Enhancing the Functionality of Delactosed Whey by Enzymatic Hydrolysis Using Response Surface Methodology Approach (RSM)

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ABSTRACT

Delactosed whey (DLW), which has ~24% protein, is a by-product of lactose production and is mostly used as animal feed. DLW applications can be expanded to include food products, and its economic value improved by amplifying its protein functionality. Moderate enzymatic hydrolysis can significantly enhance protein functionality. It is, therefore, hypothesized that subjecting DLW to enzymatic proteolysis will lead to enhanced functionality. To amplify and improve the functionality of DLW via enzymatic hydrolysis, hydrolysis conditions need to be optimized. The infinite number of possible combinations of factors and their levels to be tested makes the task quite challenging. Using response surface methodology (RSM), the number of experimental units necessary to find the optimal point can be tailored depending on the time and resource constraints of the experimenter.

The first objective was, therefore, to amplify the functional properties of the protein component of DLW by enzymatic hydrolysis using response surface methodology (RSM). The second objective was to monitor the functional properties and sensory quality of beef patties fortified with selected DLW hydrolysates as compared to those fortified with whey protein isolate (WPI) and whey protein concentrate 34% (WPC 34).

In order to determine the optimal hydrolysis conditions, a Box-Behnken design with 4 independent variables at 3 levels was generated and implemented. The variables chosen, temperature (x_1), enzyme-to-substrate (e/s) ratio (x_2), time (x_3) and pH (x_4), are known to have the most significant influence on the degree of hydrolysis (DH) and protein functionality. The design had 27 experimental units that included 3 center points to assess the pure error. Each experimental unit was run in triplicate and the means of DH and measured functional properties were recorded as the y responses. The behavior of each response was explained by a quadratic equation. The effect of the independent variables on each response was evaluated and tridimensional response plots were generated. The conditions at which the responses were maximal/optimal were then determined. To validate the model, experimental data was obtained using the predicted optimized levels.

In triplicate, ground beef patties were formulated using selected DLW hydrolysates, with optimized functionality, non-hydrolyzed DLW, WPC34 or WPI. A control sample was formulated without the addition of any whey protein ingredient. Cook loss due to losses in water and fat was assessed. Texture analysis of the cooked patties was done using a TA-XT2 texture analyzer. Sensory analysis was conducted to determine differences in various attributes among the different beef patties.

Several response surface models were compared to determine the best fit for the RSM data collected. The point within the range of experimentation with the highest fitted value was determined. Using the model with the best fit (high R^2 and $P \leq 0.05$) for each response, prediction equations were generated and used to determine optimal hydrolysis conditions. Within the range tested, the emulsification capacity and activity of the DLW hydrolysates, produced under optimal conditions, were comparable to, if not greater, than that of WPI, which is known for its exceptional functionality attributes. The RSM approach provided an understanding of the effect of each hydrolysis parameter on the DH and on the assessed emulsification properties. Results highlighted that the extent of hydrolysis had a significant effect on the final functionality. While emulsification properties were enhanced significantly, hydrolysis of DLW was detrimental to the gelation property.

Beef patties formulated with hydrolyzed DLW, with optimal emulsification properties, lost about half as much moisture as the control. The fat loss in the beef patties formulated with hydrolyzed DLW was about 5 times less than that of the control. The beef patty formulated with Alcalase hydrolyzed DLW had significantly ($P < 0.05$) higher compression force than the control. The functional performance of DLW hydrolysates in the formulated beef patties was comparable to that of WPI. While instrumental measurements indicated functional differences, the formulation with hydrolyzed DLW did not affect the sensory quality of the beef patties.

Results of this work showed, for the first time, that the functionality of the protein component of DLW can be amplified upon limited and controlled hydrolysis. The tested DLW hydrolysates can be incorporated into meat products to amplify the functional properties without jeopardizing the overall sensory quality. Therefore, functionally

enhanced DLW have a great potential to reduce processing cost by replacing WPI, while maintaining acceptable quality.

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1. LITERATURE REVIEW

1.1. Introduction and Objectives

The dairy industry produces a tremendous amount of whey, which is a by-product of cheese and casein production. The increasing production of whey has raised issues related to pollution because of its high biochemical and chemical oxygen demand. In an effort to find a solution to the problem, many researchers have looked for ways to not only safely reuse whey, but also to increase the profit margin of the already struggling dairy industry.

Depending on the process used, there are three different types of whey: sweet, acid and salt. Sweet whey is produced either during cheese manufacture (cheese curdling) when using rennet enzyme or during casein production. Acid whey is the by-product of cheese and casein production when acid is used to curdle or precipitate the casein. Salty whey is the additional whey that exudes out during the process of salting the cheese curd. Due to the technological developments, whey is used to produce several value added ingredients such as whey protein isolates (WPI), whey protein concentrate (WPC), whey protein hydrolyzate (WPH), and lactose (food or pharmaceutical grade). In the US, the production of whey and lactose products has reached 1.1 million metric tons per year.

Whey protein has a remarkable nutritional value. In fact, it is a high quality protein rich in all essential amino acids. Its biological value is 15% greater than that of the former benchmark egg protein. To benefit from their nutritional value, whey protein ingredients are used in several food applications such as infant formula and protein bars/drinks. Additionally, WPI and WPC products are used for their great functional attributes, namely solubility, gelation, emulsification capacity (EC), emulsification stability (ES) and activity (EA), as well as water holding capacity (WHC). The emulsification properties, for example, are important in emulsion-based products, such as beef patties. Incorporation of whey protein in the formulation of beef patties results in reduced fat loss. Whey protein ingredients are also used by the food industry in cost

effective applications aiming to reduce water loss during processing, to benefit from their water holding capacity.

Another cheese by-product is delactosed whey (DLW). DLW is in fact the by-product of lactose separation from sweet whey. Because of its relatively low protein content (24%) as compared to WPC and WPI, DLW has limited applications in the food industry and is mostly used for animal feed. For instance, DLW is mixed with brewer grains by some farmers, as an alternative to corn for milking cows. Considering that WPH was proven to have amplified functional properties, it is hypothesized that the protein functionality of DLW can be amplified upon limited hydrolysis. DLW with improved functionality could thus be used in various food applications replacing expensive ingredients such as WPI or even meat and egg proteins.

Limited enzymatic hydrolysis with degree of hydrolysis (DH) between 2-8% is generally preferred to avoid liberation of bitter peptides. Limited and controlled enzymatic hydrolysis can result in a targeted enhancement of functionality. Several enzyme systems have been utilized to produce hydrolyzed whey protein with improved functionality. Type of enzyme and hydrolysis conditions, including pH, enzyme-to-substrate ratio and incubation time, influence the properties of the final product. To amplify and improve the functionality of DLW via enzymatic hydrolysis, hydrolysis conditions need to be optimized. There is an infinite number of possible combinations of factors and their levels to be tested, which makes the task quite challenging.

A practical approach to this problem is to implement Response Surface Methodology (RSM) as the experimental design. Using RSM, the number of experimental units necessary to find the optimal point can be tailored depending on the time and resource constraints of the experimenter. After running statistically selected combinations of the different levels of the independent variables of interest, RSM plots are constructed. A polynomial equation is then generated for each dependent variable tested. These equations are used to predict the optimum conditions for the most improved functionality attributes of interest.

Therefore, the overall objective of this study is to enhance the functionality of DLW by enzymatic hydrolysis using RSM approach, with a focus on processed meat application. The specific objectives are:

1) Optimize the functionality of DLW using enzymatic hydrolysis by either alcalase or papain following RSM approach.

2) Determine the functional properties and quality of beef patties fortified with selected DLW hydrolysates in comparison to DLW, WPI, and WPC 34.

1.2. Whey Protein

1.2.1. Significance

The global market size of functional foods is witnessing an 8% increase per year and is estimated to reach a value of $100 billion by 2012 (Smithers, 2008). Therefore, the food industry is facing the challenge of choosing healthier ingredients that are economical and can sustain or even improve texture and overall quality of the final product. Whey proteins and its hydrolysates, in particular, serve as an excellent choice for many food applications due to their exceptional functional and biological properties. Because of these properties, researchers have intensified efforts to expand the utilization of whey proteins as food ingredients.

Today, whey protein ingredients are used to replace other proteins or to improve the functional and biological properties of many food products such as baby food, luncheon meat, ice cream, baked goods, and beverages (Spellman et al., 2005). The U.S. production of WPI and WPC was 15.6 million kilograms and almost 170 million kilograms, respectively in 2005. Eighty six percent of the produced whey protein was for human consumption (Onwulata and Huth, 2009).

1.2.2. Composition, Structural and Physicochemical Characteristics

The whey fluid obtained after cheese or casein/caseinate production constitutes approximately 93% water, 0.6-0.9 % protein (representing 20% of the total milk proteins), 4.5-5.1 % lactose, 0.5-0.7 % minerals and 0.1-0.3 % lipids (Foegeding et al, 2002). Specifically, whey protein constitutes approximately 58 % β-lactoglobulin (β-lg), 25 % α-lactalbumin (α-la), 3 % bovine serum albumin (BSA), 4.5 % immunoglobulin and 1% lactoferrin (Blaschek, Wendorf and Rankin, 2007). Because β-lg and α-la are the two major whey protein components, they contribute the most to the functional properties of whey proteins.

Amino acid composition, molecular structure and physicochemical characteristics of β-lg and α-la influence the functional behavior of these proteins. Mature α-la constitutes 123 amino acids and has 2 predominant genetic variants, A and B, which have a molecular weight of 14,147 Da and 14,175 Da, respectively (Fennema, 1996). The difference between the 2 variants is that the A variant contains a Glu at position 10 of the mature protein whereas the B variant has an Arg substitution at that position (Farrell et al., 2004). Alpha-lactalbumin (Figure 1a) is a very compact globular protein that contains four α-helices, several 3_{10}-helices and an antiparallel β-sheet. On the other hand, β-lg (Figure 1b) is composed of 162 amino acid residues and is about 18,000 Da (Onwulata and Huth, 2009). Beta-lactoglobulin also has two genetic variants, A and B. These variants differ in the substitution of a glycine in Variant B for an aspartate in Variant A (http://class.fst.ohio-state.edu/FST822/lectures/Milk2.htm). Beta-lactoglobulin exists as a dimer from about pH 5 to 7 (Vyas et al, 2002), an octamer at pH values between 3.5 and 5.0, and as a monomer at pH values below 3.5 or above 8.0 (Onwulata and Huth, 2008, http://class.fst.ohio-state.edu/FST822/lectures/Milk2.htm). Beta-lactoglobulin is also a globular and amphiphilic protein (Bouaouin et al., 2006) that folds up into an 8-stranded, antiparallel β-barrel with a 3-turn α-helix on the outer surface and a ninth β-strand flanking the first strand (Kontopidis et al., 2004). The center of the β-barrel forms a hydrophobic pocket. A hydrophobic pocket is also present on the surface between the α-helix and the β-barrel (Fennema, 1996). For α-la and β-lg, the sequence distribution of hydrophobic, polar and charged is uniform causing them to fold intramolecularly. Most of the hydrophobic residues are, therefore, buried preventing interaction with other proteins.

Because α-la and β-lg differ in structure, they have different physicochemical properties. Specifically, β-lg and α-la have different denaturation temperatures. This difference is mainly attributed to the secondary structure of the α-la, which comprises less β-sheets (which is more thermally stable than α-helices) and more α-helices than that of β-lg. Since α-helices are more sensitive to temperature than β-sheets, β-lg denatures at higher temperature (78˚C), greater than that of α-la (64˚C) (Ju et al., 1999). Interestingly, although α-la denatures at relatively lower temperature, it unfolds and refolds reversibly

except at very high temperatures. Consequently, unlike β-lg, α-la does not undergo irreversible denaturation under most milk processing conditions (Fennema, 1996). These are important considerations to take into account during processing of whey, especially when heat treatment is involved.

a. Alpha-lactoglobulin b. Beta-lactoglobulin

Figure 1. Three-dimensional structure the two major components of whey: α-la and β-lg (Fennema, 1996)

Denaturation of α-la is highly reversible because it contains four disulfides and no free cysteine. The positions of these disulfides are Cys 6-Cys 120, Cys 28-Cys 111, Cys 61-Cys, Cys 73-Cys 91 (Fennema, 1996). According to Vasbinder and de Kruif (2003), the process of denaturation resembles a polymerisation process, in which the unfolding step represents the initiation. Alpha-lactalbumin cannot initiate the polymerization process due to the absence of a free thiol group. However because it has four disulfide bridges it is irreversibly denatured in the presence of β-lg due to thiol group-disulfide bridge exchange reactions.

Beta-lactoglobulin, contains two disulfides and one free cysteine. The two disulfides are Cys 106 - Cys 119 and Cys 66 - Cys 160. The free cysteine, which is Cys 121, is partially buried in the hydrophobic core of β-lg (Fennema, 1996), more specifically at the sheet-helix interface (strand H and helix 130 to 140). When the globular structure opens as a result of harsh conditions such as high temperature or hydrostatic pressure, the SH becomes exposed and available to participate in intra- or inter molecular reactions, leading to irreversible denaturation and subsequent protein aggregation (Liu et al., 2008).

Calcium binding is crucial for α-la stability against denaturation (Fennema, 1996). The protein actually possesses two binding sites and their occupation strongly influences the transition temperature and denaturation enthalpy. Studies have shown that, when the calcium is sequestered by EDTA, significant decreases in temperature and enthalpy of denaturation occur (Ju et al., 1999). Beta-lactoglobulin was also found to be extremely sensitive to the presence of calcium in solution. Some authors believe that calcium ions induce protein aggregation by participation in intermolecular salt bridges between negatively charged amino acid. Calcium might play a role in the screening of surface charges, rather than bridging of two proteins. Other researchers believed that calcium-induced aggregation was due to ion-induced conformational changes leading to altered hydrophobic interactions (Damodaran, Parkin and Fennema, 2008)

The denaturation state of α-la and β-lg can also be affected by pH. Lowering the pH significantly increases the denaturation temperature of β-lg (85 °C at pH 3), while it decreases the denaturation temperature of α-la (58.6 °C at pH 3.5) (Ju et al., 1999). Both proteins are more sensitive to thermal denaturation at a pH close to their iso-electric point (pI). For instance, β-lg is most heat sensitive near pH 4.0, with a maximum stability at pH 6.0 and decreasing stability in the higher pH range (Wong et al., 1996).

1.2.3. Nutritional and Physiological Benefits

Whey protein is considered a high quality protein since it is rich in all essential amino acids, and has a biological value that is 15% greater than the former benchmark egg protein (Foegeding et al., 2002). Specifically, whey protein is rich in branched chain amino acids (leucine, isoleucine, and valine), which play a role in weight control by acting as metabolic regulators in protein and glucose homoeostasis and in lipid metabolism (Smilowitz et al., 2005). Leucine in particular, is crucial for protein synthesis. Furthermore, whey protein can improve muscle strength and reduce bone breaking. For these reasons, whey protein is generally preferred over other proteins by body builders, athletes, health conscientious individuals, and others with nutrition system disorders (Madureira et al., 2007).

In addition to its high nutritional quality, whey protein has been recognized for several physiological functions, such as antimicrobial activity, growth promotional activity and immune activity, attributed to the naturally occurring lactoferrin, growth factors and immunoglobulin peptides, respectively (Kitts and Weiler, 2003). Apart from the inherent bioactivity of the native whey peptides, derived peptides from the primary amino acid sequences of whey proteins have been shown to have biological activity such as antihypertensive, opioid, anticancerous, immunomodulating, bactericide and hypocholesterolemic activities (Ferreira et al., 2007; Mine and Shahidi, 2006; Gauthier et al., 2006; Gobbetti et al., 2004; Meisel and FitzGerald, 2000). These peptides are inactive within the primary amino acid sequence, but they can be released during enzymatic digestion or food processing (Korhonen et al., 1998).

1.2.4. Functionality

Whey protein gained popularity not only for its nutritional and biological value but also for its unique physicochemical characteristics, which allow it to have versatile functionality, such as gelation, foaming and emulsification (Foegeding,et al., 2002). As compared to other food proteins, whey protein has great solubility under acidic conditions (Pelegrine and Gasparetto, 2006), which makes it the protein-of-choice for protein-fortified acidic beverages. In addition to their high solubility over a wide range of pH, whey protein possesses water holding and thickening attributes. Because of all these functional properties, the applications of whey protein ingredients have increased tremendously to include a variety of food products such as high protein sport and meal replacement beverages, baked products, processed meat, sauces and dressings (Ryan et al., 2011; Onwulata and Huth, 2009).

1.2.4.1. Solubility

The solubility of a protein is generally defined as the amount of protein present in the liquid phase in relation to the total amount of protein in liquid and solid phases (Pelegrine

and Gasparetto, 2006). Thus, determination of protein solubility mainly consists of making a protein solution, centrifuging it and separating the supernatant which is subjected to protein content determination. The methods usually used for protein determination are Kjeldahl and Dumas.

Whey protein solubility is affected by the denaturation state and several environmental factors such as pH, ionic strength and temperature. Particularly, pH influences whey protein's nature and net charge. High (~8) or low pH (~2) values lead to an excessive amount of same charges, therefore increasing protein-water interactions and repulsion among molecules. As a result, solubility reaches greater values at these pH values (Pelegrine and Gasparetto, 2006). Whey protein is soluble and clear in water in acidic solutions of pH < 3.5, while it forms opaque solutions at or greater than neutral pH. Around the pI (pH 4.3-5.2), the electrostatic forces as well as the level of interactions with water are at minimal. However, the protein remains relatively soluble at this pH because of its low surface hydrophobicity. Upon heating at this pH, the protein denatures and exposes its hydrophobic interior leading to protein-protein interactions and subsequent aggregation. Due to its relatively good solubility under acidic conditions (pH < 3.4) compared to other food proteins, whey protein is the best candidate for protein acidic beverages. A major drawback is the thermal stability of pasteurized whey protein beverages.

Around the pI of whey proteins, addition of salt facilitates their solubility. Salts impart charges to the molecules, keeping them apart from each other. This is generally called "salting in" (de Wit and van Kessel, 1996). However, near neutral pH, salts have an opposite effect. At neutral pH values and without salt, the electrostatic repulsion keeps the protein molecules apart. In that case, addition of salts shields the charges initially present on the molecule and causes aggregation. This effect is called "salting out" and leads to the formation of gels called "soluble aggregates" (Ryan et al., 2012). The same effect was observed at very low pH values. Mate and Krochta (1994) observed precipitation of several whey proteins upon addition of 7% of NaCl to solutions initially brought to pH 2.

1.2.4.2. Gelation and Water Holding Capacity (WHC)

Proteins are characterized by their aptitude to gel and to hold water. Gelation is defined by some researchers as a balance of protein–protein and protein–water interactions, enabling the formation of a three dimensional network (Onwulata and Huth, 2009). Heat treatment at a critical protein concentration causes whey proteins to unfold, aggregate, and form a three dimensional network of a protein matrix entrapping large amounts of water. Commercially, gelation of whey protein is a very important functional property utilized by the bakery and the meat industries, where whey protein is added to improve water retention (Ju et al., 1995; Onwulata and Huth, 2009). Typically, whey protein could gel at protein concentrations ranging from 2 to 8% upon heating at 95˚C for 20 min (Modler and Emmons, 1977).

Gel formation of whey protein is mostly dictated by the β-lg component (Davis, Doucet and Foegeding, 2005). Several factors such as pH, protein concentration, heating rate, and ionic strength influence gelation of β-lg (Wong et al. 1996). Between pH 4 and 6, heating of β-lg causes the formation of opaque rigid gels. Above or below the latter pH range, the gels obtained upon heating are transparent and elastic. High pH values are not suitable for gelation. For instance, heating β-lg at pH 8.0 results in poor strength gels (10% protein solution, 3˚C/min to 90˚C). This is attributed to the occurrence of excessive negative charge, resulting in electrostatic repulsion that interferes with the protein ability to form a strong network.

According to Wong et al. (1996), the gelation temperature of β-lg solutions varies with both pH and the rate of heating. Between pH 4 and 6, gelation started at temperatures varying from 15 to 30 °C, at 12% protein, 5˚C/min to 30˚C, 0.1˚C/min to 90˚C. Above and below the previously mentioned pH range, the gelation temperature is above the denaturation temperature. At high heating rates (10˚C/min), gel formation only occurs in a narrow range boundary below pH 4.5 and above 6.5. High heating rates are detrimental to gelation because the denatured proteins do not have sufficient time to rearrange and associate to form an ordered network. The resulting protein association gives a precipitate instead of a gel.

Ionic strength also has a significant impact on the gelling ability of β-lg by influencing the balance between attractive and repulsive forces of the unfolded protein molecules. For instance, low concentrations of NaCl interact with the counter ions on the protein, reducing the repulsion between molecules and facilitating protein-protein interactions. These interactions then lead to the formation of a fine matrix. Higher concentrations result in shielding of the charges on the protein molecule, thus allowing hydrophobic forces to become predominant. This leads to an extensive aggregation and collapse of the gel network into precipitates. Additionally, ion type affects the rheological properties of the gels: monovalent cations decrease the shear stress of the gels while the divalent cations increase it (Wong et al. 1996).

The required whey protein concentration to form a gel between pH 4 and 6 is 1% w/w. However, at low and high pH values, the critical gel concentration ranges between 5 and 10%. For instance, a 5% β-lg solution is necessary to form a gel at pH 6.5 (Wong et al., 1996).

1.2.4.3. Emulsification

Proteins play an important role in the formation and stabilization of food emulsions. Because, whey proteins are soluble over a wide range of pH and comprise hydrophobic and hydrophilic regions, they can stabilize emulsions by forming interfacial films between hydrophobic and hydrophilic food components (Onwulata and Huth, 2009). Emulsification properties are commonly identified as emulsification capacity (EC), emulsification activity (EA) and emulsification stability (ES).

The EC can be defined as a parameter that denotes the maximum amount of oil that is emulsified under specified conditions by a protein of interest (Pearce and Kinsella, 1978). According to Schmidt et al. (1984), the emulsification properties of a protein are a function of its ability to diffuse to the water-oil interface, unfold, and orient in order for the hydrophobic groups to associate with the oil while hydrophilic groups associate with the water phase. In general, EC measurements can be done using various techniques including centrifugation procedures (Wong and Kitts, 2003; Ahmedna et al., 1999),

determination of particles size distribution, determination of the change in electrical resistance upon collapsing of the emulsion (Pearce and Kinsella, 1978; Webb et al., 1970), or addition of an oil soluble dye, like Sudan Red, that facilitates the recognition of the phase inversion that occurs when EC of the protein is exceeded during homogenization of the oil and protein solution (Rickert et al., 2004).

