Creation of a Sticky Coating of Dairy Proteins Containing Bioactive Peptides to Reduce Dental Caries

by

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Abstract

A sticky coating composed of dairy proteins and bioactive peptides (caseinophosphopeptides, CPPs) to prevent dental caries was formulated. The coating consisted of 10% CPPs, 70% glycerol and 20% casein (or hydrolysate) and/or whey in ratios from 0:100 to 100:0 casein:whey. Sodium caseinate was hydrolysed with three different enzymes, Alcalase, trypsin and α-chymotrypsin, yielding three hydrolysates classified as CN-A, CN-T and CN-C, respectively. The degree of hydrolysis reached levels of 18.04, 13.63 and 11.40% for CN-A, CN-T and CN-C respectively. Degree of hydrolysis was proportional to molecular weight determined by FPLC. CN-C coatings were the stickiest based on probe-tack test and capable of withstanding up to 22.46 N of force. Attempts to correlate glass transition temperatures to stickiness of the coatings proved inconclusive. All coatings were capable of binding enough calcium to remineralise tooth enamel. Results indicated that 100:0 CN-C:whey produced the stickiest coating; however this combination also increased bacterial adhesion.

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List of Abbreviations

ACP	Amorphous calcium phosphate
AHS	Adhesive hard sphere
AN_1	Amine nitrogen prior to hydrolysis
AN_2	Amine nitrogen after hydrolysis
Asp	Aspartic acid
CFIA	Canadian Food Inspection Agency
CN-A	Casein hydrolysed by Alcalase
CN-C	Casein hydrolysed by α-chymotrypsin
CN-T	Casein hydrolysed by trypsin
CPP	Caseinophosphopeptide
DH	Degree of hydrolysis
DMTA	Dynamic mechanical thermal analysis
DSC	Differential scanning calorimetry
FPLC	Fast protein liquid chromatography
FTIR	Fourier transform infrared spectroscopy
Glu	Glutamic acid
GMP	Glycomacropeptide
HAP	Hydroxyapatite
MCP	Micellar calcium phosphate
MDSC	Modulated differential scanning calorimetry
MS	Mass spectroscopy
NaC	Sodium caseinate
N_{pb}	Nitrogen content of peptide bonds

pI	Isoelectric point
RP HPLC	Reverse phase high pressure chromatography
S. mutans	*Streptococcus mutans*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser(P)	Serine phosphate
S_o	Surface hydrophobicity
STP	Sticky temperature point
T	Temperature above glass transition temperature at which stickiness occurs
T_g	Glass transition temperature
THYE	Todd Hewitt yeast extract
TMA	Thermal mechanical analysis
TNBS	2,4,6-Trinitrobenzene sulfonic acid
T_p	Transition phenomenon
USFDA	United States Food and Drug Act
WPI	Whey protein isolate

1.0 Introduction

Bovine milk is a common staple in the human diet providing essential nutrients such as calcium and protein. Approximately 3.5% of bovine milk is protein but this percentage may fluctuate depending on cow breed, stage of the lactation cycle and age (Huppertz, 2013; Singh & Flanaghan, 2005). Milk proteins are known for their high functionality particularly their ability to form gels. The gelation of milk proteins occurs at a certain temperature and pH and, depending on the protein ratio, contributes to the textural properties of foods such as cheese and yoghurt (Vasbinder *et al.*, 2003). Sports nutrition and infant formula use milk proteins as well, due to their high nutritional properties, but they are often used as powders where they are prone to stickiness (Liu *et al.*, 2012). The stickiness is dependent on environmental conditions such as relative humidity, presence of amorphous sugars and the type of milk protein in the powder.

Milk protein is divided into two groups based on solubility at pH 4.6 where whey is soluble and casein is insoluble. Each class has four different main proteins. Whey consists of α-lactalbumin, β-lactoglobulin, bovine serum albumin and immunoglobulin and casein consists of α_{S1}-, α_{S2}-, β- and κ-casein (Huppertz, 2013). Whey proteins have well-defined secondary and tertiary structures, unlike caseins, and exist as individual globular proteins. Caseins lack structure and are unfolded proteins that co-exist in milk as “micelles”. The term “micelle” is a traditional term rather than an accurate description of casein as casein lacks true micellar structure. An accurate description of casein in an aqueous medium is a supra-molecule since

casein forms a well-defined complex of non-covalently bound casein and calcium phosphate (McMahon & Oommen, 2008). The caseins may be separated using various pHs and temperatures as well as hydrolysis.

Although hydrolysis can be used to treat separate caseins, it is becoming more widely implemented to generate casein hydrolysates for use in food products. Hydrolysates have different functional properties than pure isolates and produce lower allergenic responses in sensitive individuals (Liu *et al.,* 2012). Functionality changes, primarily due to increased hydrophilicity, creates a hygroscopic powder. In the presence of moisture, the casein hydrolysate will plasticize more than intact casein resulting in a stickier powder. It has been suggested that this is due to a decrease in the glass transition temperature of casein which would reduce the temperature and relative humidity required to induce sticky behaviour (Hogan *et al.*, 2010).

When hydrolyzed for long periods of time, casein generates bioactive peptides called caseinophosphopeptides (CPPs). CPPs are the section of casein containing three phosphoserines followed by two glutamic acid residues which are capable of binding the calcium phosphate present in milk (Kitts, 2005). CPPs were first discovered in cheese where they exhibited anticariogenic effects in humans and animals. Milk products, in general, showed a decrease in dental caries when consumed but the result with cheese was more profound. This is due to the sticky nature of cheese which allowed it to adhere to the teeth. When held in close proximity to the tooth enamel, CPPs act as a mineral reserve replenishing the tooth's calcium and phosphate lost due to acid erosion from food or oral bacteria (Yamaguchi *et al*., 2006).

CPPs have the added effect of reducing the adhesion of cariogenic bacteria thereby further decreasing dental caries (Schupbach *et al.*, 1996).

In this study, casein was hydrolyzed by three different enzymes, Alcalase, trypsin and α-chymotrypsin, and added to whey in different ratios. Glycerol and CPPs were added to create a sticky coating with anticariogenic properties. The stickiness of the coatings was determined using a texture analyzer (TA.XT-2). Calcium content of each coating was measured to ensure the coating acted as a mineral reserve and microplates were used to test the efficacy of the coatings on *Streptococci mutans*, a cariogenic bacterium.

2.0 Literature Review

2.1 Whey

Whey is classified as the soluble protein fraction of milk at a pH of 4.6. Whey consists of four major proteins; α-lactalbumin, β-lactoglobulin, bovine serum albumin and immunoglobulin which account for 95% of whey (Singh & Flanagan, 2005). α-lactalbumin, β-lactoglobulin and bovine serum albumin contribute to the majority of whey protein and contain disulphide bonds and free SH groups. The quantity of disulphide bonds and free SH groups in each protein denotes their function which individually and together represent whey functionality (Lacroix & Cooksey, 2005). One such function is in the creation of films through denaturation of the whey proteins, typically by heating. Denaturation causes the whey protein to unfold exposing hydrophobic regions and free SH groups (Adhikari *et al*., 2007b; Lacroix & Cooksey, 2005). In solution, these groups may interact to form bonds but the effect is more significant when the whey is subsequently dried. Drying a heated whey solution forms disulphide bonds and instigates hydrophobic interactions on the surface creating a non-sticky film around the droplets resembling a water balloon (Adhikari *et al*., 2007b).

Stickiness in whey is not commonly a desired quality either in a dried food powder or a coating, where minimal interactions between whey and itself or other food compounds are the main focus. Whey protein stickiness is particularly an issue in terms of fouling and scaling in heat-exchanger plates, filters and spray driers. This results in costly maintenance for companies as they must stop production to remove the films (Adhikari *et al*., 2007a). Storage of whey powders also has its problems due to the tendency of whey to stick and clump together,

referred to as caking, reducing its usability and perceived quality to the customer (Adhikari *et al*., 2007b; Boonyai *et al*., 2004). Research into the creation of a sticky whey coating is rare as non-sticky whey would be more beneficial to the industry to solve the common issues experienced, for example, in spray drying. Therefore, research into prevention of stickiness can be analyzed to determine the best conditions for non-sticky whey films and coatings and inversely used to create a sticky whey protein coating. Adhikari *et al.* (2007a) performed multiple drying tests with whey protein isolates (WPI) to determine the point where WPI would not adhere to test probes mimicking the surface of a spray drier. It was found that tensile strength, as a measure of stickiness or tackiness, correlated well with the surface tension, the magnitude of attraction between surface molecules and contact molecules, of the droplets, even under varying temperatures and WPI concentrations. A decrease in surface tension resulted in a decrease in adherence, or tensile strength, to the testing probes. The authors stated that increased WPI concentration did not solve sticking in spray drying despite these results due to cohesive failure occurring before adhesive failure. Cohesive failure occurs when the interactions between molecules of the same substance fail and cause separation. Adhesive failure is similar but the interactions occur between two separate substances such as test probes and whey (Adhikari *et al.*, 2001). The cohesive failure experienced by the protein-water solutions made by Adhikari *et al.* (2007a) was caused by the WPI disrupting and weakening the hydrogen bonds of the water in the solution leaving WPI on the test probe and base rather than a full separation from the test probe. Cohesive failure is desirable for a sticky whey coating as the bioactive peptides need to remain on the teeth to be functional.

Whey may lose this sticky behaviour and tendency to experience cohesive failure if it is allowed to dry at elevated temperatures, up to 130 ^{o}C (Adhikari *et al.*, 2007b). As previously mentioned, when it is heated, whey reveals hydrophobic regions and free SH groups which interact during drying to form a non-sticky film around the droplet. This flexible surface will transition from a rubber to a glassy film with continued drying creating a non-stick surface that demonstrates adhesive failure (Adhikari *et al*., 2007b). For use as a sticky coating these conditions must be avoided during processing but should not be an issue at room temperature. Research performed by Adhikari *et al.* (2007b) confirmed that cohesive failure still occurs at room temperature as room temperature does not facilitate the unfolding of the whey protein which prevents the formation of the non-sticky film. Whey proteins are known best for their sticky behaviour and caking during storage in the form of food powders. Caking can lead to collapse or the loss of structure of the powder which is exhibited more in whey powders than for casein. This form of stickiness is driven by moisture and temperature specifically through their effect on the glass transition temperature. Sticky behaviour of both casein and whey has been shown to occur around 20 ^{o}C above the glass transition temperature making temperature and moisture crucial conditions to be monitored (Mauer *et al.*, 2000).

2.2 Casein

Casein is classified as the fraction of milk protein that precipitates at 30^{o}C and pH 4.6 while whey remains soluble. Casein constitutes 80% of the milk protein and is composed of four separate caseins in a micellar formation. The four types of casein are α_{S1}-, α_{S2}-, β- and κ-casein which represent 37%, 10%, 35% and 12% of the total casein respectively (Singh & Flanagan, 2005). Caseins are, in general, unfolded proteins lacking in rigid tertiary structure to facilitate

casein's biological function of binding calcium. By binding calcium, casein provides the neonate with calcium and prevents calcification and precipitation within the mammary gland (McMahon & Oommen, 2008). Calcium occurs in milk at a concentration of 30 mM and is part of the casein micellar structure (Farrell Jr. *et al.*, 1988; Zittle *et al.*, 1958). The casein micelles exist as a highly stable colloidal system but the exact model of casein and calcium phosphate nanocluster associations and overall structure remain widely disputed (de Kruif, 1999; Horne, 2006). The primary issue with casein micelle models is their inability to encompass casein's behaviour under all conditions that invoke aggregation/flocculation and changes in viscosity and stickiness. These behaviours are generally considered positive as they are responsible for the creation of products such as yogurt and cheese. The exception is stickiness especially for casein in powder form which is problematic in milk processing and protein isolation, similar to whey. Stickiness is affected by enzymatic hydrolysis, calcium addition, acidification, temperature and moisture content (de Kruif, 1998). To understand stickiness in casein, it is necessary to have an accurate model of the casein micelle. The exact relation between these conditions, mentioned above, and behaviours are partially solved by current micelle models but there has yet to be an exact model which encapsulates the entire range of casein's behaviour.

2.2.1 Casein Micelle Structure and Models

2.2.1.1 Adhesive Hard Sphere Model

The first model created was based on casein's behaviour during renneting and acidification; it was called the adhesive hard sphere (AHS) model. In this model, casein micelles are hard spheres of non-covalently bound α_{s1}-, α_{s2}- and β-casein which are stabilized in milk by short-ranged steric repulsion between the layer of extended κ-caseins on the periphery of the

micelle. This layer of κ-casein is referred to as the "hairy layer" by some researchers as each κ-casein extends from the micelle like hair. Other researchers prefer the term "polyelectrolyte brushes" or "salted brushes". This term is based on the glycomacropeptides (GMPs) attached to the κ-casein. GMPs have fourteen carboxylic acids, which in the high ionic strength of milk (approximately 0.08M) become charged (de Kruif, 1998). The salted brushes extend out into the continuous phase to increase the entropy of the system which increases the solvation of κ-casein (de Kruif, 1999). The spheres are called "adhesive" because the range of interaction is small when compared to the radius of the micelles, approximately 100 nm. When casein micelles come into contact with each other, the hairs increase the protein concentration at the contact points increasing free energy. This results in the steric stabilization of the micelles keeping them in solution (de Kruif, 1999; Ettelaie *et al*., 2014; Horne, 2003). The casein micelles flocculate if the steric repulsion from κ-casein is lost. This occurs if the κ-casein hairs are cleaved by an enzyme or lose their charge and collapse to the surface. In this manner, the behaviour of the micelles in response to environmental changes is based on the κ-casein layer remaining extended, collapsing or being cleaved (de Kruif, 1998).

Flocculation by enzymatic hydrolysis and acidification can therefore be explained by this model. The introduction of enzymes to casein micelles has two potential effects; cleavage of GMP, causing κ-casein to collapse, or removal of the κ-casein from the micelle. This results in cessation of steric repulsion which decreases the entropy of the system and reduces the solvency of κ-casein. Eventually van der Waals forces become prevalent in the system leading to the flocculation of casein. Alternatively, acidification neutralizes the charge of the carboxylic

acids on the GMP causing the full brush to collapse against the micellar surface. Flocculation would then occur similar to enzymatic hydrolysis (de Kruif, 1999).

Beyond the collapse or cleavage of κ-casein brushes, the AHS model cannot explain any other behaviour such as changes in viscosity. This was demonstrated by Olivares *et al*. (2013) and Dahbi *et al.* (2010) who researched the effect of shearing rate on casein viscosity. Casein micellar behaviour was explained by the AHS model under low shearing but widely deviated under high shearing in both studies. They concluded in both experiments that higher shear rates induced a change in the casein from a suspension of hard spheres to soft spheres. The unexpected increase in viscosity was caused by the compression of the soft spheres. It was postulated that the spheres could be compressed due to high shear rates forcing water from the loose casein micelle structure increasing the malleability. This allows for greater interaction between micelles as the κ-casein layer supposedly collapses leading to an increase in viscosity and potentially stickiness. Dahbi *et al*. (2010) focused on the volume fractions of casein and found that the AHS model held up to volume fractions of 0.25 but at the volume fraction range of 0.55-0.61 behaviour was more appropriately described as soft spheres.

The AHS model also lacks a key component to casein's biological function, calcium binding. When formed in the mammary gland, casein is an unfolded protein lacking a rigid tertiary structure. This allows for efficient uptake of calcium phosphate to accommodate the nutritional needs of the neonate and prevent calcification or precipitation within the gland. The casein micelle present in milk therefore contains calcium phosphate in its structure (McMahon & Oommen, 2008). Flocculation in high concentrations of calcium is also unexplained by the

AHS model as κ-casein does not precipitate in the presence of calcium and has very little binding affinity (Huppertz, 2013). Other models, such as the nanocluster and dual-binding models, have managed to solve these issues to a certain extent by incorporating calcium phosphate into the micelle and identifying the structure as porous to enhance calcium interactions (Dalgleish *et al*., 2004; Holt *et al*., 1998).

Despite the limited range of behaviours AHS can explain, the basic model of casein is appropriate. Subsequent models maintained the peripheral location of κ-casein for stabilization with a core of α_{s1}-, α_{s2}- and β-casein. Studies have shown the micelles can only be formed using either α_{s1}-, α_{s2}- or β-casein isolates if κ-casein is present. One such study by Kumosinski and Farrell Jr. (1991) studied the micelles of α_{S1}-casein and κ-casein in calcium solutions to determine if calcium was a stabilizing factor or κ-casein. Each casein was added individually and then together to note their stability and interaction with calcium. They found that calcium caused α_{S1}-casein to become insoluble and precipitate but it remained soluble in the presence of κ-casein. It was concluded that the κ-casein was not binding calcium but α_{S1}-casein to form a complex that altered α_{S1}-casein's affinity to calcium allowing the production of stable colloids.

2.2.1.2 Nanocluster Model

The nanocluster model addresses some of the issues with the AHS model, primarily calcium binding. The nanocluster model is based on micelle formation through the interaction of colloidal calcium phosphate and the caseins. This works well to explain the formation of the casein micelle in the mammary gland as the casein molecules would move through the gland and sequester calcium phosphate (Holt, 2004). This model would also be thermodynamically

stable and no assumptions or adjustable parameters are required to predict casein behaviour (Holt, 2004). In general, the casein micelle can be considered a three dimensional network formed from nanoclusters of colloidal calcium phosphate linked by various caseins.

The colloidal calcium phosphate nanoclusters are called micellar calcium phosphate (MCP) and are comprised of amorphous calcium phosphate in a hydrated state bound to the phosphoseryl groups of a casein molecule (Holt *et al*., 1998). The non-phosphoseryl regions of the caseins act as bridges between the MCPs, which forms a lattice. Different caseins have different sizes of phosphoseryl regions and therefore different quantities of phosphate centres. A phosphate centre on casein refers to at least two phosphorylated residues capable of forming a nucleus with the colloidal calcium phosphate. Each casein has one or more phosphate centres except κ-casein which has only one phosphoserine and therefore cannot adequately bind colloidal calcium phosphate, limiting micellar growth (Holt, 2004; Horne, 2006). β-casein, with only one phosphate centre, can also limit the growth of the casein micelle. This allows expansion in only one direction whereas α_{S1}- and α_{S2}-casein have two and three phosphate centres, respectively, allowing expansion in multiple directions as more than one MCP can link to these caseins (Horne, 2006). MCPs bind to the phosphate centres which are the hydrophilic regions of the caseins. When the hydrophilic portion is bound, the hydrophobic tail is forced to extend out into the continuous phase. The extended tails then become entangled and create a dense, hydrophobic region that is postulated to provide the stabilization force between the micelles (de Kruif *et al*., 2012; Horne, 2006).

This model is prevalently used in research as it is thermodynamically stable, capable of explaining casein behaviour in response to pH, and requires no adjustable parameters or arbitrary values to work (Holt 2004). There are some grey areas that exist in this model though, mainly a lack of experimental evidence. De Kruif *et al*. (2012) refute the model as the amount of colloidal calcium phosphate does not account for the scattering seen by small angle neutron scattering and that large variability in scattering also makes any results highly unreliable. Despite the hydrophobic outer sphere, the nanocluster model does not include any hydrophobic interactions within the micelle. Saturation of phosphate centres is also ignored but if saturation occurs on caseins with only one phosphate centre, networking is not possible. The nanocluster model also fails to provide a role for κ-casein. Growth is also not adequately limited and the model cannot be used for other species of milk besides bovine. The energetics of folding and rearranging of the casein micelle are also not discussed, leaving more uncertainties in the micelles' structure (Horne, 2006).The model suggested to replace the nanocluster model is the dual-binding model which seeks to fill these holes.

2.2.1.3 Dual-Binding Model

The dual-binding model of casein micelles is very similar to the nanocluster model as it incorporates calcium phosphate and maintains the three dimensional lattice structure. The models differ in terms of the interactions taking place as dual-binding has the micelle held together by hydrophobic interactions as well as binding between caseins and colloidal calcium phosphate (Horne, 2003). To accommodate the addition of hydrophobic interactions, the caseins acts as "block copolymers" because they have hydrophilic and hydrophobic regions which possess different functionalities allowing for two methods of polymerization through the

hydrophobic interactions and colloidal calcium phosphate linkages (Horne, 2003). Another key difference between the two models is that the κ-casein exists on the periphery of the micelle in dual-binding model serving the purpose of steric repulsion to stabilize the micelles in solution as it does in the AHS model (de Kruif *et al*., 2012). The benefit of including the hydrophobic interactions is the previous limitations of the nanocluster model are prevented, such as loop closures and saturation of casein phosphate centres (Horne, 2006). Hydrophobic interactions also explain the formation of micelles without calcium phosphate or the maintenance of the structure if calcium phosphate is solubilized (McMahon & Oommen, 2008).

2.2.2 Calcium Role in Casein Micelles and Flocculation

Despite the fact that micelles can form without calcium phosphate in the dual-binding model, calcium binding remains caseins' biological function and influences casein's structure and behaviour. α_{S1}, α_{S2}, β and κ-caseins each have a different affinity and ability to bind calcium based on their primary structure. Three amino acids provide the binding sites for calcium: glutamic acid (Glu), aspartic acid (Asp) and phosphorylated serine (Ser(P)). The negatively charged carboxyl groups and phosphate on Glu, Asp and Ser(P) respectively, attract the positively charged calcium ion. Ser(P) binds calcium the strongest and the number of Ser(P) groups is strongly correlated with calcium affinity and binding capacity (Mekmene & Gaucheron, 2011; Park & Allen, 1998). The Ser(P) residues are present in each casein at 8-9, 10-13, 5 and 1 for α_{S1}-casein, α_{S2}-casein, β-casein and κ-casein, respectively (Huppertz, 2013; Park & Allen, 1998). Calcium binding is typically stronger in α_{S2}-casein due to the higher number of Ser(P) but it accounts for only 10% of casein whereas α_{S1}-casein and β-casein account for 38 & 36%, respectively (Farrell Jr. *et al*., 1988). For this reason, β-casein and α_{S1}-casein are more

prominent in calcium binding studies as larger quantities can be isolated and presumably contribute more to calcium binding in micelles.

Isolated caseins respond differently to calcium binding than in micellar form. Individual caseins are more prone to aggregation at lower concentrations of calcium than is naturally present in milk with the exception of κ-casein which is insensitive to calcium. The type of bond between isolated caseins and calcium varies depending on the concentration of calcium which varies per casein. At low concentrations, binding is exothermic so calcium binds to the Ser(P) via hydrogen bonds and the carboxyl groups remain unbound. As calcium concentration increases, binding becomes endothermic and hydrophobic interactions are attributed to the binding of calcium to Ser(P) and the carboxyl groups. If calcium concentration increases further calcium binding becomes extremely endothermic as most of the Ser(P) are bound leaving only the unbound carboxyl groups. Binding of calcium to Ser(P) residues alters the casein's structure and, once saturated, the affinity to the hydrophobic regions increases to the point of causing aggregation and eventual precipitation of casein (Huppertz, 2013).

In bovine milk, bridging between casein proteins and free calcium phosphate has been proposed as a secondary method of keeping the casein micelles in suspension. This is shown when the pH of a casein suspension is decreased to the point where calcium phosphate solubilizes. After solubilisation, the casein micellar structure loosens and susceptibility to aggregation increases (Ruis *et al.*, 2007). Calcium phosphate may not be responsible for this effect but the change in κ-casein's charge may, although it does contribute to the limitation of micellar size. κ-casein reacts very little with calcium phosphate due to its one Ser(P) group

which impedes calcium phosphate's ability to branch (Ettelaie *et al.*, 2014). If the κ-casein is removed or collapsed, calcium phosphate cannot maintain casein micelle dispersion alone therefore they interact and flocculate (Horne, 2003). Similar to calcium phosphate bridging, Antipova *et al*. (2002) proposed that the micellar structure of casein was stabilized by calcium acting as a link or bridge between caseins potentially even during flocculation. The authors found that calcium ion addition to casein solutions increased the stickiness up to 5 mM calcium ions before its effect no longer significantly increased stickiness. The authors proposed that calcium ions neutralized the charge on casein micelles thereby increasing aggregation. The change in density and thickness of the casein micelles by calcium ion addition would inversely decrease aggregation, meaning there would be an equilibrium between these two reactions unseen in acidification. Overall, Antipova *et al*. (2002) concluded that pH had a greater effect on the stickiness of casein than the concentration of calcium ions added due to the κ-casein collapsing.

