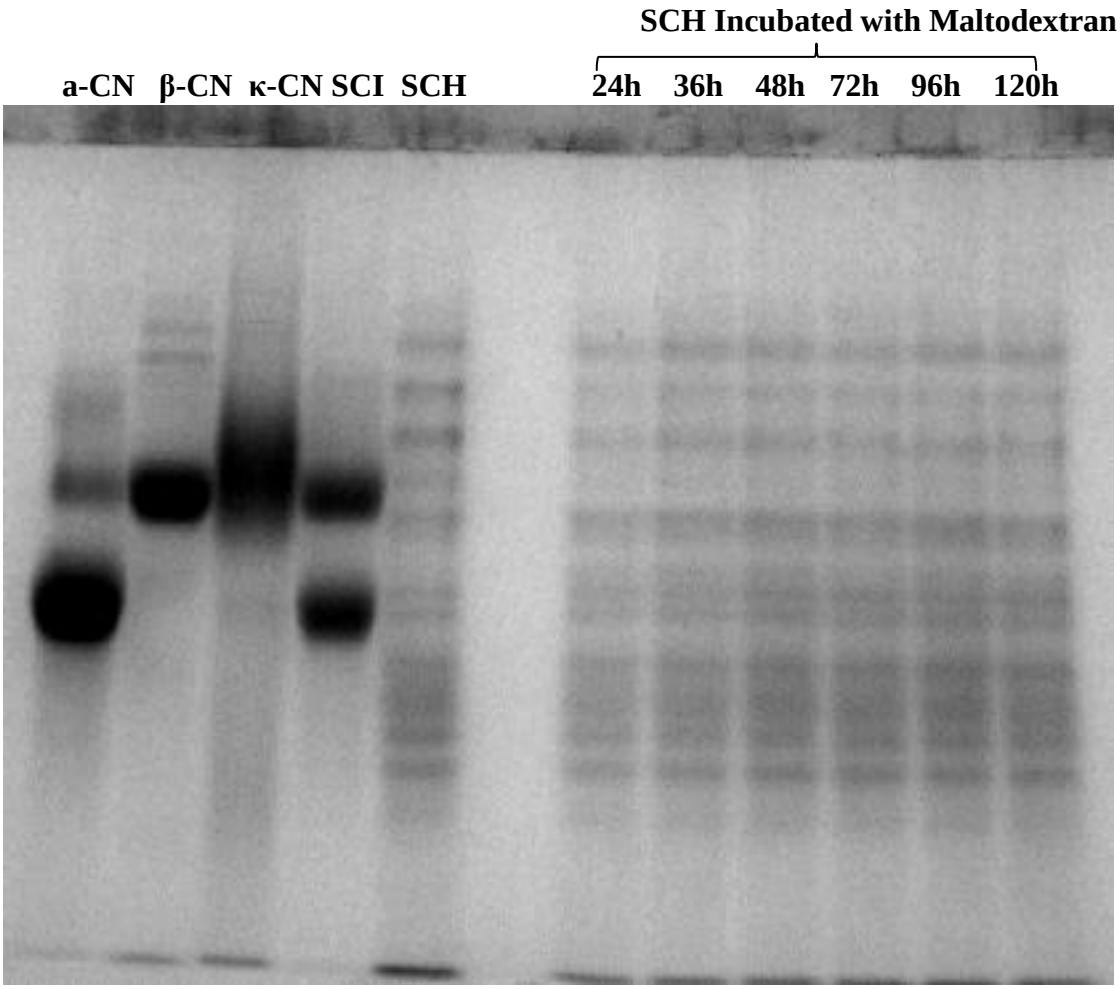

Figure 12. Urea-PAGE visualization with Coomassie Blue Staining of the hydrolysis pattern of prepared sodium caseinate (SCI), SCH, produced by trypsin at pH=7, 37°C, 45min, e/s=0.8:100 with the DH of 5.0 %, α-, β-, κ- caseinate standards and the same SCH glycated with maltodextran for 24 to 120 h at 60˚C and 0.63 a_w . The amount of protein loaded in each lane was 20 μg.

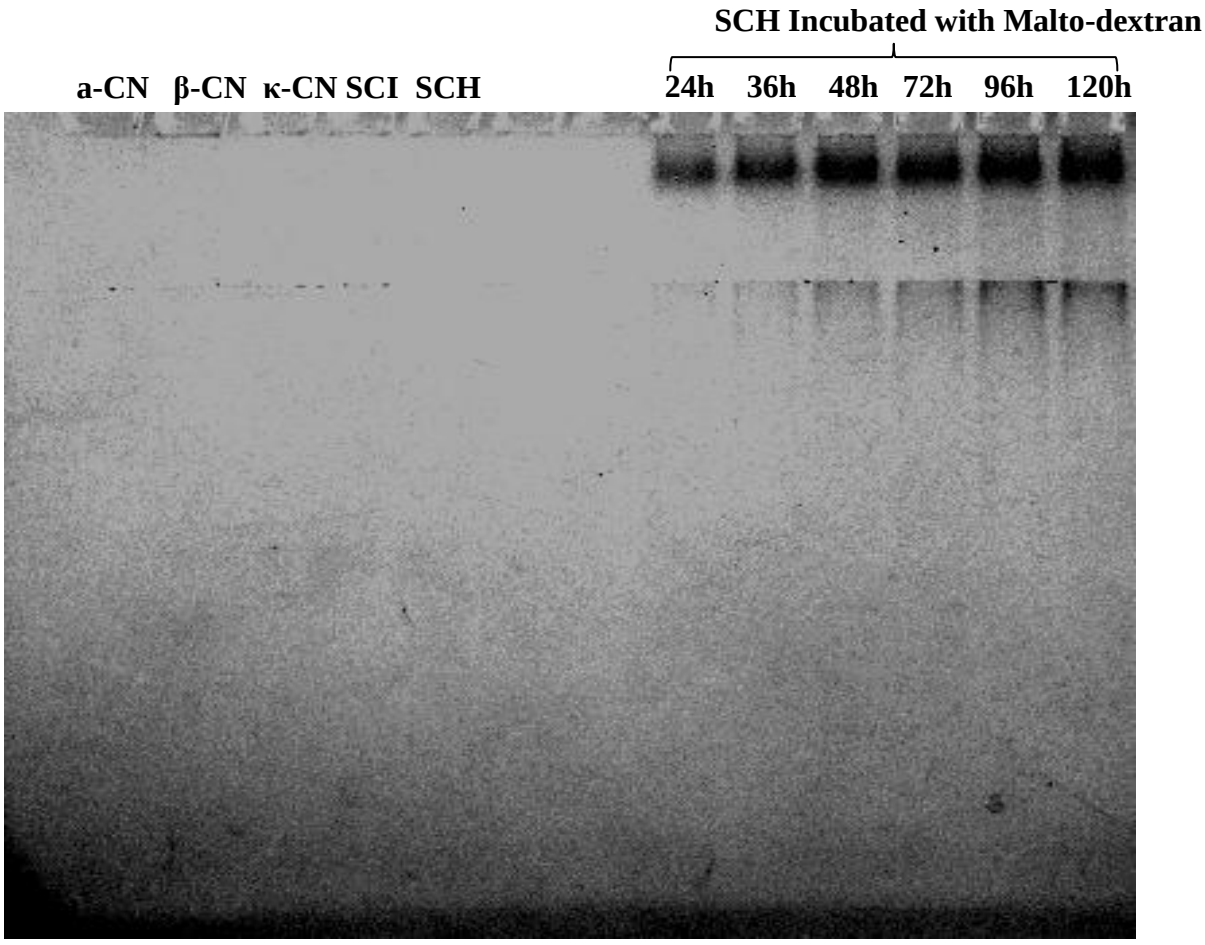

Figure 13. Urea-PAGE visualization with glycoprotein staining of the hydrolysis pattern of prepared sodium caseinate (SCI), α-, β-, κ-caseinate standards, SCH, produced by trypsin at pH=7, 37°C, 45min, e/s=0.8:100 with DH of 5.0 %, and the same SCH partially glycated with maltodextrin for 24 to 120 h at 60˚C and 0.63 a_w. The amount of protein loaded in each lane is 20 μg.