The EA is a measure of the area of interface that can be stabilized per unit weight of a specific protein. It is a function of oil volume fraction, protein concentration, and the type of equipment used to produce the emulsion (Pearce and Kinsella, 1978). Commonly, the EA is determined by measuring the absorbance of the emulsion at 500 nm immediately after it is formed (Tang et al., 2003). The emulsions are usually diluted in a SDS solution to avoid creaming from occurring.

The ES, on the other hand, is a measure of how stable an emulsion is over time. This is an important concept in emulsion-based products such as salad dressings and mayonnaise (Gauthier et al., 1993). According to Pearce and Kinsella (1978), the ability of a protein to aid in the formation of an emulsion is related to its ability to adsorb at and stabilize the oil-water interface. In other words, the capacity of a protein to stabilize an emulsion is expected to be related to the interfacial area that can be coated by the available protein. Therefore, the stability of an emulsion is related to the constancy of the interfacial area. Kerker (1969) used the Mie theory (for light scattering by dispersed spherical particles) to demonstrate the existence of a simple relationship between the turbidity and the interfacial area of an emulsion when certain conditions are met. Furthermore, Pearce and Kinsella (1978) indicated that the turbidity is proportional to the concentration of the diluted emulsion for absorbances up to about 0.4. At higher emulsion concentrations and higher absorbances, the relationship becomes non-linear because of multiple scattering of light by the dispersed oil globules, especially when oil globules are small. Turbidity measurements are generally done at 500nm using common laboratory spectrophotometers. Microscopic examination of the emulsions revealed that these turbid solutions contained small flocs (Pearce and Kinsella, 1978).

It is crucial to control the droplets size to achieve good emulsification stability values. According to Klink et al. (2011), the initial rate of coalescence of an emulsion, is strongly

dependent upon the presence of large drops. As the number fraction of large droplets within the distribution increases, the rate of coalescence also increases. The size of the droplets depends on several factors such as emulsifier-to-dispersed phase ratio, time required for the emulsifier to move from the bulk phase to the droplet surface, probability of adsorption, aptitude in reducing the interfacial tension, and effectiveness of emulsifier membrane to prevent coalescence.

Whey proteins have a great potential in stabilizing water/oil emulsions. They are able to produce highly charged and thick interfaces that increase the repulsive interactions among droplets and prevent flocculation. When adsorbed, whey proteins produce very visco-elastic interfaces that resist deformation, preventing coalescence. Additionally, non-adsorbed whey proteins serve as thickening or gelling agents and, therefore, prevent the droplets from coming into close contact of each other for extended period of time (Onwulata and Huth, 2009). However, since whey proteins are globular, they do not move to or rearrange at the oil/water interface, as readily as proteins such as caseins. Caseins, which are fibrous proteins, have a more open structure. Also, in whey proteins' primary structure, hydrophobic and hydrophilic groups are distributed uniformly (Wong et al., 1996; Schmidt et al., 1984) as opposed to caseins where these groups are rather concentrated in discrete regions, providing a highly amphiphilic structure ("soap-like" structure). In order to be able to compete with casein and other proteins with good interfacial properties (egg protein), whey proteins need to be modified in a fashion that exposes the buried hydrophilic and hydrophobic groups. These groups would then be available to interact with water and the oil phases during the formation of the interface. Whey proteins are commonly used in applications such as ice creams, soufflés, frothed drinks, and other food foams and emulsions where they serve as surface-active agents.

The surface activity of whey globular proteins and β-lg in particular, is affected by the pH of the system. At low pH, they have a compact and rigid conformation that does not favor movement to the interface. However at pH values ranging from 3 to 7, the resulting increase of the surface pressure leads to a better arrangement of the molecule at the interface. Finally, near the pI and specifically at pH 4.9, the electrostatic repulsion is

minimal among protein molecules and facilitates high adsorption and rearrangement at the interface.

1.3. Whey Protein Modifications to Enhance Functionality

1.3.1. Need for Protein Modifications

Even though whey proteins have great functional properties, it quickly became imperative to expand the whey protein market in order to compete with meat proteins (that have excellent gelation property), egg proteins (that have excellent foaming and emulsification properties), and other functional protein ingredients. In general, processing imparts some challenges pertaining to whey protein solubility (Sava et al., 2005), gelation (Foegeding et al., 2002), foaming and emulsion stability (Ye and Singh, 2006).

The behavior of whey protein in heated systems poses a challenge in protein-fortified beverages applications. Because of their globular structure, whey proteins are relatively heat labile. Specifically, denaturation can occur at temperature as low as 60 ºC leading to aggregation. Additionally, FDA requires a protein concentration of at least 4.2% in order to make a label claim of a "high protein beverage" (CFR, 2008). Producing stable beverages with $\geq$ 4.2% protein is dependent on the whey protein solubility and thermal stability. Upon heating, whey protein beverages tend to lose their clarity unless the pH is < 3 (Etzel, 2004). Because of the resulting excessive sourness and astringency, producing beverages with pH < 3 is generally undesirable. Maintaining clarity at high protein % (>4.2%) in processed beverages over an extended period of time, is therefore, a challenge.

Interfacial properties are negatively affected by the globular structure of whey proteins. As opposed to caseins which are fibrous proteins, whey globular proteins are not great at quickly moving to the interface or rearranging. Modification of whey protein is therefore necessary to improve their interfacial properties, namely foaming and emulsion (capacity and stability).

The minimal concentration of whey protein necessary to form a gel also constitutes a major limitation. In general whey proteins form gel at relatively high protein concentration compared to meat proteins such as gelatin. Gelatin can form a gel at protein concentration as low as 0.6%, whereas whey protein gels requires about 5 to 6% at least (Hongsprabhas and Barbut, 1999). Finding ways to enhance the gelling properties of whey proteins will result in significant economic benefits.

On the other hand, whey protein has been shown to be involved in the moisture-induced aggregation that result in undesirable hardening of intermediate-moisture food matrixes, such as nutrition bars, due to protein-protein covalent and non-covalent interactions that occur during storage (Liu et al., 2008). This phenomenon reduces shelf-life and adversely affects acceptability. It is therefore crucial to find ways to reduce protein-protein interactions in such systems.

1.3.2. Recently Researched Protein Modifications

The most researched protein modifications techniques to improve the functional properties of proteins include Maillard conjugation (Zhu et al., 2008; Mishra et al., 2001); physical modification utilizing high pressure treatment (Bouaouin et al., 2006, Chicón et al., 2009), ultrasound treatment (Jambark et al., 2008), supercritical carbon dioxide treatment (Zhong and Jin, 2008) and enzymatic modification, which is extensively researched (Spellman et al., 2005; Davis et al., 2005; Creusot and Gruppen, 2008; Britten et al., 1994; Sinha et al., 2007). The use of enzymatic modification to improve the functionality of whey protein is the focus of this work; therefore, it will be discussed in details in the following section.

1.3.3. Enzymatic Modification

Whey protein hydrolysates (WPH) are produced using enzymatic hydrolysis rather than chemical hydrolysis. Chemical hydrolysis causes loss of some essential amino acids, such as tryptophan, and can result in a product with high amount of free amino acids,

which can encumber the body's osmotic balance (Spellman et al., 2009). Enzymatic hydrolysis, on the other hand, is performed under mild conditions, and results in improved digestibility, and enhanced bioactivity. Endoproteases are generally used to ensure low levels of free amino-acids. The nutritional quality of the enzymatically hydrolyzed protein is comparable to that of the native protein. Upon enzymatic hydrolysis bioactive peptides can be released from the primary sequence (Mahmoud, 1994), which is an added benefit. Enzymatic hydrolysis is also preferred over chemical hydrolysis due to the availability of a wide range of enzymes that are considered safe (GRAS) and natural. Several enzymes are currently used in industry and others are being researched for the production of WPH with tailored functionality and biological activity. The most researched and used enzymes for the production of WPH are digestive enzymes, namely trypsin, pepsin and chymotrypsin (Pouliot et al., 2009; Ju et al., 1995; Groleau et al., 2003; Konrad et al., 2005. Hernández-Ledesma et al., 2005), and plant enzymes such as papain and bromalin (Nakamura et al., 1993). Bacterial proteases have also been used, mainly those originating from *Bacillus licheniformis* (Spellman et al., 2005, Davis, Doucet and Foegeding, 2005; Creusot and Gruppen 2008, Creusot and Gruppen, 2007) and *Bacillus subtilis* (Madsen et al., 1997), or a mixture of some of these enzymes (Kim et al., 2007).

Functionality of whey protein can be improved via limited hydrolysis that impart changes in the molecular size, conformation, and strength of inter- and intramolecular interactions (Guan et al., 2007). The functional and biological properties of the resulting WPH depend to a great extent on the type of enzyme used (specificity and selectivity), hydrolysis conditions employed (enzyme-to-substrate ratio, incubation temperature, pH and time), and the source of the protein, i.e. native vs. denatured, WPI vs. WPC, membrane or ion-exchange product, etc. However, excessive degree of hydrolysis could be detrimental to the functional properties. For instance, excessive hydrolysis is detrimental to the interfacial properties of whey proteins. At high degree of hydrolysis, many short peptides are produced, which result in poor stability during storage and upon thermal treatment (Ye and Sing, 2006; Kilara and Panyam, 2003; Agboola et al., 1998). In a comparative study of 44 different hydrolysates, the hydrolysates containing high

percentage of large molecular weight fragments (> 7 kDa) had the best foam stability (van der Ven et al., 2002). Hydrolysates with larger peptides can be produced by limiting the degree of protein hydrolysis (Deeslie and Cheryan, 1988). Uncontrolled and extensive hydrolysis of whey protein can also impair its ability to gel (Huang et al., 1999).

Extensive hydrolysis could also be detrimental to flavor. High DH result in the release of bitter tasting peptides, which in many cases limit the use of WPH to low concentrations at which bitterness is not detected (Sinha et al. 2007). A DH value less than 8% is often targeted to limit the production of bitter peptides (Rios et al. 2004). Thus, enzymes and proteolysis conditions must be carefully chosen and controlled in order to achieve the desired functionality (Guan et al., 2007), without altering the taste of the products

1.3.3.1. Effect of Hydrolysis on Solubility

Proteolysis has led in many cases to increased solubility over a wide range of pH (Mutilangi et al., 1996; Jost and Monti; 1977; O'Keefe and Kelly, 1981), due to the reduced molecular weight and the increased hydrophilicity resulting from the increase in free carboxyl and amine groups. Heat stability also was improved upon partial hydrolysis due to the loss of secondary structure, thus contributing to reduced structural changes upon heating (Foegeding et al., 2002). The nature of the peptides released influences solubility. Solubility was reduced in cases when the enzymes used resulted in the release of hydrophobic peptides that promoted the formation of aggregates (Otte et al, 1996).

1.3.3.2. Improvement of Gelation

Limited enzymatic hydrolysis has led to improved gelation property. Hydrolysis causes the exposure of buried hydrophobic residues and/or release of specific peptides that promote peptide-peptide and peptide-protein aggregation (Creusot and Gruppen, 2007). Enhanced aggregation and gelation is desired in systems such as cold set gels and yogurts (Onwulata and Huth, 2009). Specifically, yogurts with more compact structure,

consisting of robust casein particles and large aggregates, can be obtained with the addition of whey protein (Aziznia et al., 2008). Therefore, hydrolysates with enhanced gelation properties are desirables in these products.

The effect of hydrolysis on the gelation ability of whey protein is dependent not only on the source of enzyme but also on the environmental conditions and the degree of hydrolysis. Although limited trypsin hydrolysis was shown to enhance solubility of whey proteins at neutral pH (Jost and Monti, 1977), in other cases trypsin hydrolysis of β-lg and WPI resulted in gelation under controlled acidic, neutral and basic conditions (Groleau et al., 2003; Pouliot et al., 2009). Hydrolysis conditions, source of protein, enzyme-to-substrate ratio, and degree of protein denaturation varied between the former study and the more recent studies.

Bacillus licheniformis protease (BLP) is the most researched, among the enzymes investigated for the production of WPH with enhanced gelation properties (Creusot and Gruppen, 2007; Davis, Doucet and Foegeding, 2005; Spellman et al., 2005; Otte et al., 2000). Commercially available BLP preparations, such as Alcalase and Prolyve are often utilized (Spellman et al., 2005). Subtilisin Carlsberg, which is an endoprotease with a preference for large uncharged residues, and glutamyl endopeptidase (GE), which cleaves peptide bonds on the C-terminal side of glutamate and aspartate residues, are the main BLP enzymes with the ability to produce hydrolysates with enhanced gelation property. A solution of BLP-hydrolyzed whey protein, with a concentration as low as 2% (w/w), has been shown to form a gel at 50 ºC under neutral conditions (Creusot and Gruppen, 2007). Additionally, cold gelation of BLP-hydrolyzed whey protein was found to be induced by salt addition or acidification (Rabiey and Britten, 2009 a and b). Limited hydrolysis (DH ~ 2.2%) of WPI by BLP resulted in the formation of a soft gel with similar characteristics to a heat-induced gel (Otte et al.1996). Although extensive enzymatic hydrolysis of whey proteins usually improves solubility and decrease gelling ability, non-heat set gelation occurred at high solid content (20% w/v) of an extensively hydrolyzed WPI with Alcalase 2.4L® (Doucet et al., 2003). Gelation occurred at DH of 18 %, with gel characteristics similar to that of a heat-induced gel. Doucet et al., (2003)

showed that extensive hydrolysis of whey proteins by Subtilisin Carlsberg led to aggregation and formation of a gel that was stable over a wide range of pH.

The BLP glutamyl endopeptidase cleaves hydrophilic segments in the substrate and, therefore, preserves hydrophobic segments that aggregate with each other and with other intact proteins present in the solvent, resulting in a gel network (Ipsen et al., 2000). The uneven partition of the Glu residues in β-lg and α-la results in the release of large fragments with high average hydrophobicity upon hydrolysis with GE, and causes a specific conformational change that exposes hydrophobic residues. Therefore, gelation is not only a function of DH, it is also influenced by both DH and the type of peptide released (Ipsen et al., 2000).

Enhancing gelation upon protein hydrolysis is directly influenced by the characteristics of the hydrolysate produced using specific combinations of substrates, enzymes and degrees of hydrolysis. The peptides responsible for peptide-peptide and peptide-protein aggregation are quite diverse in their intrinsic factors, namely molecular mass, pI, average hydrophobicity, amino acid composition and net charge at a given pH. It must be emphasized, though, that peptide aggregation does not always lead to gelation. Along with the mentioned intrinsic characteristics, extrinsic factors such as protein concentration, pH, and ionic strength play a major role in gel formation. Moreover, different gelation mechanisms are involved due to limited vs. extensive hydrolysis, and native vs. denatured substrate. For example, enzyme-induced aggregation and gelation of native whey proteins was obtained only with enzymes exhibiting GE activity. The type of enzyme chosen is important since the specificity is different from one enzyme to another. Subtilisin Carlsberg is the main proteolytic component in Alcalase 2.4L. However, GE was found to be present in Alcalase 2.4L as well (Doucet et al., 2003). Spellman et al. (2005) showed, after isolating subtilisin and GE activities from Alcalase 2.4L, that the GE is the enzyme responsible for the peptide aggregation in WPH obtained from Alcalase 2.4L. Aggregation of WPH could occur with two different mechanisms. Aggregation of hydrolysates prepared with GE occurred at relatively limited hydrolysis (2-7% DH, pH 7) and involved rather large peptides (2-10 kDa); whereas aggregation of hydrolysate prepared with both GE and subtilisin activities (Alcalase 2.4L) occurred after

extensive hydrolysis and involved small peptides (<2 kDa) (Doucet et al., 2003). The aggregation that occurred at limited hydrolysis was possibly due to the narrow specificity of the enzyme for polar residues and a substrate containing hydrolphobic segments. Whereas the aggregation that occurred after extensive hydrolysis was the result of enzymes with specificity for both polar and nonpolar residues that caused the cleavage of most hydrophilic and hydrophobic segments resulting in oligopeptides, with a net charge of 0 at pH 6, that can physically aggregate at high concentration. These results highlighted the influence of the protease specificity on the characteristics of the hydrolysates generated with commercially available protease preparations.

1.3.3.3. Effect of Hydrolysis on Interfacial Properties

Because of their globular nature, whey proteins have a relatively poor ability to create an oil/water interface. To achieve better emulsification properties, their structure needs to be modified in order to have greater flexibility, smaller average molecular weight, increased number of charged groups and exposure of hydrophobic and other reactive groups. Researchers have successfully controlled hydrolysis conditions of whey proteins to produce hydrolysates with enhanced interfacial properties of whey protein, namely foaming and emulsification properties (Kitts and Weiler, 2003; Dalgleish and Singh, 1998; Caessens et al., 1999; Kilara and Panyam, 2003). Factors such as enzyme specificity, denaturation state of the substrate, DH and environmental conditions influence the interfacial properties of the resultant WPH.

Mild hydrolysis generally improves whey protein emulsifying and foaming properties, mainly due to the reduced size of peptides, which promotes a more rapid adsorption at the interface (Turgeon et al., 1992). However, excessive hydrolysis may be detrimental to the interfacial properties of whey proteins, because of the production of many short peptides, which result in poor stability during storage and upon thermal treatment (Kilara and Panyam, 2003; Dalgleish and Singh, 1998; Agboola et al., 1998;). Hydrolysates with larger peptides can be produced by limiting the degree of protein

hydrolysis, or by separation of hydrolysates using an ultrafiltration reactor system (Deeslie and Cheryan, 1988; Gauthier et al., 1993).

Apart from molecular size, the relative hydrophobicity of the hydrolysates is directly related to foaming, emulsification capacity and stability. Peptide hydrophobicity was correlated with improved foamability and foam stability (Caessens et al., 1999), and with improved emulsifying properties (Chaplin and Andrew, 1989). Konrad et al. (2005) studied the effects of limited hydrolysis of whey protein concentrate by pepsin on surface properties in model systems. Peptic WPH showed improved emulsification and foaming properties at two optimal degrees of hydrolysis (DH 1-1.5% and DH 5.8%). The first was primarily related to molecular unfolding of whey protein in the starting phase of hydrolysis by pepsin, and the second to the symbiotic effects of intact β-lg and large peptides, liberated during further proteolysis. Deterioration of surface properties was noted at DH > 6.5%, due to the release of very small peptides and the drastic increase in surface hydrophobicity. Increased hydrophobicity reduces the repulsive forces needed to avoid coalescence. Turgeon et al. (1992) demonstrated that β-lg hydrolysates with peptides containing distinct zones of hydrophobic and hydrophilic regions, within a minimum molecular weight allowing this distribution, had the best interfacial properties and stability. Rahali et al (2000) also showed that adsorption of β-lg peptides of known sequences at the oil/water interface is more influenced by the amphiphilic character than the peptide length. Therefore, while the DH and molecular weight distribution are good indicators of hydrolysate properties, the functional properties are determined by the specific peptides produced.

The use of highly specific proteases may have an impact on the hydrophobicity and on the size of peptides. Hydrolysis of whey protein with BLP endoprotease specific for Glu and Asp residues resulted in a hydrolysate with improved foam overrun and stability (Ipsen et al., 2001). These enzymes cleave hydrophilic segments in the substrate and, therefore, preserved hydrophobic segments. These hydrophobic segments interact more readily with the oil phase of the emulsion.

Davis, Doucet and Foegeding (2005) investigated the foaming and interfacial rheological properties of β-lg hydrolysates produced using three different proteases,

pepsin, trypsin and Alcalase®. As compared to unhydrolyzed β-lg, all hydrolysates resulted in lower interfacial tension indicating that they adsorbed more rapidly at the interface. Hydrolysis of β-lg by pepsin was less extensive than its hydrolysis by trypsin or Alcalase. Native β-lg resists hydrolysis by pepsin, which has a broad specificity with a preference for cleaving after hydrophobic residues (Konrad et al., 2005). Although pepsin and Alcalase hydrolysates had different DH, both had similar foaming characteristics, which were slightly better than those of trypsin hydrolysates. The authors hypothesized that hydrophobic interactions between minimally charged peptides were present at the interface. However, due to the different specificity and activity of the enzymes, relating DH to adsorption rate and peptide association at the interface was not possible. Another study, which highlighted the impact of enzyme specificity on interfacial properties of WPH, showed that plasmin-hydrolyzed β-lg had better interfacial functionality than trypsin- or *Staphylococcus aureus* V8 endoprotease-hydrolyzed β-lg (Caessens et al., 1999). The plasmin hydrolysates had larger molecular weight and more hydrophobic peptides than the other two hydrolysates. Other researchers have shown that tryptic peptides from whey proteins have better emulsifying and interfacial properties than chymotryptic peptides (Turgeon et al., 1992). Thus, the emulsifying properties depend not only on DH and molecular weight distribution, but also on the type of enzyme used and the produced peptides.

1.4. Whey Protein Ingredients

There are numerous whey ingredients available in the market (Table 1). Among the whey protein ingredients, WPI, WPC and WPH are the most commonly used in various food applications. Because of its relatively good protein content (24%), DLW can be used in food applications if its protein functionality is amplified. The following section will focus on whey protein ingredients and DLW.

Table 1. Composition (%) of selected whey protein ingredients (Tamime, 2009)

Whey Protein Ingredient	Protein	Lactose	Minerals
Regular dried whey powder	12.5	73.5	8.5
Demineralized (70%) whey powder	13.7	75.7	3.5
Demineralized (90%) whey powder	15.0	83.0	1.0
Special whey protein concentrate	35.0	50.0	7.2
Whey protein concentrate (WPC)	65.0 – 80.0	4.0 – 21.0	3.0 – 5.0
Whey protein isolate (WPI)	88.0 – 92.0	<1	2.0 – 3.0
Delactosed whey (DLW)	24	56	5

1.4.1. Whey Protein Concentrates and Isolates

Whey protein concentrate is obtained by subjecting liquid whey to ultra-filtration (UF). Ultra-filtration allows to selectively concentrate proteins, which are subsequently dried. Commonly, WPC is 35 % protein (WPC 35), but UF and diafiltration (DF) can be combined in the treatment of the retentate in order to achieve protein content greater than 50% (up to 80%), by further removal of minerals and lactose (Onwulata and Huth, 2009). Membrane filtration does not cause denaturation of the proteins, however the drying step may.