Despite having only one Ser(P) residue, κ-casein is capable of binding calcium up to 3 mM through the utilization of its carboxyl groups (Huppertz, 2013). The primary purpose of κ-casein is for stabilization of the casein micelle which was demonstrated in a study by Kumosinski and Farrell Jr. (1991). To test the calcium association with the individual caseins and micelles, micelles were formed using only α_{S1}-casein and κ-casein. They found that calcium caused α_{S1}-casein to become insoluble and precipitate but remained soluble in the presence of κ-casein. It was concluded that the κ-casein was not binding calcium but α_{S1}-casein to form a complex that altered the affinity to calcium allowing the production of stable colloids. This supports the dual-binding model where κ-casein existed as a peripheral stabilizing component

and α_{S1}-, α_{S2}- and β-casein bind calcium phosphate nanoclusters within the micelle (de Kruif, 1999).

The peripheral κ-casein, which remains in the dual-binding model, explains renneting and acidification similar to the AHS model. Peripheral κ-casein is commonly disputed as the amount of κ-casein required to coat the outside of a casein micelle is much greater than the amount found in milk (de Kruif *et al.*, 2012). Imaging of the casein micelle performed by cold field emission scanning electron microscopy by Dalgleish *et al*. (2004) found a potential explanation. The images showed casein micelles to be constructed of tubules or cylinders leaving open pores into the micelle. These protrusions extended 10 to 20 nm from the micelle and had a much lower surface area than if the micelle was a solid sphere. They postulated that if the κ-casein existed on the ends of these tubules much less would be required (~30 to 40 per tubule) closely reflecting the content in milk. Having the κ-casein on the ends of these tubules in lesser quantities also made the protein more accessible for enzymes and whey to bind. Even when collapsed or hydrolyzed, κ-casein may interact with whey creating a casein micelle with a whey coating (Vasbinder *et al.*, 2003). These whey coated casein micelles have the potential to create a strong gel as whey increases the isoelectric point (pI) of casein from 4.6 to approximately 5.2. As pI increases, the pH of gelation increases therefore stickiness will occur at a higher pH just before gelation (Vasbinder *et al.*, 2003). Vasbinder and de Kruif (2003) examined the pH effect on the formation of a whey coating around casein micelles using denatured whey and intact sodium caseinate. The denatured whey formed soluble aggregates at high pH values with very little being incorporated on the surface of the casein micelles. They found that a pH of 6.55 generated a homogenous coating of whey on the casein micelle and

reduced the level of soluble whey aggregates. A pH lower than 6.55 increased the quantity of whey on the micelle but the coating was not homogenous, creating inconsistencies in micellar interactions (Vasbinder & de Kruif, 2003). Therefore to form an acceptable coating, a pH around 6.5 should be used and the pI and temperature of gelation should be avoided to prevent aggregation and subsequent gelation. To create a coating that is stickier than whey or casein alone, whey should be added to a casein solution at a pH higher than the pI.

2.2.3 Enzymatic Hydrolysis of Casein and Relation to Stickiness

In its intact state, casein is not as sticky as whey but hydrolysis makes casein stickier. Enzymes are capable of cleaving more than just the outer layer of κ-casein, potentially due to the gaps left between the casein tubules. The extent of hydrolysis is commonly measured by the degree of hydrolysis (DH) which is the amount of amine nitrogen released by the protein after hydrolysis. Greater DH values result in shorter peptide chains and more free amino acids which have been linked to greater levels of stickiness in dairy protein powders (Netto *et al*., 1998). Hogan and O'Callaghan (2013) speculated that this is due to a sharp decrease in hydrophobicity causing the hydrolyzed protein to be highly hygroscopic. This was shown by large changes in the moisture sorption curves suggesting that hydrolysis creates more binding sites for water. The change in water binding between intact and hydrolyzed caseins is from structural changes which also affect their viscous flow. Mounsey *et al*. (2012) found that intact casein exhibited Newtonian flow behaviour and was more viscous than the hydrolyzed casein. After hydrolysis, casein exhibits shear thinning possibly due to the exposure of hydrophobic regions leading to the formation of weak complexes. The increase in viscous flow from plasticization also causes a decrease in the glass transition temperature (T_g) compared to intact

whey or casein. The T_g is the shift of a material from a glassy state to a rubbery state and is frequently used to define when a protein powder is sticky. The sticky temperature point (STP) occurs at a certain range above the T_g both specific to the protein or mixture of compounds. While the range is relatively unaffected by external factors such as temperature and relative humidity, the T_g is very susceptible (Hogan *et al.*, 2010). Increasing the water content of casein powder from 0 to 20% can shift its T_g from 140 to 40^{o}C (Kalichevsky *et al*., 1993). Even higher quantities of plasticizer would be expected to depress the T_g further, causing stickiness to occur at room temperature.

The T_g is a more useful measure of stickiness for powders and polymers whereas other methods are more effective for measuring stickiness of products such as coatings. The predominant method for the measurement of stickiness is the probe-tack test due to its simplicity and diversity. The basic premise of a probe-tack test is to place a certain quantity of sample on a platform and apply a known pressure with a probe. The probe is then lifted at a specified speed and the force and distance of the probe is recorded until contact is lost or a predetermined distance is reached. The diversity of this test comes from the ability to program specific parameters (force, distance, hold times etc…) into a test and the wide variety of probe shapes, sizes and materials available. For stickiness, a cylinder probe is generally used to provide a constant contact area and the peak force attained prior to breakage is measured as stickiness. This replicates the sensation experienced by the tongue, teeth or fingers when attempting separation from the food product (Chen *et al.*, 2008). The probe-tack test has the added benefit of showing which type of failure was experienced by the sample, which is crucial to processing and consumer acceptance. Failure is the point at which the sample ceases

elongation and breaks the connection between the probe and the bulk of the sample. Three types of failure exist; adhesive, cohesive and adhesive-cohesive. Adhesive failure is the clean separation between the product and the probe whereas cohesive failure involves the product separating from itself, leaving remnants on the probe and platform. Adhesive-cohesive failure is a combination of the two where some product remains on the probe but it is not covered entirely (Adhikari *et al*., 2001). The properties of the food determine the type of failure where semi-solid or solid foods experience any of the three failures, liquid foods primarily experience cohesive failure (Chen *et al.*, 2008).

Casein and whey have been extensively researched using probe-tack tests as gels but only the gel strength is measured not the stickiness. When used as coatings, casein and whey are typically dried as films and tested for mechanical strength using tests involving elongation or puncturing the films. This is because protein coatings are used for foods such as produce or to coat cardboard for packaging materials where stickiness is unacceptable and not commonly experienced after drying. A few studies do exist measuring stickiness of casein-whey mixtures for spray drying purposes. Adhikari *et al*. (2007b) and Werner *et al*. (2007) used probe-tack tests on protein formulations containing WPI to determine when a non-sticky film was formed to prevent agglomeration during drying. Both experiments found that a concentration of 20% (w/w) WPI in water changed the stickiness of the formulated solutions and the mode of failure changed from cohesive to adhesive with drying. Werner *et al.* (2007) found there to be a peak stickiness during drying at 78 ^{o}C immediately before the WPI solution became non-sticky at a moisture content of 56%. Adhikari *et al*. (2007b) found similar results at 80 ^{o}C but when drying was performed at 25 ^{o}C WPI was sticky and experienced cohesive failure.

These experiments were performed using proteins in water but plasticizers, such as glycerol, are more commonly used in food coatings. Glycerol is a USFDA and CFIA approved food additive used to improve the mechanical properties of casein and whey coatings (Siew *et al*., 1999). The major setback of dairy protein coatings is their water solubility and tendency to form hard, brittle films which are unsuitable as coatings. For these reasons, dairy proteins are usually hydrolysed, cross-linked or added to plasticizers to modify their hydrophobicity and form more flexible films (Somanatha & Sanjeevi, 1994). Stickiness is not commonly measured for coatings as they are dried after application to prevent adhesion. Viscosity and elongation are frequently measured though, which may be used to infer potential stickiness. When glycerol is added to sodium caseinate there is a higher probability of stickiness as the viscosity decreases and the elongation increases. The decrease in viscosity is due to glycerol disrupting the protein-protein and protein-solvent interactions of sodium caseinate and water. As glycerol is a small, hydrophilic molecule, it is capable of entering between the side chains of sodium caseinate and generating hydrogen bonds with the hydrophilic locations on the protein's backbone. By making these sites unavailable to both the protein and water molecules, their interactions are reduced creating a looser protein network which is capable of greater elongation and flow. The solution becomes less hydrophilic because the bound glycerol has only one hydroxyl group remaining to interact with water molecules compared to the original hydrophilic sites on the protein (Siew *et al.*, 1999). The same effect is seen when glycerol is added to whey. Unlike casein, whey is capable of forming disulphide bonds which create a more rigid structure. Disulphide bonds still form in the presence of glycerol but viscosity is lower than a whey solution without glycerol (Galietta *et al.*, 1998). In both cases, glycerol was shown to

depress the T_g, due to the reduction in protein-protein interactions, which could cause stickiness to occur at room temperature depending on the quantity of glycerol added.

For use in an oral coating, glycerol would be beneficial to decrease the mechanical strength of the dairy protein coatings temporarily for ease of application. In their decreased viscosity state, the coatings could be easily applied and adhere to the teeth. To dry the coating only mild heat or air drying would be required as the coating would be very thin. It is expected that the resulting coating would be water resistant, flexible and strong after drying (Chen, 1995).

To determine the stickiness of an oral coating containing casein and whey, a probe-tack test is reasonable, particularly if the probe material is similar to tooth enamel, such as acrylic. Modulated differential scanning calorimetry is commonly used to measure the T_g which could monitor the effect of glycerol and protein hydrolysis on T_g depression and potentially estimate the occurrence of stickiness. In this way, the stickiest coating formulation could be determined and later tested for effectiveness in oral use.

2.3 Bioactive Peptides

Enzymatic hydrolysis of casein will also produce short peptide chains called caseinophosphopeptides (CPPs) that are bioactive peptides. Bioactive peptides are peptide fragments from parent proteins that, when released through hydrolysis, provide beneficial functions to the human body, positively influencing health (Korhonen & Pihlanto, 2006). Bioactive peptides from milk proteins gain their positive influence from the phosphate groups bound to certain amino acids, typically serine residues (Cross *et al.*, 2005; Southward, 2002).

Phosphorylation is crucial to the function of casein bioactive peptides as it determines the negative charge on the peptide and the quantity of calcium that may be bound. The four main proteins of casein have varying degrees of glycosylation and phosphorylation. α_{s2}-casein has the highest level of phosphorylation ranging from 10-13 phosphate residues per mole of protein and β-casein has the least ranging from 1 to 3 residues per mole (Singh & Flanagan, 2005). The phosphorylated serine present in the casein proteins binds calcium phosphate nanoclusters, creating a stable bridge between casein micelles to keep them in suspension (Huppertz, 2013). α_{s1}-, α_{s2}- and β-casein contain a special sequence of amino acids known as CPPs identified by the unique amino acid sequence of SerP-SerP-SerP-Glu-Glu (Cross *et al.*, 2005; Kitts, 2005). This particular combination allows for a higher rate of phosphorylation of the active sites of the CPP than other peptides created by hydrolysis (Cross *et al.*, 2005). Phosphoserine is negatively charged, and in these clusters forms areas of high negativity in comparison to the rest of the casein molecule. Other sections of the casein are positively charged, lending the amphipathic nature of α_{s1}-, α_{s2}- and β-casein. The CPPs' highly charged state makes α_{s2}-casein the most hydrophilic of the casein proteins (Huppertz, 2013).

CPPs are isolated from casein using acid or enzyme hydrolysis or natural digestion (Kim *et al*., 2007; Korhonen, 2009; Korhonen & Pihlanto, 2006; McDonagh & FitzGerald, 1998). The highly negative phosphoseryl residue areas allow for the binding of amorphous calcium phosphate (ACP) which restricts the size of the clusters preventing nucleation and precipitation (Wong *et al*., 2011; Zidane *et al*., 2012). This unique ability to bind amorphous calcium phosphate forms a complex referred to as CPP-ACP that functions as a perfect anticariogen (Korhonen, 2009).

2.3.1 Anticariogenic Properties of Caseinophosphopeptides

Many factors are involved in the development of dental caries, from pH to microflora, making it difficult to prevent. Any imbalance to the mineral gradient between the plaque fluid and teeth causes minerals to be lost from the tooth, softening the enamel and leading to dental caries (Fejerskov *et al.,* 2004). Acid, either from food or produced by bacteria, is considered the primary cause of caries as it dissolves the hydroxyapatite of the dentine (Kumar *et al.,* 2008). CPP has been theorized to prevent dental caries by either preventing bacteria from adhering to the teeth, reducing acid production, or acting as a mineral reserve to remineralise depleted dentine.

It is important to note that CPPs are not antibiotic nor antimicrobials. CPPs reduce the adherence of acid-producing cariogenic bacteria such as *Streptococci mutans*, thereby slowing the progression of dental lesions (Schüpbach *et al.*, 1996). The exact mechanism behind reduced adhesion of bacteria is not known but is thought to be from the CPP incorporation into the salivary pellicle. The amino acid sequence attributed to CPP's anticariogenicity, Ser(P)-Ser(P)-Ser(P)-Glu-Glu, has a high affinity to the hydroxyapatite surface of teeth arising from its negative charge (White *et al.,* 2011). The CPP displaces human serum albumin from the pellicle and becomes incorporated into the protective layer (Neeser *et al.,* 1994; Schüpbach *et al*., 1996). To the author's knowledge, it is unknown whether the human serum albumin is the receptor for streptococci bacteria or if the CPP is binding to the receptor (Neeser *et al.,* 1994; Schüpbach *et al*., 1996; White *et al.,* 2011).

Most anticariogenic claims for CPPs have been based on remineralisation where even pre-existing erosion from certain foods or tooth brushing are reversed to a certain degree through the application of CPPs on teeth. A few products already exist for this purpose in the form of toothpastes and mousses to act as a mineral reserve suppressing demineralization (Ranjitkar *et al*., 2009; Kumar *et al*., 2008; Wong *et al*., 2011; Yamaguchi *et al*., 2006). The mineral reserve is composed of ACP held in solution by the CPP. The mineral reserve is only effective when it is held within proximity of the enamel and dentine of the tooth altering the mineral gradient and enabling remineralisation (Kumar *et al*., 2008). Ranjitkar *et al*. (2009) found that in enamel and dentine the use of CPP in a mousse form reduced wear by 1.15 µm (approx. 48% of lesion depth regained through remineralization) and 3.59 µm (approx. 62% of lesion depth regained through remineralization) respectively. Kumar *et al*. (2008) also suggests that besides remineralisation caused by the CPPs, their ability to neutralize acids may also hinder the process of lesion formation to a greater extent than that of fluoride toothpaste. The authors noted that using CPPs topically after brushing with fluoride toothpaste provides a synergistic effect increasing the reduction in dental caries. Cross *et al*. (2004) attributed this synergism to the CPP and calcium phosphate complex incorporating fluoride as well increasing the concentration of calcium, phosphate and fluoride ions in plaque increasing the ions availability for remineralisation. Reynolds *et al*. (2008) confirmed the synergistic effect with fluoride by comparing fluoride at 1100 ppm with and without CPP-ACP. They found that when CPP-ACP was added, remineralisation increased by 156% and fluoride ion uptake increased by 30%.

To be beneficial, the CPPs must be in contact with the teeth to act as a mineral reserve which is why rinses, gums and mousses are typically used. Despite the short contact time of these products, research by Yamaguchi *et al*. (2006) proved them to be beneficial. By using ultrasound transmission velocity, they found that even small amounts of CPP-ACP over time still promoted the increase in remineralisation compared to the controls. Furthermore, changes in the surface of the teeth were found using scanning electron microscopy, indicating that calcium and inorganic phosphate concentration increased in the supragingival plaque and adhered to bacteria present in the plaque biofilm. This effect may be enhanced if the product was able to hold the CPP-ACP to the enamel for longer time periods which would require a sticky coating. The sticky coating must not only be able to hold the CPP-ACP to the tooth but also maintain its functionality. Since CPPs act as a mineral reserve for the tooth enamel, the calcium phosphate must remain in the coating and in contact with the enamel to be readily available. Measuring the calcium content in the coatings would ensure that the CPPs have not been altered through the addition of casein and whey. Therefore a sticky coating formulated with whey, hydrolyzed casein and CPPs could remain in contact with the tooth enamel and prevent dental caries.

3.0 Materials and Methods

3.1 Casein Hydrolysis, Degree of Hydrolysis, Bioactive Peptide Production and Coating Formulations

3.1.1 Source Material

Bovine sodium caseinate, trypsin, α-chymotrypsin and Alcalase were purchased from Sigma-Aldrich (MO, USA). Whey protein isolate was purchased from Bulk Barn (Winnipeg, MB, Canada); the intended use of this product is for nutritional supplementation. To improve the solubility of whey, soy lecithin was added to the powder by the manufacturer and the whey may also be hydrolyzed. Bovine casein (Sigma Chemical Co, MO, USA) was used for production of caseinophosphopeptides (CPPs) instead of sodium caseinate to avoid sodium blocking calcium receptor sites on the CPPs.

3.1.1 Enzymatic Hydrolysis

The casein, in the form of sodium caseinate (Sigma-Aldrich, MO, USA), was hydrolyzed with three enzymes: trypsin (Sigma-Aldrich, MO, USA), α-chymotrypsin (Sigma-Aldrich, MO, USA) and Alcalase (Sigma-Aldrich, MO, USA). Casein was dissolved in distilled water (5% w/v). The pH was adjusted with 1M NaOH (Fisher Scientific, NJ, USA) to the optimal value for each enzyme using a pH meter (Accumet Basic AB15 Plus, Fisher Scientific, ON, Canada). For trypsin, the optimal pH was 9 and α-chymotrypsin and Alcalase have the optimal pH of 8. Hydrolysis was performed in triplicate according to the following procedure to reach a degree of hydrolysis of approximately 18-20% for Alcalase, 10-12% for α-chymotrypsin and 12-14% for trypsin. Alcalase was added at 2% (v/w), trypsin and α-chymotrypsin was added to the casein

solution at 8% (w/w). The samples were incubated at the optimal temperature for the enzymes. Alcalase was incubated at 50 ^{o}C for 15 min, trypsin at 37 ^{o}C for 4 h and α-chymotrypsin at 50 ^{o}C for 2 h. The samples were then boiled in a beaker on a hot plate (Corning Hot Plate-Stirrer, PC-351) at 95-100 ^{o}C for 10 min to stop the reaction. The samples were then allowed to cool to room temperature and stored in brown bottles in a refrigerator (at 4^{o}C) for further testing.

3.1.2 Degree of Hydrolysis

The degree of hydrolysis of the samples was measured using the 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) (Pierce, IL, USA) method (Alder-Nissen, 1979). A 0.2125M sodium phosphate buffer at pH 8.2 was made by dissolving two buffers in deionized distilled water. One of the buffers was 0.2125M sodium phosphate monobasic (Fisher Scientific, NJ, USA) and the other 0.2125M sodium phosphate dibasic (Sigma-Aldrich, Steinheim, Germany), which were added together at a ratio of 1:20, respectively. To reach the appropriate pH, the monobasic buffer was added to the dibasic buffer. Samples and the standards at the desired concentrations were added to a 1% (w/v) solution of sodium dodecyl sulfate (Sigma-Aldrich, ON, Canada) in the sodium phosphate buffer. Sodium dodecyl sulfate is used to prevent protein aggregation rather than mercaptoethanol in the samples as TNBS reacts with mercaptoethanol creating sporadic results (Adler-Nissen, 1979). The samples were added at a concentration of 4 mg/mL for the trypsin and α-chymotrypsin and 2mg/mL for the Alcalase hydrolysates based on the original concentration of sodium caseinate dissolved in distilled water (80 and 40 µL, respectively). The standard curve was generated using D-Lysine (Sigma Chemical Company, MO, USA) at the following concentrations: 0 mM, 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM and 2.5 mM. An aliquot of 250 µL of samples or standards was added to labeled glass test tubes with a ten mL

capacity. Two mL of sodium phosphate buffer were added to the test tubes and mixed well before being incubated in a water bath (Magni Whirl UL 543H Constant Temperature Bath, Blue M Electric Company, IL, USA) at 50 ^{o}C for 15 min. Immediately before use, the 0.1% TNBS in sodium phosphate buffer was made in the dark and wrapped in foil. The solution was kept in the dark and in foil at all times and all work was carried out under low lighting conditions. TNBS is a very reactive substance which reacts with numerous functional groups such as hydroxyl groups which can cause an increase in the reading of the blank over time. This reaction is accelerated in the presence of light which is why low lighting conditions are required for the reaction and absorbance measurement (Adler-Nissen, 1979). Each tube had 2 mL of 0.1% TNBS solution added and the test tubes remained in the water bath at 50 ^{o}C for 60 min. During this interval, the TNBS reacts with the primary amines of the hydrolyzed proteins under alkaline conditions producing a chromophore. Immediately after incubation, 4 mL of 0.1M HCl (Fisher Scientific, ON, Canada) were added to each test tube to stop the reaction. The samples were cooled for 20 min in the dark before measuring the absorbance at 340 nm using an Ultrospec 4300 Pro spectrophotometer (Biochrom, England; Model # 80-2112-43) and the computer software SWIFT II. Although the colour intensity has been considered correlated to the concentration of α-amines, better reproducibility has been found measuring the absorbance at 340 nm rather than 420 nm, a more common wavelength for yellow solutions (Adler-Nissen, 1979). The degree of hydrolysis was calculated as follows:

$$Degree\ of\ Hydrolysis\ (\%) = \frac{AN_2 - AN_1}{N_{pb}}$$

AN_1 is the amino nitrogen content before hydrolysis in mg/g

AN_2 is the amino nitrogen content after hydrolysis in mg/g

N_{pb} is the nitrogen content of the peptide bonds in mg/g; 156.7 mg/g for casein protein based on the N conversion factor for milk proteins of 6.38.

3.1.4 Bioactive Peptide Production

Bioactive peptides called caseinophosphopeptides (CPPs) were created using the method of McDonagh and FitzGerald (1998). 32 g of casein from bovine milk (Sigma Chemical Co, MO USA) was dissolved in 400 mL of distilled water. The pH was then adjusted to a pH of 8 using 2M NaOH (Fisher Scientific, NJ, USA). 4% (v/w) Alcalase (Sigma-Aldrich, MO, USA) was added and the solutions were then incubated in a water bath (Magni-whirl Constant Temperature Batah, UL5434, Blue M Electric Company, IL, USA) at 50 ^{o}C for 2.5 h. The pH was checked and adjusted back to pH 8 using 2M NaOH every 15 min. To deactivate the enzyme after hydrolysis, the pH was dropped to 4.6 using 1M HCl. The hydrolysate was then centrifuged for 10 min at 12000g. The supernatant was collected and the pH was adjusted to 7 using 2M NaOH. 1% calcium chloride (Fisher Scientific, NJ, USA) (w/v final concentration) was added prior to the addition of 50% (v/v final concentration) ethanol (Commercial Alcohols, ON, Canada). The solution was left for an hour to allow for the CPPs to agglomerate. The solution was then

centrifuged again at 12 000g for 10 min. The precipitate was then collected and freeze dried prior to grinding.

3.1.5 Peptide Molecular Weight Determination using Fast Protein Liquid Chromatography (FPLC)

The molecular weight of the proteins: whey, sodium caseinate, sodium caseinate hydrolysates and CPP were determined using fast protein liquid chromatography (FPLC). The buffer used for both the column and the samples was a 50mM phosphate buffer with 0.15M NaCl. The buffer was made by combining 61% (v/v) 0.01M Na_2HPO_4 with 39% 0.01M NaH_2PO_4. The same volume of Milli-Q water was used to dilute the sodium phosphate and 0.15M NaCl was added. The pH was adjusted to 7 using the NaH_2PO_4. The buffer was then filtered with a vacuum filter using 0.22 µm GS filter (Millipore Corporation, MA, USA).

The samples were prepared at 10mg/mL using the buffer as a solvent. They were then filtered using a 5 mL syringe (BD, NJ, USA) with an attached 0.2 µm filter (Whatman, Puradisc^{TM} 25mm, PES filter, GE Healthcare UK Limited, Buckinghamshire, UK). An exception was made for the casein hydrolysate derived from α-chymotrypsin hydrolysis which used a 0.22 µm filter (Millex-Or, nylon, Millipore, Cork, Ireland) as sufficient quantities could not be filtered using filters with 0.2 µm pores.