3.7. Effect of Glycation on Emulsification Properties of Casein Proteins

To investigate the effect of glycation on the emulsification properties of sodium caseinate, EC, ES and EA were evaluated for original SCI, non-incubated SCH (produced using trypsin, DH of 5%) and PGSCH (incubated at 0.79 and 0.63 a_w, 60˚C and 96 h) at pH 7 (Table 9).

Table 9. Emulsification properties of SCI, SCH, and PGSCH

Sample ± SE[A]	EC[B] ± SE	ES[C] ± SE	EA[D] ± SE	Color/Form
SCI[E]	1867 ± 17[a*]	15 ± 0.1[d]	0.11 ± 0.001[d]	Free flowing, pure white
SCH[F]	1070 ± 11[b]	20 ± 0.8[a]	0.19 ± 0.019[b]	Free flowing, pure white
PGSCH[G] (0.79 a_w)	1872 ± 91[a]	17 ± 0.6[b]	0.23 ± 0.007[a]	Caked, dark brown
PGSCH[H] (0.63 a_w)	1076 ± 64[b]	16 ± 0.1[c]	0.17 ± 0.001[c]	Free flowing, light yellow

[A] Standard Error; [B] EC: Emulsification capacity; [C] ES: Emulsification stability; [D]EA: Emulsification activity; [E]SCI: Sodium caseinate isolate; [F]SCH: Sodium caseinate hydrolysate produced using trypsin at pH=7, 37°C, 45 min, e/s=0.8:100 with DH of 5.0 %; [G]PGSCH (0.79 a_w): Partially glycated sodium caseinate hydrolysate produced upon incubation with maltodextrin at 60°C, 0.79 a_w for 96 h; [H] PGSCH (0.63 a_w): Partially glycated sodium caseinate hydrolysate produced upon incubation with maltodextrin at 60°C, 0.63 a_w for 96 h; * Means followed by the same lower case letter are not statistically different according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

At 0.79 $a_{w,}$ PGSCH had significantly higher EC compared to SCH and was comparable to that of unmodified SCI. The recovered EC upon glycation of SCH could be attributed to the increased chain length of SCH after conjugation with maltodextrin, which was confirmed by the large molecular weight conjugates formed at the top part of the urea-PAGE gel (Fig. 12 and 13). Large molecular size has been positively correlated with enhanced emulsification properties (Kuehler and Stine, 1974; Gauthier et al., 1993). In addition, conjugated maltodextrin improves the overall hydration of proteins by increasing the surface hydrophilicity (Shepherd et al., 2000), thus enhancing solubility, which is a determining factor for a protein's ability to form an emulsion (Kinsella & Whitehead, 1989). Poor solubility negatively impact emulsification capacity of the protein by limiting its ability to migrate to the interface and its efficiency in reducing the interfacial tension during droplet break up (Dickinson, 2003a).

Apart from EC, PGSCH produced at 0.79 a_w also had improved EA compared to SCH and SCI by a factor of 1.2 and 2.1, respectively (Table 9). The improved EA might have resulted from improved amphiphilicity upon glycation. According to Oliveira et al. (2014), conjugates are more surface reactive than polysaccharide or protein by combining the hydrophobic residues of proteins and the hydrophilic saccharide. Thus, conjugates adsorbed more readily at the oil-water interface. Similar result was reported by Kato et al.

(1992), who found that casein-dextran conjugated at a_w of 0.79, 60°C for 24 h significantly increased the EA of casein by a factor of 1.5.

On the contrary, the ES of PGSCH produced at 0.79 a_w was inferior to that of SCH (Table 9). This result is contradictory to several studies that showed an increase in ES upon glycation. The reported increase was attributed to many factors such as increased steric hindrance, enhanced electrostatic repulsion of the bulky and hydrophilic polysaccharide moiety, higher extent of glycation, longer chain of carbohydrates attached, and different types of proteins utilized (Akhtar and Dickinson, 2003; Akhtar & Dickinson, 2007; Oliveira et al., 2014). The reduced ES found in this study might have resulted from the unabsorbed protein and maltodextrin in the emulsion system. According to Akhtar and Dickinson (2003), conjugated emulsifier is not a single species. At low concentration, non-glycated maltodextrin was found to destabilize emulsion through depletion flocculation (Dickinson & Galazka, 1991; Kruif and Tuinier, 2001). Depletion flocculation is caused by the presence of excess free polymers (e.g. maltodextrin) in aqueous solution. Instead of distributing uniformly, polymers are precluded between adjacent emulsion droplets where the distance is less than the polymers' diameters. The resulting osmotic pressure leads to a net attraction between the emulsion droplets, thus leading to reversible flocculation (Dickinson and Golding, 1997a). At a higher concentration of protein, unabsorbed sodium caseinate may aggregate with each other through hydrophobic interactions. These resulted sub-micelles also destabilized emulsion through depletion flocculation (Dickinson and Golding, 1997a). Similarly, unabsorbed SCHs might associated with each other and destabilize the emulsions. Therefore, an additional separation step of unabsorbed maltodextrin, intact casein proteins, and peptides may be used in future research in order to have a better evaluation of glycation on the emulsification properties of SCH.

Another possible reason for the reduced ES of PGSCH could be the protein to sugar ratio of 2:1 utilized in this study. An optimum ratio is often required to achieve better emulsification properties in the Maillard reaction (Oliver et al., 2006). For instance, the emulsification properties of bovine serum albumin-galactomannan (22 kDa) conjugates reacted (60° C, 0.79 a_w) at 1:6 had better ES than conjugates reacted at 1:3,

1:9, 1:12 and 1:15 albumin : galactomannan mole ratio (Kim et al., 2003). The best ES of whey protein isolate-dextran (DX, 500 kDa) conjugates (80°C, 0.79 a_w) was achieved at whey protein isolate: dextran weight ratio of 1:3 (Akhtar and Dickinson, 2003). The ratio of SCH to maltodextrin of 2:1 may destabilize the emulsion due to the presence unadsorbed sodium caseinate and free maltodextrin. Therefore, the ratio of SCH to maltodextrin should be optimized in future research for potential enhancement of emulsification properties of PGSCH.

Emulsification properties of PGSCH glycated at 0.63 a_w for 96 h were also investigated (Table 9). Compared to SCI, PGSCH only improved the EA, with minor enhancement in ES. Compared to non-incubated SCH, ES and EA were significantly lower while EC was not significantly different. Compared to PGSCH glycated at a higher a_w of 0.79 PGSCH, EC, EA and ES were all significantly lower for PGSCH glycated at 0.63 a_w. The difference in emulsification properties between different glycation conditions might be related to the extent of glycation. Based on the loss of free amino groups result, PGSCH glycated at 0.79 a_w for 96 resulted in 25.6% of lysine blockage, while PGSCH glycated at 0.63 a_w for 96 glycation had only 9.7% of lysine blockage. According to Oliver et al. (2006) through a comprehensive review, improved emulsification properties is normally reported in protein conjugate with more than 25% of lysine modification and 1.5–4 moles polysaccharide attached per protein (Oliver et al., 2006). Therefore, it is likely that glycation at 0.63 a_w and 60 °C for 96 h did not provide sufficient degree of glycation to improve the emulsification properties. It should be noted that PGSCH at 0.79 a_w, although had higher emulsification properties, displayed significantly visual browning and caking, indicating that the Maillard reaction was progressed into advanced stages. While improving emulsification properties is appealing, loss in overall quality due to browning and other undesired outcomes of advanced Maillard reaction should not be over looked. Therefore, optimization of the glycation conditions is still necessary to achieve enhancement in functionality while maintaining overall quality.

3.8. Effect of Glycation on ACE Inhibitory Activity of Sodium Caseinate Hydrolysates

The ACE-inhibitory of PGSCH (96h, 60°C and 0.63 a_w) and SCH were compared and investigated (Fig. 21 and 22, Appendix J). A regression line with a steep slope indicates high enzyme activity, and thus a reduced slope indicates an inhibition of the enzyme activity. The slope of both SCH and PGSCH were considerably lower than that of the control (SCI), confirming ACE inhibitory activity of both SCH and PGSCH. In addition, the slopes of PGSCH and SCH were similar, confirming that controlled and limited glycation of bioactive SCH, performed in this study, did not negatively impact bioactivity of SCH. Glycation could have potentially occurred at the site of interaction with the ACE enzyme, thus prohibiting its competitive inhibition. Results indicated, however, that the glycation did not occur at a prohibitive site. These findings were similar to the recent work by Lasky (2015), who also determined that glycation of whey protein hydrolysate under mild conditions had no negative effect on the ACE inhibitory activity.