Whey protein isolate (>90%) can be produced using ion-exchange (separates components by ionic charge instead of molecular size), membrane filtration, or a combination of both. Ion-exchange allows quasi-complete removal of the lactose (Goodall et al., 2009; Onwulata and Huth, 2009). When ion-exchange is used, the resulting WPI contains no casein glycomacropeptide and less immunoglobulins (Ounis et al., 2008), lactoferrin and α-la (Mercier et al. 2004), but more β-lg (Mercier et al., 2004) than WPI prepared by microfiltration or ultrafiltration. Membrane produced WPI has variable amounts of glycomacropeptides with molecular weights ranging from 2–10 kDa,

and a small amount (<6%) of peptides with molecular size < 2 kDa. Ion exchange-WPI, however, are mostly composed of large molecular weight components (> 10 kDa) (Spellman et al., 2005). The technique used to produce whey protein ingredients, therefore, affect the composition of the final product, which in turn affects the functionality.

Ion-exchange-produced WPI has less calcium and lactose content than membrane-produced WPI. Calcium plays a significant role in whey protein coagulation and gelation (Ju and Kilara, 1997). Even though calcium has a negative impact in thermally processed beverages where gelation is not desired, it was shown to improve gelation properties in systems where gelation is desired (Ispen and Otte, 2007).

Bonnaillie and Tomasula, (2008) reported that the protein in ion-exchange-produced WPI is slightly more denatured than the protein of a membrane-produced WPI. Spray-drying may also cause denaturation because of the high temperatures involved during the process. Drum drying results in even higher extent of denaturation. The thermal conditions involved in the latter are generally extreme. As a result, the functionalities of WPC and WPI are highly dependent on the process used.

Because of its high protein purity and solution clarity, WPI is generally preferred over WPC in beverages and nutritional supplements (Foegeding and Luck 2003). Whey protein concentrates with lower protein content tends to be used in lower-value food products, such as dairy and bakery items, whereas WPC with higher protein % is generally used in higher-value products such as meat and seafood (http://www.fas.usda.gov/dlp2/circular/1999/99-12Dairy/uswhey.html).

Common applications of WPC and WPI, in general, include baked goods and baking mixes; cakes and pastries; candy, chocolate, and fudge; coffee whiteners; crackers and snack foods; diet supplements; fruit beverages; gravies and sauces; infant formulas and baby food; mayonnaise; pasta; pie fillings; processed dairy products including ice creams; processed fruits and vegetables; salad dressings; soups, meats, and sausage; and sports drinks (Onwulata and Huth, 2009).

1.4.2. Whey Protein Hydrolysates

Because native whey protein is globular and resists digestion, hydrolysates produced particularly via enzymatic hydrolysis, have raised a lot of interest. They were found to be of equivalent nutritional quality to that of their intact counterpart, with added benefits (Potter and Tome, 2008). Whey protein hydrolysates provide an excellent protein source for infants with underdeveloped digestive tract (Zeiger et al., 1986), and for patients with digestive tract disorders, such as impaired luminal hydrolysis, reduced absorptive capacity and or specific gastric or hepatic failure (Potter and Tome, 2008). Additionally, WPH is a nutritious protein source for individuals with whey protein allergy (Guadix et al., 2006). Allergic reactions are caused by specific amino acid sequences in the native form of β-lg (Otani, 1987). Such sequences can be hydrolyzed by appropriate proteases, and thus reduce antigenicity by several magnitudes (Nakamura, 1993). Research is still ongoing to ensure maximal reduction of antigenicity through efficient processes and controlled enzymatic reactions, while maintaining a high quality product (Kim et al., 2007; Guadix et al., 2006).

As discussed earlier, WPH has improved functionality compared to its intact counterpart. Based on the enzyme used, protein source and hydrolysis conditions, a variety of WPH products with specific functionality and physiological contributions can be produced. Uses of WPH as discussed earlier include, baby formula, fortified drinks, energy bars, and meat products.

1.4.3. Delactosed Whey

Delactosed whey is a by-product of lactose production during which lactose is separated from sweet whey. The powdered DLW is subsequently obtained by submitting the remaining liquid delactosed whey to spray-drying. Delactosed whey is also known as ‘reduced lactose whey’. The final product still contains significant amount of lactose (55 %), since only a portion of lactose is separated out. Sweet whey or unprocessed whey contains 75 % lactose on a dry matter basis.

Because of its relatively low protein content (24 %) compared to WPC (34-80%) and WPI (~ 90 %), DLW is mainly used for animal (cattle and swine) feed either in liquid or dried form (Schingoethe, 1975). For instance, some farmers mix wet brewers grains with an equal amount of delactosed whey, as a corn alternative for milking cows. DLW sodium content however, is around 2.4%, which puts limitations on the amount that can be fed before exceeding animal requirements and tolerances for sodium (www.whminer.com/Research/WHM-06-1.pdf).

Because the dairy industry annually generates more than one billion pounds of whey solids as a by-product of cheese manufacturing, low cost whey solids such as DLW are being utilized as a filler or extender in urethane foam formulations, as a replacement to wood resins, starch and British gum dextrin. Extenders are one of the best approaches to reducing costs of urethane foams (Hustad and Richardson, 1972). Polyurethanes applications are diverse and include, but are not limited to, manufacture of flexible and high-resilience foam seating, rigid foam insulation panels, microcellular foam seals, and gaskets.

Since DLW contains a relatively good amount of protein (24%), there is an opportunity of increasing its value by amplifying the functional properties of its protein content. Enhanced functionality of DLW would potentially expand its uses to include food applications. This is also a great opportunity to increase the profit margin of the dairy industry.

1.5. Enhancing the Functionality of DLW

Given that limited hydrolysis can improve the functional properties of whey protein in general, it is hypothesized that subjecting DLW to enzymatic proteolysis will lead to enhanced functionality. In order to achieve the desired functionality enhancement, hydrolysis parameters and conditions need to be optimized. Enzymatic hydrolysis is mostly impacted by several factors including type of enzyme, temperature, time, enzyme-to-substrate ratio and pH. Therefore, there are a lot of possible combinations of these parameters. One way to determine the optimal hydrolysis condition would be to conduct

a full factorial design. However, in order to save time and resources, a more practical approach is following Response Surface Methodology (RSM), which will be discussed in the following section.

1.6. Response Surface Methodology

Response surface methodology is a very effective systematic approach for making quantitative models where multiple parameters vary simultaneously. Most importantly, it allows for optimization of these parameters (Inoue et al., 2008). The actual response function is approximated with a quadratic polynomial model that consists of the main terms, second-order terms, and interaction terms (Eq. 1). Therefore, the RSM consists of a set of mathematical and statistical methods developed for modeling phenomena and finding combinations of a set of experimental factors that will lead to an optimal response (Nielsen et al., 1973).

$$\boldsymbol{Y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2} \quad \text{(Eq. 1)}$$

where: Y is the response variable,
b_o is the intercept,
b_1, b_2 and b_3 are the first order coefficients,
b_{12}, b_{13} and b_{23} are the interactions coefficients, and
b_{11}, b_{22} and b_{33} are the second order coefficients.

Response surface methodology allows running fewer experimental units than a full factorial design. The optimum responses generated for the variables of interest are then used to produce a polynomial equation, which not only allows the determination of the optimal conditions but also is used to produce tri-dimensional response plots (Cruz et al., 2010). In fact, several experiments are conducted, using the results of one experiment to provide direction to the next (Myers et al., 2009). The next action could be to focus the

experiment around a different set of conditions, or to collect more data in the current experimental region in order to fit a higher-order model or confirm what was predicted. In other words, a first order is employed until the experimenter observes a lack of fit and decides to expand the model to a second order. A significant lack of fit is an indication that the experimenter gets closer to the maximal or minimal point (saddle point).

Running RSM allows to significantly save time and resources by eliminating factors and levels that have no effect. For example a three-level full factorial design of an experiment involving four independent variables would result in 81 experimental units, which can be reduced to a number as low as 27 (depending on the constraints), when RSM is used.

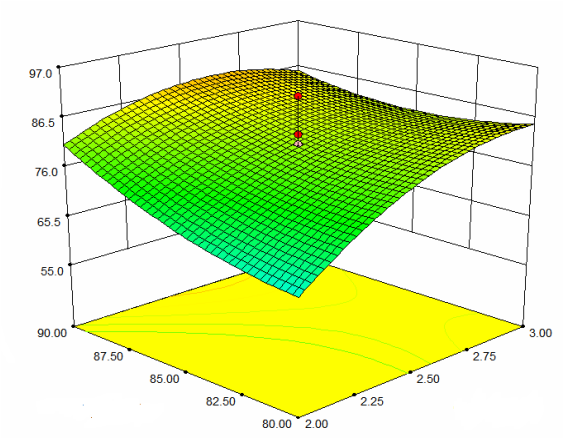


Figure 2. Example of RSM plot.

To optimize a process, one has to establish the independent variables or the factors that impact the process the most. For instance, protein hydrolysis is influenced by factors such as pH, time, enzyme-to-substrate ratio and temperature. On the other hand, the response or dependent variables are the ones the experimenter is looking to optimize (for example emulsification attributes or water holding capacity).

The RSM steps include coding the independent variables levels, generating a standard design (central composite or Box-Behnken), simplification of the specification of the standard response-surface models, visualizing a fitted response surface, and finally

exploring further experimentation, for example, along the steepest ascent (Myers et al., 2009).

The data is coded to allow easier manipulation (Table 2). The coded levels of the independent variables are (-1, 1) and (-1, 0, 1) for 2-level and 3-level designs, respectively. In order to determine the experimental units (combinations of the independent variable at their different levels), Central Composite Design (CCD) or Box-Behnken designs can be generated using a software such as SAS or R. Both designs can be blocked but the CCD's blocks are of two types: the cube and star blocks. The cube blocks contain design points from a two-level factorial or fractional factorial design (±1,±1, . . . ,±1), plus center points (0, 0, . . . , 0), while star blocks contain axial points [(±α, 0, 0, . . . , 0), . . . , (0, 0, . . . ,±α)], plus center points (Myers et al., 2009). Generally, α is fixed at 1.414 but its value is usually selected based on considerations of rotatability (the variance of the prediction depends only on the distance from the center) and orthogonality of blocks (to prevent coefficients of the fitted response-surface equation from correlating with block effects).

Table 2. Example of natural and coded data.

Factor	Coded data			Natural data		
e/s (%)	-1	0	1	0.5	1	1.5
Time (min)	-1	0	1	15	45	75
pH	-1	0	1	6.5	7.5	8.5

RSM has been used to optimize products such as probiotic yogurts (Cruz et al., 2010), frozen dessert like ice cream (Inoue et al., 2008; Tong., 2009), infant formula (Maduko et al., 2007), cheese curd yield and protein recovery (Fagan et al., 2007). Specifically, Cruz et al. (2010) succeeded in optimizing the processing of probiotic yogurt, using CCD. They considered 2 independent variables, glucose oxidase and glucose concentrations, at two levels. Their response variables were the dissolved oxygen concentration and the count of B. longum BL 05. Their CCD contained a total of 11 experimental units, which allowed the generation of an adequate model with acceptable errors between

experimental and predicted results. Fagan et al. (2007) used a similar approach to optimize curd moisture, whey fat losses and curd yield as a function of cutting time, temperature, and calcium. Even though some of their RSM plots did not show a saddle point (minimum or maximum response), their model allowed them to determine the directions in which the process was more likely to be improved. Another example for the application of RSM was the optimization of the hydrolysis conditions of whey proteins for the production of angiotensin-I converting enzyme (ACE) inhibitory peptides (Guo et al., 2009). The considered independent variables were hydrolysis temperature, pH and enzyme to substrate (E/S) ratio. These parameters were optimized using a central composite rotatable design (CCRD) and the response variable was the ACE inhibitory activity. The highest ACE inhibitory activity was 92 %, at an E/S of 0.60, a reaction time of 8 h, a reaction temperature of 38.8 °C and a reaction pH of 9.18. Response surface methodology, therefore, represents a promising tool for the optimization of various processes that involve complex systems of factors and interactions.

1.7. Application of Enzymatically Modified DLW

One of the main applications of whey protein ingredients is processed meat. Quality improvement, nutritional optimization and cost-effectiveness are key drivers in using whey protein ingredients in processed meats. In general, whey protein ingredients, such as WPI and WPC, are used in processed meats (ground meats, emulsion products, coarse ground products and whole muscle products) in order to improve flavor, texture, emulsification, water binding, cook yield, and finished product functional performance. Response surface methodology approach will be pursued in this study to identify the best conditions that will lead to a directed functionality enhancement of DLW, including emulsification, water holding capacity and gelation, with a focus on a processed meat application.

2. ENHANCING THE FUNCTIONALITY OF DELACTOSED WHEY BY ENZYMATIC HYDROLYSIS USING RESPONSE SURFACE METHODOLOGY APPROACH

2.1. Overview

To amplify and improve the functionality of delactosed whey (DLW), it was subjected to limited and controlled enzymatic hydrolysis using Alcalase® and papain. Conditions of hydrolysis were optimized using response surface methodology (RSM). A Box-Behnken design with 4 independent variables at 3 levels was generated and implemented for each enzyme. The variables chosen, temperature (x_1), enzyme-to-substrate (e/s) ratio (x_2), time (x_3) and pH (x_4), are known to have the most significant influence on the DH and protein functionality. The design had 27 experimental units per enzyme that included 3 center points to assess the pure error. Each experimental unit was run in triplicate and the means of DH and measured functional properties were recorded as the y responses. Several response surface models were compared to determine the best fit for the RSM data collected. The point within the range of experimentation with the highest fitted value was determined. Using the model with the best fit (high R^2 and $P \leq 0.05$) for each response, prediction equations were generated and used to determine optimal hydrolysis conditions. The effect of the independent variables on each response was evaluated and tridimensional response plots were generated. The conditions at which the responses were maximal/optimal were then determined. To validate the model, experimental data was obtained using the predicted optimized levels. Within the range tested, the emulsification capacity and activity of the DLW hydrolysates, produced under optimal conditions, were comparable to, if not greater, than that of WPI, which is known for its exceptional functionality attributes. The RSM approach provided an understanding of the effect of each hydrolysis parameter on the DH and on the assessed emulsification properties. While emulsification properties were enhanced significantly, hydrolysis of DLW was detrimental to the gelation property. Nevertheless, results of this work showed, for the first time, that the functionality of the protein component of DLW can be amplified upon limited and controlled hydrolysis.

2.2. Introduction

World-wide an increasing amount of whey is available due to increasing cheese production. For decades scientists have been working on identifying beneficial components in whey that contribute to functional and nutritional enhancement in various food products, with an ultimate intention of benefiting the dairy industry/farmers. The most valuable component of whey is its protein, which delivers both enhanced functionality and nutrition to many formulations. Technological advances, including efficient and cost-effective unit processes for concentration, fractionation, dehydration as well as structure-function modifications (Smithers, 2008), resulted in the development of several whey protein ingredients including whey protein concentrates (WPC, 35-80% protein), whey protein isolates (WPI, 90-96% protein) and whey protein hydrolysates (WPH). Today, these whey protein ingredients are used to replace other proteins or to improve the functional properties of many food products such as baby food, luncheon meat, ice cream, baked goods, and beverages (Spellman et al., 2005). Other whey ingredients available in the market include whey powder, demineralized whey permeate powder and delactosed whey.

Delactosed whey is the by-product of lactose separation from sweet whey. Because of its relatively low protein content (24%) as compared to WPC and WPI, DLW has limited food applications and is mostly used for animal feed (Schingoethe, 1976). However, since the dairy industry actively seeks and evaluates new ways to reduce waste and increase profit, DLW is considered an excellent candidate for functional enhancement. Uses of DLW can be extended to include food applications, if the functionality of the protein component is amplified. Given that limited hydrolysis can improve the functional properties of whey proteins in general (Guan et al., 2007; Foegeding et al., 2002), it is hypothesized that subjecting DLW to enzymatic proteolysis will lead to enhanced functionality. DLW with improved functionality can thus be used in various food applications replacing expensive ingredients such as WPI or even meat and egg proteins.

Limited degree of hydrolysis (DH) between 2-8% is generally desired to promote functional enhancement while minimizing the release of bitter peptides (Sinha et al.,

2007; Matoba and Hata, 1972). Limited and controlled enzymatic hydrolysis can result in a targeted enhancement of protein functionality (Foegeding et al., 2002). Enzymatic modifications widened the use of whey proteins as functional food ingredients. However, the choice of enzyme and hydrolysis conditions is what determines the best application of the WPH produced (i.e. either for enhanced solubility, gelation or emulsification purposes).

A wide range of enzymes and enzymatic conditions, including pH, enzyme-to-substrate ratio and incubation time, have been tested for the production of WPH with enhanced functional and biological properties (Pouliot et al., 2009; Spellman et al., 2009; Rabiey and Britten, 2009 a & b). However, industrial feasibility for many of the successful bench scale production of the functionally enhanced WPH remains to be seen. This is mainly due to the great variability in the results obtained, which originates from the many variables that can influence the characteristics of the WPH produced. Fundamental understanding of the effect of these variables on the final characteristic of the hydrolysates could be addressed by conducting research using multivariate analysis approach, such as response surface methodology (RSM). Using RSM, the number of experimental units necessary to find the optimal point can be tailored depending on the time and resources constraints of the experimenter (Myers et al., 2009).

To amplify and improve the functionality of DLW via enzymatic hydrolysis, hydrolysis conditions need to be optimized. The infinite number of possible combinations of factors and their levels to be tested makes the task quite challenging. Therefore, RSM approach will be pursued to identify the best conditions that will lead to a directed enhancement in several functional properties of DLW, while maintaining a limited DH (2-8%).

2.3 Materials and Methods

2.3.1 Materials

Samples of DLW, WPC34 and WPI were kindly provided by Valley Queen (Milbank, SD, USA), PGP International (Woodland, CA, USA) and Main Street Ingredients (La Crosse, WI, USA), respectively. Bicinchoninic acid (BCA) protein assay kit (23235) was purchased from Pierce (Rockford, IL, USA). Food grade Alcalase 2.4 L and papain (65,600 USP/mg) were provided by Novozymes (Bagsvaerd, Denmark) and American Laboratories Incorporated (Omaha, NE, USA), respectively. Pre-stained broad-range molecular weight (MW) standard (161-0318), Laemmli sample buffer (161-0737), 10X Tris/glycine/SDS running buffer (161-0732), ammonium persulfate (161-0700), 40% Acrylamide/Bis solution, 37.5:1 (2.6% C) (161-0148), and N,N,N',N'-tetra-methyl-ethylenedimine (TEMED) (161-0800) were purchased from BioRad (Hercules, CA, USA). Sudan Red 7B and soybean oil were purchased from Sigma Inc. Other analytical reagent grade chemicals were purchased from Fisher Scientific (Pittsburg, PA, USA) and Sigma-Aldrich (St Louis, MO, USA).

2.3.2 Experimental Design

To order to determine the optimal hydrolysis conditions, a Box-Behnken design with 4 independent variables at 3 levels (Table 3) was generated and implemented. The variables chosen, temperature (x_1), enzyme-to-substrate (e/s) ratio (x_2), time (x_3) and pH (x_4), are known to have the most significant influence on the DH and protein functionality. The design was generated using SAS and had 27 experimental units that included 3 center points to assess the pure error (Tables 4 and 5). The three levels for each independent variable were coded as -1, 0, 1. Each experimental unit was run in triplicate and the means of DH and measured functional properties were recorded as the y responses. The behavior of each response was explained by a quadratic equation (Eq. 2). To evaluate the effect of the independent variables on the response and generate tridimensional response plots, R 2.14.1 was used. The conditions at which the responses were maximal/optimal were then determined. To validate the model, experimental data was obtained using the predicted optimized levels.

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j \quad \text{Eq.2}$$

where y is the dependent variable (DH, EA, EC or ES); β_0, β_i, β_{ii}, and β_{ij} are the linear, quadratic and interactions coefficients estimated by the model; x_i and x_j are the levels of the independent variables.

2.3.3 Protein Analysis

Protein analysis of DLW, WPC 34, WPI and hydrolyzed DLW samples was performed using a Nitrogen analyzer (LECO® TruSpec TM, St. Joseph, MI, USA) according to the AOAC Dumas method (AOAC method 990.03).

Table 3. Natural and coded variable levels used for the hydrolysis of DLW using Alcalase and papain following the RSM approach.

	Coded Levels			Natural Levels					
				Alcalase			Papain		
Temperature (°C), X1	-1	0	1	45	55	65	60	65	70
Enzyme-to-substrate ratio (%, v/w), X2	-1	0	1	0.5	1	1.5	0.5	1	1.5
Time (min), X3	-1	0	1	15	45	75	15	45	75
pH, X4	-1	0	1	6.5	7.5	8.5	6.5	7.5	8.5

2.3.4 Hydrolysis of DLW and Determination of DH Using the pH-STAT Method

Hydrolysis of DLW was done following the pH-STAT method (Adler-Nissens, 1986) using a Mettler Toledo titrator (DL 22 food and beverage analyzer, Mettler Toledo LLC, Columbus, OH, US). Briefly, 25 mL protein solutions (5% protein w/v) of DLW were prepared in phosphate buffer (pH 7, 0.01 M) and placed in a water bath set at the desired temperature with constant stirring. The pH was adjusted using 2 M HCl or 0.5 M NaOH to the final desired pH, and the enzyme was added to the solution at a specific e/s ratio (as per the experimental runs presented in Tables 4 and 5). The pH drop was monitored

and readjusted back to the original value using 0.5 M NaOH. The total volume of NaOH used to keep the pH constant was then recorded and DH was calculated according to Eq. 3.

$$DH = (h/h_{tot}) \times 100 = (B \times N_b)/(MP \times \alpha \times h_{tot}) \quad \text{Eq. 3}$$

Where B is the volume of NaOH (mL); N_b is the normality of the base; MP is the mass of the protein; h_{tot} is the total number of peptide bonds of the protein; α is the degree of dissociation of the α-NH2 groups as determined according the method described by Silva et al. (2010). Please see Appendix B for example calculations.