The samples were loaded manually into the FPLC (GE AKTA system, GE Healthcare, Montreal, PQ) using a 1 mL syringe (BD, NJ, USA). The column used was Superdex Peptide12 10/300 GL 10x300 mm column (GE Healthcare, Montreal, PQ). The peptide elution was carried out at 25 ^{o}C using the 50mM phosphate buffer containing 0.15M NaCl at pH 7.0 at a flow rate of 0.5 mL/min. The eluted peptides were detected at 215 nm. The standards for the column were

prepared according to the Superdex Peptide 10/300 GL high performance column manual. The final standard solution contained: 1 mg/mL cytochrome C (12384 Da), 2 mg/mL aprotinin (6512 Da), 0.1 mg/mL vitamin B_{12} (1855 Da) and 7.8 mg/mL glycine (75 Da). The known molecular weight and elution volumes of the standard proteins were used to create a linear plot of log molecular weight versus elution volume; this relationship was used to calculate the molecular weight of the samples. The molecular weight standard curve and equation used to calculate the molecular weights are available in Appendix 1.

3.1.4 Coating Formulations

Coatings consisted of 20% (w/w) protein, 10% (w/w) CPP and 70% (v/w) glycerol. Glycerol was chosen due to its frequent use in CPP-containing oral products and the tendency of casein to solidify in water. Casein and whey were added in five different ratios of 0:100, 25:75, 50:50, 75:25 and 100:0 (casein:whey) to test how their interactions would alter stickiness. Whey was added as an intact protein and was sourced from Bulk Barn (Winnipeg, MB) and contained soy lecithin to improve solvency. Separate coatings were made for intact sodium caseinate (Sigma Chemical Co, MO, USA) and each type of casein hydrolysate (Alcalase (CN-A), trypsin (CN-T) and α-chymotrypsin (CN-C)).

The coatings were mixed in glass vials using a metal spatula and left for a week to ensure the proteins and CPP were hydrated and incorporated into the glycerol.

3.2 Measurement of Coating Stickiness and Functionality

3.2.1 Modulated Differential Scanning Calorimetry of Proteins and Hydrolysates

Sodium caseinate (Sigma Chemical Co, MO, USA), whey (Bulk Barn, Winnipeg, MB, Canada), CPPs (made in laboratory), casein hydrolysates (Alcalase, trypsin and α-chymotrypsin) and the coating samples were analyzed using modulated differential scanning calorimetry (MDSC) (TA Instruments, DE, USA) to determine their glass transition temperatures (T_g) which have been shown to indicate when stickiness occurs in powders and protein solutions of dairy origins (Chuy & Labuza, 1994). Moisture content was measured using freeze drying method (data not shown). The moisture content of the protein powders was approximately 9% and the coatings were approximately 11% prior to the addition of water or glycerol depending on the sample as mentioned below. The MDSC was calibrated using a sapphire. Large volume pans (TA Instruments, DE, USA) were used as the usual hermetic alodined aluminums pans frequently burst.

Two types of samples were prepared before being weighed into the pans for DSC or MDSC. The first type of samples were pure protein samples that were dissolved in water. Sodium caseinate was measured using a 15% solution of sodium caseinate in water as higher concentrations gelled immediately and were not transferable to the pans. Whey, casein hydrolysates and CPPs were measured at a 20% concentration of pure proteins in water. The second type of samples were the coatings. Coating samples each contained 20% total protein and 10% CPPs with the remainder being glycerol and measured as is without any addition of water (Fisher Scientific, NJ, USA). The difference between plasticiser and concentration of proteins makes the two sample sets incomparable.

Prior to MDSC, normal DSC was performed on the samples to aid in the determination of the temperature range and note the samples response to the differential scanning calorimetry treatment. The sample size was between 10 and 20 mg and was scanned using a 10 ^{o}C/min heating rate over a temperature range of -30 ^{o}C to 200 ^{o}C. This range was selected based on a paper by Mizuno *et al*. (1999) but the range was extended beyond 150 ^{o}C due to events occurring around this temperature; Kalichevsky *et al*. (1993) stated that the glass transition temperature of β-casein is 164 ^{o}C. An exothermic peak occurred between 0-20 ^{o}C for proteins dissolved in water obscuring the T_g of α-casein which is reportedly located between 0 and 30 ^{o}C (Mizuno *et al*., 1999).

For MDSC, the sample size was between 10 and 15 mg with a modulation amplitude of $\pm$ 1.272 ^{o}C, period of 60 s and a heating rate of 2 ^{o}C/min (Thomas, 2005). The pure protein samples The temperature range of -30 ^{o}C to 200 ^{o}C was used as determined by the DSC curves. After the samples were run, the T_gs of all samples were determined by the presence of an endothermic peak experienced by both non-reverse heat flow and reverse heat flow. Within the area of these peaks, the T_g was identified as a region that experienced a sharp slope between two plateaus and analyzed using the TA Universal Analysis 2000 program (TA Instruments, DE, USA) glass transition temperature feature. Example MDSC graphs demonstrating T_g for selected samples are available in Appendices 2 to 6.

Table 1. DSC and MDSC method parameters for pure proteins and coatings

		DSC			MDSC				
Samples	Concentration Used	Sample Size (mg)	Heating Rate (^{o}C/min)	Temperature Range (^{o}C)	Sample Size (mg)	Heating Rate (^{o}C/min)	Temperature Range (^{o}C)	Period (s)	Amplitude (^{o}C)
Sodium caseinate	15% in water	10-20	10	-30 to 200	10-15	2	-30 to 200	60	$\pm$ 1.272
Whey, CPP, casein hydrolysates	20% in water								
Glycerol	Pure								
Coatings	Pure								

3.2.2 Stickiness Measurement of Bioactive Peptide Containing Coatings

The method to measure stickiness in the coatings was adapted from the premade program "Comparison of Surface Stickiness and Stringiness of Syrup, Honey and Treacle" (TA Instruments, DE, USA). This program was chosen as the behaviour of the coatings was reminiscent of honey.

Stickiness was measured using a TA-XT-2 texture analyzer. A 38 mm diameter acrylic cylindrical probe was used so as to compress the sample without the sample escaping out of the sides and changing the perceived stickiness. Acrylic was chosen over stainless steel because acrylic is a common material in the fabrication of dentures. Therefore, presumably acrylic has similar properties to teeth making it more relevant to the study. A TA.XT-2 Plus texture analyzer and program were used (Stable Micro Systems, NY, USA). 0.500 g of a coating was put on a stainless steel platform and the probe was lowered to compress the sample to a 1 mm thickness. Weight was used rather than volume because the coatings were too viscous for a pipette. Each sample was examined for bubbles prior to testing as bubbles would result in erratic measurements and greater adhesion. After compression and testing each sample covered approximately the same surface area on the probe and platform. The coatings did not spread beyond the probe during testing.

The test began with the application of 6 g of force on the sample for 2 s, the probe was then raised and the tension measured. The pre-test speed and test speed were 1 mm/s with a post-test speed of 8 mm/s. The return distance was changed from 80 to 30 mm as the coatings

did not experience as much stringiness as honey, syrup and treacle making the extra 50 mm unnecessary. The tests were performed at 23 ^{o}C in the open atmosphere and after each test the probe and platform were cleaned with detergent and distilled water before being thoroughly dried.

Stickiness was measured based on the maximum peak tension force in grams of force experienced by the probe upon ascent.

3.2.3 Calcium Determination

The calcium content was measured for the primary material, whey, casein and bioactive peptides, the hydrolysates and the final coating solutions using the spectroscopic method from McDonagh and FitzGerald (1998) and an indicator solution based on a method from Pollard and Martin (1956). A dilute ammonia buffer, pH 10, was made from 5% (v/v) ammonia buffer (Fluka, Sigma-Aldrich, MO, USA), 95% (v/v) distilled water and 0.75% ammonium chloride (Fisher Scientific, NJ, USA). This buffer was used to create the o-cresolphthalein complexone indicator solution and calcium standards. The calcium standard was calcium chloride (Fisher Scientific, NJ, USA) rather than the calcium carbonate used by Pollard and Martin (1956) due to complications with clouding. The calcium chloride was dissolved in distilled water (0.11g per 100 mL) and diluted with the dilute ammonia buffer to generate a standard curve from 0 to 0.3 mg/mL calcium. Calcium was calculated using the standard curve where the equation of the line of best fit was used to convert the absorbance to mg calcium/mL and multiplied by the concentration of protein or coating being measured (25 mg/mL for proteins and coatings and 0.2 mg/mL for CPP). 40 µL were transferred to 10 mL capacity glass test tubes prior to the

addition of 4 mL of indicator. Samples were dissolved in the dilute ammonia buffer in polypropylene microcentrifuge tubes and 40µL were transferred to glass test tubes of 10 mL capacity. Due to variations in the calcium content, different sample sizes were used to keep the absorbance within the range of the standard curve. 25 mg of the powder samples, whey, sodium caseinate, and protein hydrolysates were dissolved in 500 µL dilute ammonia buffer to give 1 mg in 40 µL. The CPP required 0.2 mg dissolved in 1000 µL dilute ammonia buffer to give 0.008 mg in 40 µL. Coatings required 12.5 mg dissolved in 500 µL dilute ammonia buffer. The indicator solution must be made immediately before use otherwise it will degrade within 15 min. The indicator solution was made using 28% (v/v) dilute ammonia buffer, 72% (v/v) distilled water and 0.003% (w/v) o-cresolphthalein complexone (Acros Organics, NJ, USA). 4 mL of indicator was added individually to each test tube and shaken prior to measuring at 575 nm using an Ultrospec Pro 1100 UV/Vis spectrophotometer (Biochrom, England, Model# 80-2112-00) and polysterene cuvettes.

3.2.4 Hydroxyapatite (HAP) Disk Assay for Oral Bacteria

The adhesion of *Streptococci mutans* (*S. mutans*) was measured using a HAP disk assay for planktonic growth. This test was performed for only the stickiest and least sticky coatings and two controls, glycerol and water.

Fresh *S. mutans* were made from frozen glycerol stock (ATCC, ON, Canada) by streaking the frozen mixture onto two Todd Hewitt Yeast Extract (THYE) plates. The THYE plates were made by heating 3.64% (w/v) Todd Hewitt powder (Oxoid, Hampshire, England), 0.3% (w/v) yeast extract (Difco laboratories, MI, USA) and 1.6% (w/v) agar (Difco laboratories, MI, USA) in

1L distilled water while stirring. The mixture was then autoclaved at 115 ^{o}C for 20 min and left to cool slightly before pouring the plates. The plates used were 100 mm in diameter. After streaking, the plates were placed upside down in anaerobic bags (Biomérieux, Marcy L'Etoile, France) and incubated in an ISOTemp Incubator (200 series, Fisher Scientific, NJ, USA) at 37 ^{o}C for 2 days. To create an overnight planktonic inoculum, THYE + mucin broth was made by mixing 3.64% (w/v) Todd Hewitt powder, 0.3% (w/v) yeast extract and 0.01% (w/v) mucin (Oxoid, Hampshire, England) in 1L distilled water. The pH was adjusted to 7.0 using 1M HCl (Fisher Scientific, NJ, USA) and autoclaved for 15 min at 121 ^{o}C. 40 mL of THYE + mucin broth was added to a 50 mL tube which was then heavily inoculated with *S. mutans* taken from the THYE plates. The tube were placed upright in an anaerobic bag and left to incubate for 24 h at 37 ^{o}C.

While the stock was growing, the HAP disks (Maxill Infection Control, ON, Canada) were autoclaved, coated and dried. This was done with sterile tweezers to hold the disk and dip into the coating either water, glycerol, most or least sticky coating. The coating was checked for bubbles and only a thin coat was applied by letting excess coating drip off. Each disk was placed in a labelled 15 mL sterile culture tube that contained 2 mL of the planktonic inoculum that was incubated overnight. The tubes were incubated in the anaerobic sacs for 2 and 4 h at 37 ^{o}C.

After the time point was reached, the *S. mutans* inoculum was poured out keeping the disk within the tube. 2 mL of phosphate buffer saline (pH 7.0) was used to wash the disk three times before pouring out the HAP disk carefully into a clean plate. The disks were placed in new tubes with fresh 2 mL phosphate buffer saline and were sonicated in a Bransonic Ultrasonic

CPXH series bath (Branson Ultrasonics Corporation, CT, USA) for 30 s. The tubes were then vortexed (Fisher Vortex Genie 2, Fisher Scientific, NJ, USA) for a minute each before removing the HAP discs.

Dilutions of the final wash solution were prepared using a 96 well plate with 180µL phosphate buffer saline in all but the top row where 200µL of sample were placed. The dilutions were performed by taking 20 µL from the top row and adding it to the second mixing well. 20µL were taken from the second row and mixed into the third and so on until the last row where 20µL was taken and discarded.

Each well was plated in duplicate by pipetting 10µL onto the plate and letting it dry before incubating the plates upside down in the anaerobic bags for two days at 37^{o}C. The plates were then removed and the colonies counted. Bacteria colonies were converted into CFU, the higher the CFU found the more bacteria were adhering to the coatings.

3.3 Statistical Analysis

Statistical analysis was performed using SAS version 9.3 using PROC GLIMMIX and the test of significance was the Tukey test. The results are shown as the mean $\pm$ standard deviation and the probability value (P) of the significant difference was set to $P > 0.05$.

4.0 Results and Discussion

4.1 Coating Formulation

The objective of this experiment was to create a sticky coating containing bioactive peptides for oral use to reduce dental caries. Casein and whey were chosen as the protein component of the coatings for their inherent stickiness and ability to form viscoelastic films (Chen, 1995; Mounsey *et al.,* 2012). Casein was also subjected to hydrolysis as previous studies have found hydrolysis to increase the stickiness of proteins (Goel *et al.,* 1999; Mounsey *et al*., 2012). The coating formulation was chosen based on a current product on the market, a caseinophosphopeptide (CPP) containing toothpaste. The toothpaste contained 10% CPP and the main plasticiser was glycerol. Some water was present but when water was added to the sodium caseinate (NaC) a gel was formed immediately. As gelation was a major issue, four formulations were originally tested (not shown here) to determine which was stickier and resistant to gelation for all hydrolysates, NaC and whey, with CPP added. The stickiest formulation was 20% protein (w/v), 10% (w/v) CPP and 70% (v/w) glycerol which was capable of flow and could be used as a coating.

Glycerol is a good plasticiser for a product used in the oral cavity as it is an approved food ingredient and adds viscosity to the final product (Galietta *et al.,* 1998). Glycerol is hydrophilic and is capable of developing strong hydrogen bonds with proteins which should improve after casein hydrolysis making a stronger network hence a stickier coating. In water, NaC forms a brittle, weak film that would be undesirable for a sticky coating whereas glycerol has been shown to produce a more flexible film that is considerably less brittle (Chen, 1995;

Galietta *et al.,* 1998). The small size of the glycerol molecules improves their interaction with protein as they are small enough to fit between protein chains to interact with the reactive groups on the protein forming hydrogen bonds (Galietta *et al.,* 1998). Glycerol's interactions with NaC are non-specific but alter the structure in ways that alter the viscosity. This was found by Siew *et al*. (1999) who formulated coatings of NaC and glycerol to determine the film properties. Each coating contained 10% (w/w) NaC and 0.01 to 0.26 mol glycerol/100g solution with the remainder being distilled water. Each coating was analysed by FTIR spectroscopy to identify changes in structure. Three distinct changes were found with increasing glycerol content where initially NaC contained 63% unordered structures (random and turn conformations). At 250 to 570 mol glycerol-oxygen atoms/mol NaC the random coils and β sheets formed turns. The β-turns then became random coils as plasticiser increased from 600 to 850 mol glycerol-oxygen atoms/mol NaC. From 850 to 1025 mol glycerol-oxygen atoms/mol NaC the amount of β-sheets and β-turns increased. Each change in structure aligned with a discontinuous region of viscosity in the coatings where the trend of increasing viscosity with increasing glycerol concentration would plateau or drop before rising again. Siew *et al*. (1999) also studied the effects of increasing NaC concentration from 10% to almost 12% which had the same result as increasing the glycerol concentration to 1200 mol glycerol-oxygen atoms/mol NaC. Higher levels of protein would not be desirable in the context of an oral coating as this would increase the expense of the product while negatively affecting the mouth-feel, taste and tolerable thickness of the product. At 70% glycerol and 20% NaC, hydrolysates or whey with 10% CPP, similar structural changes as seen by Siew *et al*. (1999) would be expected but the

effect of the different hydrolysates on the final stickiness, to the author's knowledge, has not been studied.

4.2 Degree of Hydrolysis

Degree of hydrolysis (DH) was determined using the Adler-Nissen (1979) method where samples were incubated with TNBS. The TNBS reacts with the primary amino acids to produce a yellow pigment which becomes darker at higher levels of hydrolysis.

4.2.1 Comparison of Proteolytic Enzymes

Sodium caseinate (NaC) was hydrolyzed with three different enzymes to produce hydrolysates with different DH and peptides. The enzymes chosen were Alcalase, trypsin and α-chymotrypsin. The original objective was to reach 15% DH as Mounsey *et al.* (2012) found this to be the stickiest DH of hydrolysed casein. Being unable to reach 15%, the time and enzyme:substrate ratio were adjusted to achieve DH values as close to 15% as possible.

The DH and hydrolysis conditions are shown in Figure 1 and Table 1, respectively. It is clear that Alcalase is the most efficient at hydrolysing casein as it took only 15 min to reach a DH of 18.04%. Trypsin had the second highest DH at 13.63% but required 4 h of hydrolysis. The lowest was α-chymotrypsin at 11.40%, the maximum achieved for α-chymotrypsin; therefore hydrolysis was stopped at 2 h as 8 h showed no further hydrolysis.

Table 2. Enzymatic hydrolysis conditions of sodium caseinate hydrolysates. Results are expressed as the mean ± the standard deviation and like characters are not significantly different (P > 0.05).

Enzyme Type	Amount Enzyme	pH	Temperature (^{o}C)	Hydrolysis Time (min)
Alcalase	2% (v/w)	8	50	15
Trypsin	8% (w/w)	9	37	240
α-chymotrypsin	8% (w/w)	8	50	120

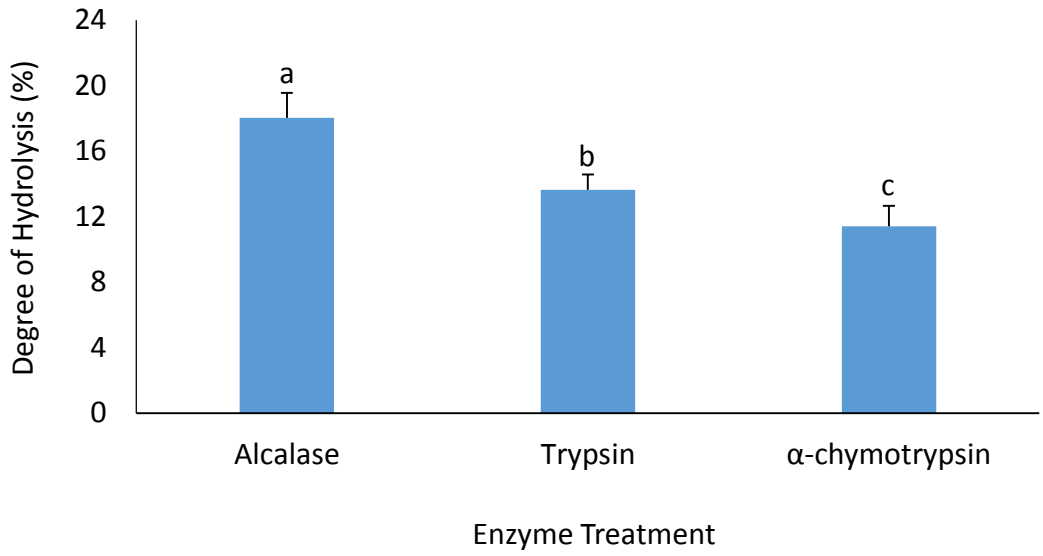

Figure 1. Degree of hydrolysis of sodium caseinate hydrolysates. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P > 0.05).

4.2.2 Hydrolysis of Sodium Caseinate

The variation in hydrolysis and DH achieved for NaC is due to the structure of NaC. Casein is a group of proteins that lack a definite tertiary structure in their natural state and are commonly referred to as flexible unfolded proteins. The unfolded structure is capable of interactions with minerals such as calcium as well as other caseins. When caseins interact with each other they form micelle-like structures which create a colloidal suspension due to steric hindrance (Vorob'ev, 2013). Hydrolyzing the micelle is a two-step process; the first step being demasking and the second hydrolysis. Demasking is the removal of steric obstacles from the casein that prevent enzymes from reaching the desired peptides buried within the protein's structure. The main masking factor for casein is the hydrophobic interactions originating from hydrophobic amino acids such as tryptophan, tyrosine, and phenylalanine. Alcalase and α-chymotrypsin are capable of hydrolyzing interactions involving these amino acids promoting faster demasking than trypsin (Kalyankar *et al.*, 2013; Vorob'ev, 2013). In turn, the speed at which NaC is hydrolysed by Alcalase and α-chymotrypsin is much greater than trypsin as seen by the hydrolysis time compared to the achieved DH (Table 2 and Figure 1).

4.2.2.1 Alcalase Hydrolysis

Casein hydrolysates from Alcalase hydrolysis (CN-A) had a significantly higher DH in much less time than hydrolysates obtained from trypsin (CN-T) and α-chymotrypsin (CN-C) hydrolysis (Figure 1) potentially due to demasking and the broad specificity of Alcalase (Zhao & Li, 2009). Alcalase lacks specificity as it is a mixture of different enzymes (Park & Allen, 1998). Alcalase is obtained from *Bacillus licheniformis* and is a protease preparation which contains subtilisin and glutamyl endopeptidase activity (Kalyankar *et al.,* 2013). Generally, Alcalase is

highly specific to aromatic amino acids and aliphatic amino acids (Zhao & Li, 2009). This is primarily due to glutamyl endopeptidase which is a serine protease, like trypsin and α-chymotrypsin. This particular enzyme cleaves after acidic residues, such as glutamic acid, aspartic acid, phenylalanine and alanine, on the C terminal side similar to what has been reported for trypsin and α-chymotrypsin (Kalyankar *et al*., 2013). Glutmayl endopeptidase, in low quantities, is specific as to which amino acid is at position P1 for cleavage to occur; hydrolysis will not take place if alanine, leucine, phenylalanine, tyrosine or lysine are at P1. This appears very limiting but nonspecific cleavage has been reported at concentrations above 1.2 μM glutamyl endopeptidase although this has been attributed to the presence of other enzymes such as chymosin and plasmin left behind after purification or attained by the addition of ribonuclease (Kalyankar *et al*., 2013).

The broad specificity of Alcalase led to CN-A having the highest DH at 18.04%; this was achieved in only 15 min. Alcalase and α-chymotrypsin share the ability to rapidly demask casein, unlike trypsin, due to their ability to cleave aromatic amino acid residues. Aromatic amino acids participate in hydrophobic interactions which mask a large quantity of amino acids. By cleaving at these sites, demasking occurs rapidly allowing for further hydrolysis by Alcalase and α-chymotrypsin (Mahmoud *et al.*, 1992).

4.2.2.2 α-chymotrypsin Hydrolysis

α-chymotrypsin should be the second most hydrolyzed due to this demasking ability and larger array of cleavage sites including aromatic amino acids, phenylalanine, tyrosine and tryptophan and occasionally methionine, leucine, glutamine and asparagine, compared to

trypsin (Vorob'ev, 2013), but was the least hydrolyzed. The greater number of cleavage sites enabled α-chymotrypsin to hydrolyze the sodium caseinate (NaC) faster than trypsin which required 4 h to reach only 13.63% DH and α-chymotrypsin required only 2 h to achieve 11.40% DH. By this reasoning, α-chymotrypsin should be capable of a much higher DH but the maximum reached was 11.40%. A possible explanation is the production of CPPs which have an inhibitory effect on α-chymotrypsin and trypsin. The effect on trypsin is very low but Ohtani *et al*. (2003) found the inhibition of α-chymotrypsin by CPPs was significant. The exact mechanism of inhibition was not discussed but was eluded to as being a competitive inhibition.

4.2.2.3 Trypsin Hydrolysis

The low DH reached by trypsin is due to the limited specificity for peptide cleavage sites which are restricted to the C-terminal end of only arginine and lysine. This explains the limited DH reached as for the average casein micelle, consisting of 37% α_{S1}-, 10% α_{S2}-, 35% β-, and 12% κ-casein, there are only 79 potential cleavage sites for trypsin and well over a hundred for α-chymotrypsin and Alcalase (Post *et al.,* 2012).