3.9. Effect of Glycation on Immunoreactivity of Sodium Caseinate

Glycation was reported to effectively reduce protein allergenicity (Babiker et al., 1998; Lagemaat et al., 2007; Nakamura et al., 2008) by shielding the epitopes with bulky carbohydrate groups (Hattori et al., 2004), and disrupting conformational epitopes through inducing tertiary-structural changes of the protein (Arita et al., 2001). However, former studies employed adverse glycation conditions that may negatively impact overall quality. Therefore, it was of interest to investigate whether combining controlled glycation with limited enzymatic hydrolysis will have a dual effect on reducing the immunoreactivity of casein.

The effect of glycation on modifying the allergenicity of sodium caseinate was determined by comparing the reductions in % immunoreactivity of SCH, PGSCH (0.79 a_w or 0.63 a_w, 60°C and 96h) and PGSCI (0.63 a_w, 60°C and 96h), using sera from individuals allergeic to milk proteins (Table 3). PGSCI was utilized to determine the effect of glycation alone on modifying allergenicity of casein proteins. Unlike SCH,

PGSCI produced at 0.63 a_w had increased the immunoreactivitywhen using sera from all participants, except for participant 2 serum, indicating glycation alone increased the allergenicity of casein proteins. Compared to non-glycated SCH, PGSCH produced at 0.63 a_w did not further reduce immunoreactivity when using sera from participants 1, 2, 3 and 4, but showed further reduction when using sera from participant 5 (Fig. 14). Similarly, no further reduction in immune-reactivity for PGSCH produced at 0.79 a_w when using sera from participants 1, 2, and 3, with additional reductions noted when using sera from participants 4 and 5. These results suggested that glycation of SCH with maltodextrin did not have a dual effect on modifying the immunoreactivity of SCH when using sera from different participants. Change in the immunoreactivity was a function of the sera used.

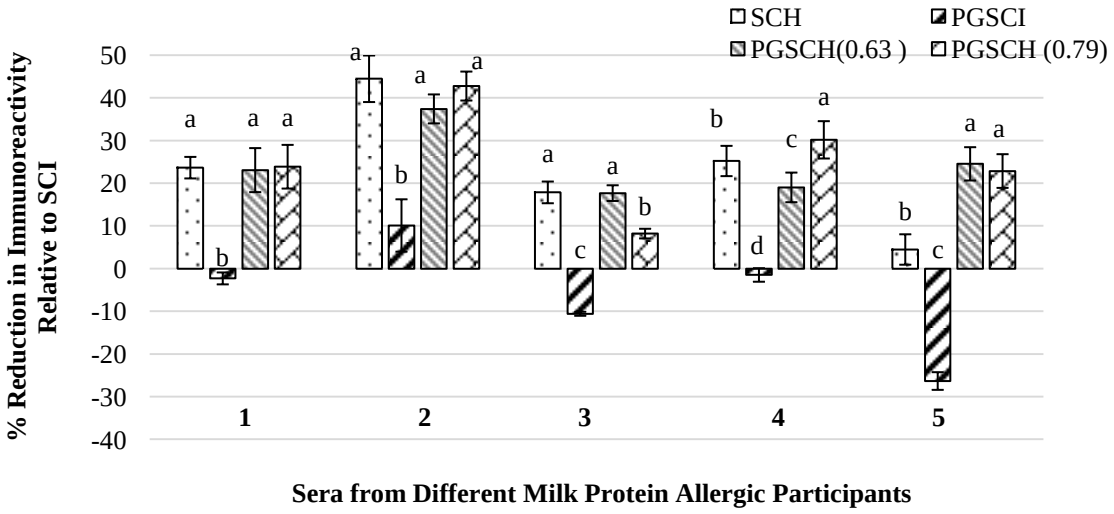

Figure 14. % Reduction in Immunoreactivity of sodium caseinate hydrolysates (SCH), produced by trypsin at 37°C, e/s=0.8:100, 45 min, pH=7 with DH of 5.1% and partially glycated sodium caseinate hydrolysates (PGSCH) with maltodextrin for 96 h at 60 °C and a_w of 0.63 (PGSCH 0.63) and a_w of 0.79 (PGSCH 0.79) relative to sodium caseinate isolate (SCI) using sera from all participants. PGSCI referred to glycated sodium caseinate isolate with maltodextrin for 96 h at 60 °C and a_w of 0.63. Error bars represent standard errors (n=3). Different lower case letters indicate significant differences among the samples within each serum according to Tukey-Kramer multiple means comparison test ($P < 0.05$).

The mechanisms involved in the modification of the immunoreactivity of proteins upon glycation are very complex. Apart from shielding and disrupting existing epitopes, glycation can expose buried epitopes and generate new conformational epitopes during protein rearrangement (Hattori et al., 2000; Hattori et al., 2004). The increased immunoreactivity in PGSCI produced at 0.63 a_w in this study might have resulted from new epitopes exposed upon glycation. In fact, these different mechanisms might take place individually or concurrently under the glycation condition studied, leading to the observed mixed results. The sensitivity and specificity of casein specific IgE in each serum differed as well. Some allergic individuals may have IgE specific for certain epitopes that could have been exposed due to protein unfolding upon glycation, while others do not, which further complicated the immunoreactivity results. Wang and Ismail (2012) and Wang et al. (2013) confirmed protein partial unfolding upon glycation.

Compared to PGSCH produced at 0.63 a_w, PGSCH produced at 0.79 a_w resulted in increased immunoreactivity when using sera from participants 2 and 4, while showing decreased immunoreactivity when using serum from participant 5, and had marginal difference in immunoreactivity when using sera from participants 1 and 3 (Fig. 14), indicating that degree of glycation did not positively or negatively correlate with immunoreactivity for all sera tested. Previous reports showed that a higher degree of glycation is associated with a higher reduction in immunoreactivity. For instance, β-Lg-carboxymethyl dextran conjugates with a higher amount of dextran attached showed a higher reduction of allegenicity (Hattori et al., 2000). Although more carbohydrates were attached to proteins at a higher degree of glycation, there was no evidence that more epitopes on the proteins were blocked or disrupted. Instead the locations of glycation might take place randomly within protein structures. In addition, the effect of glycation on immunoreactivity is affected by many other factors, such as different protein-sugar combination, reaction conditions (time and temperature) and protein structures that were utilized.

Based on the findings, glycation cannot be easily controlled to reduce protein's allergenicity across different sera. Future work would be directed at monitoring the structural changes of proteins during glycation to have a better understanding of the

effect of glycation on immunoreactivity. In any case, given the variability between different allergenic individuals, it might be extremely challenging to produce a hypoallergenic protein ingredient for all consumers.

4. Overall Conclusions, Implications, and Recommendations

Trypsin was better than Alcalase and Neutrase in producing sodium caseinate hydrolysate (SCH) with retained emulsification properties. This finding suggested that hydrolyzing sodium caseinate using an enzyme with narrow specificity could maintain the functionality due to minimal formation of short chain peptides with impaired amphiphilicity. Limited hydrolysis (%DH ~ 5) of sodium caseinate by trypsin also resulted in pronounced ACE inhibitory activity and a substantial reduction of the immunoreactivity (up to 45% depending on the serum used). However, In order to be claimed as a hypoallergenic ingredient, further modification of SCH is required.