2.3.5 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein distribution of DLW, WPC 34 and WPI, and hydrolysis pattern of the hydrolyzed DLW samples were monitored following SDS-PAGE under non-reducing conditions as outlined by Laemmli (1970). Each sample was dissolved in DDW (~50 mg protein/mL), and an aliquot (100 µL) was mixed 1:2 (v/v) with Laemmli buffer and boiled for 5 min. Pre-stained broad range MW standard, protein standards, controls and hydrolyzed samples (8 µL; containing ~ 133 µg protein) were loaded onto 18-well hand-cast 20% acrylamide and 4% stacking gels. The gels were electrophoresed at a constant voltage of 200 V for approximately 1 h, then were stained using Coomassie Brilliant Blue (G-250) for another hour followed by de-staining. Molecular Imager Gel Dox^{TM} XR system (Bio-Rad Laboratories, Hercules, CA) was used to scan the gels.

Table 4. The 27 experimental units (runs) performed for DLW hydrolysis using Alcalase following the Box-behnken design.

Run	Coded Levels				Natural Levels			
	X_1	X_2	X_3	X_4	X_1	X_2	X_3	X_4
1	-1	0	-1	0	45	1	15	7.5
2	0	0	0	0	55	1	45	7.5
3	-1	1	0	0	45	1.5	45	7.5
4	1	0	0	1	65	1	45	8.5
5	1	0	-1	0	65	1	15	7.5
6	0	1	0	1	55	1.5	45	8.5
7	0	0	1	1	55	1	75	8.5
8	-1	0	0	-1	45	1	45	6.5
9	1	-1	0	0	65	0.5	45	7.5
10	0	-1	0	1	55	0.5	45	8.5
11	1	0	0	-1	65	1	45	6.5
12	0	1	-1	0	55	1.5	15	7.5
13	-1	0	0	1	45	1	45	8.5
14	0	-1	0	-1	55	0.5	45	6.5
15	0	-1	1	0	55	0.5	75	7.5
16	0	0	0	0	55	1	45	7.5
17	0	0	-1	1	55	1	15	8.5
18	0	1	1	0	55	1.5	75	7.5
19	-1	0	1	0	45	1	75	7.5
20	0	-1	-1	0	55	0.5	15	7.5
21	1	1	0	0	65	1.5	45	7.5
22	0	1	0	-1	55	1.5	45	6.5
23	1	0	1	0	65	1	75	7.5
24	0	0	0	0	55	1	45	7.5
25	0	0	1	-1	55	1	75	6.5
26	-1	-1	0	0	45	0.5	45	7.5
27	0	0	-1	-1	55	1	15	6.5

Table 5. The 27 experimental units (runs) performed for DLW hydrolysis using papain following the Box-behnken design.

Run	Coded Levels				Natural Levels			
	X_1	X_2	X_3	X_4	X_1	X_2	X_3	X_4
1	-1	0	-1	0	60	1	15	7.5
2	0	0	0	0	65	1	45	7.5
3	-1	1	0	0	60	1.5	45	7.5
4	1	0	0	1	70	1	45	8.5
5	1	0	-1	0	70	1	15	7.5
6	0	1	0	1	65	1.5	45	8.5
7	0	0	1	1	65	1	75	8.5
8	-1	0	0	-1	60	1	45	6.5
9	1	-1	0	0	70	0.5	45	7.5
10	0	-1	0	1	65	0.5	45	8.5
11	1	0	0	-1	70	1	45	6.5
12	0	1	-1	0	65	1.5	15	7.5
13	-1	0	0	1	60	1	45	8.5
14	0	-1	0	-1	65	0.5	45	6.5
15	0	-1	1	0	65	0.5	75	7.5
16	0	0	0	0	65	1	45	7.5
17	0	0	-1	1	65	1	15	8.5
18	0	1	1	0	65	1.5	75	7.5
19	-1	0	1	0	60	1	75	7.5
20	0	-1	-1	0	65	0.5	15	7.5
21	1	1	0	0	70	1.5	45	7.5
22	0	1	0	-1	65	1.5	45	6.5
23	1	0	1	0	70	1	75	7.5
24	0	0	0	0	65	1	45	7.5
25	0	0	1	-1	65	1	75	6.5
26	-1	-1	0	0	60	0.5	45	7.5
27	0	0	-1	-1	65	1	15	6.5

2.3.6 Emulsification Capacity (EC)

Emulsification capacity was measured following the method outlined by Rickert et al. (2004) with some modifications. Briefly, sample dispersions (10 mL of a 1% protein solution, w/v) of DLW, WPC 34, WPI and hydrolyzed DLW were prepared in DDW and adjusted to pH 7.0 with 2N HCl or NaOH. Soybean oil dyed with 4 µg/mL of Sudan Red 7B was continuously blended into the dispersion at 37 mL/min flow rate using a homogenizer (IKA® RW 20 Digital, IKA Works, Inc., NC, US) with the stirrer (R 1342 propeller stirrer, 4-bladed) rotating at 830 rpm. This was done until phase inversion was observed. Emulsification capacity was expressed as g oil/g protein.

2.3.7 Emulsification Activity (EA) and Stability (ES)

The ES index and EA were measured according to the methods outlined by Tang et al. (2003) and Chove et al. (2001), with modifications. An aliquot (6 mL) of protein solutions (0.1% protein w/v), prepared in DDW, was added to 2 mL of soybean oil and homogenized using a homogenizer (Power Gen 700, Fisher Scientific, Pittsburg, PA, US) set at the least rotational speed (~ 10,000 rpm) of the instrument for 2 min. Right after the homogenization, 50 µL of the emulsion (pipetted from the bottom of the beaker) was diluted in 5 mL of 0.1% SDS, vortexed for 5 sec, and absorbance measured at 500 nm using a spectophotometer (Beckman DU 640 B, Beckman Coulter, Inc., CA, US). The addition of 0.1% SDS was to avoid creaming from occurring. At t=0, the absorbance value was recorded as the EA. Another measurement was taken at t=10 min after which the ES was determined using Eq. 4.

$$ES = (A_0/A_0\text{-}A_{10}) \times 10. \quad \text{Eq. 4}$$

Where A_0 is the absorbance at t=0, and A_{10} the absorbance at t=10min.

2.3.8 Heat-induced Gelation

Heat-induced gelation was monitored following the method outlined by Kong et al. (2008), with some modification. Protein solutions (10% protein, w/v) were prepared in DDW and transferred to a mold (1.2 cm x 3 cm). The mold was filled to three quarters, heated at 95 °C for 30 min in a water bath, and cooled to room temperature (~ 23 °C) on an ice bath. The gel formed was compressed using a Texture Analyzer (TA-TX Plus, Stable Micro Systems LTD, Surrey, UK) until ruptured at room temperature, and the force was recorded. The probe used was 10 mm in diameter and the speed was set at 1 mm/s.

2.3.9 Water holding capacity (WHC)

Measurement of WHC was conducted as outlined by Sternberg et al. (1976), with some modifications. Protein solutions (10% protein, w/v) were prepared in DDW and adjusted to pH 7 using 2 M HCl or NaOH. One mL of each prepared solution was pipetted into a microcentrifuge tube and the weight was recorded (T_1). The samples were then heated at 100 °C for 10 min. After heating the tubes were weighed (T_2), inverted for 10 min, and weighed again (T3). The WHC was determined using Eq. 5.

Eq. 5

$$WHC = 100 \times [(T_3 - T_1)/(T_2 - T_1)]$$

2.3.10 Statistical analysis

Several response surface models, first order, first order with two-way interactions, and second order, were compared to determine the best fit. Polynomial regression equations were generated and used to construct RSM plots. The point optimization method was used to optimize each variable level for maximum response. Experimental data obtained using the predicted optimized levels were analyzed using analysis of variance (ANOVA) utilizing XLSTAT for Windows (2010). Significant ($P \leq 0.05$) differences between the means were determined using Tukey-Kramer multiple means comparison test.

2.4 Results and Discussion

2.4.1 Protein Content and Profile

The protein content of DLW and hydrolysates ranged between ~ 20-23 %, and was, as anticipated, less than that of WPC 34 and WPI (Table 6). Protein profiling revealed the presence of the two major whey proteins, β-lactoglobulin and α-lactalbumin (β-lg and α-la) among other whey proteins and glycomacropeptides in DLW, WPI and WPC (Figures 12 and 13, Appendix A). After hydrolysis of DLW using either Alcalase or papain under the various hydrolysis conditions, major protein bands faded or disappeared, and new peptide bands with molecular weight less than 6 kDa were visualized. Some qualitative differences in the intensity of the intact protein and the released peptides can be visualized among the different DLW hydrolysates. This observation is in line with the observed differences in DH, as will be discussed in the following sections.

Table 6. Protein content of DLW, hydrolyzed DLW, WPI and WPC34.

Sample	**Protein content (%)**
DLW	22.49
Alcalase-hydrolyzed DLW (runs 1 to 27)	20.99 – 23.68
Papain-hydrolyzed DLW (runs 1 to 27)	20.65 – 23.26
WPI	90.02
WPC34	34.00*

* As provided on the specification sheet of the product.

2.4.2 Response Surface Modeling

Several response surface models were compared to determine the best fit for the RSM data collected (Tables 7 and 8). First order, first order with two-way interactions, and second order were the models tested (Tables 9 and 10). When the most complex model didn't fit significantly better at $P \leq 0.05$, the next simplest one was chosen for further analysis. The point within the range of experimentation with the highest fitted value was

determined. Using the model with the best fit (high R^2 and $P \leq 0.05$) for each response, prediction equations were generated (Table 11). For EC, Log (ES) and EA of Alcalase hydrolysates, the first order with two-way interactions models were selected, while for DH second order model was selected (Tables 9 and 11). For EC, Log (ES), EA and DH of papain hydrolysates, second order, first order, first order with two-way interactions, and second order models were selected, respectively (Tables 10 and 11). The predicted response obtained when using the generated equations, were close to the actual responses, more so for the DH and EA (Tables 7 and 8).

2.4.3. Effect of the Hydrolysis Parameters on the DH of DLW

Using Alcalase and papain, the experimental DH values ranged from 3.02% to 16.2% and 2.39% to 15.4%, respectively (Tables 7 and 8). When using Alcalase, temperature and time appeared to be the major driving factors (Figure 3, see DH quadratic equation when using Alcalase Table 11, and Table 20 in Appendix C). As incubation temperature and time increased, DH increased. Maximum DH was obtained at the highest temperature and longest incubation time. The optimum temperature for Alcalase, per the specification sheet and reported literature (See and Babji, 2011), is between 55-70 °C. Thus, it is anticipated that higher DH values can be reached beyond the range of experimentation. Based on the results obtained within the range of experimentation, Alcalase had the highest activity at 65 °C. At this temperature there was a constant increase in DH as time increased. When hydrolyzing a protein, the number of cleaved peptide bonds increases as a function of time.

Table 7. Actual (A) and predicted (P) responses for the 27 DLW hydrolysates obtained using Alcalase.

Run	Coded Variables*				EC (g oil / g protein)		ES (min)		EA		DH (%)	
	X_1	X_2	X_3	X_4	A	P	A	P	A	P	A	P
1	-1	0	-1	0	1586	1451	28.9	28.8	0.58	0.60	3.02	2.02
2	0	0	0	0	2006	1731	53.9	41.7	0.49	0.49	8.02	7.94
3	-1	1	0	0	1994	1847	37.7	34.7	0.70	0.66	6.57	6.57
4	1	0	0	1	1587	1428	79.3	95.5	0.32	0.29	9.18	9.06
5	1	0	-1	0	2027	1989	39.8	33.1	0.54	0.53	5.88	5.45
6	0	1	0	1	1425	1637	143	118	0.26	0.32	11.7	11.09
7	0	0	1	1	1848	1690	63.3	91.2	0.35	0.30	10.6	9.80
8	-1	0	0	-1	1309	1579	21.8	20.4	0.51	0.58	6.75	7.38
9	1	-1	0	0	1612	1883	35.2	36.3	0.45	0.50	7.32	7.22
10	0	-1	0	1	1872	1798	36.9	33.1	0.56	0.60	6.66	6.48
11	1	0	0	-1	1897	1911	33.7	36.3	0.41	0.45	14	13.99
12	0	1	-1	0	1874	1927	28.7	37.2	0.55	0.60	6.39	6.30
13	-1	0	0	1	1911	2007	38.4	40.7	0.64	0.64	6.4	6.91
14	0	-1	0	-1	2110	1824	22	26.3	0.48	0.46	9.92	10.06
15	0	-1	1	0	1998	2109	39.4	34.7	0.53	0.53	7.51	8.12
16	0	0	0	0	1767	1731	47	41.7	0.49	0.49	7.68	7.94
17	0	0	-1	1	1678	1745	39.6	42.7	0.7	0.63	5.53	6.68
18	0	1	1	0	1290	1376	84.8	87.1	0.28	0.29	11.2	12.08
19	-1	0	1	0	2079	2135	30	28.8	0.59	0.62	6.63	6.63
20	0	-1	-1	0	1435	1513	23	25.7	0.49	0.53	3.11	2.78
21	1	1	0	0	1444	1456	96.7	95.5	0.22	0.24	11.2	11.29
22	0	1	0	-1	1667	1666	26.1	28.2	0.57	0.57	13.2	12.91
23	1	0	1	0	1197	1350	134	105	0.20	0.21	11.4	11.97
24	0	0	0	0	2000	1731	42.8	41.7	0.54	0.49	8.14	7.94
25	0	0	1	-1	1912	1795	27.6	33.1	0.59	0.53	16.2	14.94
26	-1	-1	0	0	1626	1739	24.5	24.0	0.57	0.56	3.33	3.18
27	0	0	-1	-1	1588	1695	24.8	22.4	0.57	0.50	6.23	6.94

* Refer to Table 3 for natural variable levels.

Table 8. Actual (A) and predicted (P) responses for the 27 DLW hydrolysates obtained using papain.

Run	Coded Variables*				EC (g oil / g protein)		ES (min)		EA		DH (%)	
	X_1	X_2	X_3	X_4	A	P	A	P	A	P	A	P
1	-1	0	-1	0	1850	1832	31.9	38.0	0.51	0.54	4.39	3.41
2	0	0	0	0	1941	2002	44	49.0	0.41	0.39	6.88	5.72
3	-1	1	0	0	1861	1940	81.6	67.6	0.34	0.34	6.88	6.86
4	1	0	0	1	2000	2017	51.3	47.9	0.47	0.48	8.81	7.01
5	1	0	-1	0	1953	1928	42.7	51.3	0.44	0.42	4.96	4.95
6	0	1	0	1	1918	1958	43.9	56.2	0.47	0.50	7.06	7.31
7	0	0	1	1	1956	2062	38.5	45.7	0.63	0.57	6.73	6.53
8	-1	0	0	-1	1979	1909	29.3	33.9	0.49	0.46	10.2	11.47
9	1	-1	0	0	1861	1933	43	39.8	0.41	0.40	4.18	4.18
10	0	-1	0	1	1823	1647	37.7	42.7	0.55	0.58	3.03	4.03
11	1	0	0	-1	1981	1875	58.5	67.6	0.25	0.26	12.4	12.39
12	0	1	-1	0	1908	1796	52.4	53.7	0.49	0.45	6.29	6.29
13	-1	0	0	1	1826	1879	56	51.3	0.59	0.55	4.63	4.15
14	0	-1	0	-1	1979	1842	26.2	24.5	0.57	0.55	8.85	9.10
15	0	-1	1	0	1696	1754	29.1	28.8	0.52	0.55	4.96	4.48
16	0	0	0	0	2062	2002	60.6	49.0	0.38	0.39	4.88	5.72
17	0	0	-1	1	1821	1780	59.6	53.7	0.58	0.58	3.92	5.15
18	0	1	1	0	1954	1749	101	102.3	0.23	0.25	9.64	9.57
19	-1	0	1	0	1965	1893	50.2	46.8	0.41	0.45	5.30	5.81
20	0	-1	-1	0	1519	1672	35.9	36.3	0.59	0.56	2.67	2.25
21	1	1	0	0	1539	1659	99.1	81.3	0.23	0.24	8.15	8.49
22	0	1	0	-1	1571	1650	91.4	95.5	0.25	0.23	15.4	14.94
23	1	0	1	0	1980	1901	66.0	63.1	0.32	0.30	6.57	8.05
24	0	0	0	0	2003	2002	62.4	49.0	0.39	0.39	5.4	5.72
25	0	0	1	-1	1550	1741	56.7	64.6	0.33	0.32	15.5	14.24
26	-1	-1	0	0	1518	1548	27.7	26.3	0.59	0.58	2.39	2.03
27	0	0	-1	-1	1945	1988	41	36.3	0.47	0.53	9.95	10.13

* Refer to Table 3 for natural variable levels.

Table 9. ANOVA for the various responses using Alcalase RSM data.

Response	Model	DF[d]	SS[e]	MS[f]	F	p-value	Lack of Fit F	Lack of Fit p-value	R^2
EC	FO[a]	4	126169	31542	0.79	0.55	4.39	0.20	0.07
	FO + 2WI[b]	6	1052276	175379	4.39	0.01	2.25	0.35	0.65
	SO[c]	4	147521	36880	0.92	0.48	2.36	0.33	0.73
	Residuals	12	478908	39909					
	Lack of fit	10	441558	44156					
	Pure error	2	37351	18675					
Log (ES)	FO	4	1.10	0.28	36.86	0.00	5.52	0.16	0.79
	FO + 2WI	6	0.17	0.03	3.82	0.02	3.10	0.27	0.92
	SO	4	0.03	0.01	0.88	0.50	3.31	0.25	0.94
	Residuals	12	0.09	0.01					
	Lack of fit	10	0.08	0.01					
	Pure error	2	0.01	0.00					
EA	FO	4	0.27	0.07	24.45	0.00	12.31	0.08	0.56
	FO + 2WI	6	0.17	0.03	10.37	0.00	3.47	0.25	0.91
	SO	4	0.01	0.00	0.97	0.46	3.61	0.24	0.93
	Residuals	12	0.03	0.00					
	Lack of fit	10	0.03	0.00					
	Pure error	2	0.00	0.00					
DH	FO	4	214.19	53.55	81.03	0.00	58.93	0.02	0.76
	FO + 2WI	6	12.80	2.13	3.23	0.04	68.34	0.01	0.80
	SO	4	47.38	11.85	17.92	0.00	13.55	0.07	0.97
	Residuals	12	7.93	0.66					
	Lack of fit	10	7.82	0.78					
	Pure error	2	0.12	0.06					

[a] FO: first order; [b] FO + 2WI: first order plus two-way interaction; [c] SO: second order; [d] DF: degrees of freedom; [e] SS: sum of squares; [f] MS: mean sum of squares.

Table 10. ANOVA for the various responses using papain RSM data.

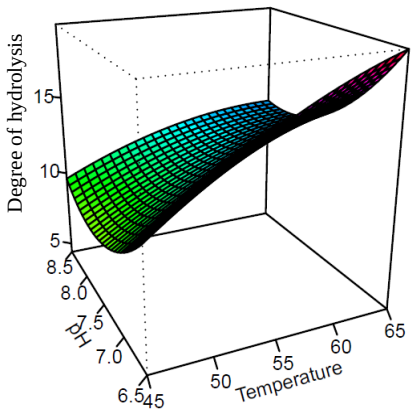
Response	Model	DF[d]	SS[e]	MS[f]	F	p-value	Lack of Fit F	Lack of Fit p-value	R^2
EC	FO[a]	4	29193	7298	0.34	0.84	9.52	0.10	0.04
	FO + 2WI[b]	6	258088	43015	2.02	0.14	8.60	0.11	0.39
	SO[c]	4	196281	49070	2.30	0.12	6.73	0.14	0.65
	Residuals	12	256026	21335					
	Lack of fit	10	248639	24864					
	Pure error	2	7387	3694					
Log (ES)	FO	4	0.46	0.11	18.09	0.00	1.62	0.45	0.65
	FO + 2WI	6	0.15	0.03	3.97	0.02	0.80	0.68	0.87
	SO	4	0.02	0.00	0.69	0.61	0.87	0.64	0.89
	Residuals	12	0.08	0.01					
	Lack of fit	10	0.06	0.01					
	Pure error	2	0.01	0.01					
EA	FO	4	0.28	0.07	45.27	0.00	18.63	0.05	0.75
	FO + 2WI	6	0.04	0.01	4.16	0.02	15.47	0.06	0.85
	SO	4	0.04	0.01	5.74	0.01	7.30	0.13	0.95
	Residuals	12	0.02	0.00					
	Lack of fit	10	0.02	0.00					
	Pure error	2	0.00	0.00					
DH	FO	4	216.73	54.18	40.85	0.00	4.29	0.21	0.70
	FO + 2WI	6	4.90	0.82	0.62	0.71	5.80	0.16	0.71
	SO	4	73.07	18.27	13.77	0.00	1.29	0.51	0.95
	Residuals	12	15.91	1.33					
	Lack of fit	10	13.78	1.38					
	Pure error	2	2.14	1.07					

[a] FO: first order; [b] FO + 2WI: first order plus two-way interaction; [c] SO: second order; [d] DF: degrees of freedom; [e] SS: sum of squares; [f] MS: mean sum of squares.

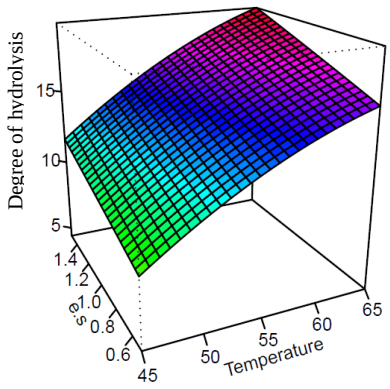
Table 11. Equations derived using RSM models with the best fit for the prediction of the dependent variables (Y_1-Y_8), with temperature as X_1, enzyme-to-substrate (e/s) ratio as X_2, time as X_3 and pH as X_4.