4.2.2.4 Comparison of Hydrolysis by Alcalase, α-chymotrypsin and Trypsin

The high degree of hydrolysis reached by Alcalase may also be related to the enzyme:substrate ratio. For trypsin and α-chymotrypsin the ratios were known (8% w/w) as they were powders; Alcalase was in liquid form with no indication as to the dosage in each millilitre. The enzyme activity noted could not be related or converted to that of the other enzymes and 2% v/w was decided upon based on a previous study (Otte *et al*., 1997) using the same product for a similar experiment. Time and the amount of enzymes were evaluated but

yielded very little difference in terms of the degree of hydrolysis (results not shown). The original target was to reach 15% DH as Mounsey *et al.* (2012) found this to be the stickiest level of hydrolysis in hydrolysed casein but this was not possible so conditions which produced DH values as close to 15% as possible were chosen. For Alcalase, the lowest value achievable was 18.04% DH and the highest value for trypsin and α-chymotrypsin were 13.63% and 11.40%, respectively. This created a range of DH to test the stickiness through glass transition temperature and probe-tack test, calcium binding and bacteria adhesion based on peptide size. Other studies managed to achieve higher DH values with trypsin and α-chymotrypsin, up to 20% in some cases, but differences in the casein's primary structure and composition (ratio of α_{S1}-, α_{S2}-, β- and κ-casein) influence the achievable DH. Structure and composition can vary depending on a number of factors such as cow breed, age and lactation cycle (Huppertz, 2013; Luo *et al.*, 2014). The hydrolysis conditions, including use of buffers and salts, also have a significant effect, particularly for demasking the protein. In this experiment, a buffer was not used, as pH was adjusted using only sodium hydroxide; this may have reduced the achieved DH. Even with buffers and salts, the time required to achieve the higher DH values in previous studies for trypsin and α-chymotrypsin were not suitable to produce a commercial product as hydrolysis would require more than 24 h to complete (Vorob'ev, 2013).

4.3 Evaluation of Proteins and Peptides by Fast Protein Liquid Chromatography (FPLC)

In general, it is expected that the peptide lengths, composition and charges will affect the resultant stickiness and interaction with the added bioactive peptides, CPPs. The length of peptides would be expected to impact the stickiness by varying the viscosity of the mixtures as longer chains would become entangled. A very high DH value would have smaller peptide

chains and therefore be less sticky (Mounsey *et al*., 2012). Since DH does not necessarily correlate to peptide length, fast protein liquid chromatography (FPLC) was used to determine the peptide length for each hydrolysate and protein. The peptides were separated based on size where higher molecular weight peptides eluted first followed by low molecular weight peptides. Absorbance as a function of elution volume has been plotted in Figure 2 and the molecular ranges calculated from the peaks are presented in Table 3. The elution volume was then converted to molecular weight by creating a linear plot of log molecular weight versus elution volume for the standards which covered a range of 75 to 12500 Da (Appendix 1).

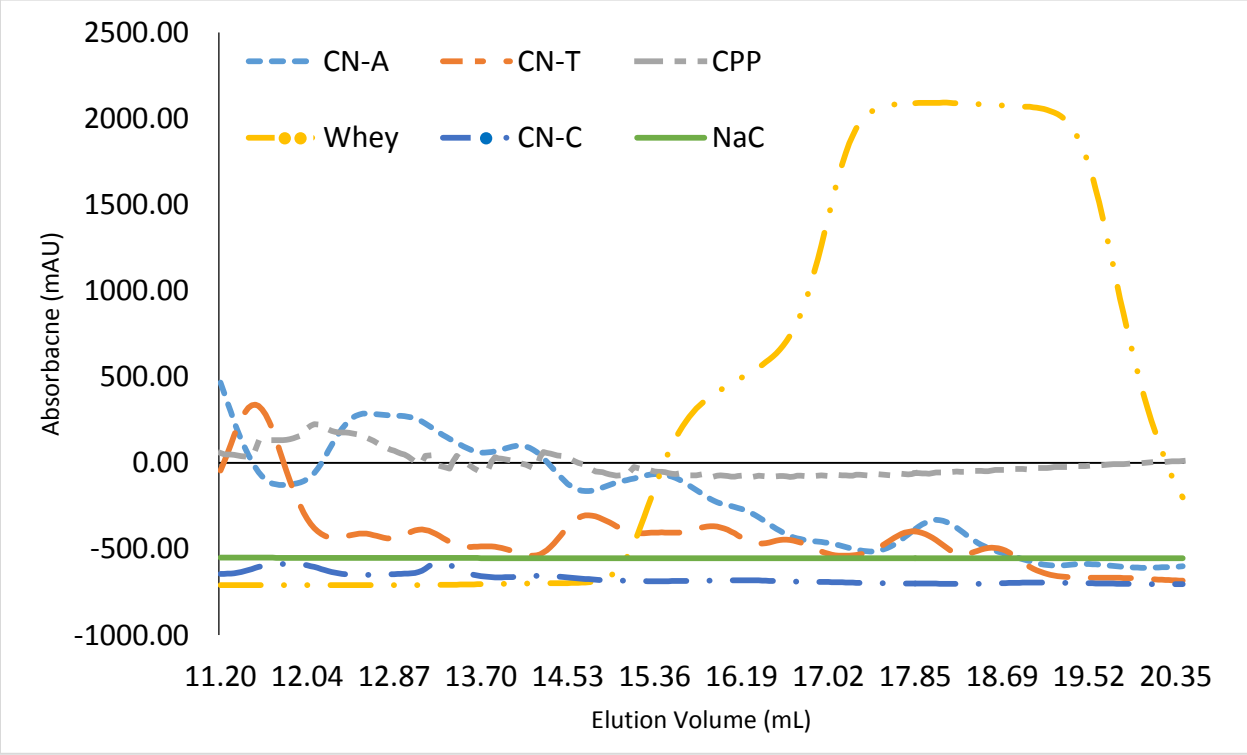

Figure 2. FPLC results for CN-A, CN-T, CN-C, CPP, NaC and whey between 75 and 12500 Da determined using a Superdex Peptide 10/300 GL column.

Table 3. Molecular weight ranges between 75 and 12500 Da of CN-A, CN-T, CN-C, CPP, NaC and whey from FPLC.

Protein Type	Molecular Weight Range			
	Peak 1	Peak 2	Peak 3	Peak 4
NaC	Out of Range			
Whey	81-1200			
CPP	76-89	1900-2300	2600-2900	3400-10500
CN-A	2200-7100	10700-12500		
CN-T	8900-12100			
CN-C	Out of Range			

4.3.1 Molecular Weights of Milk Proteins and Hydrolysates

As seen in Table 3, CN-A consisted mainly of low molecular weight peptides ranging from 2270 to 7110Da with a spike around 12100 Da. However there was absorbance beyond the range established by the standards used. If the same equation is used, CN-A can be extrapolated, to show material at 22700 Da which would represent either an intact casein fragment or the formation of aggregates. CN-T had the second highest molecular weight peptides with a peak at 10480 Da and a secondary extrapolated peak (not shown) which was much higher than CN-A but could not be accurately defined. Whey was surprisingly the smallest peptide, at ~250 Da, despite the fact that it was not expected to be hydrolyzed. As the whey used was a commercial variety and not laboratory grade, it may have been hydrolyzed or treated to improve solubility. CN-C and NaC were too large and eluted from the column well before the standard range and are therefore noted as being out of range in Table 3. The CPP produced a large variety of different molecular weights which was unexpected. Since the casein was hydrolyzed with Alcalase for 2.5 h, the CPP was expected to have the smallest peptides yet the range was from 2220 to 9920 Da. The cause of this phenomenon may be from self-association amongst the small peptides as well as clusters formed with the additional calcium. A

study by Cruz-Heurta *et al*. (2015) analyzed the molecular weight of various types of CPP using a RP HPLC and a MS/MS and found the weight range to be from 1220 to 3120 Da. This would explain the distinct peaks seen in Figure 2 which corresponded to the molecular weights of 2220, 2880 and 3530 Da, as reflected in Table 3, but the rest is unknown.

4.3.2 Relation between Peptide Length and Degree of Hydrolysis and Implications for Stickiness

From these results, with the exception of CPP, the DH appears to be closely related to the peptide length. It would therefore be expected that whey and CN-A would be the least sticky followed by CN-C, CN-T and NaC. Previous studies (Mounsey *et al.,* 2012; Netto *et al.,* 1998) have shown that NaC is not as sticky as its hydrolysates potentially due to steric hindrance preventing the caseins from interacting to form a cohesive network. Once hydrolyzed the steric hindrance may be reduced or removed entirely allowing for protein-protein interactions to take place (Mounsey *et al.,* 2012; Netto *et al.,* 1998). It is in this manner that the charge and composition of peptides would be expected to alter the rankings of stickiness a part from peptide length. The charge and composition dictate protein-protein, protein-CPP and protein-glycerol interactions changing the resultant network. For whey and glycerol interactions with NaC and the hydrolysates, surface hydrophobicity may have an effect. Hydrolysis of NaC has been shown to reduce the surface hydrophobicity (S_o) by generating charged peptides which are hydrophilic. In a study by Luo *et al.* (2014), it was found that casein hydrolyzed by trypsin had a significant drop in S_o from 546.3 to 351.6 as the DH increased from 16% to 20.68%, after which the DH values plateaued as did the S_o values.

In this experiment, it would be expected that Alcalase would have the lowest S_o because it had the highest DH. The hydrophilic CN-A should be more soluble in glycerol than CN-T and CN-C but CN-A's hydrophilicity may limit the protein-protein interactions as most of the available sites may be bound by glycerol. CN-T and CN-C may be stickier due to limited hydrolysis exposing hydrophobic regions which facilitate polypeptide associations, increasing viscosity and cohesion within the network (Townsend & Nakai, 1983). In terms of CPP addition, extensive hydrolysis reduces metal chelation which may increase the free calcium in the coating making it more available for use as a mineral reserve.

4.4 Glass Transition Temperatures

4.4.1 Glass Transitions of Pure Samples of Coating Ingredients

A common approach to determine structural changes that may influence stickiness in powders or edible films is through measurement of the glass transition temperature (T_g) (Chen, 1995). Stickiness in this context is defined by agglomeration or caking in powders and increased viscosity in edible films. T_g is the temperature at which a material transitions from a "glassy" state, where it is inert and resistant to flow, to a "rubbery" state, where flow and interactions are more likely to occur. Stickiness does not occur at this temperature but at a temperature above the T_g, denoted as T. Most commonly, the range between these temperatures is 10-20 ^oC and is referred to as the sticking point temperature, expressed as $T\text{-}T_g$ (Chen & Özkan, 2007; Mounsey *et al.,* 2012; Netto *et al.,* 1998). Ergo, for a sticky coating to exist at room temperature, a T_g below 20 ^oC is required. For this reason, Modulated Differential Scanning Calorimetry (MDSC) was used to determine the T_g of whey, NaC, CN-A, CN-T, CN-C, CPP, glycerol and each coating within the temperature range of -30 to 200 ^oC.

MDSC was chosen as it is more sensitive to small changes in heat flow experienced during a glass transition than conventional DSC. Although, to the author's knowledge, no literature exists on the T_g of whey, casein or glycerol as determined by MDSC, other methods such as TMA and DSC will be used for comparison, where applicable. T_g values from literature sources as well as the requirement of a sticky coating's T_g to reside below 20 ^oC were taken into account when selecting the temperature range of -30 to 200 ^oC. Most studies ran the samples twice to remove a peak that formed around 50-60 ^oC which was thought to be caused by the hysteresis effect of thermal relaxation of the protein (Ghanbarzadeh & Oromiehi, 2008; Mauer *et al.,* 2000; Netto *et al*., 1998). Unlike previous experiments, no peak was formed at 50-60 ^oC but a large endothermic peak was found in the pure protein samples at 0-20 ^oC (Figure 3). This peak impeded the identification of any T_g within this region. Running the samples twice saw no differences in the peaks or T_g found despite similar conditions. The samples that were run twice showed only a slight decrease in the peak's height and T_g continued to be masked. Adjustments of protein to solvent ratio, temperature range, sample size and running samples twice did not shift or reduce this peak. Interestingly, this peak, while present in pure protein samples, vanished in the coating samples. The peak was most likely the results of freezing and subsequent melting of water in the pure protein samples as water was present in the protein samples, but not the coating or pure glycerol (Figure 4). The transitions for pure CN-C and whey are shown in Appendices 2, 3 and 4 and Appendices 5 and 6, respectively to show the appearance of the transitions.

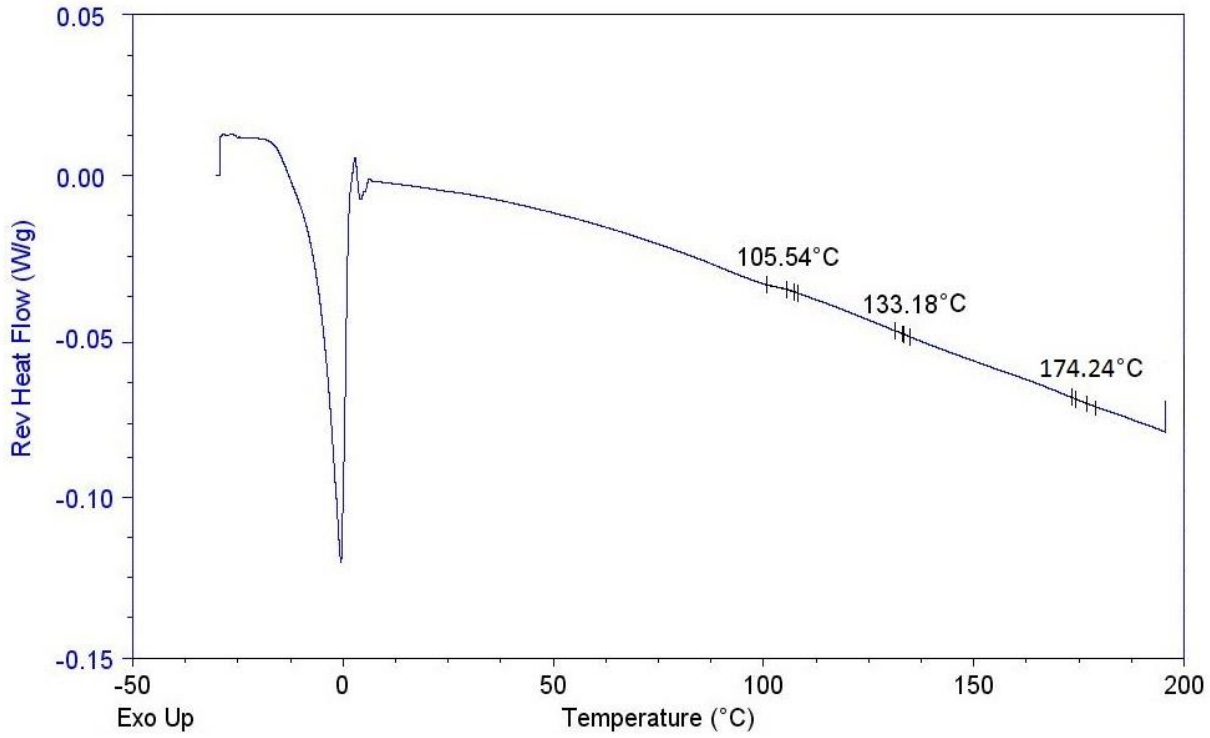

Figure 3. MDSC curve of 20% pure CN-C in water where transitions are indicated based on reverse heat flow.

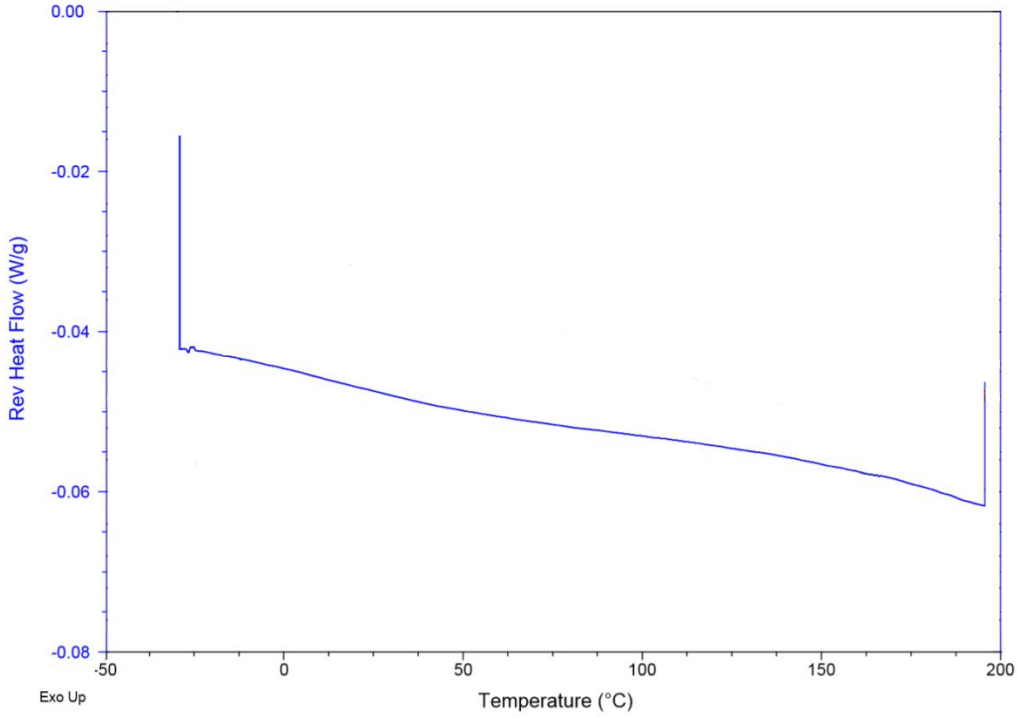

Figure 4. MDSC curve of 100% glycerol transitions not indicated.

4.4.1.1 Results for Glycerol

The only literature values found for the T_g of glycerol were -107.57 ^oC (Ghanbarzadeh & Oromiehi, 2008) and -83 ^oC (Noirez & Baroni, 2010), which were below the temperature range used in this experiment. These values make sense as glycerol is a liquid at room temperature placing glycerol in a rubbery state. The transition for glycerol that was produced consistently as seen by the MDSC was at 149.05^oC (Figure 5) which is considerably higher than the literature values. The closest value this transition could possibly be related to is the flash point or the boiling point of glycerol which are 160 and 182 ^oC, respectively (Sigma Aldrich, MO, USA). Although this phenomenon has not been mentioned in any previous literature the transition appeared too consistently in pure glycerol samples to be considered as an error.

4.4.1.2 Identification of Glass and Other Transitions

Other potential transitions observed for the coatings were also not consistent with literature values for glass transitions and it was decided they were not likely T_g but other phenomena caused by the structural alterations resulting from protein-protein and protein-glycerol interactions. These will be referred to as "transition phenomena" (T_p) as their cause can only be speculative. The pure protein samples of NaC, CN-A, CN-T, CN-C, CPP and whey did reveal any transitions that were similar to literature values but will be still be discussed in terms of T_p as the majority of results did not appear to be true tansitions.

4.4.1.3 Transitions for Sodium Caseinate

In Figure 5, the T_p presented for each protein sample and glycerol greatly varied and for whey, NaC and CN-C, sometimes more than one transition was found. As the proteins used are

made up of sub-units, it is possible that each subunit could have its own T_p. On this basis, hydrolysis would create more T_ps as the protein is broken down into smaller peptides; however, most peptides would not be present at great enough concentrations to produce a distinctive T_p even using the sensitive MDSC technique. Surprisingly, of the caseinates, only NaC and CN-C generated more than one T_p; it was expected NaC would have a single T_g as seen in literature. The two T_gs of NaC could be from interactions between different caseins such as α_{S1}-, α_{S2}-, β-, and κ-casein. The first T_p at 142.2 ^oC is similar to results found by Netto *et al*. (1998) who stated casein had a T_g of 140 ^oC determined using DSC with a heating rate of 10 ^oC/min. The second T_p at 162.38 ^oC is similar to the T_g of β-casein found by Mauer *et al*. (2000) of 164 ^oC using DSC at a heating rate of 5 ^oC/min. It is also possible that since NaC is casein with the colloidal calcium phosphate removed and replaced with sodium, this may have led to dissociation of the inner caseins resulting in two T_ps rather than one. β-casein represents on average 35% of the casein (Singh & Flanagan, 2005), a high enough concentration to result in a defined T_p. The highest quantity of casein in the micelle is α-casein (α_{S1} and α_{S2}), comprising 47% of the total protein. The first T_p of 142.2 ^oC, however, is not from α-casein according to results found in literature. Mizuno *et al*. (1999) found the T_g of α-casein to be within the range of 0 to 30 ^oC as the moisture content varied from 0 to 20%. Their result was found using DSC with a heating rate of 6 ^oC/min and a temperature range of -30 to 150 ^oC. If α-casein did have a peak in this range it would have occurred within the obscured range for the current study and was not recorded. Literature for the T_g of κ-casein could not be found by the author and is present in such a low quantity in NaC, approximately 10%; therefore a distinct T_g would not be expected. The first

transition could then be attributed to the interaction between all four caseins to produce 142.2 ^{o}C similar to the 140 ^{o}C found by Mauer *et al.* (2000) for complete casein.

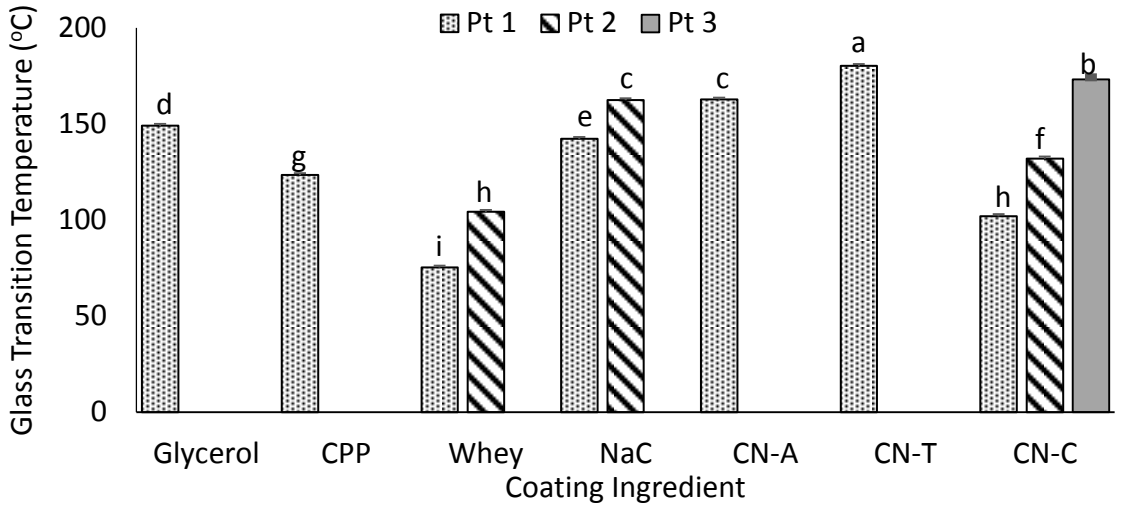

Figure 5. Glass transition temperatures of coating ingredients consisting of glycerol, CPP, whey, sodium caseinate (NaC) and its hydrolysates (CN-A, CN-T and CN-C). Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05). Error bars are present but too small to be seen.

4.4.1.4. Transitions for Casein Hydrolysates

The multiples T_gs for CN-C is understandable when compared to CN-A and CN-T which have only one T_g due to the lower DH of CN-C which yielded a smaller variety of peptides with higher molecular weights than the other hydrolysates. Although the exact quantity of peptides was not measured, enough of these peptides were present to create three distinct T_ps at 102.0 ^{o}C, 132.0 ^{o}C and 173.2 ^{o}C. Each T_g was significantly different from those found for the other hydrolysates and that obtained for NaC; this was expected as the materials were different. The second T_p of CN-C, 132.0 ^{o}C, was not significantly different from that for CPP at 123.5 ^{o}C. As previously mentioned, the limited hydrolysis of NaC by α-chymotrypsin was potentially due to the production of CPPs that inhibited further hydrolysis. While the similar T_p does not verify this

theory it is worth noting. The T_p may also be a coincidence from the coalescence of other peptides formed from α-chymotrypsin hydrolysis. SDS-PAGE or MS would be required to explore this further. T_gs for CN-T and CN-A were significantly different from each other and CPP as expected by their different molecular weights generated due to differences in cleavage sites.

Molecular weight has been found to be connected to the T_g of food materials where reduction in molecular weight results in a reduced T_g (Netto *et al*., 1998). Based on this theory, it follows that CN-A should have the lowest T_g followed by CN-T, CN-C and NaC, respectively. This was not found to be the case as the T_p of CN-A, 162.7 ^{o}C, was not significantly different from the highest T_p for NaC when it was expected to be below both 162.4 ^{o}C and 142.2 ^{o}C. The lowest T_p found was, in fact, from CN-C at 102.0 ^{o}C. The T_p of CN-T was significantly higher than both CN-A and CN-C and also significantly higher than NaC. A possible explanation is the peptides formed from hydrolysis with trypsin were interacting to form high molecular weight aggregates increasing the T_g, although this is unlikely as it is not supported by the FPLC results. It is most likely that, unlike NaC and whey, the T_gs found were not actually glass transitions. To the author's knowledge, a T_g for CPP has never been determined. Whether or not the T_g found is correct and resultant from the same peptides present in CN-C is unknown and requires further research.