Maillard-induced glycation of SCH and SCI was successfully carried out in a controlled and limited fashion upon incubation with maltodextrin in a 2:1 ratio at 60°C and 0.63 a_w for 96 h, confirmed by the moderate formation of Amadori compounds and marginal loss in free amino groups. Minimal fluorescence and browning confirmed that the Maillard reaction was limited mostly to initial stages of the Maillard reaction. The produced PGSCH, while still bioactive, showed no significant improvement in emulsification properties compared to SCH. On the other hand, PGSCH produced under more adverse reaction conditions had improved emulsification properties, yet the product underwent browning and caking. Therefore, glycation conditions should be further optimized for targeted enhancement in the emulsification properties of casein, while maintaining quality. An additional removal step should also be adopted to remove free maltodextrin and unabsorbed sodium caseinate that might destabilize the emulsion through depletion flocculation. Removal of unreacted components may also provide a better idea of the emulsification properties of glycated proteins.

PGSCH generated after incubation with maltodextrin at both 0.63 and 0.79 a_w for 96h did not further reduce the allergenicity of SCH, suggesting that the degree of glycation is not the only factor that determines the immunoreactivity of the modified proteins. In fact, the structural changes should occur at specific regions that mask/inactivate linear and/or conformational epitopes. Future work should be directed at monitoring the structural changes of proteins/peptides during glycation to have a better

understanding of the effect of glycation on immunoreactivity. Techniques such as epitope mapping and tandom mass spectrometery may provide information regarding the site of glycation, epitopes that got hydrolyzed, and/or epitopes that got exposed. Therefore, structural characterization can be utilized to better explain dual effects of the combined protein modification methodologies, hydrolysis and glycation. Additionally, optimization of Maillard-induced glycation conditions is necessary to result in significant reductions in immunoreactivity across different sera obtained from allergic individuals.

In addition to optimization of Maillard-induced glycation for enhanced functionality and reduced allergenicity, it is also important to determine the nutritional quality of the product by assessing its digestibitly. Maintaining nutritional quality is essential to meet the consumer's demand for not only safe and functional protein, but also nutritious.

This work is a step forward toward understanding the impact of a dual protein modification approarch on functionality, bioactivity, and immunoreactivity of casein protein. While results are promising, further work is needed to characterize the impact of each modification on the structure and function of the protein. Immunoreactivity data revealed the complexity of achieving hypoallergenic ingredient for all allergic individual. While a product could be made hypoallergenic for some allergic individual, it may not be so for others. Continued trials and screening of larger serum pool may result in identification of optimal modification conditions. If successful, the development of functional and hypoallergenic casein ingredients can result in substantial benefit to consumers and economic gains to producers.

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Appendix A: Enzyme Volume Calculation

For alcalase and neutrase, the final enzyme to substrate ratio was 0.2:100.

$$\frac{0.2mL}{100g\ protein} = \frac{x\ mL\ of\ enzyme}{7.5g\ protein}$$

$$x = 0.015mL = 15\mu L$$

Therefore, the volume of enzyme applied is 15uL

Trypsin's unit is 14.8 U/g enzymes and alcalase's unit is around 2.4 U/g of enzymes or 0.216 U/mL. In order to make 1mL of 0.216 U/mL of trypsin, x g of trypsin enzyms was weight out and dissolved in 1mL DDW

$$\frac{14.8\ U/g \times xg}{1mL} = 0.216\ U/mL$$

Therefore x=14.6mg

When a total of 60 μL of trypsin enzyme solution was utilized in hydrolysis, the final e/s was calculated as following:

$$\frac{E}{S} = \frac{60\mu L}{1000\mu L}/(7.5\ g\ protein) = \frac{0.8}{100}$$

Appendix B: Example Calculation for Degree of Hydrolysis (DH)

$DH = (h / h_{tot}) \times 100 = [(B \times N_b) / (MP \times \alpha \times h_{tot})]*100$

Where B is the volume of the NaOH necessary to keep the pH constant = 3.9mL

N_b = Normality of the NaOH = 0.2N

MP is the mass of the protein (g N x 6.38) = 7.5g

h_{tot} = 8.2 meq/g (for casein protein)

α is the degree of dissociation of the α-NH2 groups and is expressed as:

$\alpha = 1 / (1 + 10^{pK-pH})$

The pK value varies significantly with temperature and can be estimated as follows:

pK = 7.8 + (298-T) / (298 x T) x 2400

where T is the absolute temperature (K).

At 37°C, pK = 7.8 + [((298-(37 + 273) / 298*(37 + 273))] = 7.48

Therefore, at pH 7.5, $\alpha = 1/ (1 + 10^{6.95-7.5}) = 0.25$

Finally, DH = ((3.9mL x 0.2 moles/L) / (7.5g x 0.25 x 8.2 meq / g)) x 100 = 5.07%

Appendix C: Example Calculation for Determination of % Lysine Blockage

The % amine blockage is determined using the following steps:

Lysine concentration is determined from the lysine standard curve

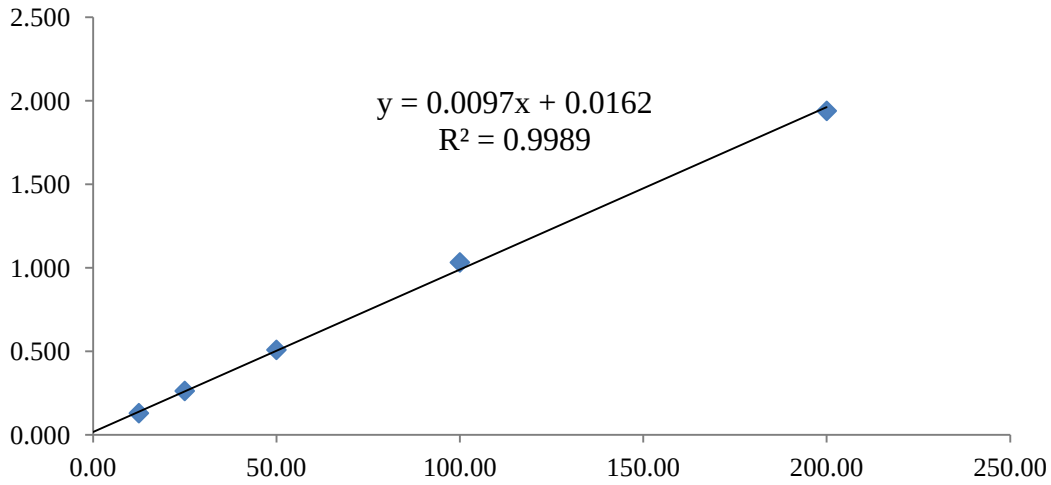


Figure 15. Example standard curve using lysine as standard

y = 0.0097x + 0.0162 with y representing the absorbance at 340 nm and x the lysine concentration in μg/mL

0.352 – 0.0162 / (0.0097) = 34.584 μg/mL lysine for PGSCH (0.63 a_w, 60°C, and 96 h)

The % lysine corrected is determined first by determining the protein content of the sample using the BCA assay standard curve

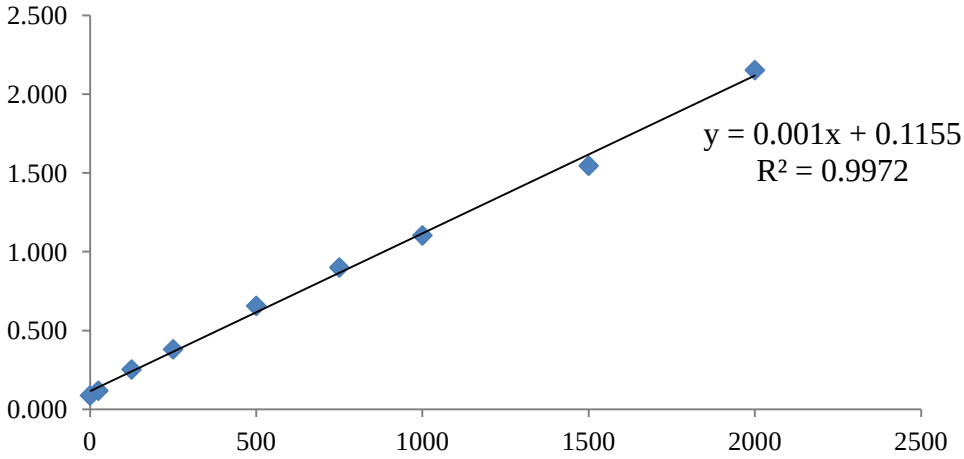


Figure 16. BCA standard curve using bovine serum albumin as (BSA) the standard

y = 0.001x + 0.1155 with y representing the absorbance at 562 nm and x representing the protein concentration in µg/mL