Enzyme	Response	Quadratic Polynomial Equations	R^2	P-value
Alcalase	**EC** (FO[a] + 2WI[b])	$Y_1=1731.17 - 61.79X_1 - 79.91X_2 + 11.23X_3 - 13.60X_4 - 133.62X_1X_2 - 330.89X_1X_3 - 228.03X_1X_4 - 286.69X_2X_3 - 1.05\ X_2X_4 - 38.51\ X_3X_4$	0.65	0.01
	Log (ES) (FO + 2WI)	$Y_2=1.62 + 0.15X_1 + 0.14X_2 + 0.12X_3 + 0.18X_4 + 0.06X_1X_2 + 0.13X_1X_3 + 0.03X_1X_4 + 0.06X_2X_3 + 0.13X_2X_4 + 0.04X_3X_4$	0.92	0.02
	EA (FO + 2WI)	$Y_3=0.49 - 0.12X_1 - 0.04X_2 - 0.07X_3 - 0.02X_4 - 0.09X_1X_2 - 0.09X_1X_3 - 0.06X_1X_4 - 0.08X_2X_3 - 0.10X_2X_4 - 0.09X_3X_4$	0.91	0.00
	DH (SO[c])	$Y_4=7.94 + 2.19X_1 + 1.87X_2 + 2.78X_3 - 1.35X_4 + 0.17X_1X_2 - 0.48X_1X_3 - 1.12X_1X_4 - 0.11X_2X_3 + 0.44X_2X_4 - 1.22X_3X_4 - 0.84X_1^2 - 0.04X_1^2 - 0.59X_1^2 + 2.23X_1^2$	0.97	0.00
Papain	**EC** (SO)	$Y_5=2001.93 + 25.97X_1 + 29.69X_2 + 8.70X_3 + 28.30X_4 - 166.7X_1X_2 - 22.08X_1X_3 + 43.06X_1X_4 - 32.53X_2X_3 + 125.7X_2X_4 + 132.4X_3X_4 - 43.22X_1^2 - 188.66X_2^2 - 70,37X_3^2 - 38.75X_4^2$	0.65	0.12
	Log (ES) (FO + 2WI)	$Y_6=1.69 + 0.07X_1 + 0.18X_2 + 0.04X_3 + 0.005X_4 - 0.03X_1X_2 - 0.002X_1X_3 - 0.08X_1X_4 + 0.09X_2X_3 - 0.12X_2X_4 - 0.08X_3X_4$	0.87	0.02
	EA (SO)	$Y_7=0.39 - 0.07X_1 - 0.10X_2 - 0.05X_3 + 0.08X_4 + 0.02X_1X_2 - 0.005X_1X_3 + 0.03X_1X_4 - 0.05X_2X_3 + 0.06X_2X_4 + 0.05X_3X_4 - 0.01X_1^2 - 0.01X_2^2 + 0.05X_3^2 + 0.06X_4^2$	0.95	0.01
	DH (SO)	$Y_8=5.72 + 0.94X_1 + 2.28X_2 + 1.37X_3 - 3.17X_4 - 0.13X_1X_2 + 0.17X_1X_3 + 0.48X_1X_4 + 0.26X_2X_3 - 0.64X_2X_4 - 0.68X_3X_4 - 0.21X_1^2 - 0.12X_1^2 + 0.05X_1^2 + 3.25X_1^2$	0.95	0.00

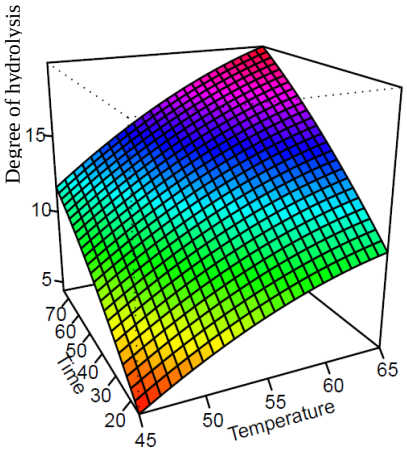
[a] FO: first order; [b] FO + 2WI: first order plus two-way interaction; [c] SO: second order



Slice at e.s=1.5, time=75



Slice at pH=6.5, time=75



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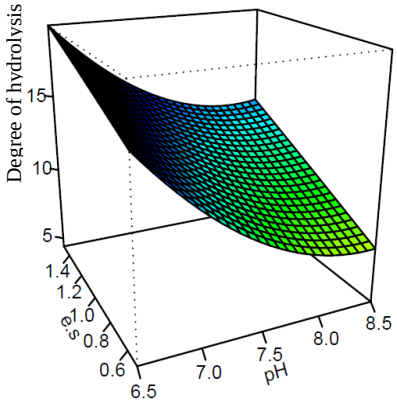
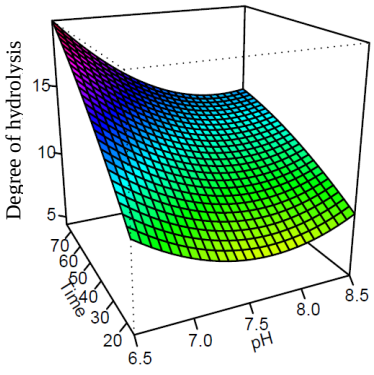
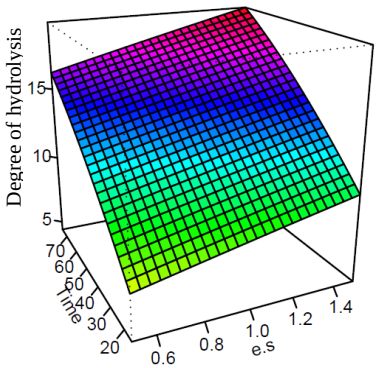


Slice at temperature=65, time=75



Slice at temperature=65, e.s=1.5



Slice at temperature=65, pH=6.5

Figure 3. RSM plots for the effect of temperature, enzyme-to-substrate ratio (e.s), time and pH on DH when Alcalase was used. Each plot shows the effect of two variables while keeping the other two constant at a level that elicits the maximum response.

Within the parameters of the hydrolysis conditions tested using Alcalase, as e/s ratio increased DH increased (Figure 3). The substrate concentration used was kept at levels exceeding the K_m of the enzyme, maintaining a zero order reaction with respect to the substrate. Thus, with an increase in e/s ratio, higher enzyme activity was achieved, as demonstrated by the observed increase in DH. The DH response to pH change, on the other hand, was mostly a saddle response, with an overall negative correlation, as shown in the quadratic equation (Table 11). Under most of the conditions tested, minimum DH was observed when the pH approached 7.5.

When using papain, pH and e/s ratio were the most driving factors (Figure 4, see also DH quadratic equation when using papain in Table 11, and Table 24 in Appendix D). The quadratic equation showed an overall negative correlation between the DH and pH (Table 11). The highest DH was obtained at the lowest pH level tested. The optimal pH range of papain is from 5.0 to 7.0 (Whitehurst and Oort, 2009). Thus, a higher DH values can be reached at pH lower than 6.5, which is beyond the range of experimentation. Although when using papain, e/s had a higher impact than when using Alcalase, the noted trend in the DH response was similar.

The effect of temperature on DH when using papain was not as significant as when using Alcalase. This is mostly attributed to the fact that the range of temperature tested falls within the actual optimum temperature for papain (See and Babji, 2011). Finally, and as expected, DH increased as incubation time increased, within the range tested.

2.4.4 Effect of the Hydrolysis Parameters on the Emulsification Properties of DLW

2.4.4.1 Effect on Emulsification Capacity

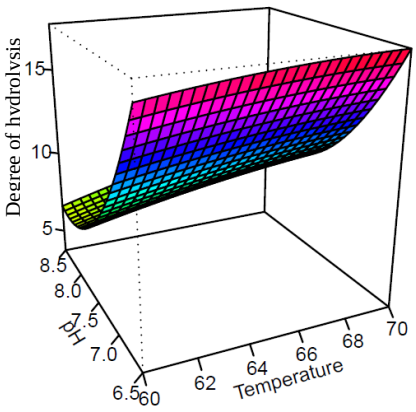
Of the four hydrolysis parameters, when Alcalase was used, temperature and e/s ratio had the greatest effect on EC (refer to the quadratic equation for EC using Alcalase in Table 11, and Table C in Appendix 17). And the most pronounced interaction was between temperature and time. At a constant pH (8.5) and e/s ratio (0.5), The highest incubation temperature and longest time resulted in minimum EC (Figure 5). Also, at the

highest pH (8.5) and the longest time (75 min), the EC decreased as e/s ratio and temperature increased. On the other hand, EC increased as hydrolysis temperature decreased, reaching maximum levels at the lowest temperature, lowest e/s ratio, longest time and highest pH.

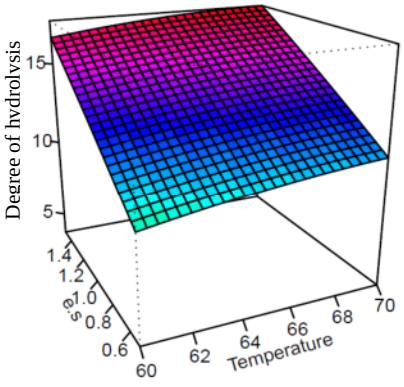
When papain was used, none of the individual variables had a significant effect on EC, however the interaction effect of temperature and e/s, and that of time and pH were significant (Table 21 in Appendix D). The second order model, which was significant only when $P \leq 0.12$, was selected to generate the quadratic equation since it had the highest R and lowest *P*-value among the tested models. As incubation pH increased EC increased, reaching a high values at pH 8.5 when the temperature was in the low 60s °C, e/s between 1-1.5%, and when time was at the highest level (Figure 6).

The conditions that elicited high EC, when using either Alcalase or papain, corresponded to a moderate DH, 5-10%, while the conditions that elicited low EC, corresponded to a relatively high DH, 10-17 %. For example, at pH 8.5, 60-65 °C, 70-75 min and e/s ratio of 1-1.5 %, when papain was used, the DH was 5-10% (Figure 4), while EC was at maximum (Figure 6). While, at pH 6.5, at the highest temperature, and at the highest level for time and e/s ratio, DH was greater than 15% (Figure 4) and EC was at a minimum (Figure 6). Similarly, when Alcalase enzyme was used, at the highest temperature and time levels and lowest pH and e/s ratio, DH was > 15% (Figure 3) and EC was in the mid range (~ 1,700 g oil per/1 g protein) (Figure 5). The EC was ~ 2,200 g oil per/1 g protein and DH was less than 10% at the lowest temperature and highest e/s ratio time and pH levels (Figures 3 & 5). Thus, higher DH is detrimental to the EC.

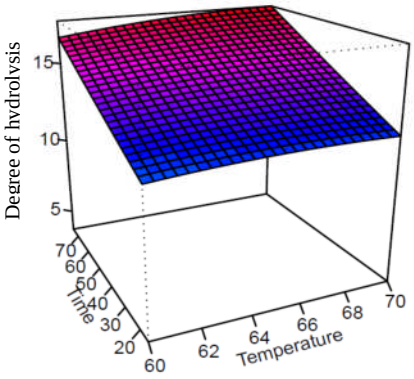
A positive correlation exists between emulsification properties and peptide chain length. It was suggested that a peptide with a minimum length of 20 residues possesses good emulsifying and interfacial properties (Gauthier et al, 1993). Higher DH indicates an elevated levels of short-chain peptides, and hence a reduced EC. In general, desired functionalities are obtained by controlling the DH. A moderate DH imparts increased molecular flexibility to the globular whey protein, reduced average molecular weight, increased number of charged groups and increased surface hydrophobicity (Kitts and Weiler, 2003), allowing faster movement and rearrangements at the interface.



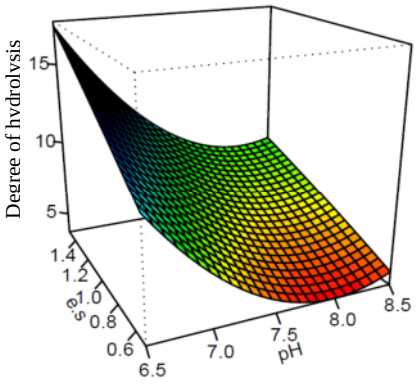
Slice at e.s=1.5, time=75



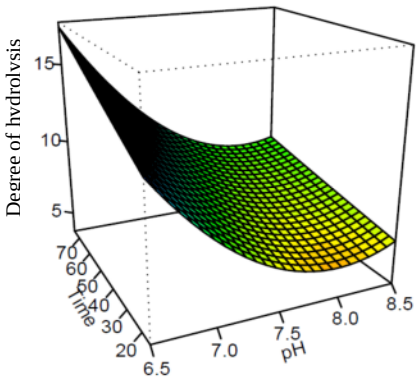
Slice at pH=6.5, time=75



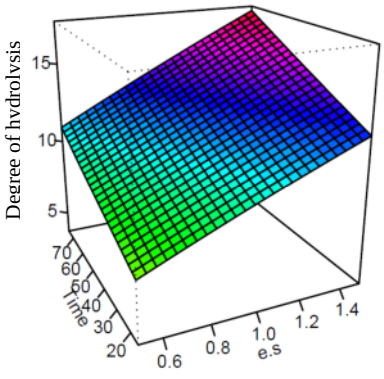
Slice at pH=6.5, e.s=1.5



Slice at temperature=70, time=75

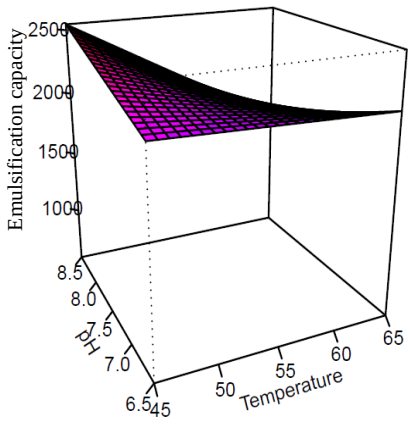


Slice at temperature=70, e.s=1.5

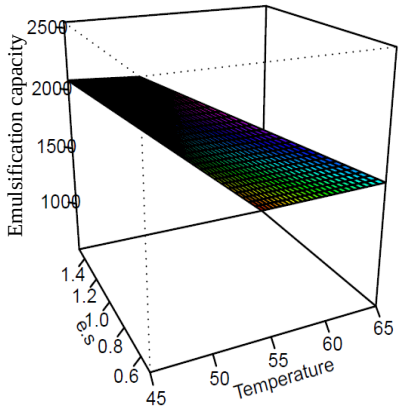


Slice at temperature=70, pH=6.5

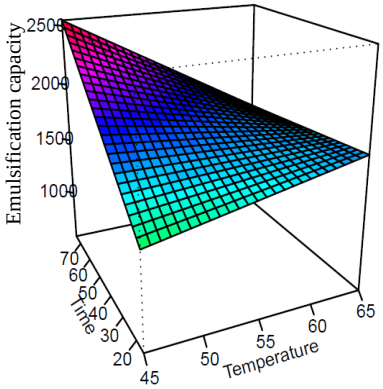
Figure 4. RSM plots for the effect of temperature, enzyme-to-substrate ratio (e.s), time and pH on DH when papain was used. Each plot shows the effect of two variables while keeping the other two constant at a level that elicits the maximum response.



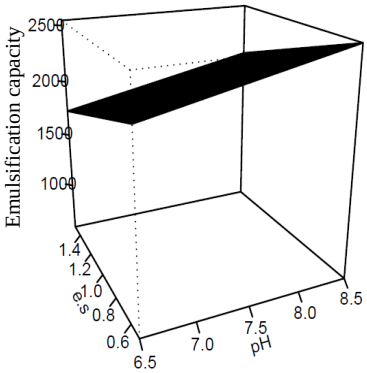
Slice at e.s=0.5, time=75



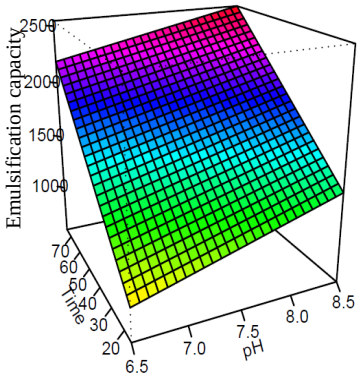
Slice at pH=8.5, time=75



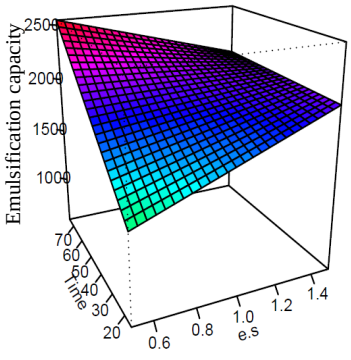
Slice at pH=8.5, e.s=0.5



Slice at temperature=45, time=75

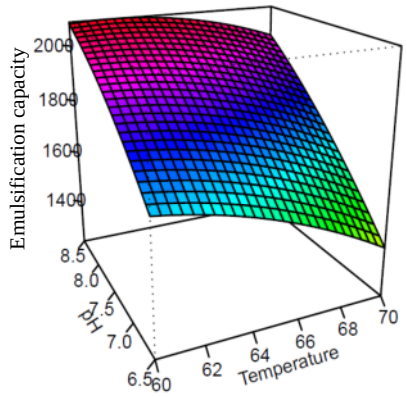


Slice at temperature=45, e.s=0.5

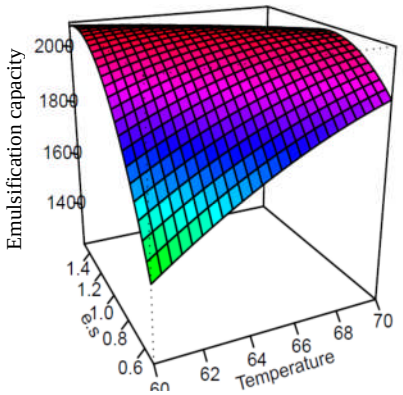


Slice at temperature=45, pH=8.5

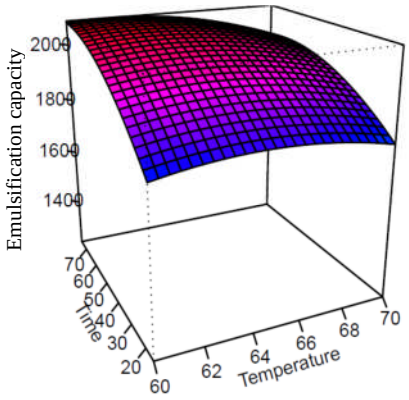
Figure 5. RSM plots for the effect of temperature, enzyme-to-substrate ratio (e.s), time and pH on the emulsification capacity when Alcalase was used. Each plot shows the effect of two variables while keeping the other two constant at a level that elicits the maximum response.



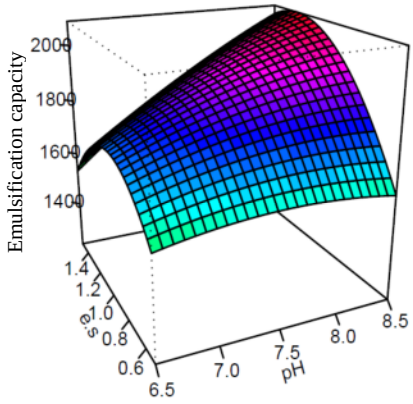
Slice at e.s =1.3, Time = 75



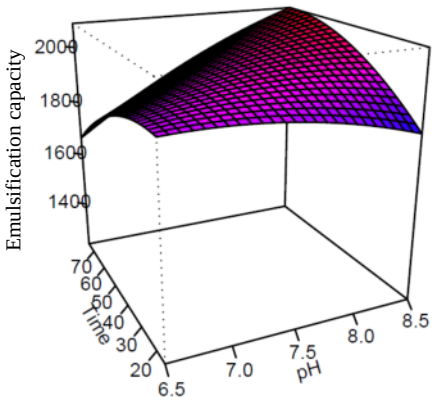
Slice at pH =8.5, time =75



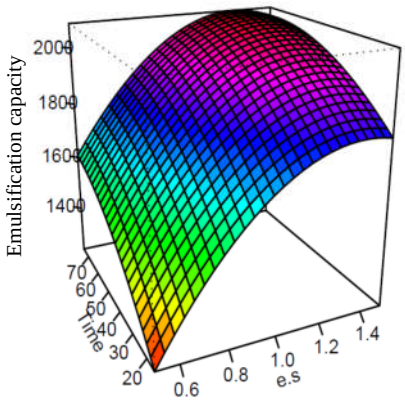
Slice at e.s =1.3, pH=8.5



Slice at temperature=62, time=75



Slice at temperature=62, e.s=1.3



Slice at Temperature =62, pH = 8.5

Figure 6. RSM plots for the effect of temperature, enzyme-to-substrate ratio (e.s), time and pH on the emulsification capacity when papain was used. Each plot shows the effect of two variables while keeping the other two constant at a level that elicits the maximum response.

2.4.4.2 Effect on Emulsification Stability and Activity

When Alcalase was used all of the tested variables and interactions were positively correlated with the ES response (after converting the data to log values). However, when papain was used some of the interactions were negatively correlated with the ES response (refer to the quadratic equations for ES in Table 11) (Also see Tables 18 and 22 in Appendix D). Within the experimental range tested, a maximum value can not be predicted since the ES seemed to continue increasing with the increase in the levels of the independent variables (Figures 7 and 8). Therefore, the true optimal point was probably not within the range tested since none of the plots showed an inflection or a saddle point.

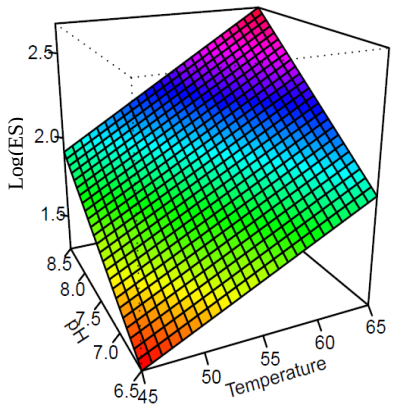
The effect of DH on the ES response at the various conditions tested is not as apparent as that on EC. A Pearson correlation test was performed, using both Alcalase and papain data (a total of 54 observations), to determine the significance of the association. A positive correlation (R= 0.368) was found between the DH and ES, indicating that the ES response tends to increase as DH increases. Although the R value is relatively low, it is statistically significant ($P = 0.006$).

The DLW hydrolysates that had really high ES had very low EA (Tables 7 and 8). At low EA, coalescence occured almost immediately after homogeneization, leading to abnormaly high ES values. For most of the highly hydrolyzed samples, coalescence caused the denominator ($A_0 - A_{10}$) in Eq.4 to have very low values as a result of A_{10} being too close to A_0, thus giving an erraneous high ES value. Hence, more focus was given to the EA response.