4.4.1.5 Transitions for Whey

As mentioned above, the transition found for whey at 75.35 ^{o}C was similar to values found in the literature of 75 ^{o}C (Zhou & Labuza, 2007) and 76.17 ^{o}C (Ghanbarzadeh & Oromiehi, 2008) and was accepted as a T_g, but unlike previous studies a second transition was found at

104.2 ^{o}C. The second transition could be from the 5% soy lecithin in the whey. Since the whey used was a commercial product it possibly contained soy lecithin to improve the solubility of whey. The glass transition of soy lecithin could not be found to confirm this speculation. The transition could also be an event occurring due to water similar to the 0-20 ^{o}C peak, but instead of melting it represents the temperature where the water is entering the vapour state. As MDSC literature data for whey could not be found, the sensitivity may be high enough to find another T_g not previously attributed to whey when analyzed via the DSC method. A study by Galietta *et al*. (1998) of whey films found a similar T_g for a whey film consisting of 10% whey protein isolate in distilled water. The experiment was performed using Dynamic Mechanical Thermal Analysis (DMTA) with a heating rate of 3 ^{o}C/min and a temperature range of -20 to 220 ^{o}C. The whey protein isolate film produced a T_g ranging from 93 to 98 ^{o}C. This T_g is lower than the 104.2 ^{o}C found in the current study. However, Ghanbarzadeh & Oromiehi (2008) compared DSC to DMTA results for whey, glycerol and whey-glycerol coatings and concluded that DMTA produced T_gs that were close but lower than the DSC. For example, in the whey-glycerol coating, the T_g shifted from 43.65 ^{o}C as measured by DSC to 31.2 ^{o}C measured by DMTA. The authors concluded the difference came from the differences in sensitivity to the molecular mobility of the samples as DSC measures heating rate and DMTA uses frequency (Ghanbarzadeh & Oromiehi, 2008). Whether or not this second T_p is a glass transition is unknown and requires further research.

4.4.2 Glass Transitions of Coatings

The addition of plasticisers alters the T_g due to changes in molecular interactions; the addition of low molecular weight compounds, the plasticizer, causes a drop in combined T_g. For

the sake of this experiment, glycerol will be referred to as a plasticizer as it forms the solvent phase of the coating in which the proteins are incorporated.

4.4.2.1 Glass transitions in a Glycerol CPP Mixture

Since glycerol is hydrophilic and small enough to reach the backbone of the protein, it is able to interact and form a network. The protein-glycerol interactions are expected to increase with hydrolysis as the peptides become more hydrophilic (Luo *et al*., 2014). CPPs were the smallest of proteins being added to the coatings; therefore they were expected to interact with glycerol the most. For this reason, a control coating was made of 90% glycerol (v/w) to 10% CPP (w/v) to determine if a complex was formed that created a prominent T_g in the coatings. Two glass transitions were found for this control coating at 100.3 ^{o}C and 161.7 ^{o}C. The first glass transition could be from plasticization depressing the T_p of CPP from 123.46 ^{o}C to 100.3 ^{o}C. The second transition occurred at a temperature significantly higher than the T_ps of CPP and glycerol. Based on the ingredients present, this transition could not be explained but it appeared in other coatings when the ratio of casein:whey was between 0:100 to 50:50 (Figures 4, 5, 6 and 7).

4.4.2.2 Coating with Whey Only

The 0:100 casein:whey coating has a similar phenomenon to that seen with glycerol and CPP, where the T_p exceeded the expected values for whey, glycerol and CPP. This T_p, at 178.4^{o}C, was significantly higher than the control and has not been reported in previous research. As mentioned, it was expected for the T_g to be depressed by the addition of glycerol and CPP making these T_gs unprecedented and most likely not a T_g but an unexplainable T_p. As these high

T_ps appear only if glycerol is present it is possible that at high temperatures the bonds between the glycerol and proteins are being broken and glycerol separates. If the T_p is, in fact, indicating that the glycerol is entering a gaseous state, the proteins appear to be altering when this occurs. Otherwise these T_ps must be considered as unknown events caused by rearrangement of the molecular structure forming new complexes. More research is required to determine the exact interactions between the proteins and glycerol in the coatings; this could be done using either scanning electron microscopy or FTIR.

4.4.2.3 Coatings with Caseinate, Caseinate Hydrolysates and Whey

Compared to the pure proteins, lower transitions were found for all coatings; this was most likely from the dairy protein being plasticized by the glycerol and can therefore be considered a T_g. The T_g depression in this experiment was not as large as that seen in a study by Fabra *et al*. (2010) where glycerol incorporation into NaC reduced the T_g to 59.7 ^{o}C from 112 ^{o}C but this was measured using DSC, making direct comparison difficult. Since only the lowest transitions found were considered to be T_g, only these values will be discussed further.

A pattern emerged for most of the coatings, as seen in Figure 6, 7, 8 and 9, in relation to the effect of the ratio of casein to whey on T_p. The lowest T_p for each coating occurred at a 50:50 casein:whey ratio. The addition of any casein hydrolysate (25:75 casein:whey) caused a decrease in the T_p (Figures 4, 5 and 6) compared to the 100% whey coating. NaC (Figure 9) has the opposite effect with a significant increase in the T_p for the 25:75 ratio.

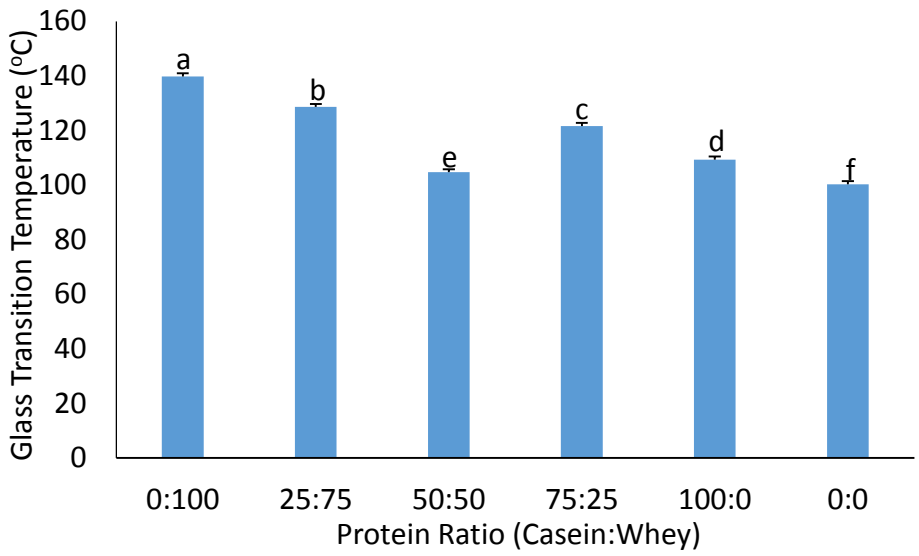

Figure 6. Glass transition temperatures coatings containing 20% dairy proteins with a ratio CN-A:whey from 0:100 to 100:0 including a control coating without dairy proteins. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05). Error bars are present but too small to be seen.

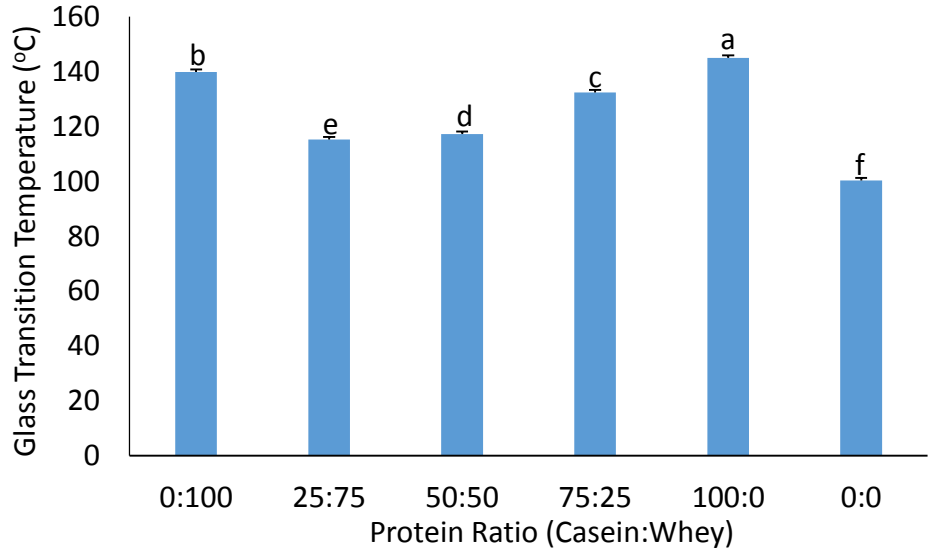

Figure 7. Glass transition temperatures (°C) of CN-T coatings comprised of 70% glycerol, 20% dairy protein (CN-T:whey) and 10% CPP compared to the control coating (90% glycerol 10% CPP). Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05). Error bars are present but too small to be seen.

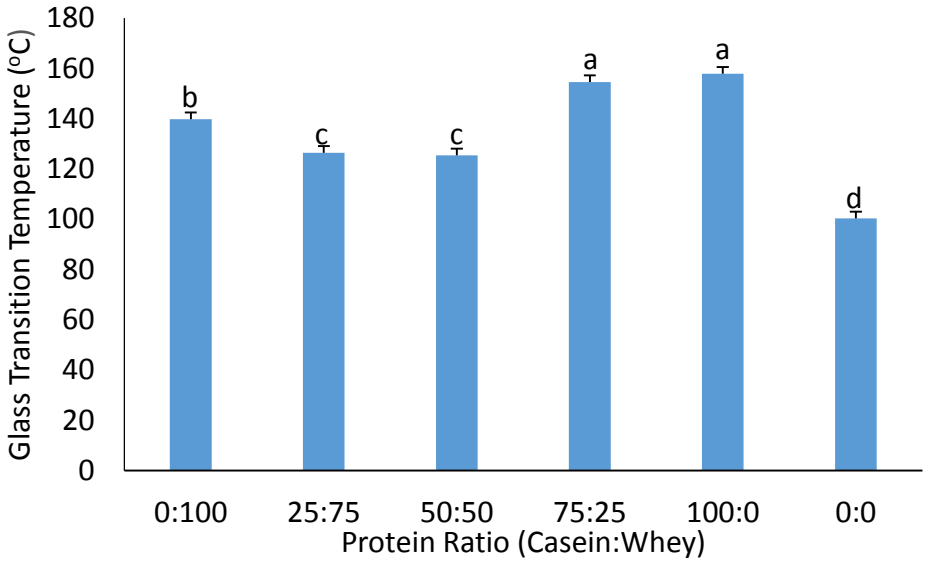

Figure 8. Glass transition temperatures (°C) of CN-C coatings comprised of 70% glycerol, 20% dairy protein (CN-C:whey) and 10% CPP compared to the control coating (90% glycerol 10% CPP). Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05). Error bars are present but too small to be seen.

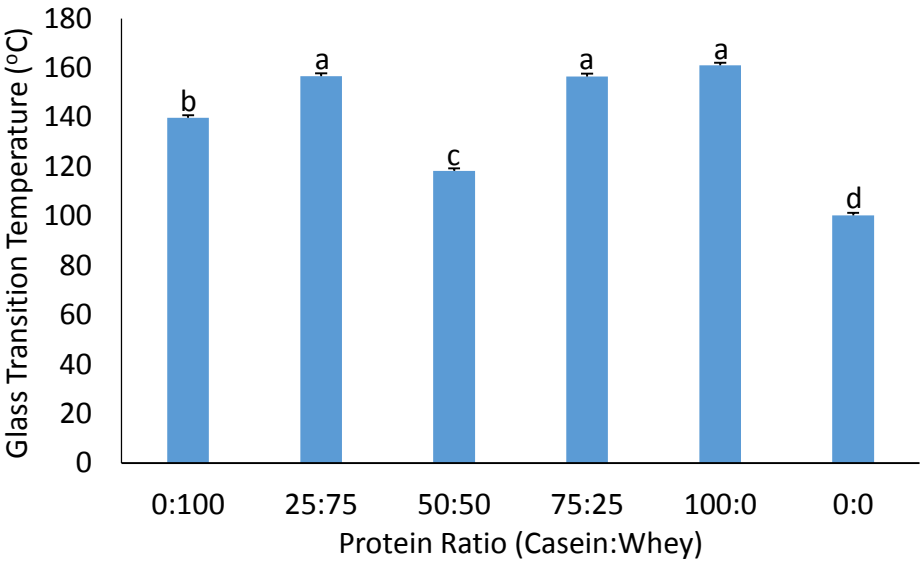

Figure 9. Glass transition temperatures (°C), measured by MDSC, of NaC coatings comprised of 70% glycerol, 20% dairy protein (NaC:whey) and 10% CPP compared to the control coating (90% glycerol 10% CPP). Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05). Error bars are present but too small to be seen.

For the CN-C and CN-T, there was a pattern of increasing T_p as the level of casein hydrolysate increased. A similar trend was seen for NaC between 50:50 and 100:0 casein:whey ratios. This demonstrated that there were changes in the protein-protein interactions between casein and whey as the ratio was shifted. Glycerol is known to disrupt protein-protein interactions through the formation of hydrogen bonds with the reactive sites of proteins. This disruptive bonding would be expected to be greater in the hydrolysates as the demasking of casein would expose more reactive sites (Siew *et al.,* 1999). As glycerol is at a constant percentage in the coatings, the alteration in T_p is more likely attributable to interactions between whey and casein. Whey and casein are known for their ability to form films and gels when introduced into an aqueous environment (Lacroix & Cooksey, 2005; Vasbinder & de Kruif, 2003).

As NaC is not in a micellar form, aggregation of whey and casein can occur through hydrophobic interactions, between α_{S1}- and β-casein and whey, resulting in small aggregates (Sakuno *et al*., 2011). Whey is attracted to highly charged molecules and although κ-casein is the most hydrophobic of the caseins, it is glycosylated with charged glycomacropeptides which preferentially interact creating aggregates separate from the other caseins. Unlike the other caseins, κ-casein is capable of forming soluble complexes with whey primarily through disulphide linkages which are crucial to gel formation but require heat to expose the active thiol group in whey (Donato *et al.*, 2007). The variation in T_p was potentially caused by the formation of these aggregates which would vary in size and bond strength as the casein:whey protein

ratio was altered. Since MDSC utilizes heat to measure the glass transition, disulphide bonds could be formed.

4.4.2.3 Influence of Protein Aggregation on Glass Transition

Larger molecular weight compounds are expected to undergo a glass transition at a higher temperature than low molecular weight compounds; therefore, alteration in aggregate size could alter the T_g. A study by Guyomarc'h *et al.* (2009) found that increased concentrations of NaC added to whey produced smaller aggregates. The aggregate size reduction was from the increased charge density on the surface of the whey once casein bound, causing steric repulsion similar to that seen in casein micelles. This reduction in the molecular weight per aggregate would be expected to result in depression of the T_g. With the exception of NaC, this appeared to be the case for ratios of 25:75 and 50:50 casein:whey; at higher casein to whey ratios the T_p increased. With NaC there was an increase in T_p seen at a ratio of 25:75 casein to whey which could have been due to the large molecular weight of NaC in comparison to whey, which was the lowest. The hydrolysates were also larger than whey, but, with the exception of CN-C whose weight was closer to that of NaC, the difference in size was not as large as that seen with NaC. The depressed T_p with the hydrolysates could result from the hydrolysates dismantling existing whey aggregates to form smaller casein-whey aggregates. The various charges and reactive groups on the hydrolysates could also contribute to the varied response. Rather than using hydrophobic interactions to reduce the aggregate size, large aggregates could be formed via protein-protein interactions or even calcium bridges from the excess calcium present in the CPP (Sakuno *et al*., 2011). Little research is available as to the interaction of casein and whey in a glycerol environment or the effect of this interaction on the observed T_p.

4.4.2.4 Value of Glass Transitions in Relation to Stickiness

The lack of MDSC data for milk proteins and glycerol hinders the interpretation of the results and comparisons between literature sources. DSC was attempted (no data shown) but it was not sensitive enough to detect any glass transitions in the proteins, hydrolysates or coatings. All considered, the measurement of the T_g was not helpful in determining the stickiness of the coatings. Most of the transitions found could not be considered glass transitions and were therefore labelled as T_p, and the general pattern seen in the coatings with changing casein:whey ratios remained inadequately explained. If the T_ps found were, in fact, glass transitions the coatings would not be sticky at room temperature as all were above 100 ^oC except whey which had a T_g of 75.35 ^oC.

4.5 Probe-Tack Test: Stickiness

Stickiness measured by a probe-tack test has a different definition than stickiness measured by MDSC. Rather than defining stickiness by a region beyond the glass transition, in a probe-tack test stickiness refers to the food material undergoing cohesive failure. Cohesive failure occurs when the cohesive strength is weaker than the adhesive strength resulting in the elongation of the food material as the probe is lifted. Once the force of the probe removal overcomes the cohesive strength, the food material divides leaving residue on the platform and on the probe (Gay & Leibler, 1999). Cohesive strength refers to the bonds and interactions between components within the coating holding it together (Hoseney & Smewing, 1999). A material with strong cohesion does not experience as much extension as a material with weak cohesion and requires more force to cause cohesive failure. Adhesive strength refers to the bonds and interactions between the coating and another material such as the probe (Werner *et*

al., 2007). For a sticky coating, the adhesive strength must be greater than the cohesive strength to remain attached to the probe. All of the coatings experienced cohesive failure and the magnitude of stickiness varied with both the ratio and the hydrolysate. The magnitude of stickiness was taken as the maximum force (N) achieved prior to cohesive failure. The larger the magnitude, the more likely that the coating will adhere to the teeth yet have enough cohesion to create a coating. Adhesive failure, separation from the probe, was not witnessed for any of the coatings. The coatings were checked for bubbles prior to the application of the probe to ensure maximum contact area was attained for maximum adhesion; no inconsistencies in structure were present so maximum cohesion was achieved. Each coating left a thin layer on the probe without any gaps or portions missing, indicating only cohesive failure occurred.

The probe-tack test used on the coatings was performed at room temperature and open to the atmosphere. Probe materials are crucial to the test method as factors such as surface roughness and hydrophobicity alter the response of materials as it changes their bonds and interactions (Adhikari *et al*., 2007b). An acrylic cylinder probe was selected as acrylics are commonly used in dentures making them the most tooth-like material available as a probe. The method chosen to measure stickiness was for testing stickiness and stringiness in honey and syrups as it involved similar parameters required to test a sticky coating. Most foods respond like pressure sensitive adhesives where increased pressure and contact time increases the magnitude of stickiness. The length of time that the materials remain in contact also affects bond formation and consequent stickiness as longer periods of time would allow for more bond formation thereby increasing the adhesive strength (Ben-Zion & Nussionvitch, 2002). The honey and syrup method used had a probe pressure of 6g applied to the coating and held for 2 s

before retracting at a speed of 8 mm/s. The time period and pressure are small to mimic the expected consumer behaviour. The coatings potentially would be stickier with a longer contact time and higher pressure but these would be unrealistic. For consumer use, the coating would be applied with a finger or brush in a slow painting stroke rather than pressed on and held. The probe was applied to the coating to reach 1 mm thickness prior to force being applied to the probe; this was done to mimic the thin layer of coating that would be needed to achieve maximum contact area. Probe removal resembled the lifting of a finger or brush during application to determine whether the coating would adhere firmly to the teeth (probe) while separating smoothly from the application device (platform) without the production of long strings or clumps.

The measure of stringiness, which is built into the test method, was not used to evaluate the stringiness of the coatings as the inhomogeneous nature of the coatings impeded precise measurements of the strings compared to homogenous syrup and honey. The coatings also had stronger cohesive forces than honey or syrup hindering string measurement. The stringiness was therefore based on the maximum height associated with the maximum force. The strong cohesion caused the probe to jump up once the coatings failed due to the sudden release of tension. This created a wide array of string measurement with coefficients of variation upwards of 80%. Based on visual assessment, stickier coatings produced smaller fibrils/strings upon probe removal as the force required to separate the probe from the coating exceeded cohesive forces before extension could occur. Coatings with weaker cohesive strength experienced greater extension resulting in necking, where the amount of material in the middle of the string was less than at the probe or platform (Gay & Leibler, 1999). Eventually

a new surface formed in the middle of the string causing cohesive failure at a lower force than for coatings with strong cohesion.

4.5.1 Stickiness of Coatings Containing Whey, Sodium Caseinate or Hydrolysates and Caseinophosphopeptides.

To the author's knowledge, research has not been performed on the stickiness of coatings containing the combination of whey, NaC, casein hydrolysates, bioactive peptides and glycerol. Due to the influx of natural coatings research, some studies have been performed on glycerol coatings containing NaC and whey as well as whey and NaC films created with water but combinations of the dairy proteins has only been found for gels. To explain the cohesion and stickiness of the coatings, these studies on gels were used, taking into account that the interactions between whey and NaC may vary from their behaviour in a heat or acid-induced gel system. Overall, CN-C coatings were the stickiest followed by CN-T, NaC and CN-A. Coatings containing 100% whey for the protein fraction were the least sticky even compared to the control coating which contained only 90% glycerol (v/w) and 10% CPP (w/v) and produced a stickiness value of 5.23 N (Figure 10). The addition of CN-C caused an unprecedented jump in stickiness compared to the other materials; stickiness values for CN-C were consistently above 20 N, with other materials had values below 10 N.

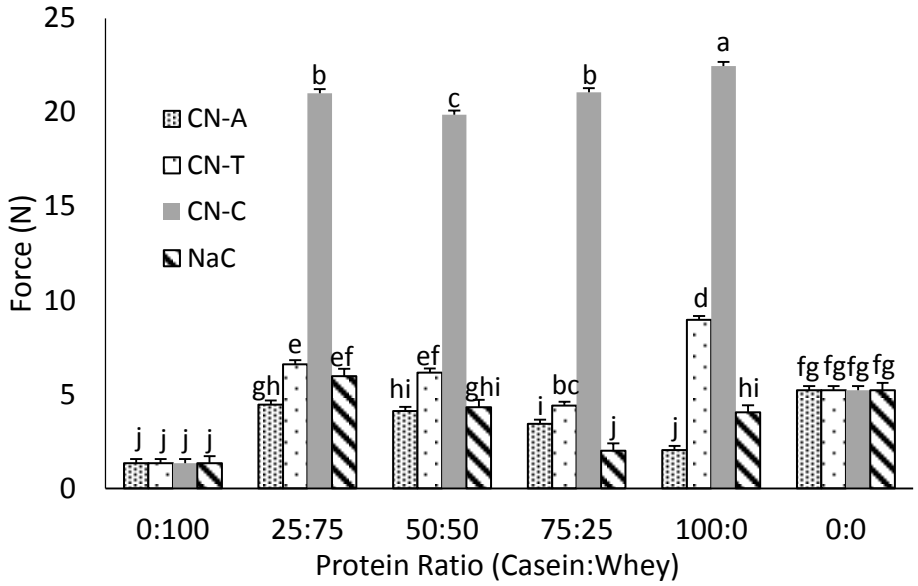

Figure 10. Stickiness of coatings containing 20% dairy protein with different ratios of casein to whey. Casein is either sodium caseinate, CN-A, CN-T or CN-C. A control coating, 0:0, without dairy proteins is included. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different.

4.5.1.1 Stickiness of Coating Containing Only Whey

The coating containing only whey as its dairy protein content was originally hypothesized to be similar in stickiness to NaC as it was thought to be unhydrolysed. The low molecular weights found using FPLC suggest the whey was broken down into very small peptides averaging 250 Da; this could be a mixture of amino acids and di- and tri-peptides. To a certain extent, hydrolysis of whey, like NaC, increases its ability to form bonds. The strongest bonds possible for whey to form are disulphide bridges, linkages arising from exposure of reactive thiol groups (Vasbinder *et al*., 2004). Without denaturation, whey is capable of gelation near its isoelectric point or in solutions of high ionic strength. The resultant gel is weak and compact with much smaller pores than when hydrolysed or heated due to the inability to form disulphide bridges (Rabiey & Britten, 2009). Whey may gel within the coating systems due to the presence of the CPP which contain high levels of calcium phosphate but the gel would

remain weak and have weak cohesive strength compared to the coatings with casein or hydrolysates incorporated. The behaviour of whey in this study was not as expected and appeared to be similar to that for CN-A due to the similarities in their peptide sizes which reduced their network forming abilities and allowed them to interact more with glycerol than CPPs (Adhikari *et al*., 2007b).