0.537 - 0.1155 / (0.001) = 421.5µg/mL protein for PGSCH (0.63 a_w, 60°C, and 96 h)

$$\% \text{ corrected lysine} = \frac{(\text{lysine concentration } (\mu g/mL)}{Protein\ concentration(\mu g/mL)} \times 100$$

= (34.584 / 421.5) * 100

= 8.205 %

The % lysine remaining for glycosylated samples is determined by

$$\% \text{ lysine remaining} = 100 - \frac{\%\ \text{Lysine}_{PGSCH0h} - \%\ \text{Lysine}_{PGSCH96h}}{\%\ \text{Lysine}_{PGSCH0h}} \times 100$$

= 100 – (9.153 – 8.205) / (9.153) * 100

= 89.64%

% lysine blockage got glycosylated samples is determined by

% lysine blockage = 100 - % lysine remaining

= 100 – 89.64 %

= 10.36%

Appendix D: Example Calculation for Determination of Fluorescence Intensity (FI)

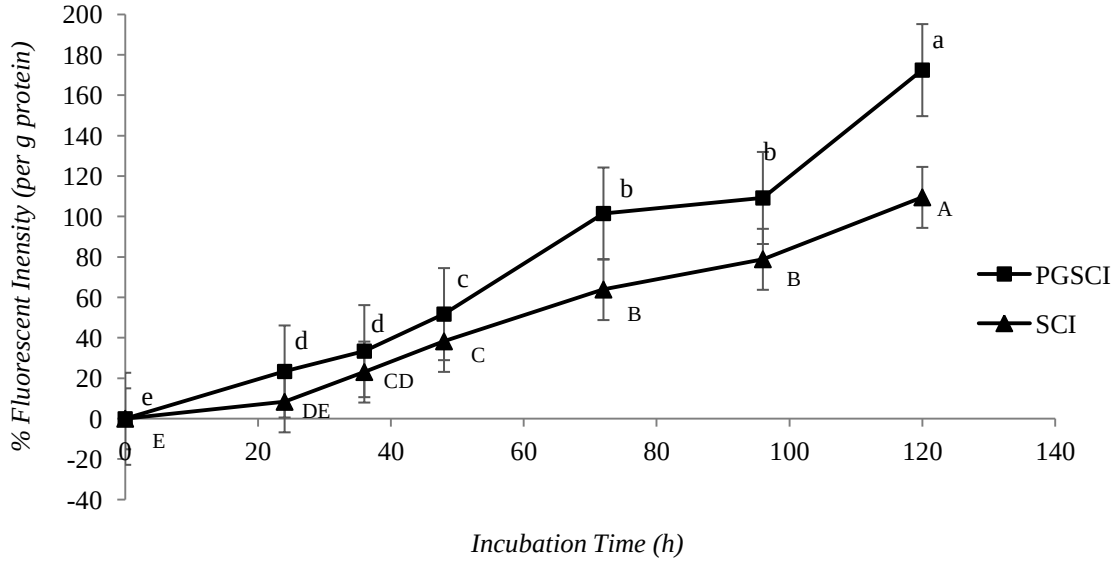


Figure 17. Fluorescent compound formation for Sodium Caseinate Isolate (SCI) incubated with maltodextran and control SCI incubated without maltodextran (n) at 60°C for 0-120 h at a_w of 0.63 as determined by fluorescent intensity quantification. Error bars represent standard errors (n=3). Different uppercase and lowercase letters above or below the shapes indicate significant differences between different time points according to the Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

The FI is determined using the following steps:

$\text{Emision}_{\text{PGSCI 96h}}$=749

$\text{Emision}_{\text{PGSCI 0h}}$=380

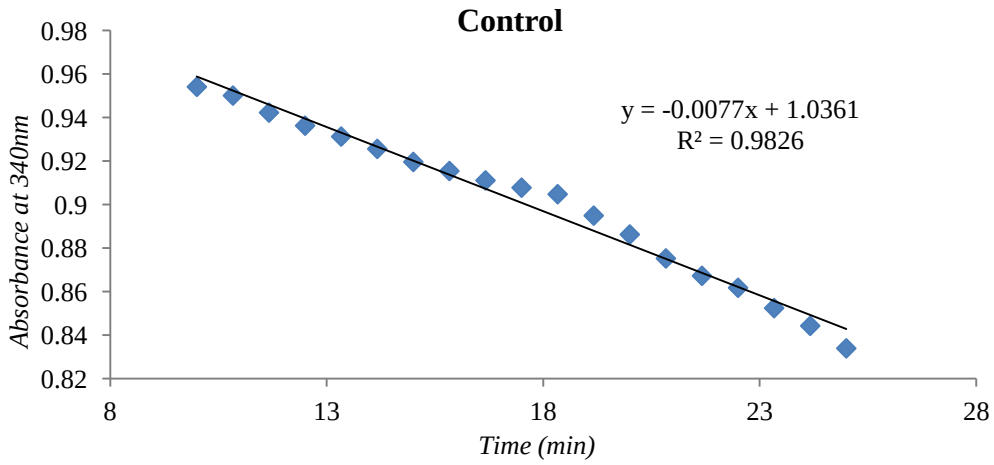
$\text{Emision}_{\text{5ppm quinine sulfate}}$=346

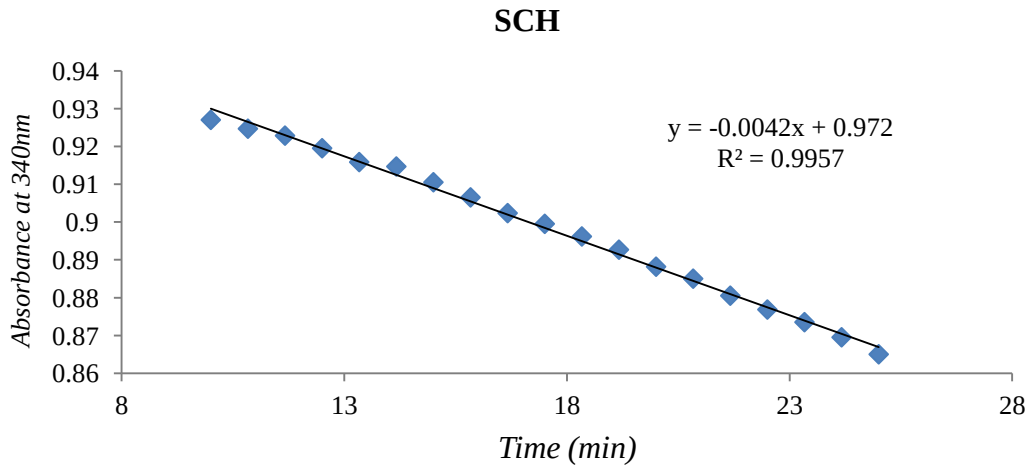
$$\%FI = \frac{\text{Emision}_{\text{PGSCI 96h}} - \text{Emision}_{\text{PGSCI 0h}}}{\text{Emision}_{\text{5ppm quinine sulfate}}} = \frac{(749-380)}{346} = 1.1$$

The FI of $\text{PGSCI}_{\text{96h}}$ is corrected by the protein content of the sample calculated using nitrogen analyzer

Therefore, $\%\frac{FI}{protein} = \frac{1.1}{0.01} = 110$

Appendix E: Sample Calculation for ACE





$$\%\text{ACE inhibition} = \frac{\text{Slope}_{\text{control}} - \text{Slope}_{\text{inhibitor}}}{\text{Slope}_{\text{control}}} \times 100\%$$

$$\%\text{ACE inhibition} = \frac{0.0077 - 0.0042}{0.007} \times 100\% = 45.45\%$$

Determining Protein Concentration of the Sample:

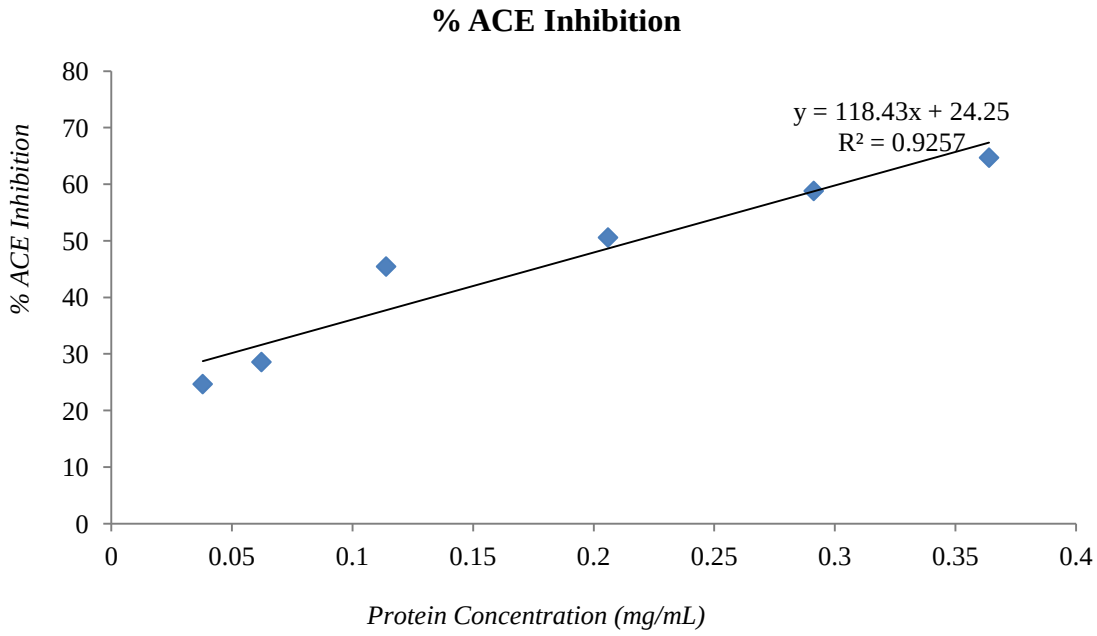
$$\text{Conc. of protein in the well} = \frac{Protein\ Concentration\ from\ BCA\ (1.922\ mg/mL) \times 10\mu L}{170\ \mu L}$$

$$= 0.113\ mg/mL$$

Where:

Protein concentration from BCA (mg/mL) is the protein concentration determined by BCA for each corresponding inhibitor solution added in the ACE assay

Determining IC_{50} value: IC_{50} value can be determined based on the plot between % ACE inhibitions and the protein contents for each sample.



$$y = 118.43x + 24.25$$

$$IC_{50}\ value = x = \frac{(50 - 24.25)}{118.43} = 0.217$$

Where:

X = protein concentration (mg/mL) of WPH or PGWPH in the well needed for 50% inhibition

y = % ACE inhibition; y = 50 to determine concentration of WPH or PGWPH needed to result in 50% inhibition.

Appendix F: Sample Calculation for % Reduction in Allergenicity

% Reduction in Allergenicity

$$= (\text{ Corrected ABS}_{\text{SCI}} - \frac{\text{Corrected ABS}_{\text{SCH or PGSCH}}}{\text{Corrected ABS}_{\text{SCI}}}) \times 100$$

Where:

ABS is the absorbance at 405nm

Corrected ABS_{SCI} is equivalent to ($ABS_{\text{SCI positive sera}} - ABS_{\text{2nd antibody}}$) = (1.7527-0.7255)

Corrected ABS_{SCH} is equivalent to ($ABS_{\text{SCH positive sera}} - ABS_{\text{2nd antibody}}$) = (0.8793-0.226)

Corrected ABS_{PGSCH} is equivalent to ($ABS_{\text{PGSCH positive sera}} - ABS_{\text{2nd antibody}}$) = (0.7705-0.128)

Therefore, % reduction in allergenicity $= 100 \times \frac{(1.7527-0.7255)-(0.8793-0.226)}{(1.7527-0.7255)} = 36.4\%$

Appendix G. Analysis of Variance (ANOVA) Tables for Determining Significant Effects of Treatments

Table 10. Analysis of variance for effect of various hydrolysis conditions on the degree of hydrolysis and emulsification properties

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
DH	Hydrolysis conditions	7	147	5793	< 0.001
	Error	40	0.025		
EC	Hydrolysis conditions	7	184×10^4	6732	< 0.001
	Error	40	273		
ES	Hydrolysis conditions	7	31.2	127.5	< 0.001
	Error	40	0.244		
EA	Hydrolysis conditions	7	0.009	142.8	< 0.001
	Error	40	0.000		

Table 11. Analysis of variance for effect of pH on the emulsification properties of SCH produced by trypsin at pH=7, 60°C, e/s=0.8:100, 45min with DH of 5.0 %

Sample Analysis	Emulsification Properties	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SCI	EC	pH	2	838×10^4	7621×10	< 0.001
		Error	33	110		
	ES	pH	2	13.9	43.17	< 0.001
		Error	33	0.323		
	EA	pH	2	0.011	555.7	< 0.001
		Error	33	0.000		
SCH	EC	pH	2	529×10^3	539.4	< 0.001
		Error	33	980		
	ES	pH	2	604	114.9	< 0.001
		Error	45	5.26		
	EA	pH	2	0.149	197.2	< 0.001
		Error	33	0.001		

Table 12. Analysis of variance on the effect of types of enzyme on bioactivity (ACE Inhibition) of SCH with similar DH (3.6 %-5.3 %)

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SCH	Type of enzyme	2	0.004	116.5	< 0.001
	Error	33	0.000		

Table 13. Analysis of variance on the effect of sera from different milk allergic subjects on % reduction in Immunoreactivity of SCH produced by trypsin at pH=7, 60°C, e/s=0.8:100, 45min with DH of 5.0 %

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SCH	Sera from different milk allergic subjects	4	1881	176	< 0.001
	Error	40	10.65		

Table 14. Analysis of variance on the effect of incubation time on 304 nm absorbance of SCH incubated with or without maltodextrin at different activitys and 60°C

Incubation Water activity	Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
0.49	SCH incubated with maltodextrin	Incubation Time	5	0.006	49.01	< 0.001
		Error	30	0.000		
	SCH incubated without maltodextrin	Incubation Time	5	0.016	314×10^3	< 0.001
		Error	30	0.000		
0.79	SCH incubated with maltodextrin	Incubation Time	6	0.080	230	< 0.001
		Error	35	0.000		
	SCH incubated without maltodextrin	Incubation Time	6	0.008	96.3	< 0.001
		Error	35	0.000		
0.63	SCH incubated with maltodextrin	Incubation Time	6	0.009	418	< 0.001
		Error	35	0.000		
	SCH incubated without maltodextrin	Incubation Time	6	0.002	119	< 0.001
		Error	35	0.000		

Table 15. Analysis of variance on the effect of incubation time on 304 nm absorbance of SCI incubated with or without maltodextrin at different activitys and 60°C

Incubation Water activity	Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
0.79	SCI incubated with maltodextrin	Incubation Time	6	0.075	4863	< 0.001
		Error	35	0.000		
	SCI incubated without maltodextrin	Incubation Time	6	0.004	46.38	< 0.001
		Error	35	0.000		
0.63	SCI incubated with maltodextrin	Incubation Time	6	0.002	34.81	< 0.001
		Error	35	0.000		
	SCI incubated without maltodextrin	Incubation Time	6	0.000	12.43	< 0.001
		Error	35	0.000		

Table 16. Analysis of variance on the effect of incubation time on 420 nm absorbance of SCH incubated with or without maltodextrin at different activitys and 60°C

Incubation Water activity	Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
0.79	SCH incubated with maltodextrin	Incubation Time	6	0.001	24.08	< 0.001
		Error	35	0.000		
	SCH incubated without maltodextrin	Incubation Time	6	0.001	13.71	< 0.001
		Error	35	0.000		
0.63	SCH incubated with maltodextrin	Incubation Time	6	0.000	158.2	< 0.001
		Error	35	0.000		
	SCH incubated without maltodextrin	Incubation Time	6	0.000	137.3	< 0.001
		Error	35	0.000		

Table 17. Analysis of variance on the effect of incubation time on 420 nm absorbance of SCI incubated with or without maltodextrin at different activitys and 60°C