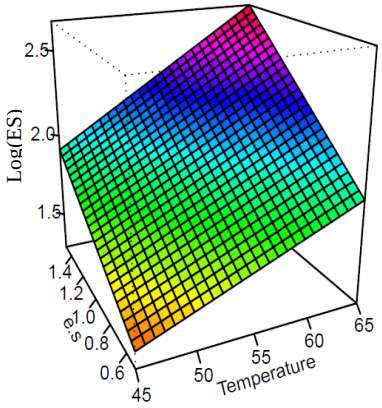
When Alcalase was used, all of the tested variables and interactions were negatively correlated with the EA response. However, when papain was used, the pH and some of the interactions were positively correlated with the EA response (refer to the quadratic equations for EA in Table 11) (Also see Tables 19 and 23 in Appendix D). The effect of pH on EA was not statistically significant when Alcalase was used. The effect of hydrolysis time on EA, however, was dependent on the pH and temperature. Emulsification activity approached maximum at lower temperature and shorter time (Figure 9). When papain was used, the EA seemed to increase as pH moved away from

7.5 (Figure 10). Similar to when Alcalase was used, EA approached maximum at lower temperature and time. However, at higher pH (8.5), as time increased EA increased when temperature was held at 60 °C.

Similar to what was observed of EC, EA was improved upon limited hydrolysis by both enzymes. When Alcalase was used, the EA of the hydrolysates with relatively high DH was lower than that of the non-hydrolyzed DLW. Non-hydrolyzed delactosed whey had an EA value of 0.365, however the hydrolysates of runs 6 and 18 had EA values of 0.260 and 0.280, and a DH of 11.7% and 11.2%, respectively (Table 7). At the highest pH 8.5, e/s ratio temperature and time, the DH was between 10-15% (Figure 3), which corresponded to very low EA (Figure 9). Maximal EA responses where observed at the lowest temperature and time combinations (Figure 9), which corresponded to low to moderate DH values of 2-8%, (Table 7 and Figure 3). Similarly, when papain enzyme was used, conditions that elicited a relatively high DH % resulted in reduced EA, and visa versa (Table 8, Figures 4 and 10). When running a Pearson correlation test, a negative correlation (R= - 0.481) was found to be significant at $P < 0.001$. While at moderate DH the hydrophobic/hydrophylic balance was most likely improved, at high DH it was distrupted. At lower/moderate DH, moderate size peptides are generated that are more likely to contain both hydrophobic and hydrophilic moieties. These peptides can rearrange at the interface more easily than the globular protein (Gauthier et al., 1993). Characterization and profiling of the peptides produced using either Alcalase or papain, would further explain the observed effect of DH on the EA of hydrolyzed DLW.



Slice at e/s=1.5, time=75



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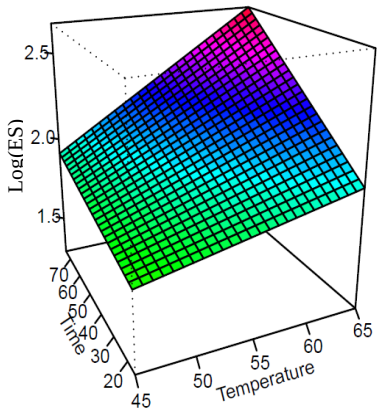

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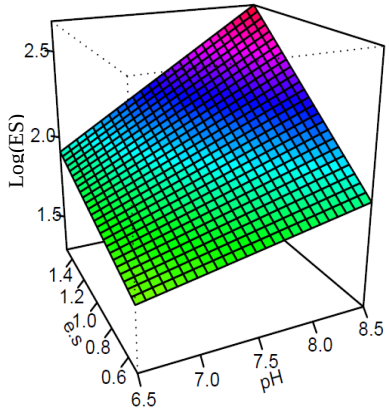
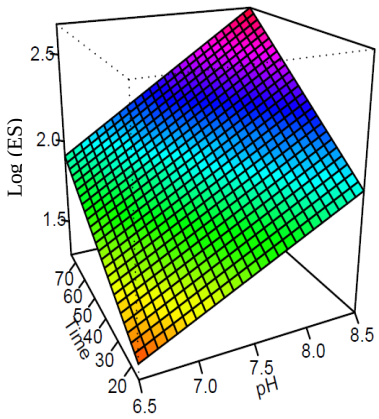


Slice at temperature=65, time=75



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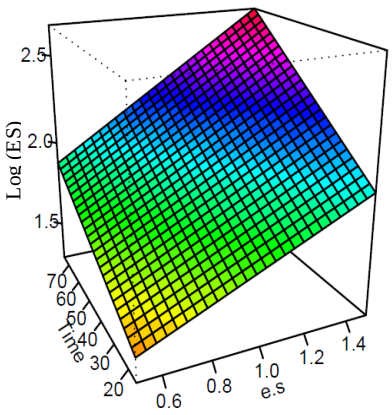
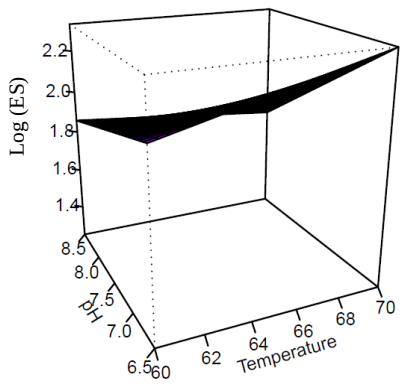
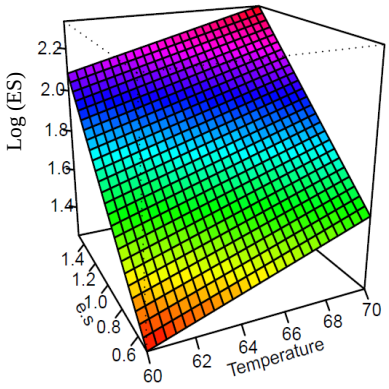


Slice at temperature=65, pH=8.5

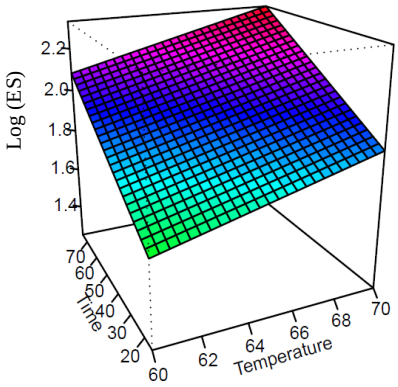
Figure 7. RSM plots for the effect of temperature, enzyme-to-substrate ratio (e.s), time and pH on the Log (ES, emulsification stability) when Alcalase was used. Each plot shows the effect of two variables while keeping the other two constant at a level that elicits the maximum response.



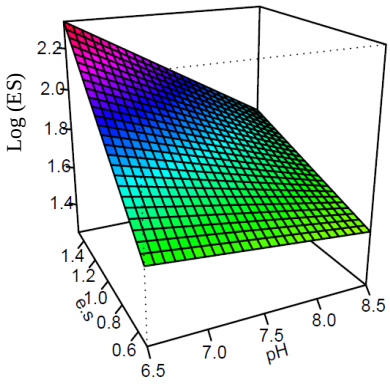
Slice at e.s=1.5, time=75



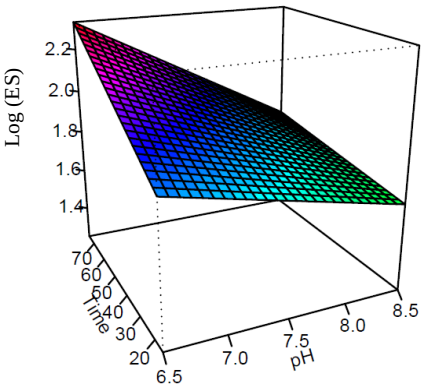
Slice at pH=6.5, time=75



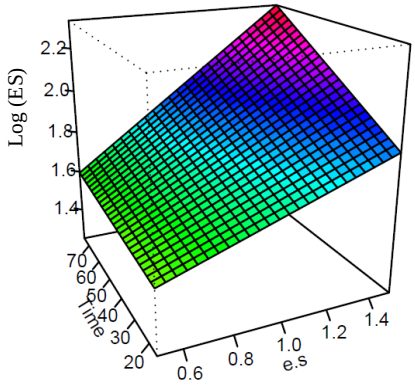
Slice at pH=6.5, e.s=1.5



Slice at temperature=70, time=75

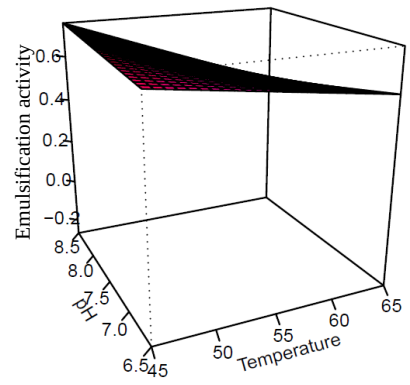


Slice at temperature=70, e.s=1.5

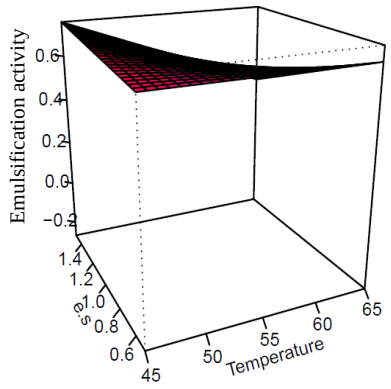


Slice at temperature=70, pH=6.5

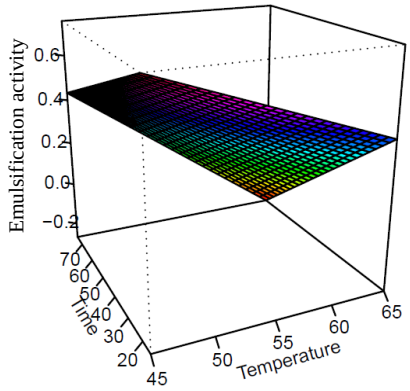
Figure 8. RSM plots for the effect of temperature, enzyme-to-substrate ratio (e.s), time and pH on the Log (ES, emulsification stability) when papain was used. Each plot shows the effect of two variables while keeping the other two constant at a level that elicits the maximum response.



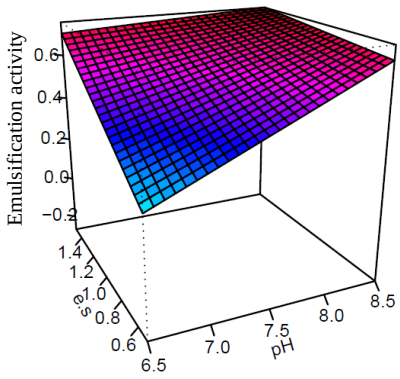
Slice at e/s=1.5, time=15



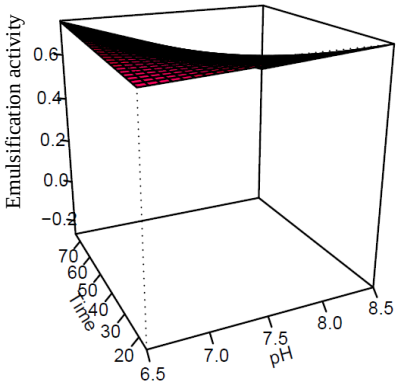
Slice at pH=8.5, time=15



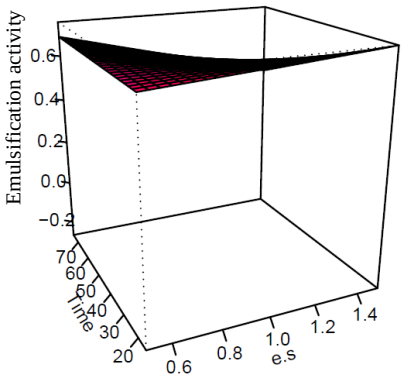
Slice at pH=8.5, e/s=1.5



Slice at temperature=45, time=15

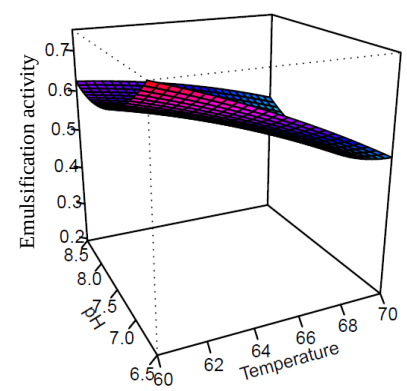


Slice at temperature=45, e/s=1.5

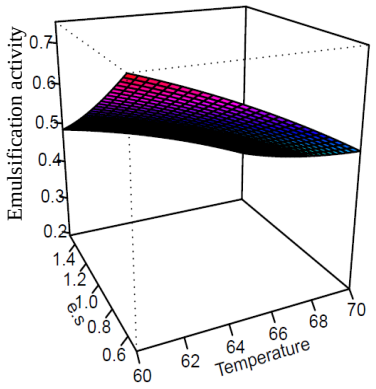


Slice at temperature=45, pH=8.5

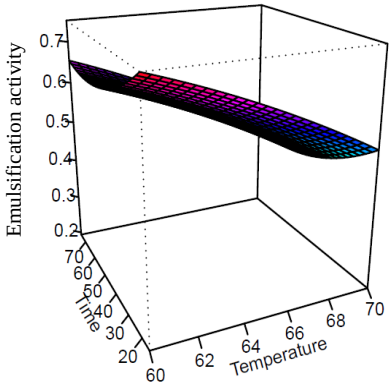
Figure 9. RSM plots for the effect of temperature, enzyme-to-substrate ratio (e.s), time and pH on the emulsification activity when Alcalase was used. Each plot shows the effect of two variables while keeping the other two constant at a level that elicits the maximum response.



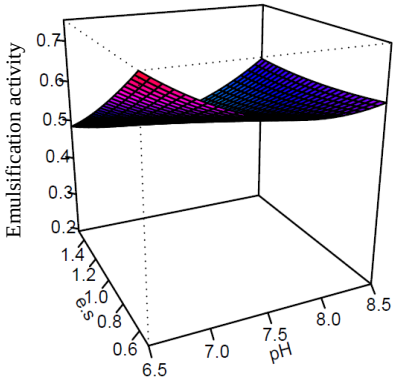
Slice at e.s=0.5, time=15



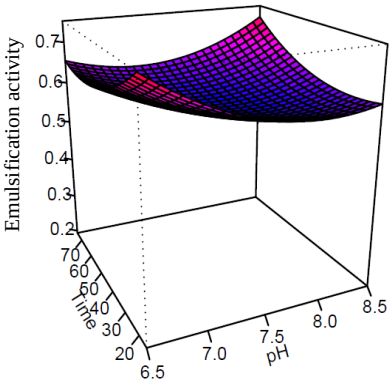
Slice at pH=6.5, time=15



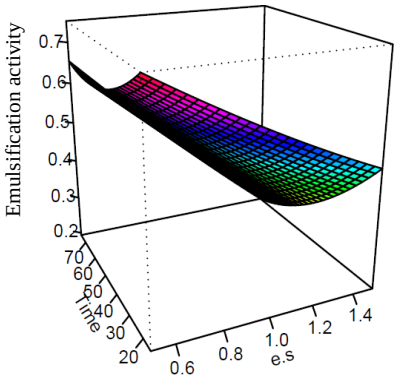
Slice at pH=6.5, e.s=0.5



Slice at temperature=60, time=15



Slice at temperature=60, e.s=0.5



Slice at temperature=60, pH=6.5

Figure 10. RSM plots for the effect of temperature, enzyme-to-substrate ratio (e.s), time and pH on the emulsification activity when papain was used. Each plot shows the effect of two variables while keeping the other two constant at a level that elicits the maximum response.

2.4.5 Effect of Hydrolysis on the Heat-Induced Gelation and WHC of DLW

The heat-induced gel formed using non-hydrolyzed DLW at 10% (w/v) protein concentration had a rupture force of 12.3 ± 1.2 N and WHC of 74.1 ± 1.25%. On the other hand, none of the DLW hydrolysates formed a heat-induced gel at 10%, 15% or even 20% protein concentration. As a result, the water holding capacity could not be determined. Hydrolysis under the conditions tested had a detrimental impact on the gelling property of DLW. Ju et al. (1995) found that whey protein hydrolysates produced using trypsin did not gel at pH 7.0 and 3.0, yet formed a gel when the pH was close to the iso-electric point (pI) of whey protein (pH 5.2). For future studies, it is recommended to monitor heat-induced gelation of DLW hydrolysates at different pH and ionic strengths.

Whey protein hydrolysates (DH 2.8-4.9%) produced using BLP enzyme (one of the enzymes found in Alcalase), however, formed strong gels at pH 7.0 (Ju et al., 1995). Contrary to their findings, our hydrolyzed DLW to a similar DH, using Alcalase, did not form a gel at pH 7. This can be partially attributed to the high lactose content of DLW that could have interfered in the formation of gels using hydrolyzed DLW. Lactose was reported to have a negative impact on gelation (Wong et al., 1996). Since the objective of the study was to find low cost and simple ways to amplify the functionalities of DLW, we did not attempt to eliminate the lactose to evaluate its impact. However, it would be interesting in future studies to investigate its effects on heat-induced gelation.

Additionally, whey protein gelation property can be affected by the source of whey. Pallavicini et al. (1988) showed that the coagulation properties of acid whey can be improved upon Alcalase hydrolysis. Sweet whey, however, did not coagulate under the same conditions, unless $CaCl_2$ was added. The DLW used in this study was produced from sweet whey, a fact that can partially explain our observation. Further studies investigating the effect of limited hydrolysis on DLW gelling property using different environmental parameters are a natural follow up to this work.

2.4.6 Emulsification Properties of Hydrolyzed DLW under the Optimized Conditions predicted by the RSM models

The predicted and actual responses of the 27 hydrolysates (Tables 7 and 8) were relatively close, indicating that the chosen models had a relatively good fit. Yet, when hydrolyzing samples under the predicted optimal conditions, actual responses drifted away from predicted responses in some cases (Table 12). Based on the RSM analysis, hydrolysis conditions that resulted in maximal response were designated as the optimal hydrolysis conditions.

Under the optimal Alcalase and papain hydrolysis conditions determined following the first order + two-way interaction model and the second order model (Table 11), respectively, the experimental (actual) values for EC were not close to the predicted values (Table 12). The R^2 of both models (0.65 for both) was relatively low, hence the observed difference between the predicted and the actual response.

Under the optimal Alcalase hydrolysis conditions determined following the first order + two-way interaction model (Table 11), the experimental (actual) value for EA was very close to the predicted value (Table 12). The R^2 of the model was relatively high (0.91) with a *P*-value of < 0.001. Surprisingly, however, the actual value for EA was far from the predicted values under the optimal papain hydrolysis conditions, although the R^2 for the second order model was relatively high with *P*-value of 0.01.

A possible reason for the variation observed between predicted and actual responses could be attributed to the viscosity of the emulsion (Pearce and Kinsella, 1978). When very viscous emulsions are formed, the mixing of oil may become inefficient or incomplete and thus lead to erroneous EC and EA values. In the present study, viscous emulsions were often obtained with hydrolysates that had low DH, while those that had high DH formed emulsions that were much less viscous. The DH of the hydrolysate produced under the optimal papain hydrolysis conditions was only 2.54%, which was relatively low.

The optimal conditions derived from the RSM models might not be the true optimal conditions due the fact that the range of levels used for each variable were deemed narrow. In several cases no inflexion or saddle point was obvious. Therefore, the optimal

conditions for the highest responses might be outside of the range tested. For future studies, it is recommended to widen the range of the levels used for each variable.

Table 12. Actual (A) and predicted (P) emulsification capacity and activity (EC and EA) responses for Alcalase and papain DLW hydrolysates produced under optimized conditions as determined by RSM analysis.

Enzyme	Parameter	pH	Temp (˚C)	Time (min)	E/S	P	A
Alcalase	EC (g oil/g protein)	8.5	45	75	0.5	2544	1571
	EA	8.5	45	15	1.5	0.75	0.77
Papain	EC (g oil/g protein)	8.5	62	75	1.3	2084	1678
	EA	6.5	60	15	0.5	0.75	0.41

2.4.7 Emulsification Properties of DLW hydrolyzed under Optimized Conditions as Compared to DLW, WPI and WPC 34

The DLW hydrolysates, produced using Alcalase under optimal conditions for maximal EC and EA, had significantly better emulsification properties than the non-hydrolyzed DLW (Table 13, see ANOVA Table 25 in Appendix F). While the EC and ES values of these hydrolysates were comparable to those of WPC 34 and WPI, their EA values were significantly higher ($P < 0.05$). Both hydrolysates had moderate DH, less than 6%, further confirming that moderate hydrolysis result in enhanced functionality.

On the other hand, the DLW hydrolysates, produced using papain under optimal conditions for maximal EA, did not show any improvement in the emulsification properties of DLW. This observation could be partially attributed to the low DH obtained (2.54%). This observation highlights the importance of considering the extent of hydrolysis when aiming at optimizing hydrolysis conditions.

The DLW hydrolysate, produced using papain under optimal conditions for maximal EC, had significantly higher EC than DLW, WPC 34 and WPI, yet low EA. Within the

range tested for papain, the derived optimal conditions did not conclusively result in enhanced emulsification properties.

Table 13. Mean values (n = 3) of DH and emulsification parameters of hydrolyzed DLW produced under selected optimized conditions, in comparison to WPI, WPC 34, and non-hydrolyzed DLW.

Protein Sample	**DH (%)**	**EC (g oil/g protein)**	**EA**	**ES (min)**
WPI	-	1738^{a}	0.662^{b}	31.93^{bc}
DLW	-	1387^{cd}	0.365^{d}	19.63^{c}
WPC 34	-	1454^{bcd}	0.485^{c}	21.37^{c}
EA optimized with alcalase hydrolysis (45 ˚C, e/s = 1.5, 15 min, pH 8.5)	5.92^{a*}	1643^{ab}	0.772^{a}	57.26^{b}
EC optimized with alcalase hydrolysis (45 ˚C, e/s = 0.5, 75 min, pH 8.5)	5.73^{a}	1571^{abc}	0.742^{a}	49.08^{bc}
EA optimized with papain hydrolysis 60 ˚C, e/s = 0.5, 15 min, pH 6.5)	2.54^{b}	1316^{d}	0.409^{d}	21.78^{c}
EC optimized with papain hydrolysis (62 ˚C, e/s = 1.3, 75 min, pH 8.5)	5.83^{a}	1732^{a}	0.239^{e}	77.69^{a}

* Means in each column followed by the same lower case letter are not significantly different according to either Duncan or Tukey-Kramer multiple means comparison test ($P \leq 0.05$); n=3.