This reduction in cohesion for whey could explain why the 0:100 coating was the least sticky even compared to the control coating. The control coating was expected to be the weakest as it contained more plasticiser than the other coatings and felt much thinner. The CPP was not expected to develop strong bonds within the glycerol as it is a small peptide, from 1200 to 3000 Da (Cruz-Huerta *et al.,* 2015), with most of its reactive groups used to hold the calcium phosphate. The molecular weights found by FPLC, however, demonstrated that CPP was capable of forming complexes with molecular weights above 12500 Da which were greater than those found for whey (250 Da). The high level of hydrolysis required to form CPP could have contributed to the cohesion seen within the control coating as the smaller peptides could be more hydrophilic and capable of forming hydrogen bonds with the glycerol. This, in combination with the complexes formed between the CPPs, most likely through calcium bridging, produce stronger cohesion than seen in whey or CN-A.

4.5.1.2 Stickiness with the Addition of Sodium Caseinate

Stickiness in protein solutions is commonly attributed to the change in viscosity caused by peptide chain entanglement or development of bonds such as hydrogen bonds and disulphide linkages (Siew *et al.,* 1999). Entanglement and bond formation increase the cohesive

strength within the coatings creating a stickier coating. The length of the peptide chains is directly linked to the DH of the protein; longer chains entangle more, thereby hindering the coating's ability to flow. The formation of bonds between the different peptides produced by hydrolysis with whey, CPP or glycerol arise from the reactive groups that are exposed during hydrolysis and these vary due to the amphipathic nature of proteins. Unhydrolysed proteins, such as NaC, retain their hydrophobic regions which remain hidden in the interior of the protein. Hydrolysis exposes these reactive groups and enables other protein chains to interact with the groups to form networks with greater cohesive strength than before hydrolysis (Pérez-Gago, 2011). Based on these parameters, it would be expected that the NaC would be the least sticky as most of the reactive groups would be masked despite NaC having the longest peptides. While the stickiness of the NaC sample without whey (ratio 100:0) was lower than for the CN-T and CN-C hydrolysate it was higer than that obtained for CN-A (Figure 10). Only at a ratio of 75:25 (casein:whey) did NaC exhibit the lowest stickiness values. NaC could potentially form micelles similar to those found in natural bovine milk due to the addition of CPPs. NaC, without CPPs would unlikely be present as micelles as it is made by removing the colloidal calcium phosphate from the micellar structure and replacing it with sodium. The colloidal calcium phosphate, according to the Holt model of the casein micelle, provides stabilizing points within the structure to connect different caseins (Holt, 2004). The removal of calcium phosphate and introduction of sodium during NaC production also collapses the micellar structure and releases individual caseins. The loss of structure enables NaC to participate in hydrophobic interactions with other caseins or whey or to generate hydrogen bonds with glycerol, thus forming a network. In this manner, NaC is capable of producing a sticky coating but the stickiness would

be less than its hydrolysate counterparts as not all hydrophobic regions or reactive groups are exposed. As noted above, this was the case for some conditions but not all. The concealment of these groups by the long peptide strands limits entanglement and availability of reactive sites for protein-protein and protein-solvent interactions reducing the cohesive strength and therefore the stickiness (Huppertz, 2013; Singh & Flanagan, 2005).

4.5.1.3 Stickiness of Coatings Made with Casein Hydrolysates

Hydrolysis of NaC produced smaller peptides, which were expected to be more reactive and form coatings with stronger cohesion. This was true to a certain extent, as seen in Figure 10, where NaC coatings at almost all ratios were less sticky than the hydrolysates; the exception was for CN-A which had similar or lower values. The highest stickiness values were for CN-C at all casein:whey ratios. CN-A, unlike the other coatings, continually decreased in stickiness with increasing amounts of CN-A (Figure 11). The high DH of CN-A produced very small peptides that were incapable of entanglement generating a more liquid coating than the other hydrolysates. The reactive groups exposed were insufficient to produce strong cohesion due to instability of the hydrolysate. Unlike the other protein materials, CN-A did not remain in solution in water or in the coating. If left for more than an hour, the CN-A would precipitate on the bottom of the container. Its inability to remain in solution was due to the high level of hydrolysis which produced peptides that are incapable of steric hindrance. Excessive hydrolysis with the incorporation of high levels of calcium from the CPPs appeared to have produced precipitates rather than soluble aggregates of protein. Alternatively, increased hydrolysis can create more hydrophilic peptides which would form hydrogen bonds with glycerol, producing a more fluid coating; this is a possibility as soluble aggregates and complexes between CN-A and whey are

not known to form nor does self-association occur (Galietta *et al*., 1998). The increased interactions with glycerol would cause an increase in elongation during the probe-tack test due to further reduce cohesion (Siew *et al*., 1999). Although not measured, the control coating and CN-A coatings were observed to have the longest fibrils leading to a high degree of necking and cohesive failure when little force was applied to separate the probe and platform.

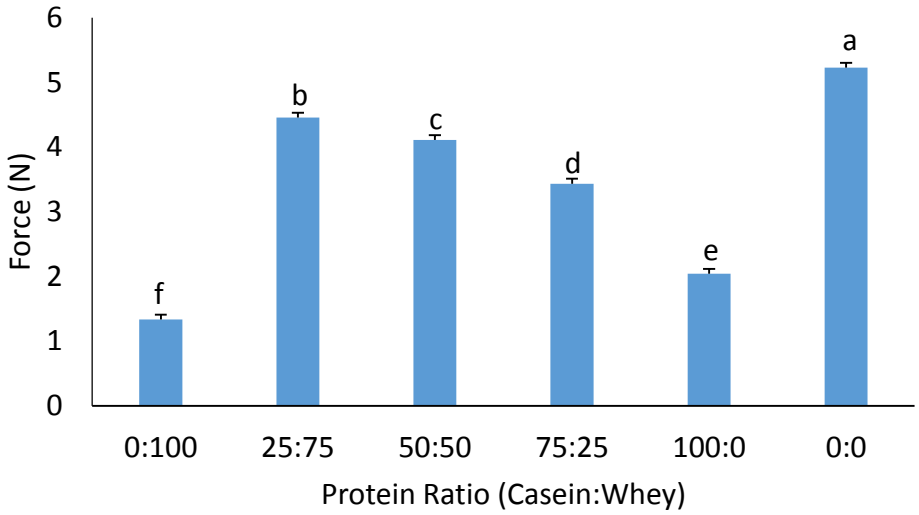

Figure 11. Stickiness of coatings containing 20% dairy proteins (w/v) with a ratio CN-A:whey from 0:100 to 100:0 including a control coating without dairy proteins. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05).

A general trend was present for all casein products where the initial addition of NaC or hydrolysates (25:75) increased the stickiness which subsequently decreased at higher levels of substitution but experienced a significant increase at a casein:whey ratio of 100:0 (Figure 10). The only exception to this trend was from CN-A which experienced a drop in stickiness at 100:0. As noted above, the all whey coating (0:100) was the least sticky. For whey to strengthen a network more than 11% of the solution must be whey and preferable it should be denatured to expose the reactive thiol groups that instigate the formation of disulphide bridges (Vasbinder *et*

al., 2004). This increase in stickiness as the amount of whey decreased signified that casein was contributing more to stickiness than whey; higher stickiness values for coatings containing NaC or hydrolysates and the control coating were observed. Casein is naturally stickier than whey due to structural differences. Casein is an unfolded protein that is highly flexible whereas whey (unhydrolyzed) are compact globular proteins which limits its ability to tangle with other peptides and readily react with ions and other proteins. Even though the whey had been denatured, the peptides that were present were too small to entangle or form a strong cohesive network (Singh & Flanagan, 2005).

4.5.1.4 Role of Casein Whey Interactions in Stickiness

The lower stickiness values at intermediate casein or hydrolysate levels was caused by protein-protein interactions. To explain the interaction between whey and casein, the general structure of casein in milk must be reviewed. Casein naturally exists as a micelle composed of the four main proteins: α_{S1}-, α_{S2}-, β- and κ-casein. α_{S1}-, α_{S2}- and β-casein form a network inside the micelle using colloidal calcium phosphate as connectors between certain protein linkages. The κ-casein is on the exterior as a "hairy layer" which extends into the aqueous phase. κ-casein is the only glycosylated casein making it highly charged which enables it to maintain the colloidal suspension of casein micelles through steric repulsion (Holt, 2004; Guyomarc'h *et al*., 2009). In milk, whey exists in two forms: whey aggregates and a coating on the casein micelles. These forms arise from whey's ability to bind to highly charged κ-casein as well as participate in hydrophobic interactions with the other caseins. In the coatings, the cohesive forces contributed by whey would consist of similar interactions and the stickiness would vary depending on the quantity of whey and its ability to interact with the other coatings materials.

If the whey is denatured, disulphide bridges are also possible as κ-casein contains cysteine (Chen, 1995; Guyomarc'h *et al.,* 2009). Similar interactions have been seen between whey and casein in gels and solutions (Chen, 1995; Guyomarc'h *et al.,* 2009; Vasbinder & de Kruif, 2003; Vasbinder *et al*., 2004). Guyomarc'h *et al*. (2009) conducted a study on the size of aggregates formed between NaC, κ-casein and whey. It was found that increased concentrations of NaC and κ-casein reduced the size of whey aggregates and prevented gelation. κ-casein had a more profound effect on whey gelation than NaC most likely due to its highly charged state and cysteine groups enabling stronger steric repulsion which prevented the whey aggregates from interacting to form a gel matrix. The formation of these small aggregates in the coatings might be the cause of the steady decrease in stickiness with the addition of more casein.

The whey in the coatings appeared to have been hydrolyzed and this may have hindered network formation by binding to necessary branching sites on the casein as the peptides were small and mobile. The prevention of network formation would produce a more fluid, liquid-like coating with poor cohesion. The effect of casein addition on stickiness was more profound for NaC than the hydrolysates, as seen in Figure 12, where the decrease in stickiness from the 25:75 to the 75:25 casein:whey ratio was much greater than for other materials. Since NaC is intact, the individual caseins have exposed regions of hydrophobic amino acids which are available for hydrophobic interactions with whey and other caseins. As the concentration of NaC increases and whey decreases, NaC will interact more with itself to form a network. The smaller peptides of whey disrupt the network formation by binding at certain sites preventing branching of the network. Once only NaC was present within the coating, the stickiness increased as there was no whey to disrupt network formation. While the effect was not as

dramatic with the hydrolysates, the same rationale would explain the lower stickiness as the amount of whey decreased. The presence of CPP could also contribute to a stronger cohesive network for 100:0 coatings as it can work as a building block between caseins to form a network. Potential calcium phosphate binding sites on the hydrolysates could be blocked by the whey. When only caseins were present, these calcium bridges could have formed creating a larger and stronger cohesive network.

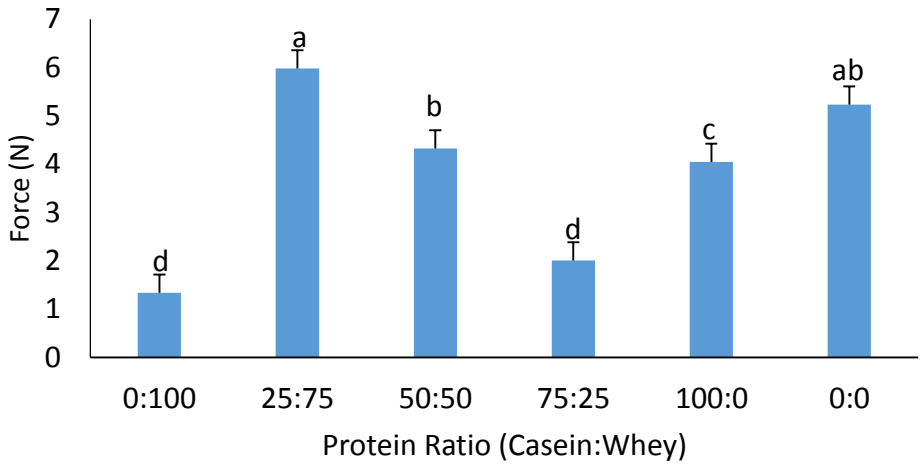

Figure 12. Stickiness coatings containing 20% dairy proteins with a ratio NaC:whey from 0:100 to 100:0 including a control coating without dairy proteins. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05).

4.5.1.5 Role of Peptide Size and Composition in Stickiness

As whey appeared to be an inhibitor to strong cohesion, changes in stickiness when comparing NaC to the hydrolysates is based on the peptide length and available reactive sites or regions. This was demonstrated, as previously mentioned, by the insolubility of CN-A resulting in these coatings being the least sticky. CN-T was stickier than CN-A, most likely due to the higher solubility and longer peptides; this combination enabled more interactions. CN-T did

not change its stickiness when the ratio of casein was increased to 50:50 from 25:75 but experienced a drop at 75:25 and then a sudden increase between 75:25 and 100:0 casein:whey (Figure 13). The differences for the CN-T:whey mixtures are much less than what was seen for NaC; this implies CN-T is not interacting as much with whey but whey is still inhibiting network formation. Once whey is removed from the coating, CN-T is free to self-associate and form a strong network as shown by the increase in stickiness to 8.96 N.

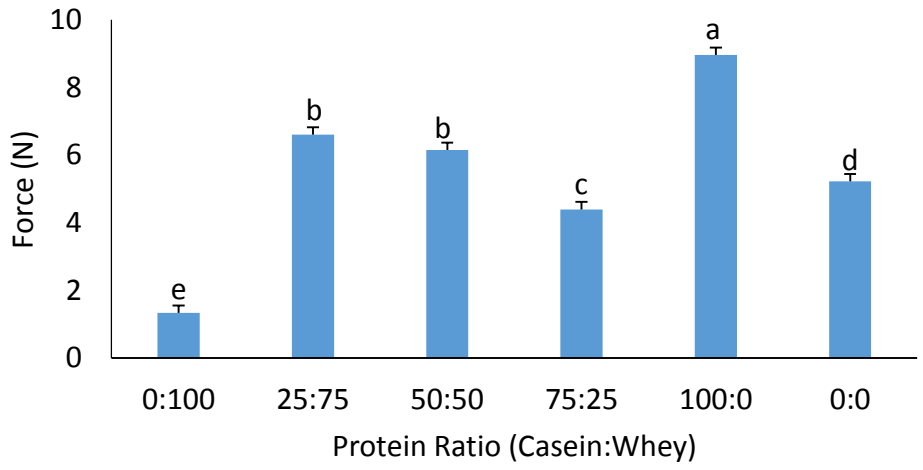

Figure 13. Stickiness 20% dairy proteins (w/v) with a ratio CN-T:whey from 0:100 to 100:0 including a control coating without dairy proteins. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05).

CN-C was the stickiest coating for reasons unknown. Although the peptides were larger, the large jump in stickiness for this hydrolysate was not proportional to the DH which was very close to that for CN-T. α-chymotrypsin cleaves hydrophobic amino acids causing the exposure of more hydrophobic regions of the peptide chains. This could potentially increase the hydrophobic interactions between CN-C and itself or with whey, thereby forming more of a gel

than a coating. Once CN-C was added, the stickiness varied little compared to the other coatings varying by 2 N at most (Figure 14).

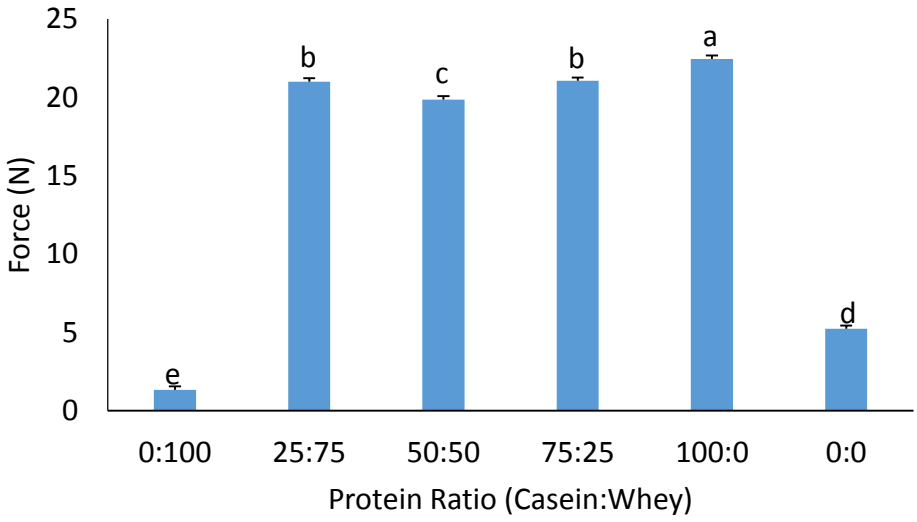

Figure 14. Stickiness of 20% dairy proteins (w/v) with a ratio of CN-C:whey from 0:100 to 100:0 including a control coating without dairy proteins. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05).

4.6 Free Calcium Content

For the coating to work as a preventative measure for dental caries, the CPP must remain functional. The exact mechanism of the reduction in dental caries by CPP is unknown but it is speculated that CPP is either acting as a mineral reserve for remineralisation of the tooth enamel (Ranjitkar *et al.*, 2009; Kumar *et al*., 2008; Wong *et al*., 2011; Yamaguchi *et al*., 2006) and/or inhibiting the adhesion of acid-producing oral bacteria such as *Streptococcus mutans* (Schupbach *et al*., 1996). To determine whether the bioactive peptides remained functional in the coatings, the free calcium and *S. mutans* adhesion were measured.

CPP can act as a mineral reserve for tooth enamel by providing colloidal calcium phosphate to replenish eroded enamel. Enamel erosion typically begins with the introduction of

acid to teeth either through the consumption of acidic foods or the production of acid by plaque bacteria. Once softened, the enamel is vulnerable to further erosion by continued exposure to acid or abrasion during tooth brushing which eventually causes lesions or caries (Ranjitkar *et al*., 2009; Reynolds, 1987). Acid demineralizes the dentine layer of the tooth by removing the calcium and dismantling the hydroxyapatite (Rose, 2000). Acid has the same effect on CPPs where any bound or solubilized calcium phosphate is removed from its structure at lower pH values, thereby neutralizing the binding sites (Rose, 2000). This free calcium and phosphate ions become available to the tooth enamel, especially if the CPP is held in close contact. As a result there can be remineralisation of the depleted surface (Reynolds, 1987).

The calcium in the coatings was measured to determine whether or not the calcium was being bound by the additional ingredients. Bound calcium would be considered more effective for cavity prevention as it would not be washed away immediately by saliva but released only when exposed to acid. The same amount of CPP was added to each coating at 10% (w/w) of the coating and provided an excess of calcium, such that it was not considered necessary to measure the total calcium. The expectation of calcium binding in the coatings was for the free calcium content to decrease with increasing casein content. This is based on the stronger affinity and greater quantity of binding sites for casein and casein hydrolysates shown in literature. Casein is capable of binding calcium via phosphoserine (Ser(P)) residues and the carboxyl groups of glutamic acid and aspartic acid whereas in whey only carboxyl groups bind calcium (Tercinier *et al*., 2014). It would therefore be expected that more free calcium would be present in coatings containing higher concentrations of whey. In addition, greater values of DH have been associated with reduced metal chelation capabilities suggesting that in coatings with

higher proportions of CN-A more free calcium would be present (Luo *et al.,* 2014). Since CPPs act as a mineral reserve by replenishing minerals under acidic conditions, lower levels of free calcium would be preferred in the coating as this would mean more calcium is in reserve to be released when needed and not washed away by saliva or bound by other agents in the oral cavity.

4.6.1 Free Calcium in Pure Protein Samples

In pure protein samples, the highest free calcium was found in whey at a level of 12.4 mg/g free calcium (Figure 15). The whey had the largest free calcium because it bound less calcium than casein leaving most calcium in the solvent. The whey used in this experiment was commercial (Bulk Barn) so calcium may have been added or not removed to improve the nutrition of the whey protein isolate. Unlike whey, the sodium caseinates hydrolysed with Alcalase, trypsin and α-chymotrypsin were better characterized and most of the calcium associated with casein had been removed to ensure protein purity. Sodium caseinate itself was stripped of calcium during manufacture and it was replaced with sodium ions meaning baseline calcium levels would be much lower than for pure casein (Tercinier *et al*., 2014). At the other end of the scale, the CPP had calcium chloride added during production to ensure the peptides were saturated. This resulted in the bioactive peptide having the most free calcium of all at 351 mg/g free calcium. CN-A had the second highest free calcium level for the protein samples with 6.21 mg/g; this was followed by CN-T, CN-C and NaC, with 0.90, 0.76 and 0.58 mg/g free calcium respectively (Figure 15). Typically longer periods of hydrolysis and higher DH values result in lower metal chelation (Luo *et al*., 2014) which could explain the high free calcium found in CN-A. This effect did not explain the results with CN-T and CN-C, as their free calcium

levels were not significantly different from those for NaC. Reduction in the ability to chelate metal ions is related to a decrease in peptide length so with a higher DH value, metal chelation should decrease. However, when the amount of free calcium was very low, this relationship did not apply (NaC, CN-T and CN-C in Figure 15). The low value found for NaC is understandable as the original calcium had been replaced with sodium. Although hydrolysis should release any remaining calcium giving higher free calcium in the hydrolysates, this release was not large enough to significantly affect the level of free calcium. The higher level of free calcium for CN-A could be due to a problem with the assay in that clouding occurred in the sample containing CN-A, whereas clear solutions were obtained for the other proteins. If clouding was responsible for this large deviation, the effect did not influence the results for the coatings, as coatings containing CN-A were as clear as the other coatings as dissolving in glycerol appeared to aid in its solubility.

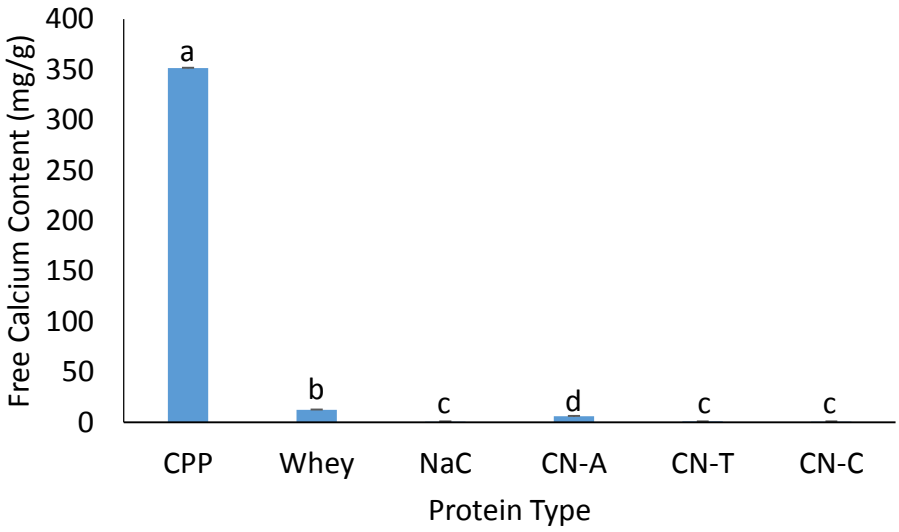

Figure 15. Free calcium content of pure whey, NaC, CN-A, CN-T, CN-C and CPP. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05).

4.6.2 Free Calcium in Coatings

As expected, the coatings contained significantly higher free calcium values than the pure proteins (Figures 16, 17, 18 and 19). The free calcium in the coatings was less than 10% of the pure CPP free calcium by a large amount, greater than 27 mg calcium/g coating was not present in measured free calcium. This meant that the additional ingredients in the coatings were binding the calcium. Glycerol was not expected to bind any calcium as its only means of interaction is through its hydroxyl groups. The hydroxyl groups preferentially form hydrogen bonds with other glycerol molecules or any water present in the sample leaving the calcium unbound (Galietta *et al*., 1998). This was not seen in the control coating, 0:0, made of 90% glycerol and 10% CPP. The control coating had a level of free calcium of 13.6 mg/g free calcium, which was much lower than the expected contribution of CPP to the coating which was 35.12 mg calcium/g. This could mean the glycerol was binding calcium or the glycerol was manipulating the structure of CPP enabling it to hold more calcium (Table 4). This means glycerol will may affect calcium binding by altering the structure and interaction capabilities of whey, casein and casein hydrolysates as well as CPP. From Table 4, it is clear that the coatings contained significantly more free calcium than was seen for the pure proteins. The free calcium content was lower than that found in CPP as well as the control coating, meaning the proteins were capable of binding calcium but to different extents. The binding of the calcium is significant because bound calcium will be held in reserve by the coating until it is exposed to an acidic environment and released, thereby improving the chances of remineralisation of the tooth enamel.

Table 4. Free calcium content (mg/g) of pure protein samples of caseinophosphopeptides, whey, sodium caseinate and hydrolysates (Alcalase, trypsin, α-chymotrypsin) and coatings. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05).