Incubation Water activity	Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
0.79	SCI incubated with maltodextrin	Incubation Time	6	0.000	2.940	< 0.050
		Error	35	0.000		
	SCI incubated without maltodextrin	Incubation Time	6	0.000	12.85	< 0.001
		Error	35	0.000		
0.63	SCI incubated with maltodextrin	Incubation Time	6	0.000	3.140	< 0.050
		Error	35	0.000		
	SCI incubated without maltodextrin	Incubation Time	6	0.000	6.289	< 0.001
		Error	35	0.000		

Table 18. Analysis of variance on the effect of incubation time on % free amino group loss of SCH incubated with maltodextrin

Incubation Water activity	Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
0.79	SCH incubated with maltodextrin	Incubation Time	6	613.3	179.5	< 0.001
		Error	35	3.417		
	SCH incubated without maltodextrin	Incubation Time	6	59.48	18.62	< 0.001
		Error	35	3.195		
0.63	SCH incubated with maltodextrin	Incubation Time	6	162.3	21.00	< 0.001
		Error	35	7.727		
	SCH incubated without maltodextrin	Incubation Time	6	1.971	1.135	< 0.050
		Error	35	1.737		

Table 19. Analysis of variance on the effect of incubation time on % free amino group loss of SCI incubated with maltodextrin

Incubation Water activity	Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
0.79	SCI incubated with maltodextrin	Incubation Time	6	486.8	42.60	< 0.001
		Error	35	11.43		
	SCI incubated without maltodextrin	Incubation Time	6	62.47	3.634	< 0.050
		Error	35	17.19		
0.63	SCI incubated with maltodextrin	Incubation Time	5	60.14	9.232	< 0.001
		Error	30	6.514		
	SCI incubated without maltodextrin	Incubation Time	5	39.01	2.669	< 0.050
		Error	30	14.62		

Table 20. Analysis of variance on the effect of incubation time on % fluorescent intensity of SCI and SCH incubated with or without maltodextrin at 0.63 a_w, 60°C

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
SCH incubated with maltodextrin	Incubation Time	6	2308 ×10	210.0	< 0.001
	Error	35	109.9		
SCH incubated without maltodextrin	Incubation Time	6	1103×10	826.3	< 0.001
	Error	35	13.35		
SCI incubated with maltodextrin	Incubation Time	6	2178×10	544.2	< 0.001
	Error	35	40.01		
SCI incubated without maltodextrin	Incubation Time	6	9555	103.0	< 0.001
	Error	35	92.73		

Table 21. Analysis of variance on the effect of sample type on % reduction in Immunoreactivity of SCH, PGSCH, and PGSCI in relation to SCI for milk allergic subjects

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
Serum from Patient 1	Sample type	3	1502	87.41	< 0.001
	Error	32	17.19		
Serum from Patient 2	Sample type	3	2388	102.7	< 0.001
	Error	32	23.25		
Serum from Patient 3	Sample type	3	1608	656.8	< 0.001
	Error	32	2.448		
Serum from Patient 4	Sample type	3	3	1743	< 0.001
	Error	32	32	10.47	
Serum from Patient 5	Sample type	3	5027	560.6	< 0.001
	Error	32	8.967		

Table 22. Analysis of variance on the effect of sample type on % reduction in immunoreactivity of SCH, PGSCH, and PGSCI in relation to SCI for milk allergic subjects

Sample Analysis	Source of Variation	Degrees of Freedom	Mean Square	F	Sig.
EC	Sample type	3	1903×10^{3}	87.41	< 0.001
	Error	32	3203		
ES	Sample type	3	41.35	151.8	< 0.001
	Error	32	0.272		
EA	Sample type	3	0.023	220.5	< 0.001
	Error	32	0.000		

Appendix H: Amadori Compound Formation of SCH Incubated with and without Maltodextrin

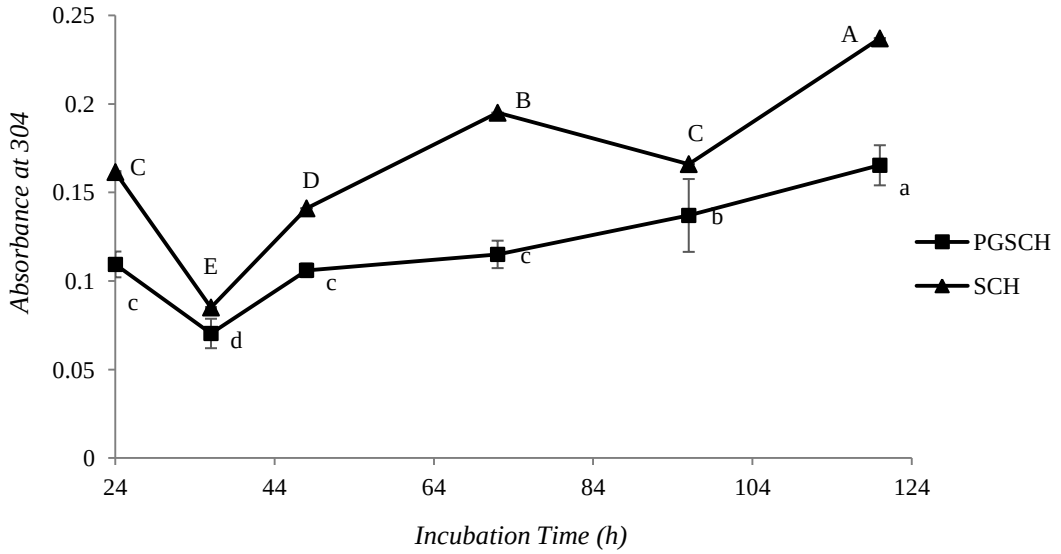


Figure 18. Amadori compound formation in samples of sodium protein hydrolysate (SCH) incubated with maltodextan and control SCH (alcalase at pH=7.5, 55°C, 45 min, and e/s=0.04:100 with DH of 3.6 %) incubated without maltodextran at 60˚C, 0.49 a_w for 24 to 120 h, as determined determined by UV-Visible difference spectroscopy at 304 nm. Error bars represent standard errors (n=3). Different lower case and upper case letters above and below the shape indicate significant differences between different time points of samples according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

Appendix I: Browning of SCH Incubated with and without Maltodextrin

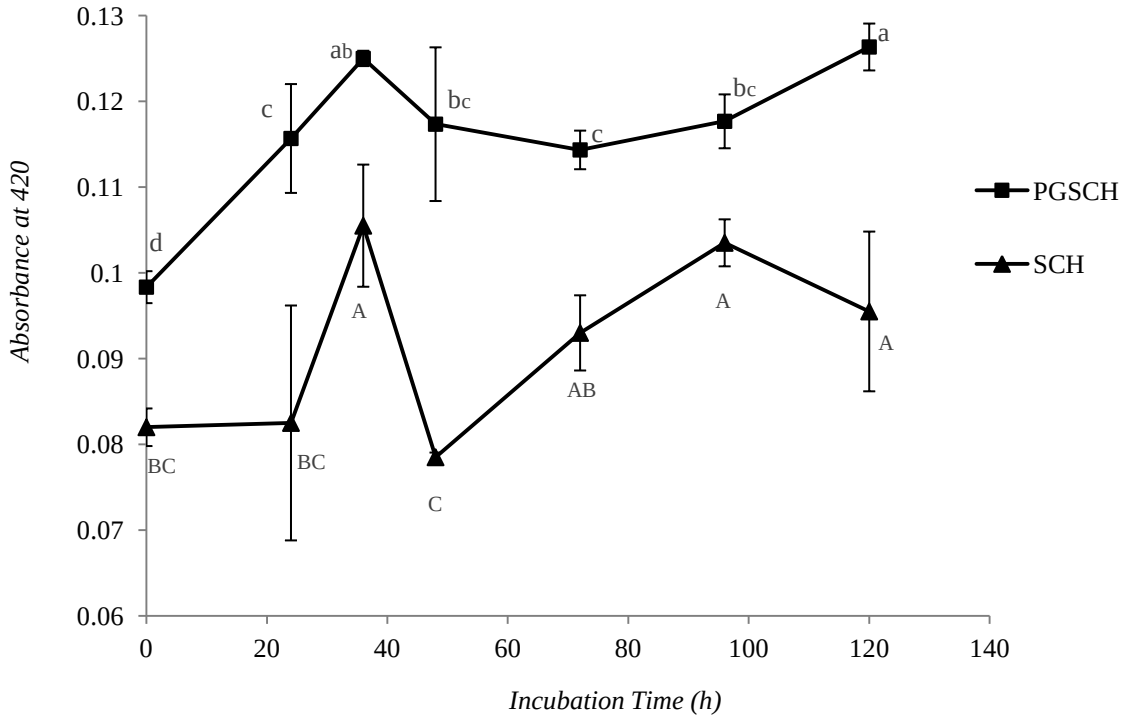


Figure 19. UV-Visible difference spectroscopy absorbance at 420 nm as an indicator of browning of (SCH) incubated with maltodextrin and control SCH (trypsin, pH =7, 37°C, 45 min, and e/s=0.8:100 with DH of 5.0 %) incubated without dextran at 60°C for 0-120 h at 0.79 $a_{w.}$ Error bars represent standard errors (n=3). Different lower case and upper case letters above and below the shape indicate significant differences between different time points of samples according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