2.5 Conclusions

Within the range tested, the emulsification capacity and activity of the optimized hydrolysates, in most cases, were comparable to if not greater than that of WPI, which is known for its exceptional functionality attributes. The RSM approach provided an understanding of the effect of each hydrolysis parameter on the DH and on the assessed emulsification properties. Results indicated that both enzymes, Alcalase and papain, can

be used to enhance the emulsification properties of DLW under optimized hydrolysis conditions and controlled DH. However, within the ranges tested, hydrolysis of DLW was detrimental to the thermally-induced gelation. Further hydrolysis optimization using RSM with wider ranges is recommended to confirm the true optimal hydrolysis conditions for a directed improvement in functionality. Nevertheless, results of this work showed, for the first time, that the functionality of the protein component of DLW can be amplified upon limited and controlled hydrolysis. Testing the functionality and acceptability of hydrolyzed DLW in a real food system, as a replacement of WPI, is a natural follow up to this study. Functionally enhanced DLW have a great potential to reduce processing cost by replacing WPI, while maintaining acceptable quality.

3. FUNCTIONAL PROPERTIES AND SENSORY QUALITY OF BEEF PATTIES FORTIFIED WITH SELECTED DELACTOSED WHEY HYDROLYSATES

3.1. Overview

In an attempt to improve the water loss and texture in beef patties, ground beef samples were formulated with delactosed whey (DLW) subjected to limited and controlled hydrolysis using Alcalase and papain. Enzymatic hydrolysis was performed following previously optimized hydrolysis conditions that ensured enhancement in the emulsification properties of the protein component of DLW. Beef patties were formulated using the hydrolyzed DLW, and also using WPI and WPC34 for comparison purposes. Cook loss due to losses in water and fat was assessed. The cooked patties were submitted to texture analysis using a TA-XT2 texture analyzer. Sensory analysis was also conducted to determine differences in various attributes among the different beef patties. Beef patties formulated with hydrolyzed DLW lost about half as much moisture as the control. The fat loss in the beef patties formulated with hydrolyzed DLW was about 5 times less than that of the control (patty formulated without any whey ingredient). The beef patty formulated with Alcalase hydrolyzed DLW had significantly ($P < 0.05$) higher compression force than the control. The functional performance of DLW hydrolysates in the formulated beef patties was comparable to if not better in some cases than WPI. While instrumental measurements indicated functional differences, the formulation with hydrolyzed DLW did not affect the sensory quality of the beef patties. The tested DLW hydrolysates can thus be incorporated into meat products to amplify the functional properties without jeopardizing the overall sensory quality.

3.2. Introduction

Retaining lean meat water is a major challenge for the processed meat industry. Water loss is primarily due to denaturation of meat proteins upon cooking (Offer et al., 1984). Since moisture is the largest constituent of lean meat (~70%), it is of a great economic importance to retain moisture during processing (Barbut, 2007). Moisture loss not only reduces production yield but also can be detrimental to flavor and tenderness. Fat loss during processing is another issue. Fat loss causes a decrease in palatability (Serdaroglu, 2005).

Whey protein is often used in processed meat to improve cook yield, texture and mouthfeel (Barbut, 2007; Onwulata and Huth, 2009). Whey protein can hold significant amount of water due to its excellent gelling property and water holding capacity. Whey protein can also act as an emulsifier, and thus enhance the fat binding properties of meat products (Hayes et al., 2005; Sun, Gunasekaran, and Richards, 2007). Incorporation of whey protein in processed meat not only reduces production loss, but also improves the nutritional quality of the products.

Whey protein ingredients commonly added to the formulation of processed meat are whey protein isolate (WPI, >90% protein) and whey protein concentrate (WPC, 34-80 % protein). Although WPI provides better functionality, WPC 34 is often used to save on cost of ingredients. Both the meat and dairy industry are in constant search of ways to reduce cost and increase profit. Therefore, researchers are intensifying efforts to find solutions for both industries through attaining the desired balance between cost reduction and revenue.

During the manufacture of several dairy products and ingredients, by-products, such as delactosed whey (DLW), are often generated. Delactosed whey is the by-product of lactose separation from sweet whey. Because of its relatively low protein content (24%) as compared to WPC and WPI, DLW has limited food applications and is mostly used for animal feed (Schingoethe, 1976). Thus, DLW is considered an excellent candidate for functional enhancement. Amplifying DLW's protein functionality adds value to the product and widens the scope of its applications. Functionally enhanced DLW can be

used in food applications such as processed meat, thus benefiting both the meat and the dairy industry.

Limited and controlled enzymatic hydrolysis can result in a targeted enhancement of protein functionality (Foegeding et al., 2002). Enzymatic modifications widen the use of whey proteins as functional food ingredients. We have recently demonstrated that the functional properties, namely the emulsification properties, of DLW were enhanced significantly upon limited and controlled enzymatic hydrolysis. Following response surface methodology, hydrolysis conditions were optimized to elicit maximal enhancement of functionality. The objective of this study was to monitor the functional properties and sensory quality of beef patties fortified with selected DLW hydrolysates as compared to those fortified with WPI and WPC 34.

3.3. Materials and Methods

3.3.1. Materials

Ground meat was purchased from Andrew-Boss Laboratory-Meat Science (University of Minnesota, St Paul, 55108). Samples of DLW (24 % protein), WPC34 (34% protein) and WPI (90 % protein) were kindly provided by Valley Queen (Milbank, SD, USA), Main Street Ingredients (La Crosse, WI, USA), PGP International (Woodland, CA, USA), respectively. Food grade Alcalase 2.4 L (2.4 AU-A/g) and papain (65,600 USP/mg) were provided by Novozymes (Bagsvaerd, Denmark) and American Laboratories Incorporated (Omaha, NE, USA), respectively. Food grade hydrochloride acid (A481-212) and sodium hydroxide (1310-73-2) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Dextrose was purchased from Science Company (Denver, CO, USA). Other formulation ingredients, including the spices, were purchased from local stores.

3.3.2. Preparation of DLW Hydrolysates

Delactosed whey was hydrolyzed using food grade Alcalase at pH 8.5, 45 °C, enzyme to substrate (E/S) ratio of 1.5 (%, v/w), for 15 min. Delactosed whey was also hydrolyzed using food grade papain at pH 8.5, 70 °C, enzyme to substrate (E/S) ratio of 1.5 (%, v/w), for 75 min. The hydrolysis conditions were optimized in our previous study to produce DLW hydrolyzed with enhanced emulsification properties. Hydrolysis of DLW with Alcalase and papain under these conditions resulted in enhanced emulsification activity (EA) and emulsification capacity (EC), respectively. The hydrolysates were produced in a food grade lab. For each enzyme treatment, one batch of 2000 mL DLW solution (5% protein, w/v) was prepared by diluting 416.7 g of DLW in water (assuming a protein content of 24% as stated on the specification sheet of DLW). The obtained solution was then heated to the required temperature using a water bath (Isotemp, Immersion Circulator, Model 730, Fisher Scientific, Pittsburg, PA, USA). After the temperature was reached, the pH of the solution was adjusted to the desired value, using 2M food grade NaOH or HCl. Then, the necessary amount of enzyme was added, and the solution was continuously stirred until the end of the hydrolysis time. The pH was maintained constant throughout the hydrolysis time by using 2M food grade NaOH or HCl. At the end of the hydrolysis time, samples were boiled for 5 min to inactivate the enzyme, and were lyophilized using a food grade lyophilizer, then were stored at -20 °C for later use. The protein content of both Alcalase and papain DLW hydrolysates was ~ 23.5% as determined by Dumas, AOAC 990.03, using a Nitrogen Analyzer (LECO® TruSpec TM, St. Joseph, MI, USA).

3.3.3. Beef Formulation

In triplicate, ground beef patties were formulated using the DLW hydrolysates, non-hydrolyzed DLW, WPC34 or WPI (Table 14). A control sample was formulated without the addition of any whey protein ingredient. The amount added of each whey ingredient was standardized based on a delivery of 15 g of whey proteins. Compared to the control more water (100 g vs. 30 g) was added to the formulations containing whey protein, to

compensate for the reduced amount of beef, which has ~ 70% moisture, and to account for the increased water holding capacity upon addition of whey protein.

3.3.4. Fat and Moisture Analysis

Fat and moisture contents of all the formulated beef patties were determined using the Sohxlet method (AOAC 960.39) and the vacuum oven method (AOAC 950.46), respectively.

Table 14. Formulation of beef patties using different whey protein ingredients.

Ingredient	Treatment									
	Control		DLW		DLWH[a]		WPC 34		WPI	
	g	%	g	%	g	%	g	%	g	%
Lean beef	1000	94.7	960	84.5	960	84.5	960	85.0	960	87.1
DLW	-	-	50	4.4	-	-	-	-	-	-
[a]DLWH	-	-	-	-	50	4.4	-	-	-	-
WPC 34	-	-	-	-	-	-	45	3.98	-	-
WPI	-	-	-	-	-	-	-	-	17	1.54
Salt	12.5	1.18	12.5	1.1	12.5	1.1	12.5	1.1	12.5	1.13
Dextrose	10	0.95	10	0.88	10	0.88	10	0.88	10	0.91
Garlic powder	1.3	0.12	1.3	0.11	1.3	0.11	1.3	0.12	1.3	0.12
[b]Na Glut	1.3	0.12	1.3	0.11	1.3	0.11	1.3	0.12	1.3	0.12
Water	30	2.84	100	8.81	100	8.81	100	8.84	100	9.07
Total	1056	100	1136	100	1136	100	1131	100	1103	100

[a]DLWH= DLW-hydrolysate, either the Alcalase or the papain hydrolysate; [b]Na Glut.= Monosodium glutamate.

3.3.5. Cook Loss Determination

Prior to cooking, the ground beef was thoroughly mixed with the ingredients (Table 14) using a Kitchen Aid® Pro 600 Mixer equipped with a KitchenAid Flat Beater. The ground beef was then mixed manually for a few minutes and refrigerated for one hour. Cook loss was determined according to the method outlined by Barbut (2010), with some modifications. Ground beef (35 g) was weighed, in triplicate, in 50- mL centrifuge tubes. The tubes were heated to 72 °C in a water bath for 75 min. The tubes were then cooled down to room temperature (23 °C) under running cold water for 15 min. The liquid released was decanted into 15 mL tubes and placed at 5-7 °C overnight. After refrigeration, separation into water, fat, and solid components was achieved. Volume of water and fat were recorded and converted to mass using their respective densities. Water and fat losses were determined as the percentage of released water and fat to the total moisture and fat content of the initial beef mix, respectively.

3.3.6. Cooking of the Beef Patties

Formulated beef samples were molded using a patty former into patties with ~ 4 in in diameter and ½ in in thickness. The patties were cooked to a core temperature of 160 °F, which took about 4 to 6 min, using a pre-heated George Foreman grill. The purpose of using a George Foreman grill was to ensure uniform cooking on each side of patties and, thus, reduce cooking-induced differences in texture and appearance.

3.3.7. Texture Analysis

Texture analysis was performed based on the method outlined by Barbut (2010), with some modifications. Prior to texture analysis, six cores (12-mm diameter, 10-mm height) were isolated from the center of each cooked patty (three patties cooked per treatment). The cores were then submitted to compression tests using a TA-XT2 analyzer (Texture Technologies Corp., Scarsdale, NY, USA). A moving flat plate descending at 1mm/s was

employed to compress the cores. The other test parameters were set as follows: pre-test speed, 1.0 mm/s; test speed, 1.0 mm/s; post-test speed, 10.0 mm/s; trigger force, 12 g; penetration distance, 8 mm. The maximum force necessary to compress the samples to the set penetration distance was then recorded.

3.3.8. Sensory analysis

3.3.8.1. Subjects

Ten subjects (9 females, 1 male, ages 23-55 years) were recruited from the trained panel maintained by the Sensory Center at the University of Minnesota. All subjects were tasters of 6-n propothiouracil (PROP) and free from food allergies or sensitivities. Subjects were paid for participating in a two-day study.

3.3.8.2. Test Samples

Formulated beef patties (Table 14) were cooked as described above. Cooking was conducted in a different room to avoid extraneous odors in the testing area. Cooked patties were cut crosswise into 4 equal pieces, and served hot to panelists in plates coded with random 3-digit numbers. The presentation order of the test samples was based on a Latin Squares design balanced to prevent bias and carryover effects.

3.3.8.3. Training session

The subjects were given a training session before they participated in the test session. During the training, the subjects were introduced and familiarized with different sensory attributes of interest, saltiness, bitterness off-flavor, juiciness, firmness and toughness of the cooked beef patties. Using an intensity rating scale from 0 = imperceptible, to 20 = extremely intense (Lawless, 2010), the subjects practiced evaluating the mentioned

sensory attributes of cooked beef patties formulated with and without whey protein. The judges were not informed about the composition of the patties.

3.3.8.4. Test Sessions

Two test sessions were held to duplicate the sensory evaluation of the beef patties by the trained panel. During each session, all 6 samples, the control, and the beef patties formulated with Alcalase hydrolysate, papain hydrolysate, DLW, WPC 34 and WPI (Table 17) were evaluated for juiciness, firmness, toughness, saltiness, bitterness and off-flavor using the intensity rating scale from 0 to 20 (see sensory evaluation form in Appendix E). Panelists were instructed to rinse their mouth with water between samples.

3.3.9. Statistical Analysis

In order to compare the functional and sensory attributes of the different beef patties, analysis of variance (ANOVA) was carried out utilizing XLSTAT for Windows (2012). The independent variable was the type of whey protein added to the beef mix. For the sensory data, judges were treated as a random factor. When the independent variable effect was found significant ($P \leq 0.05$), significant differences among the means were determined using Tukey-Kramer multiple means comparison test or Fisher's LSD test.

3.4. Results and Discussion

3.4.1. Cook Loss

Although the same beef sample was used in all formulation and water content was adjusted for consistency, slight differences in the initial moisture and fat contents were observed among the different beef formulations (Table 15). Theses variations could be attributed to some losses during handling of the beef patties and distribution to various

containers. It seems that some formulations were more prone to lose moisture and fat during handling than others.

Table 15. Initial moisture and fat content of the different beef patties and % loss in moisture and fat upon cooking.

Beef Patties Fortified with	Initial Moisture Content (%)	Initial Fat Content (%)	Water loss (%)	Fat Loss (%)
Alcalase Hydrolyzed DLW	68.30	14.89	8.37[b]	13.0[c]
Papain Hydrolyzed DLW	64.49	14.12	8.12[b]	17.9[bc]
DLW	58.97	16.41	15.3[a]	9.60[c]
WPI	58.40	17.51	19.6[a]	17.7[bc]
WPC34	57.21	16.55	15.8[a]	24.8[b]
Control	65.37	12.02	16.0[a]	62.6[a]

[*] Means in each column followed by the same lower case letter are not significantly different according to either Duncan or Tukey-Kramer multiple means comparison test ($P \leq 0.05$); n = 3. (See ANOVA Table 26 in Appendix G)

No significant difference in water loss (15-20%) upon cooking was observed among the control and beef patties formulated with DLW, WPI and WPC34 (Table 14). However, in beef patties formulated with hydrolyzed DLW, both the Alcalase and the papain hydrolysate, lost about half as much moisture (Table 15). Similarly, the fat loss in the beef patties formulated with hydrolyzed DLW was about 5 times less than that of the control, and about two times less than that of the beef patty formulated with WPC34. The percent loss in fat upon cooking of beef patties formulated with hydrolyzed DLW was not significantly different from that of the beef patties formulated with WPI. Considering both the fat and the moisture losses, the total cook loss of the control beef patty was ~ 18% while that of the beef patties formulated with hydrolyzed DLW was only ~7%. The total cook loss of the beef patty formulated with WPI was ~14%.

These observations confirmed the enhancement in the emulsification properties of hydrolyzed DLW. The enhanced emulsification properties of hydrolyzed DLW led to improved retention in both moisture and fat, similar if not greater than that of WPI. Similar findings were reported by Barbut (2007, 2010), confirming the positive impact of hydrolysates with superior emulsification properties, on cook loss of meat products.

Along with the formation of an interfacial protein film, gelation is important for fat stabilization in meat products (Zorba, Kurt and Gençcelep, 2005; Youssef and Barbut, 2010). The hydrolyzed DLW did not form a thermally induced gel (Chapter two), however, it might have formed intermolecular interactions with meat proteins and thus enhanced the formation of a gel upon cooking and better entrapment of water and fat. This assumption needs further confirmation in a model system containing both meat proteins and DLW.

Non-hydrolyzed DLW had a lower cook loss (~ 10%) than WPI and WPC34. This finding was initially thought to be the result of the high levels of lactose in DLW. However, previous work indicated that lactose had no impact on the cook loss of meat products. Barbut (2010) monitored the cook loss of batters fortified with lactose and no significant difference was found between the control and products fortified with lactose. Thus, the high lactose content of DLW was not likely the cause of the improved cook loss observed in patties fortified with DLW. The difference observed between DLW and the other non-hydrolyzed whey protein ingredients might actually be due to differences in pH, ionic strength and initial processing of the whey ingredients. The pH was not monitored, but the samples fortified with DLW contain more salt because DLW is slightly higher in mineral (5%) than WPI (2-3%). Additionally, the matching of the protein content of DLW to that of WPI in the formulations must have resulted in amplified amount of minerals in DLW patties. The higher mineral content might have facilitated improvement in the gelation and emulsification properties of the proteins in the system, thus leading to better water and fat retention.

3.4.2. Texture Analysis

The beef patty formulated with Alcalase hydrolyzed DLW had the highest compression force, which was not significantly different from that of the beef patty formulated with WPI (Figure 11). The compression force of the beef patty formulation with papain hydrolyzed DLW was not significantly different from those of the control and the beef patties formulated with WPC34 and non-hydrolyzed DLW.

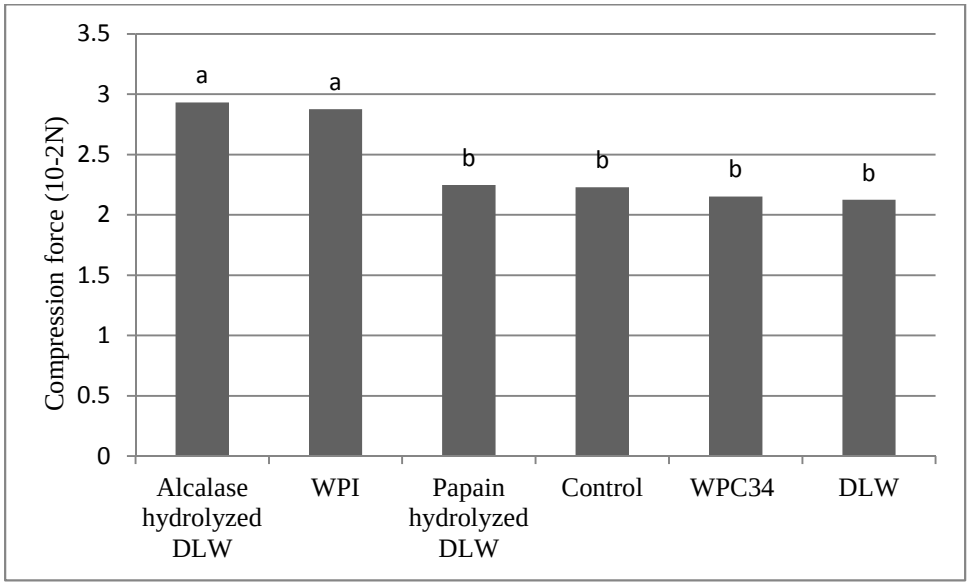


Figure 11. Compression forces of the control and of beef patties formulated with WPI, WPC34, non-hydrolyzed DLW, Alcalase hydrolyzed DLW, and papain hydrolyzed DLW. Different lower case letters above the bars indicate significant differences according to Tukey-Kramer multiple means comparison test ($P \leq 0.05$); n = 18.

A higher compression force indicates better elasticity and reduced potential to crumble upon processing (Barbut, 2010). The observed increase in compression force in beef patties formulated with Alcalase hydrolyzed DLW could be the result of either the co-gelling of whey proteins with meat proteins, or an increase in elasticity due to a greater fat retention. Previous studies suggested that, reducing the fat content significantly decreased the hardness of meat batters. Because water provides less resistance to compression (Youssef and Barbut, 2010), increased proportions of water lead to decreased hardness as well. Good emulsifiers such as caseinates were shown to increase the hardness of beef frankfurters (Barbut, 2010).

Although fat and moisture losses of beef patties formulated with Alcalase hydrolyzed DLW and those of papain hydrolyzed DLW were not significantly different (Table 15), compression forces were significantly ($P < 0.05$) different (Figure 11, and ANOVA Table 26 in Appendix G). This might be due to the differences in the strength of the matrix formed, possibly a result of the differences in DH between the two hydrolysates. The DH (5.92%) of Alcalase hydrolyzed DLW was significantly lower than that (7.76%) of papain hydrolyzed DLW (Chapter two). The higher DH coupled with the nature of the peptides released could have potentially caused the formation of a weaker matrix.

Similarly, the enhanced retention of water and fat of the beef patties formulated with non-hydrolyzed DLW did not contribute to enhanced texture, as compared to that of the control and those formulated with WPI and WPC34. This observation could be attributed to several reasons including but not limited to the salt and lactose content and their interaction effects on the protein matrix formed (Wong et al. 1996).

3.4.3. Sensory Analysis

No significant differences ($P < 0.05$) were observed in any of the evaluated sensory attributes among the different beef patties (Table 16, and ANOVA Table 26 in Appendix G). Overall, the panelists did not discriminate between the different beef samples. This observation potentially indicated that the formulation with hydrolyzed DLW did not affect the sensory quality of the beef patties, although instrumental measurements indicated functional differences.

Most importantly, no bitterness or off-flavor was detected in the samples formulated with hydrolyzed DLW. Both hydrolysates had a DH lower than 8% (Chapter two). At a DH of less than 8%, no perceived bitterness due to released peptides is expected (Rios et al. 2004).

The tested DLW hydrolysates can thus be incorporated into meat products to amplify the functional properties without jeopardizing the overall sensory quality. During reformulation, food developers aim at making favorable changes for functional and economic benefits, without incurring any perceivable changes in flavor (Lawless, 2010).

Table 16. Mean rating (±SE) of the different sensory attributes (n = 20) of the control and the beef patties formulated with Alcalase hydrolyzed DLW, papain hydrolyzed DLW, non-hydrolyzed DLW, WPI, and WPC34.