	Free Calcium (mg/g)					
Coating (casein:whey)	NaC	CN-A	CN-T	CN-C	Whey	CPP
Pure	0.58 ± 0.01^{l}	6.22 ± 0.26^{k}	0.90 ± 0.04^{l}	0.76 ± 0.03^{l}	12.4 ± 0.24^{j}	351 ± 0.36^{a}
0:100	9.65 ± 0.36^{efg}	9.65 ± 0.36^{efg}	9.65 ± 0.36^{efg}	9.65 ± 0.36^{efg}		
25:75	9.10 ± 0.44^{gh}	9.84 ± 0.69^{efg}	9.07 ± 1.03^{gh}	9.65 ± 0.67^{efg}		
50:50	10.7 ± 0.52^{def}	9.58 ± 0.30^{fg}	7.94 ± 0.50^{hi}	9.40 ± 0.36^{g}		
75:25	10.8 ± 0.42^{cde}	11.5 ± 0.68^{cd}	7.83 ± 0.40^{i}	10.6 ± 1.02^{ef}		
100:0	12.3 ± 0.39^{bc}	9.79 ± 0.69^{efg}	7.91 ± 0.27^{hi}	12.2 ± 0.63^{c}		
0:0	13.6 ± 0.40^{ab}	13.6 ± 0.40^{ab}	13.6 ± 0.40^{ab}	13.6 ± 0.40^{ab}		

4.6.2.1 Free Calcium in CN-T Coatings

Only coatings containing CN-T followed the expected pattern of decreasing free calcium with increasing casein content (Figure 16). The decrease was first seen at a ratio of 50:50 and plateaued rather than increasing as the ratio increased to 100:0 casein:whey. The decrease seen at the 50:50 ratio could be caused by the higher affinity of calcium to CN-T. The continual increase in casein may have made up for the loss of whey binding sites thus generating the plateau.

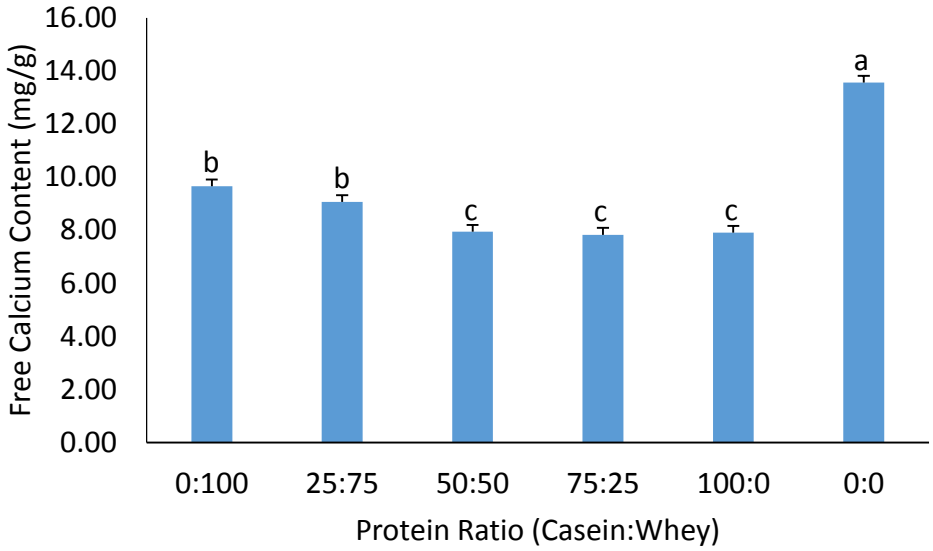

Figure 16. Free calcium content in coatings containing 20% dairy proteins with a ratio CN-T:whey from 0:100 to 100:0 including a control coating without dairy proteins. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05).

4.6.2.2 Free Calcium in CN-A Coatings

CN-A-containing coatings, Figure 17, also showed little variation in calcium binding at varying casein:whey ratios; the exception was at a ratio of 75:25 where a higher level of free calcium was observed. This peak is difficult to explain as the free calcium drops once again to

baseline levels at a ratio of 100:0. At a ratio 75:25, there may be the perfect balance between the whey and CN-A to form complexes preventing calcium from binding by altering the structure and accessibility of the binding sites on both proteins (Guyomarc'h *et al.,* 2009). As the same amount of calcium was present in each coating it is unlikely that the CN-A becoming saturated was responsible for the free calcium increase as the free calcium returned to the previous level at 100:0 CN-A. As CN-A has the highest DH value, it was expected to bind the least calcium, but this was not the case as seen in Table 4, unless it is accepted that clouding was responsible for the higher values for the protein alone. It is possible that complexes or aggregates formed with whey and CN-A were reducing binding sites, although not to the extent as was seen at the 75:25 ratio. Without heat, aggregates or complexes can form between casein or casein peptides and whey through hydrophobic interactions (Guyomarc'h *et al*., 2009).

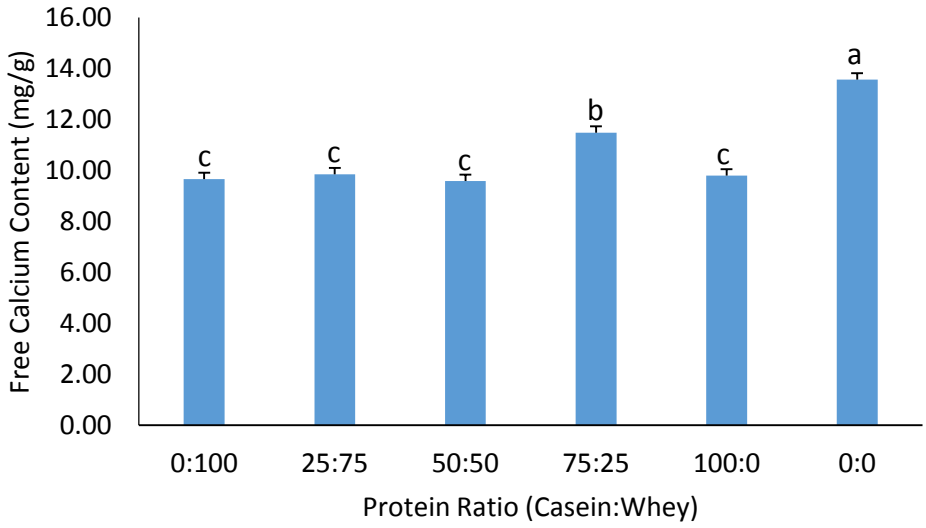

Figure 17. Free calcium content in coatings containing 20% dairy proteins with a ratio CN-A:whey from 0:100 to 100:0 including a control coating without dairy proteins. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05).

4.6.2.3 Free Calcium in CN-C and NaC Coatings

Coatings containing CN-C and NaC (Figures 18 and 19) retained a constant amount of free calcium until a ratio of 75:25 was reached, after which there was a steady increase in free calcium with increasing casein content. For these two coatings, it appears the whey was capable of binding more calcium which would explain the increase in free calcium beyond the 75:25 ratio, when the amount of whey was reduced. It was suggested previously that hydrolysis with α-chymotrypsin led to the production of CPPs which prevented further hydrolysis. CPPs are peptides generated by hydrolysis and they have been reported to contain the sequence Ser(P)-Ser(P)-Ser(P)-Glu-Glu (Zong *et al*., 2012) which has a very strong affinity for binding calcium. The presence of CCP would be expected to increase calcium binding, but a study performed by Zong *et al*. (2012) found this was not the case. They studied the effect of sequence on the strength and quantity of calcium bound using various arrays of Ser(P) and Glu peptides including discontinuous sequence of Ser(P) and unphosphorylated Ser(P). They found that natural CPP bound more calcium than the synthetic ones, but binding was weak and often in the form of calcium phosphate nanoclusters which were held together by non-covalent bonds.

For both CN-C and NaC, the large peptide sizes, denoted by DH and FPLC analysis, could account for the lack of calcium binding due to masking of binding sites. As mentioned in previously, native protein structures have intermolecular and intramolecular interactions that hide certain amino acid groups, particularly the hydrophobic groups, within the protein

molecule. Structures such as this could prevent calcium binding sites from being accessible. For both CN-C and NaC (Figures 18 and 19), the amount of free calcium at a ratio of 100:0 were 12.2 and 12.3 mg/g, respectively, values close to that of the control coating, 13.6 mg/g, demonstrating how little calcium was being bound by the casein and the CN-C casein hydrolysate.

The consistent values for free calcium between the ratios of 0:100 and 50:50 suggests a complex has been formed between the whey and the CN-C and NaC as noted above for CN-A. These interactions form through hydrophobic interactions when heat is unavailable to unmask the reactive thiol of whey (Guyomarc'h *et al*., 2009; Vasbinder, 2003). Calcium bridging could also be responsible for the increase in free calcium with increasing CN-C content as calcium bridges form between peptides generating a weak network which can be broken using shear forces (Antipova *et al*., 2002). In this manner, calcium forms a bridge between different peptides causing structural changes that hide calcium other binding sites or make them unavailable. NaC, on the other hand, is less likely to form calcium bridges but binding sites are more likely masked compared to CN-C since it has not been hydrolysed. This is because of the removal of calcium and addition of sodium during the manufacturing of NaC. If this was the case, the hydrolysates should also have limited calcium binding unless hydrolysis exposes new calcium binding sites. Due to these concerns about the ability of casein to bind calcium, it can only be concluded that for CN-C and NaC binding between casein and calcium was minor.

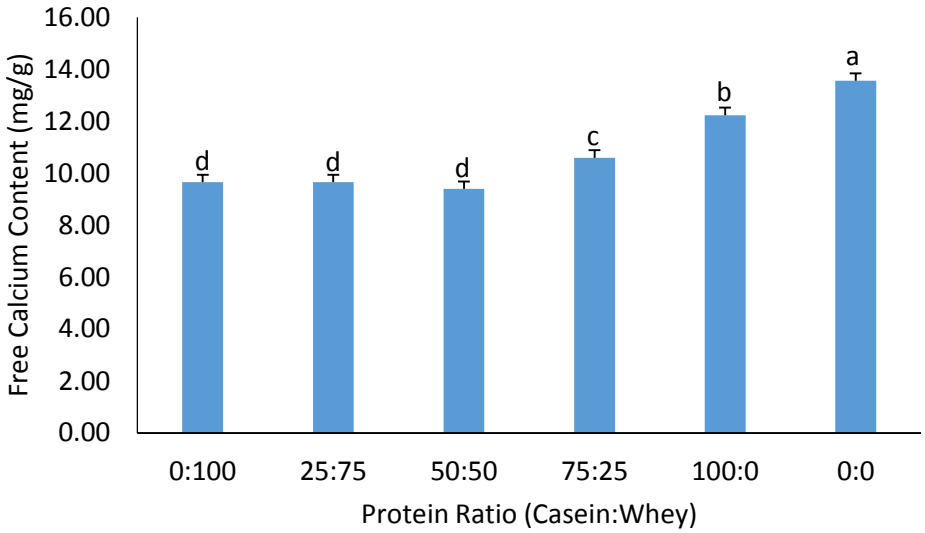

Figure 18. Free calcium content in coatings containing 20% dairy proteins with a ratio CN-C:whey from 0:100 to 100:0 including a control coating without dairy proteins. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05).

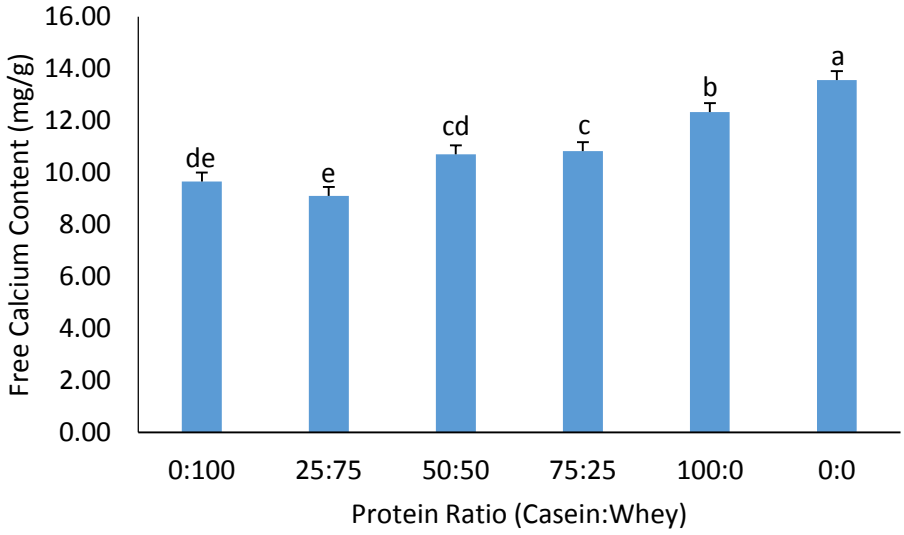

Figure 19. Free calcium content of coatings containing 20% dairy proteins with a ratio NaC:whey from 0:100 to 100:0 including a control coating without dairy proteins. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05).

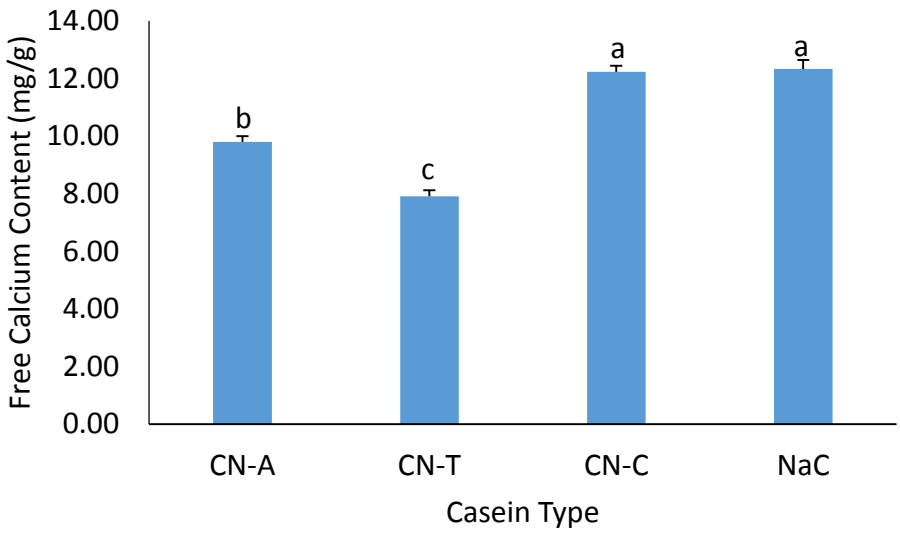

Figure 20. Free calcium content (mg/g) of coatings containing 20% NaC, CN-A, CN-T or CN-C, 10% CPP and 70% glycerol. Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05).

4.6.2.4 Free Calcium in Coatings Containing Casein Products but no Whey

Figure 20 shows a comparison between the coatings containing 20% casein or casein hydrolysates without any whey. Based on the DH, and resulting peptide sizes, CN-A should have the highest free calcium and NaC the lowest, but the free calcium content did not follow this pattern. CN-C and NaC coatings had the highest free calcium content at 12.2 and 12.3 mg/g, respectively, and were not significantly different. In contrast, CN-T bound the most calcium as only 7.91 mg/g free calcium was obtained. The variation in free calcium for coating samples is most likely a result of aggregation or complex formation due to casein-whey or casein-casein interactions rather than the size of peptides in the hydrolysates. Clearly the addition of calcium to casein or whey has the potential to change the structure of the peptides and the network achieved in the coatings.

Calcium is commonly added to milk or milk proteins to cause flocculation and destabilization of the protein so they can be harvested or processed into products such as cheeses. This is achieved by the calcium acting as a bridge between different milk proteins or peptides (Antipova *et al*., 2002). Calcium binding also alters the surface charge of the proteins which influences their conformation and availability of calcium binding sites. The dairy proteins used in this experiment had already been processed and stripped of their calcium, with the exception of whey, prior to the addition of CPP. This means they are more likely to destabilize with calcium incorporation as they were no longer part of the casein micellar structure (Farrell & Thompson, 1988). Interaction of peptides within the coatings could also negatively affect the calcium affinity causing stabilization of the protein but increasing the free calcium in the system (Kumosinski & Farrell Jr., 1991).

4.6.3 Free Calcium in Relation to Stickiness

Calcium bridging and aggregation of proteins in the coatings did not appear to affect the stickiness of the final coatings, as shown in Figure 21. Overall there was no correlation between these two parameters. This was in large part due to the high stickiness levels for CN-C while having similar levels of free calcium as the other coatings. Looking at only CN-C data, similar trends for stickiness and free calcium were seen, but this was not the case for other coatings. For the CN-C coatings, the pattern seen was opposite to the expected result as the stickiness increased with increasing free calcium. This led to the conclusion that protein-protein interactions may be more related to coating stickiness than calcium binding. As the remaining coatings did not show any relation with free calcium, a relationship between stickiness was not evident based on this experiment.

The addition of calcium to dairy proteins is commonly used in the dairy industry to cause flocculation of the proteins especially during cheese production. The calcium binds to the proteins altering their surface charge, reducing steric hindrance and causing precipitation which should increase viscosity and stickiness (Farrell & Thompson, 1988). One reason this did not happen in the coatings may be that there was not enough calcium to cause salting out of the proteins (Farrell jr. *et al*., 1988). The level of calcium used was above that normally seen in milk but the incorporation of only 10% CPP most likely enabled the coatings to remain soluble or suspended by holding the calcium as colloidal calcium phosphate, in which the phosphate portion was provided by the Ser(P) groups on the CPPs (Philippe *et al*., 2005; Zong *et al*., 2012).

In addition to altering the surface charge, the formation of calcium bridges between dairy proteins caused flocculation and destabilization of the proteins, which could affect stickiness. Antipova and colleagues (2002) measured the effect of calcium concentration on the stickiness of casein using a particle scattering apparatus with a laminar shear field. They found that calcium bridges were formed between casein particles causing them to stick together until shearing forces separated them. These bridges were theorized to alter the protein-protein interactions and net charge which in turn led to aggregation of the caseins. This aggregation increased the viscosity of the mixture and the proteins became entangled increasing the friction forces. In other words, the casein became sticky. Although there appeared to be differences observed in terms of viscosity of the coatings, these observations did not align with the amount of free calcium in the system. The calcium used in the Antipova *et al*. (2002) study was 5 mM which is much less than was added to the coatings in this study.

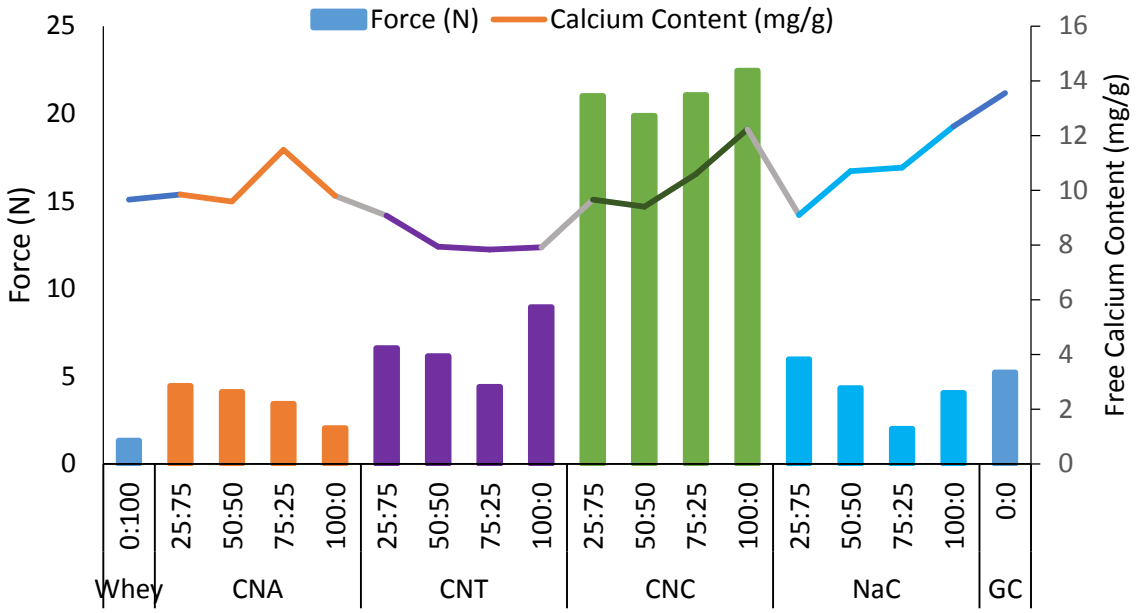

Figure 21. Comparison of coating stickiness, measured by maximum peak force (N) using a probe-tack test to free calcium content (mg/g) determined using spectrophotometric method. Coating ratio is for 20% (w/w) protein in the coating where the ratio is casein:whey. The calcium content appears as a single line but are not related outside of the respective enzyme treatment demonstrated by the changes in colour, grey lines represent a break between treatments.

4.6.4 Calcium in Relation to Enamel Remineralisation

Based on previous studies, the quantity of free calcium in these systems was enough to achieve the desired remineralisation of the tooth enamel (Kanekanian *et al.,* 2008; Kumar *et al*., 2008; Yamaguchi *et al.,* 2006), as remineralisation was shown to occur with calcium levels of 1 to 8%. The control coating had the highest free calcium at 13.6 mg/g and 100:0 casein:whey CN-T had the lowest free calcium at 7.91 mg/g, which falls in the lower region of this range and greater than 27 mg calcium/g coating is assumed bound based on the 10% CPP contribution. As previously mentioned, it is best for the calcium to be bound within the coating so that it can be

released upon introduction of acid. 100:0 casein:whey CN-T had the lowest free calcium therefore the highest bound calcium making it the best coating for remineralisation following application to acid introduction. Enough calcium was present in the coatings to act as a mineral reserve but more research is required to confirm this. To fully test the effectiveness of the coating on dental caries a test using bovine molars, similar to a study by Yamaguchi *et al*. (2006), would be required.

4.7 *Streptococcus mutans* Adhesion

Besides functioning as a mineral reserve, CPPs have also been reported to reduce the adhesion of oral bacteria to the teeth. Oral bacteria such as *Streptococcus mutans* (*S. mutans*) form colonies in the dental plaque and produce acid, dismantling the tooth enamel (Rose, 2000; Schüpbach *et al*., 1996). CPP is thought to replace human serum albumin in the salivary pellicle which reduces the adhesion of bacteria by masking the streptococci receptors (Schüpbach *et al.*, 1996). To determine whether the sticky coatings were capable of preventing bacteria from adhering to tooth enamel a hydroxyapatite (HAP) disc assay was performed using *S. mutans*. The stickiest and least sticky coatings, 100:0 casein:whey CN-C and 0:100 casein:whey respectively, were selected and HAP discs were coated for each; two control coatings, of distilled water and glycerol, were also included. The coatings were left to dry then introduced into tubes containing *S. mutans* in a Todd Hewitt yeast extract (THYE) with mucin broth. The tubes were then incubated for 2 and 4 h anaerobically at 37 ^{o}C before removing and washing the discs. The discs were vortexed in phosphate buffer saline which was subsequently diluted in a well plate then streaked onto THYE plates and incubated anaerobically for two days at 37 ^{o}C. The *S. mutans* colonies were counted and converted to colony forming units.

As seen in Figure 22, there was little variation amongst the *S. mutans* adhering to the discs with the exception of the CN-C coating. The large amount of *S. mutans* adhering to the CN-C discs is to be expected as it was the stickiest coating despite containing CPPs. It is worthy to note that after 4 h significantly less *S. mutans* adhered to the CN-C coating suggesting that longer periods of contact with *S. mutans* causes a reduction in their adhesion. The same effect was not seen for the whey coating but as previously suggested the CN-C may have produced CPPs during hydrolysis and the reduction in *S.* mutans adherence may be due to increased amounts of CPPs being released due to the acid produced by the *S. mutans*. The coatings did not appear to be effective against the adhesion of bacteria as there is little difference between the control coatings and CPP-containing coatings but the coatings did remain adhered to the HAP discs. For more conclusive results longer time periods should be explored to determine whether the reduction in *S. mutans* adhesion is influenced by CPP release.