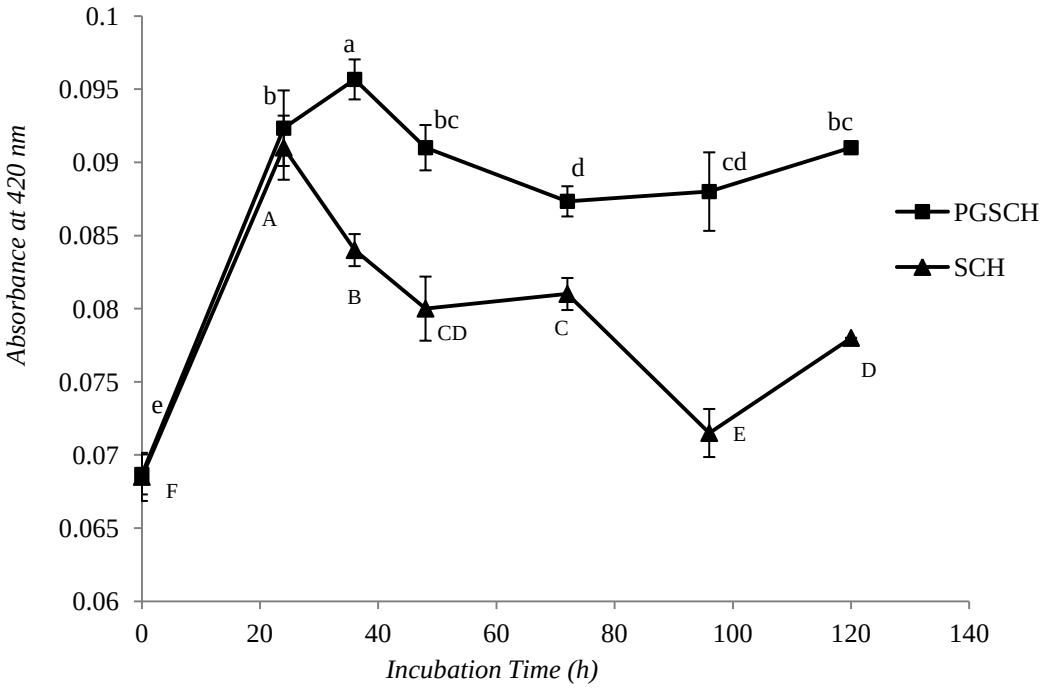


Figure 20. UV-Visible difference spectroscopy absorbance at 420 nm as an indicator of browning of (SCH) incubated with maltodextrin and control SCH incubated without dextran at 60°C for 0-120 h at 0.63 a_w. Error bars represent standard errors (n=3). Different lower case and upper case letters above and below the shape indicate significant differences between different time points of samples according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$).

Appendix J: ACE Inhibitory Activity of SCH and PGSCH

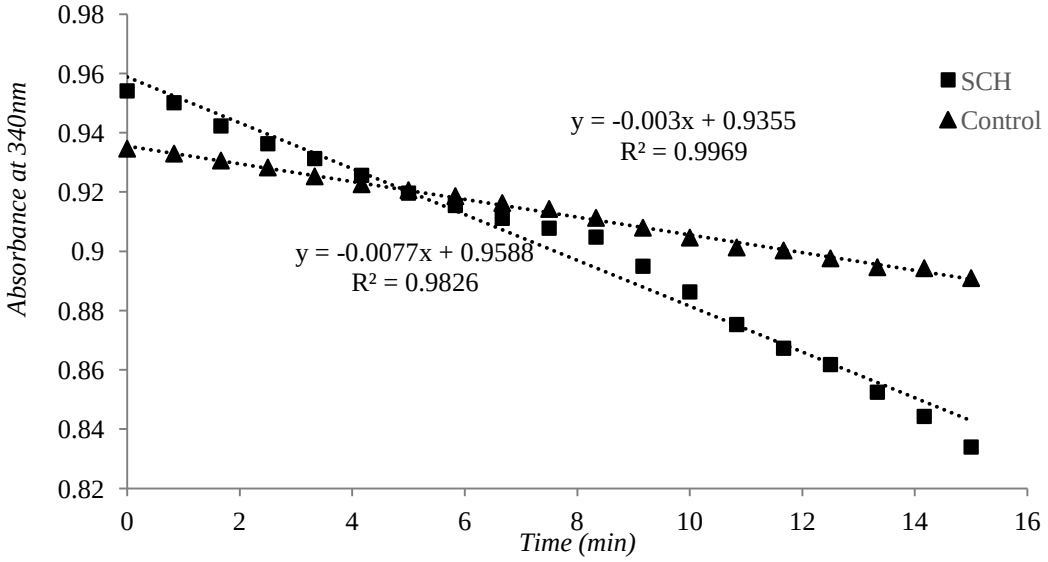


Figure 21. ACE inhibition of control and SCH (trypsin, pH=7, 37°C, e/s=0.8:100, and 45min with DH of 5.0 %) at the concentration of 10 mg/mL.

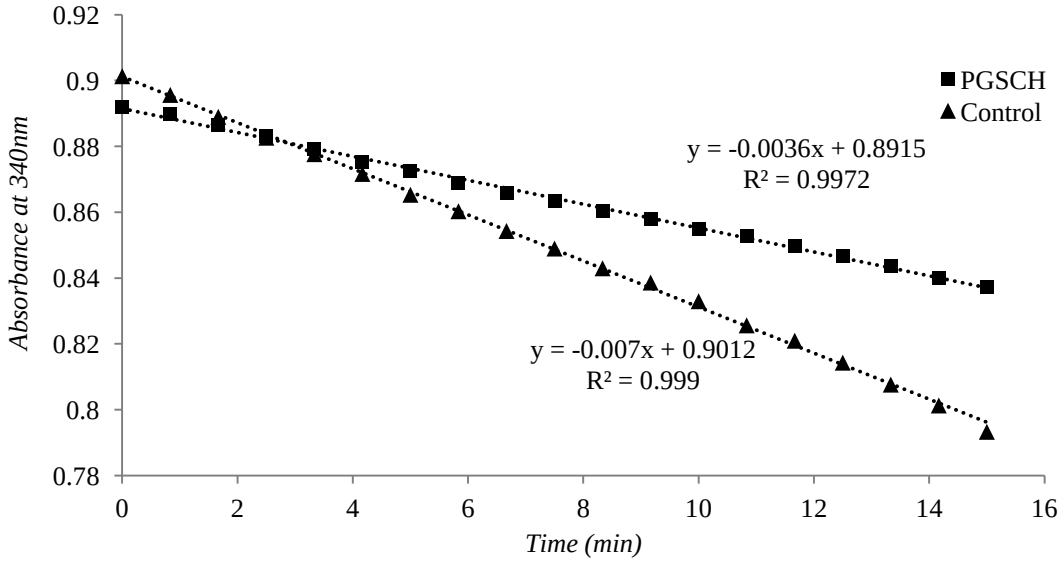


Figure 22. ACE inhibition of control and PGSCH (0.63 a_w, 60°C, and 96h) at the concentration of 10 mg/mL.