Beef Patty	Sensory Attributes*					
	Off-flavor	Bitterness	Saltiness	Juiciness	Firmness	Toughness
Alcalase Hydrolyzed DLW	0.45 ± 0.18	0.42 ± 0.15	8.13 ± 0.57	9.62 ± 0.65	7.16 ± 055	7.32 ± 0.77
Papain Hydrolyzed DLW	0.28 ± 0.16	0.12 ± 0.07	8.64 ± 0.72	9.54 ± 0.59	7.70 ± 0.60	8.33 ± 0.74
DLW	0.46 ± 0.31	0.08 ± 0.05	8.68 ± 0.74	8.39 ± 0.63	7.91 ± 0.72	8.27 ± 0.79
WPI	0.55 ± 0.22	0.16 ± 0.11	8.29 ± 0.75	9.21 ± 0.58	7.26 ± 0.45	7.94 ± 0.72
WPC 34	0.06 ± 0.05	0.21 ± 0.11	7.96 ± 0.60	8.61 ± 0.61	7.69 ± 0.52	8.14 ± 0.70
Control	1.05 ± 0.29	0.32 ± 0.14	8.12 ± 0.65	8.31 ± 0.60	7.75 ± 0.58	8.76 ± 0.72

* No significant difference ($P < 0.05$) was observed in any of the evaluated sensory attributes among the different beef patties; n = 20 (ANOVA Table 26 in Appendix G).

3.5. Conclusion

Both Alcalase and papain hydrolyzed DLW, with enhanced emulsification properties, resulted in reduced loss in moisture and fat of cooked beef patties, and thus reduced cook loss. Of the two hydrolysates, Alcalase hydrolyzed DLW enhanced the texture of the beef patty as compared to the control. The functional performance of DLW hydrolysates in the formulated beef patties was comparable to if not better in some cases than WPI, a whey protein ingredient, known for its superior functionality. Additionally the hydrolysates did not jeopardize the sensory quality of the beef patties. Overall, results indicated that DLW subjected to controlled and limited hydrolysis can easily replace WPI in processed meat applications. The use of hydrolyzed DLW in meat products will result in a dual benefit to both the dairy industry through adding value to a dairy byproduct, and to the meat industry by reducing cost.

4. OVERALL CONCLUSIONS, IMPLICATIONS, AND RECOMMENDATIONS

The RSM approach to enhance the functionality of DLW demonstrated the effect of each hydrolysis parameter on the DH and on the assessed functional properties. It also provided the optimized enzymatic hydrolysis conditions that elicited desired functional enhancement. Moderate and controlled hydrolysis of DLW using Alcalase or papain significantly amplified its functional properties. Specifically, the emulsification capacity and activity of the DLW hydrolysates produced under optimized conditions were comparable to and sometimes greater than that of WPI, which is known for its exceptional functionality. The extent of hydrolysis had a pronounced effect on the final functionality. Moderate DH levels resulted in enhanced emulsification properties, while high DH was detrimental. Additionally, within the ranges tested, samples' ability to gel was impaired by hydrolysis even at low DH.

Nevertheless, results of this work showed, for the first time, that the functionality of the protein component of DLW can be amplified upon limited and controlled hydrolysis. The functional performance of DLW hydrolysates in formulated beef patties was comparable to that of WPI. Both Alcalase and papain hydrolyzed DLW, with enhanced emulsification properties, resulted in reduced loss in moisture and fat of cooked beef patties, and thus reduced cook loss. Of the two hydrolysates, DLW hydrolyzed with Alcalase under optimized conditions enhanced the texture of beef patties as compared to a control. Additionally, the hydrolysates did not jeopardize the sensory quality of the beef patties.

Overall, results indicated that DLW subjected to controlled and limited/moderate hydrolysis can easily replace WPI in processed meat applications. The use of hydrolyzed DLW in meat products will result in a dual benefit to both the dairy industry through adding value to a dairy byproduct, and to the meat industry by reducing cost.

Further hydrolysis optimization using RSM with wider ranges is recommended to confirm the true optimal hydrolysis conditions for a directed improvement in functionality. Even though the results of the present study show that limited hydrolysis can significantly improve emulsification properties, there is still room for improvement

of the response variables and determination of their true optimized conditions. Some of the tri-dimensional plots did not show a saddle point, indicating that the response variables would potentially increase more outside the limit of the ranges tested. Therefore extension of the tested ranges is needed to determine true optimized response variables with possibly higher values. Wider ranges may lead to identifying conditions that can actually improve the gelling properties. Future work should also address the impact of lactose and salt contents on the gelation properties of DLW.

With more confirmatory work a functionally enhanced DLW may be produced that would have a great potential to reduce processing cost by replacing WPI in various food applications, while maintaining acceptable quality.

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APPENDIX A: SDS-PAGE Protein Profile Visualization of DLW and its Hydrolysates

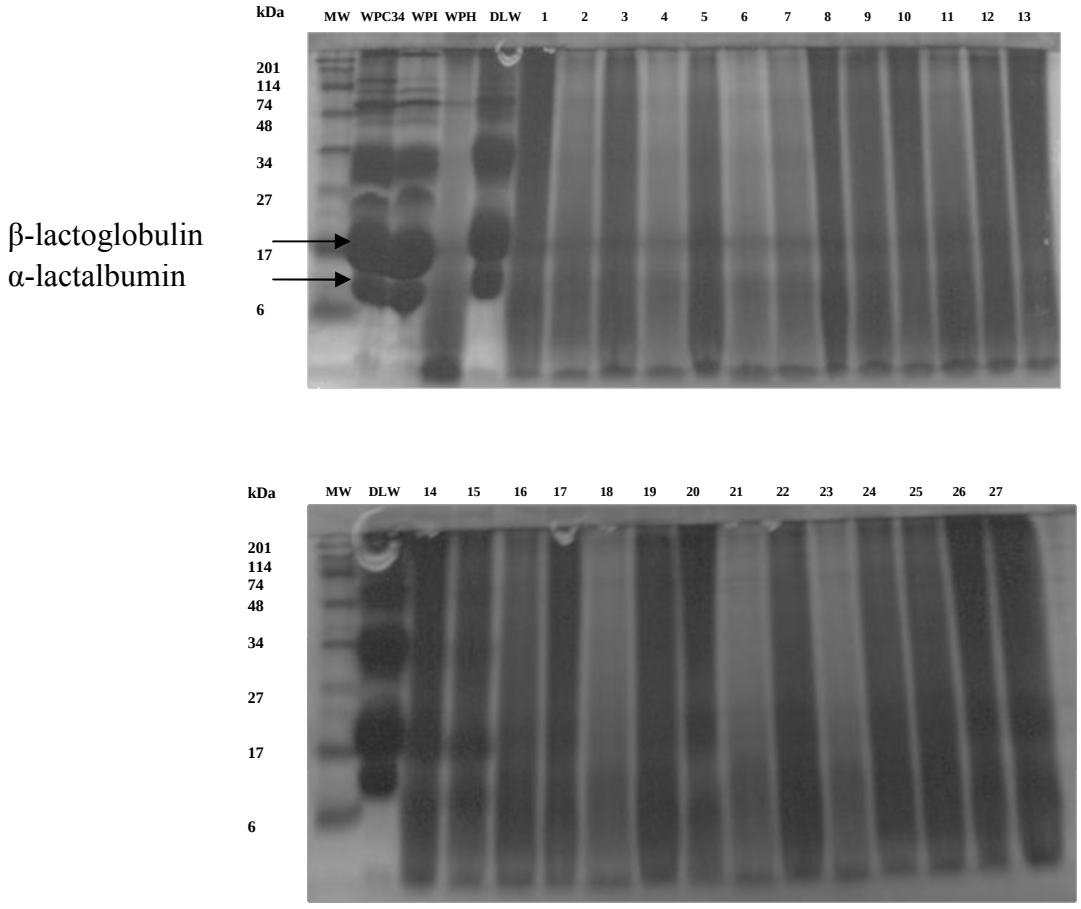

Figure 12. SDS-PAGE protein profile visualization of DLW and its alacalase hydrolysates (RSM runs 1 to 27, Table 4) as compared to WPC 34, WPI, and WPH. MW: Molecular weight marker. Amount of protein loaded in each lane was ~133 µg protein.

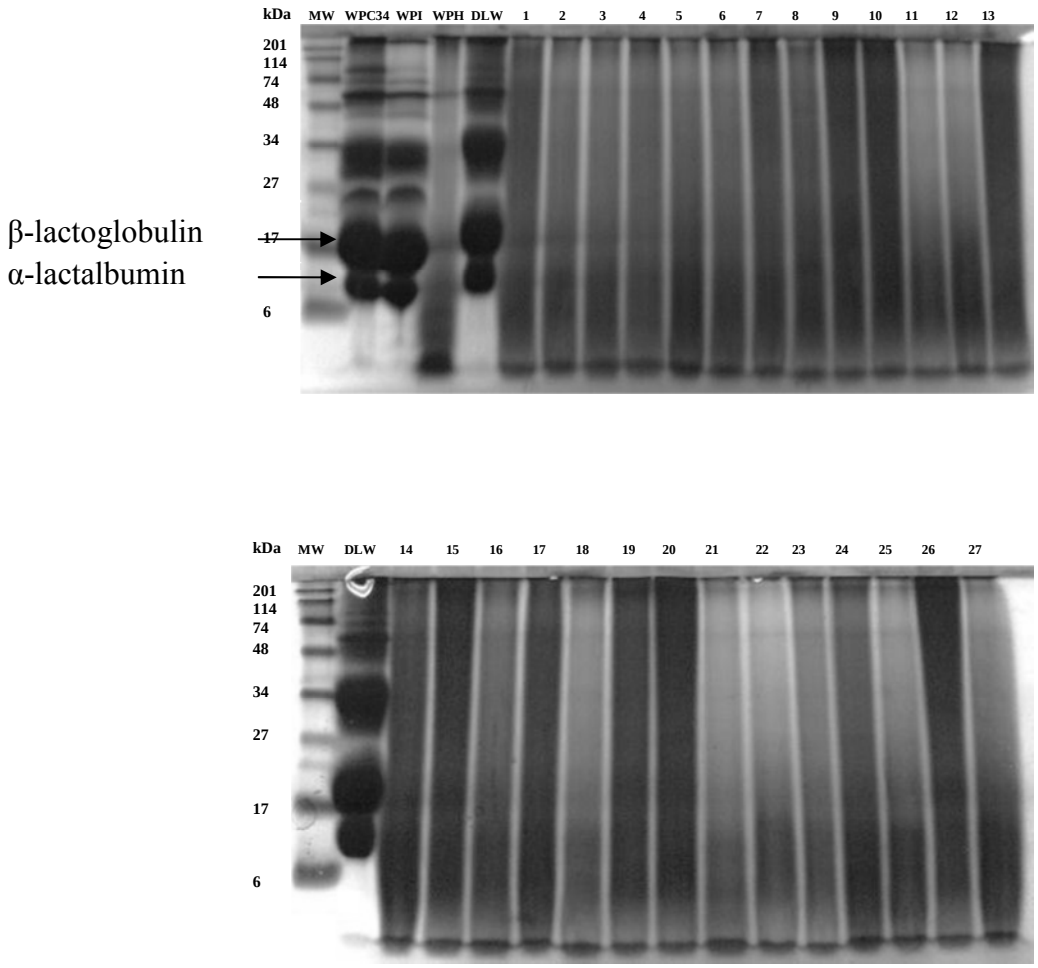

Figure 13. SDS-PAGE protein profile visualization of DLW and its papain hydrolysates (RSM runs 1 to 27, Table 5) as compared to WPC 34, WPI, and WPH. MW: Molecular weight marker. Amount of protein loaded in each lane was ~133 µg protein.

APPENDIX B: Example Calculations for DH Determination

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

Where B is the volume of NaOH necessary to keep pH constant = 0.3ml

N_b = normality of NaOH used = 0.5N

MP is the mass of the protein (g N × 6.25) = 2.5g

htot = 8.8 meq/g (for whey proteins)

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

$$\alpha = \frac{1}{1 + 10^{pK-pH}}$$

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

$$pK = 7.8 + \frac{298 - T}{298 \times T} \times 2400$$

where T is the absolute temperature (K).

At 60˚C, pK = 7.8+[((298-(60+273))/(298*(60+273))] = 6.95

Therefore, at pH7.5, $\alpha = 1/(1+10^{6.95-7.5}) = 0.78$

Finally, DH = (1.3ml*0.5mol/l)/(2.5g*0.78*8.8meq/g) = 3.78

APPENDIX C: Coefficient of the Models Tested when using Alcalase

Table 17. Model coefficients of the two-way interactions model for EC.

	Estimate	Standard error	t-value	Pr(>|t|)
(Intercept)	1731.172	38.080	45.4618	<2.2e-16***
Temp	-61.787	57.120	-1.0817	0.295416
ES	-79.909	57.120	-1.3990	0.180908
Time	11.232	57.120	0.1966	0.846583
pH	-13.599	57.120	-0.2381	0.814843
Temp:ES	-133.821	98.934	-1.3526	0.194983
Temp:Time	-330.886	98.934	-3.3445	0.004115**
Temp:pH	-228.034	98.934	-2.3049	0.034907*
ES:Time	-286.692	98.934	-2.8978	0.010489*
ES:pH	-1.051	98.934	-0.0106	0.991655
Time:pH	-38.508	98.934	-0.3892	0.702237

Table 18. Model coefficients of the two-way interactions model for log (ES).

	Estimate	Standard error	t-value	Pr(>|t|)
(Intercept)	1.615102	0.016408	98.4309	<2.2e-16***
Temp	0.154922	0.024613	6.2944	1.068e-05***
ES	0.143203	0.024613	5.8183	2.617e-05***
Time	0.124762	0.024613	5.0690	0.0001139***
pH	0.178833	0.024613	7.2659	1.889e-06***
Temp:ES	0.062980	0.042630	1.4774	0.1589944
Temp:Time	0.127707	0.042630	2.9957	0.0085565**
Temp:pH	0.031308	0.042630	0.7344	0.4733318
ES:Time	0.059438	0.042630	1.3943	0.1823000
ES:pH	0.128703	0.042630	3.0190	0.0081492**
Time:pH	0.039414	0.042630	0.9246	0.3689328

Table 19. Model coefficients of the two-way interactions model for EA.

	Estimate	Standard error	t-value	Pr(>|t|)
(Intercept)	0.488000	0.010009	48.7557	<2.2e-16***
Temp	-0.119667	0.015014	-7.9705	5.817e-07***
ES	-0.041167	0.015014	-2.7420	0.0144715*
Time	-0.074500	0.015014	-4.9622	0.0001413***
pH	-0.025000	0.015014	-1.6652	0.1153384
Temp:ES	-0.090250	0.026004	-3.4706	0.0031536**
Temp:Time	-0.085250	0.026004	-3.2783	0.0047307**
Temp:pH	-0.056500	0.026004	-2.1727	0.0451702*
ES:Time	-0.078250	0.026004	-3.0091	0.0083199**
ES:pH	-0.097000	0.026004	-3.7301	0.0018226**
Time:pH	-0.090500	0.026004	-3.4802	0.0030902**

Table 20. Model coefficients of the second order model for DH.

	Estimate	Standard error	t-value	Pr(>|t|)
(Intercept)	7.944000	0.469354	16.9254	9.697e-10***
Temp	2.191667	0.234677	9.3391	7.459e-07***
ES	1.867250	0.234677	7.9567	3.975e-06***
Time	2.780833	0.234677	11.8496	5.565e-08***
pH	-1.351417	0.234677	-5.7586	9.040e-05***
Temp:ES	0.168500	0.406472	0.4145	0.68579
Temp:Time	0.477500	0.406472	1.1747	0.26288
Temp:pH	-1.116000	0.406472	-2.7456	0.01775 *
ES:Time	0.110750	0.406472	0.2725	0.78990
ES:pH	0.441000	0.406472	1.0849	0.29926
Time:pH	-1.220750	0.406472	-3.0033	0.01100*
Temp^2	-0.839167	0.352015	-2.3839	0.03452 *
ES^2	-0.038292	0.352015	-0.1088	0.91518
Time^2	-0.585917	0.352015	-1.6645	0.12189
pH^2	2.229708	0.352015	6.3341	3.752e-05 ***

APPENDIX D: Coefficient of the Models Tested when using Papain

Table 21. Model coefficients of the second order model for EC.

	Estimate	Standard error	t-value	Pr(>|t|)
(Intercept)	2001.9317	84.3316	23.7388	1.87e-11***
Temp	25.9665	42.1658	0.6158	0.54952
ES	29.6923	42.1658	0.7042	0.49476
Time	8.7048	42.1658	0.2064	0.83991
pH	28.3038	42.1658	0.6712	0.51478
Temp:ES	-166.7112	73.0333	-2.2827	0.04148*
Temp:Time	-22.0773	73.0333	-0.3023	0.76761
Temp:pH	43.0635	73.0333	0.5896	0.56637
ES:Time	-32.5300	73.0333	-0.4454	0.66395
ES:pH	125.7303	73.0333	1.7215	0.11080
Time:pH	132.3680	73.0333	1.8124	0.09500
Temp^2	-43.2196	63.2487	-0.6833	0.50738
ES^2	-188.6649	63.2487	-2.9829	0.01142*
Time^2	-70.3673	63.2487	-1.1125	0.28769
pH^2	-38.7490	63.2487	-0.6126	0.55154

Table 22. Model coefficients of the two-way interactions model for log (ES).

	Estimate	Standard error	t-value	Pr(>|t|)
(Intercept)	1.6897164	0.0147389	114.6432	<2.2e-16***
Temp	0.0652068	0.0221084	2.9494	0.009422**
ES	0.1789593	0.0221084	8.0946	4.759e-07***
Time	0.0446553	0.0221084	2.0198	0.060473
pH	0.0049161	0.0221084	0.2224	0.826843
Temp:ES	-0.0267038	0.0382928	-0.6974	0.495589
Temp:Time	-0.0017515	0.0382928	-0.0457	0.964084
Temp:pH	-0.0847604	0.0382928	-2.2135	0.041740*
ES:Time	0.0944520	0.0382928	2.4666	0.025318*
ES:pH	-0.1193237	0.0382928	-3.1161	0.006652**
Time:pH	-0.0825119	0.0382928	-2.1548	0.046761*

Table 23. Model coefficients for the second order model for EA.

	Estimate	Standard error	t-value	Pr(>|t|)
(Intercept)	0.393667	0.022843	17.2336	7.871e-10***
Temp	-0.067833	0.011421	-5.9391	6.828e-05***
ES	-0.101167	0.011421	-8.8576	1.306e-06***
Time	-0.053583	0.011421	-4.6914	0.0005219***
pH	0.076917	0.011421	6.7344	2.090e-05***
Temp:ES	0.019500	0.019783	0.9857	0.3437321
Temp:Time	-0.005000	0.019783	-0.2527	0.8047413
Temp:pH	0.030500	0.019783	1.5418	0.1490802
ES:Time	-0.048750	0.019783	-2.4643	0.0298039*
ES:pH	0.061250	0.019783	3.0962	0.0092560**
Time:pH	0.048000	0.019783	2.4264	0.0319436 *
Temp^2	-0.014750	0.017132	-0.8609	0.4061403
ES^2	0.011500	0.017132	0.6712	0.5147761
Time^2	0.047875	0.017132	2.7944	0.0162107*
pH^2	0.060875	0.017132	3.5532	0.0039729**

Table 24. Model coefficients of the second order model for DH.

	Estimate	Standard error	t-value	Pr(>|t|)
(Intercept)	5.719333	0.664887	8.6020	1.776e-06***
Temp	0.943167	0.332443	2.8371	0.014978*
ES	2.282500	0.332443	6.8658	1.733e-05***
Time	1.374500	0.332443	4.1345	0.001384 **
pH	-3.173667	0.332443	-9.5465	5.899e-07***
Temp:ES	-0.129250	0.575809	-0.2245	0.826171
Temp:Time	0.173750	0.575809	0.3017	0.768009
Temp:pH	0.484000	0.575809	0.8406	0.417031
ES:Time	0.263750	0.575809	0.4581	0.655099
ES:pH	-0.640500	0.575809	-1.1123	0.287776
Time:pH	-0.682000	0.575809	-1.1844	0.259176
Temp^2	-0.210875	0.498665	-0.4229	0.679863
ES^2	-0.119375	0.498665	-0.2394	0.814844
Time^2	0.047625	0.498665	0.0955	0.925490
pH^2	3.245875	0.498665	6.5091	2.897e-05***

APPENDIX E: Sensory Evaluation Form

All ratings were made on scales ranging from 0 (None) to 20 (Intense)

Please bite completely through the beef at the point of the cut edges and rate the sample for each of the texture attributes below:

Juiciness – the amount of liquid released as the sample is chewed

Firmness – the amount of force required to bite through the sample with your incisors

Toughness – the amount of work required to bite through the sample with your incisors

Please take a second bite of the beef and rate the sample for the taste and flavor attributes below:

Saltiness

Bitterness

Off-flavor

APPENDIX F: Analysis of Variance for the Functional Properties of DLW Hydrolysates as Compared to WPI, WPC34 and DLW

Table 25. Analysis of variance on the effect protein powder type on the degree of hydrolysis and emulsification properties.

Sample Analysis	Source of Variation	Degree of Freedom	Mean Squares	Significance
DH	Protein Powder	3	14.1	< 0.0001
	Error	8	0.169	
ES	Protein Powder	6	4979	< 0.0001
	Error	14	306	
EA	Protein Powder	6	0.095	< 0.0001
	Error	14	0.001	
EC	Protein Powder	6	444257	< 0.0001
	Error	13	12719	

APPENDIX G: Analysis of Variance for the Functional and Sensory Properties of Beef Patties formulated with DLW Hydrolysates as Compared to WPI, WPC34 and DLW

Table 26. Analysis of Variance for the degree of hydrolysis of DLW hydrolysates as compared to WPI, WPC34 and DLW.

Sample Analysis	Source of Variation	Degree of Freedom	Mean squares	Significance
Juiciness	Protein Source	5	6.81	0.476
	Error	114	7.47	
Firmness	Protein Source	5	1.77	0.932
	Error	114	6.89	
Toughness	Protein Source	5	4.56	0.838
	Error	114	11.02	
Saltiness	Protein Source	5	1.76	0.963
	Error	114	8.99	
Bitterness	Protein Source	5	0.34	0.216
	Error	114	0.23	
Off-flavor	Protein Source	5	2.21	0.051
	Error	114	0.964	
Compression force	Protein Source	5	2.60	< 0.0001
	Error	102	0.43	
Fat loss	Protein Source	5	1128	< 0.0001
	Error	12	15.6	
Water loss	Protein Source	5	63.8	< 0.0001
	Error	12	3.98	