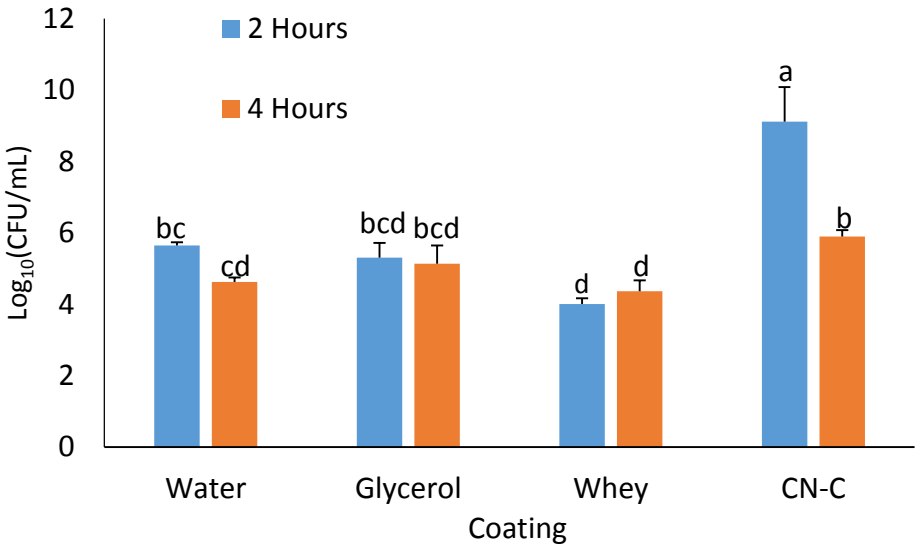

Figure 22. *Streptococcus mutans* adhered to coated hydroxyapatite discs. Coatings used were two controls (water and glycerol) and two CPP-containing coatings (20% whey and 20% CN-C). Results are expressed as the mean ± the standard deviation. Like characters are not significantly different (P>0.05).

5.0 Conclusions and Future Research

5.1 Conclusions

Overall, Alcalase was the most effective enzyme at hydrolysing sodium caseinate; a low dosage and short time period were required to generate the highest DH. Despite the high DH of CN-A, the peptides generated did not have smallest molecular weights. In fact, the commercial whey exhibited peaks with the smallest molecular weights, indicating it had been hydrolysed and was not in its natural state as expected. NaC and CN-C had peptides with the highest molecular weights; in fact they were outside of the range of standard molecular weights and could not be quantified.

Determination of glass transitions proved to be of little value as only a few transitions observed could be classified as glass transitions. A phenomenon resembling a glass transition occurred with glycerol that was also seen in coatings which contained glycerol. This transition occurred in the range between the flash point and boiling point of glycerol with such consistency that it may be associated with a transition between liquid and vapour. Attempts to relate the glass transitions, or other identified transitions to stickiness were unsuccessful.

Stickiness was successfully measured using the probe-tack test in which the 100:0 casein:whey CN-C coating was found to be the stickiest. Any coatings containing CN-C were much stickier than any other coating; the magnitude of the difference was approximately three times. The least sticky coating was that containing only whey as its dairy protein component.

Free calcium varied amongst the pure samples of protein with CN-A and whey containing the highest quantity of free calcium. The high values for free calcium in pure CN-A

were most likely due to clouding present in the sample; this clouding disappeared once CN-A was incorporated into the coatings. 100:0 casein:whey CN-T coating bound the most calcium whereas 100:0 casein:whey CN-C and NaC bound the least. Enough calcium was present in bound or free form in each coating to act as a mineral reserve for the tooth enamel, although bound calcium is the preferred form.

The adhesion of *S. mutans* varied little between the controls and the coatings. The coating containing only whey as its dairy protein component did not differ from the controls for both 2 and 4 h treatments. The 100:0 casein:whey CN-C coating showed an increase in *S. mutans* adhesion for the 2 h time stamp but demonstrated a decrease in adhesion at 4 h. This suggests the time of exposure may determine the adhesion of bacteria for this sticky coating, but it was not a factor for the other coating or in the controls.

The coating containing 100% CN-C as its dairy component proved to be the stickiest coating but its effectiveness as an anticariogenic product was less than ideal. The coating did not appear to bind calcium, hindering its ability to act as a mineral reserve and it encouraged the adhesion of bacteria. However, more research is required to determine the effectiveness within the oral cavity and whether other, less sticky coatings can achieve better results *in vitro*.

5.2 Future Research

The current research has not been able to provide evidence for the exact mechanisms behind the stickiness in the coatings especially in terms of the interactions between the casein, whey, glycerol and CPPs. Looking further into the glass transitions of the proteins and coatings could assist in understanding any structural changes; more evidence may be obtained using

different equipment or methods. This could involve increasing the temperature range well below -30 ^{o}C as the coatings may still be fluid due to the high level of glycerol. More research is also needed to determine whether the transition seen via MDSC was a true event or noise. The efficacy of the coatings require more relevant tests if they are to be used commercially. This includes a drying test to determine the application thickness, drying temperature and time for use as an oral coating. Interactions with saliva and subsequent mouthfeel should be investigated to determine whether the coatings will dissolve, form clumps or taste/feel strange in the oral cavity. If the coatings can withstand oral application, tests are required on real teeth, such as bovine molars, to determine whether remineralisation is occurring or bacteria are adhering to the surface.

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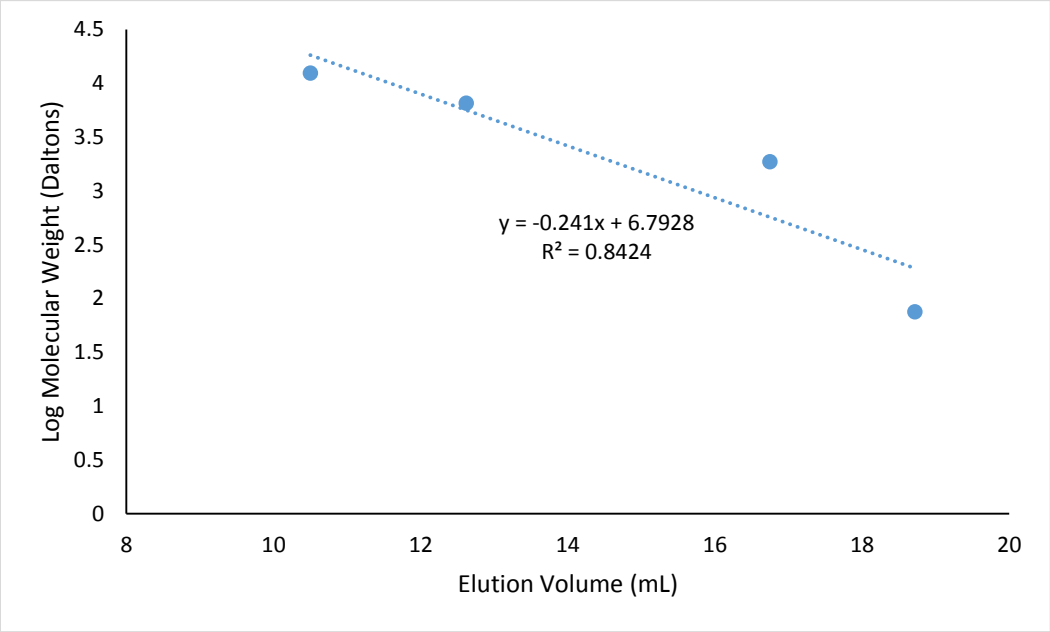
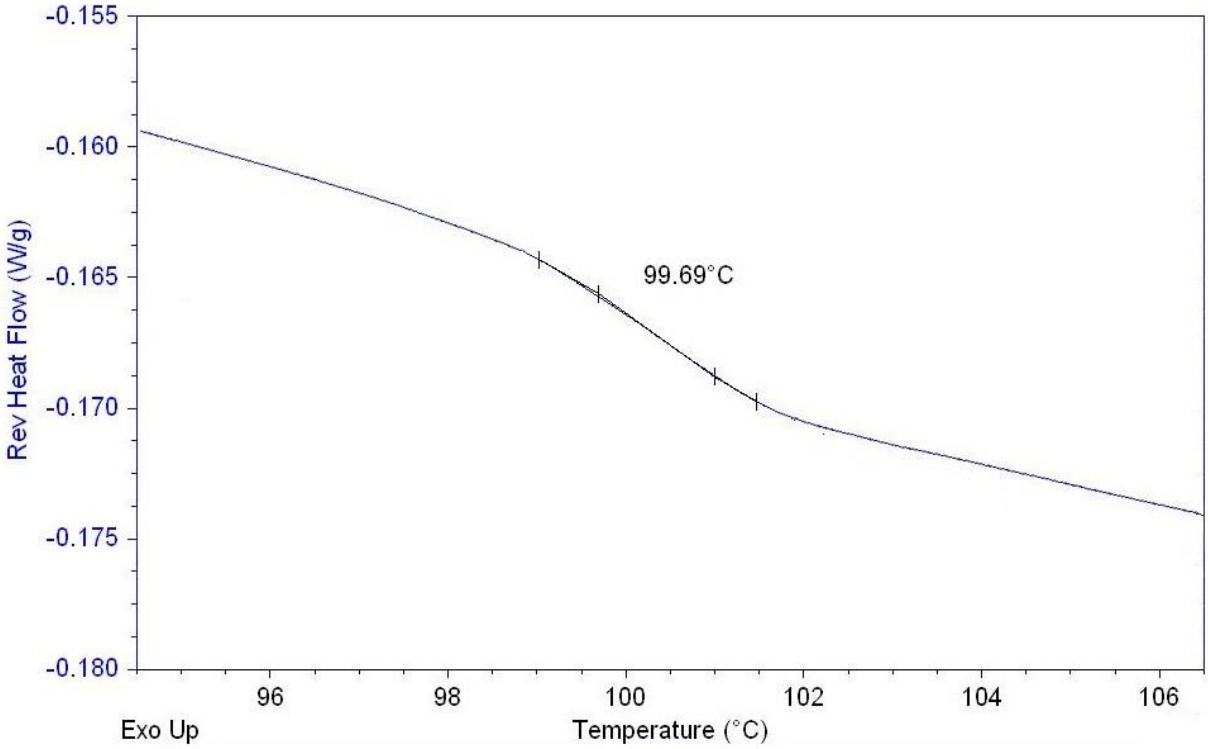
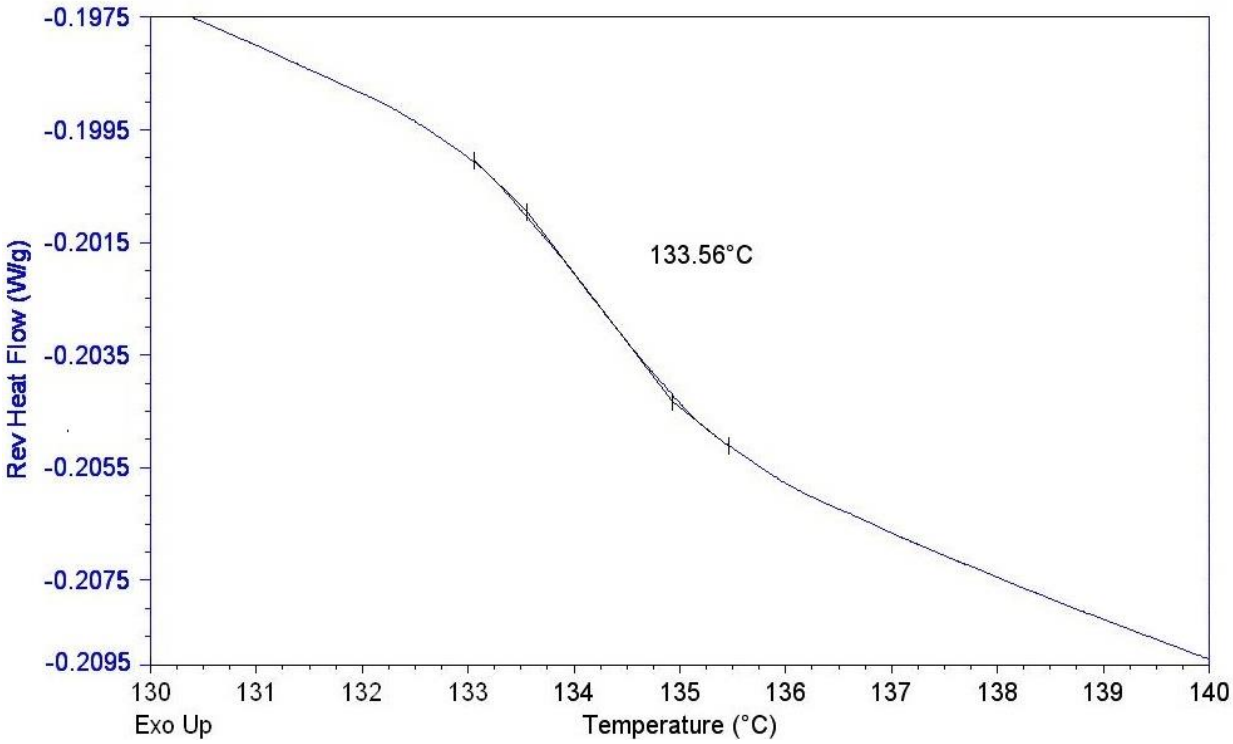
7.0 Appendices



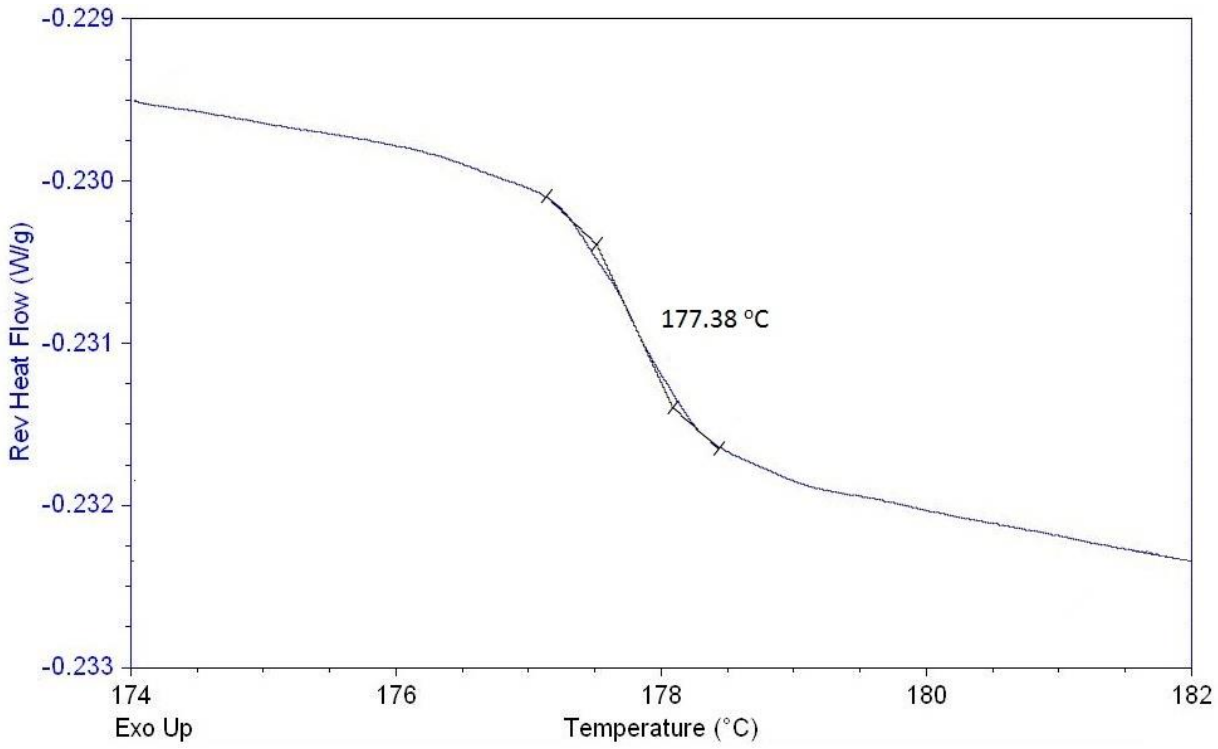
Appendix 1. FPLC standard curve of log molecular weight versus elution volume for cytochrome C (12400 Da), aprotinin (6500 Da), vitamin B_{12} (1850 Da) and glycine (75 Da). The equation to convert elution volume to log molecular weight is shown on the graph. The anti-log was taken to determine the molecular weights of the samples.



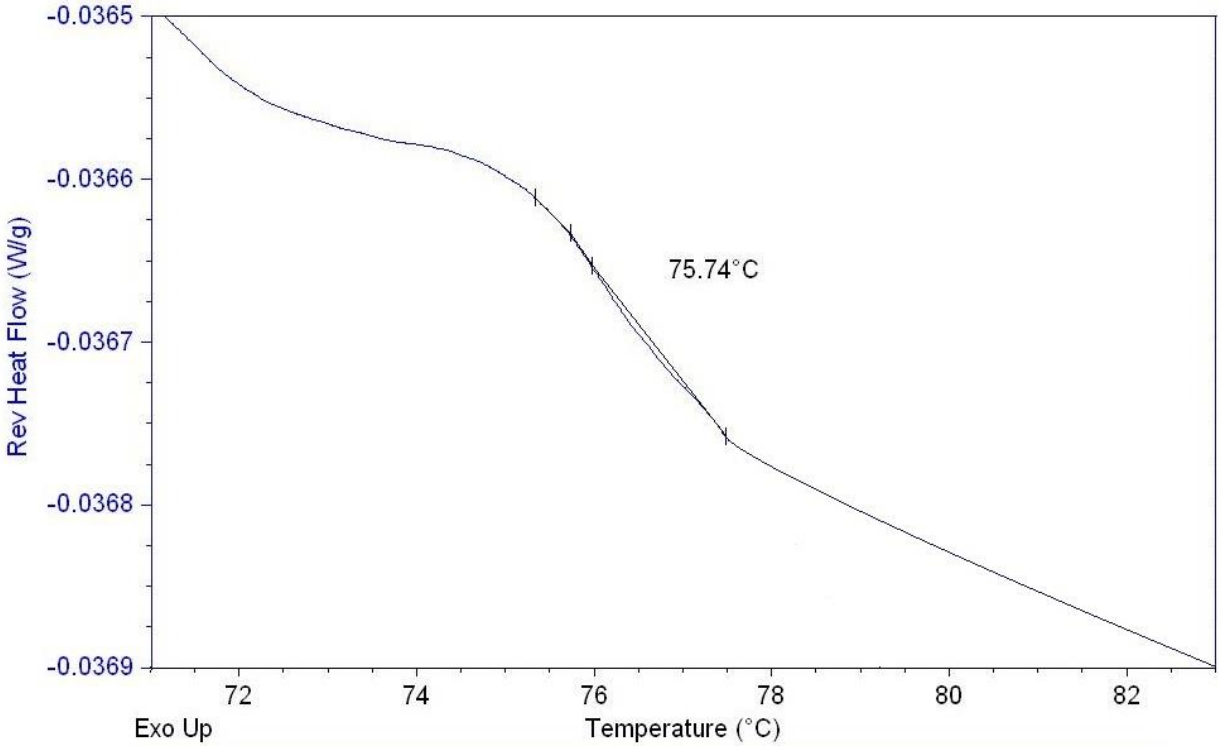
Appendix 2. First transition measured using MDSC found in pure CN-C at a concentration of 20% (w/v) water.



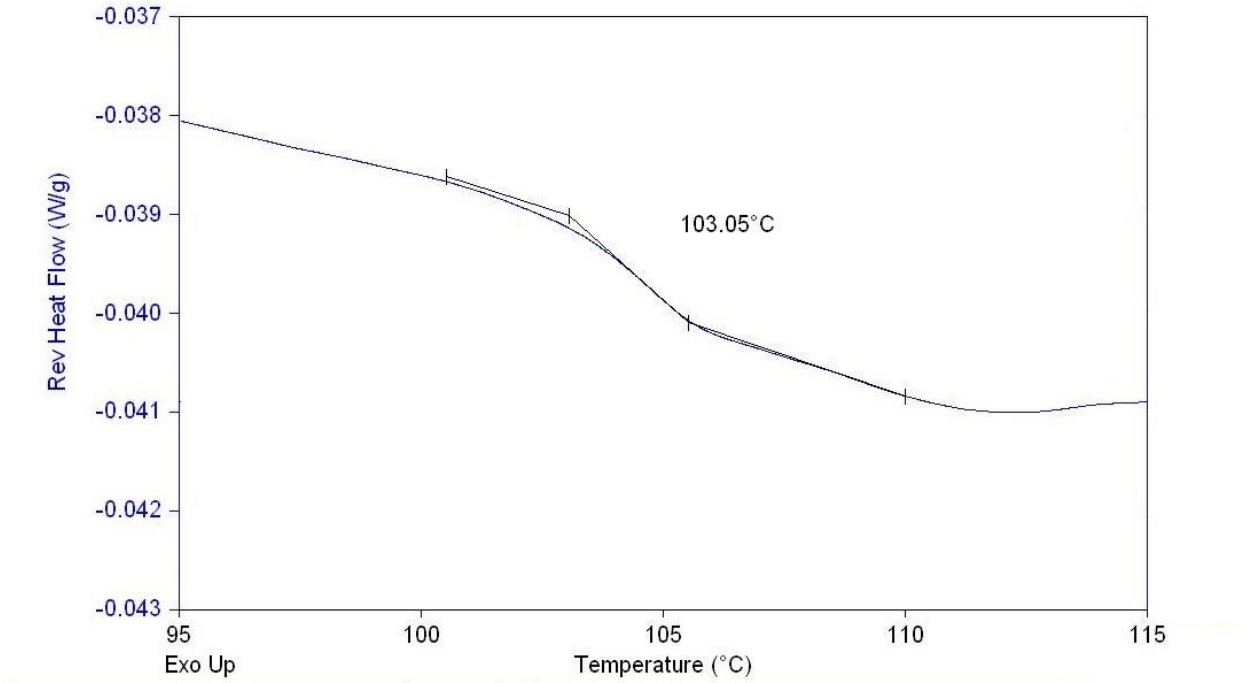
Appendix 3. Second transition measured using MDSC found in pure CN-C at a concentration of 20% (w/v) water.



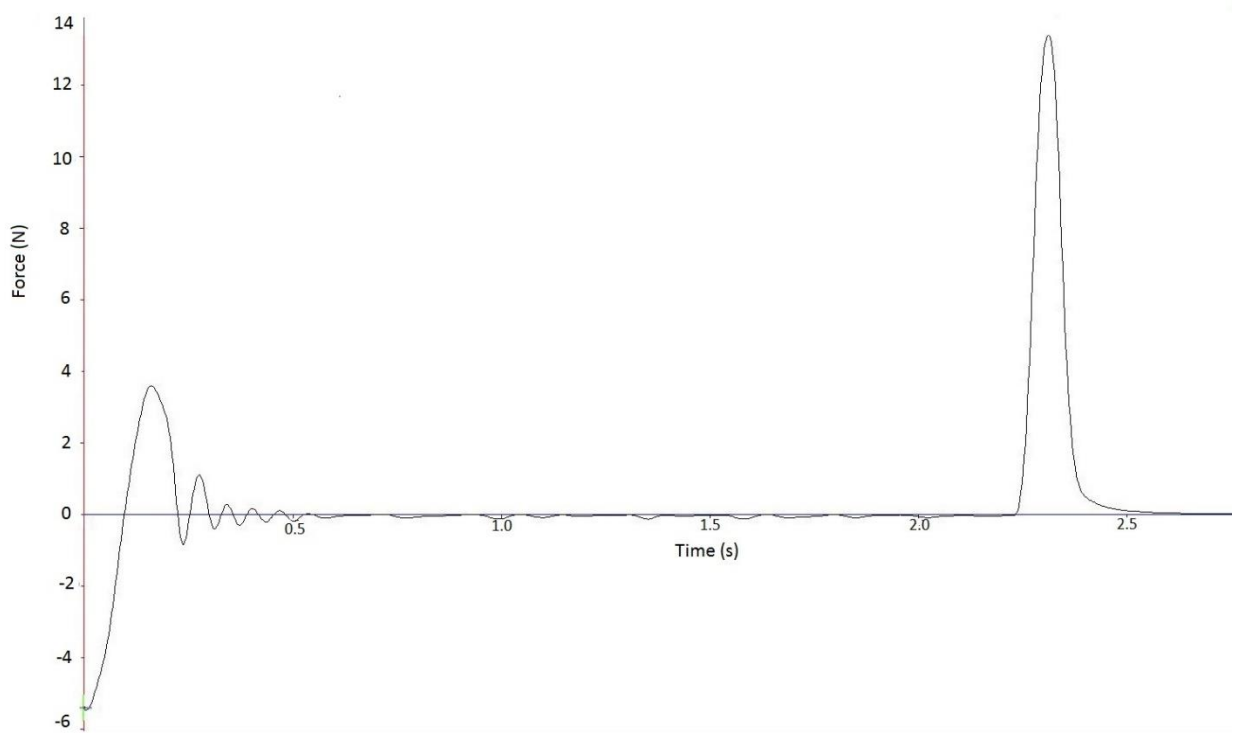
Appendix 4. Third transition measured using MDSC found in pure CN-C at a concentration of 20% (w/v) water.



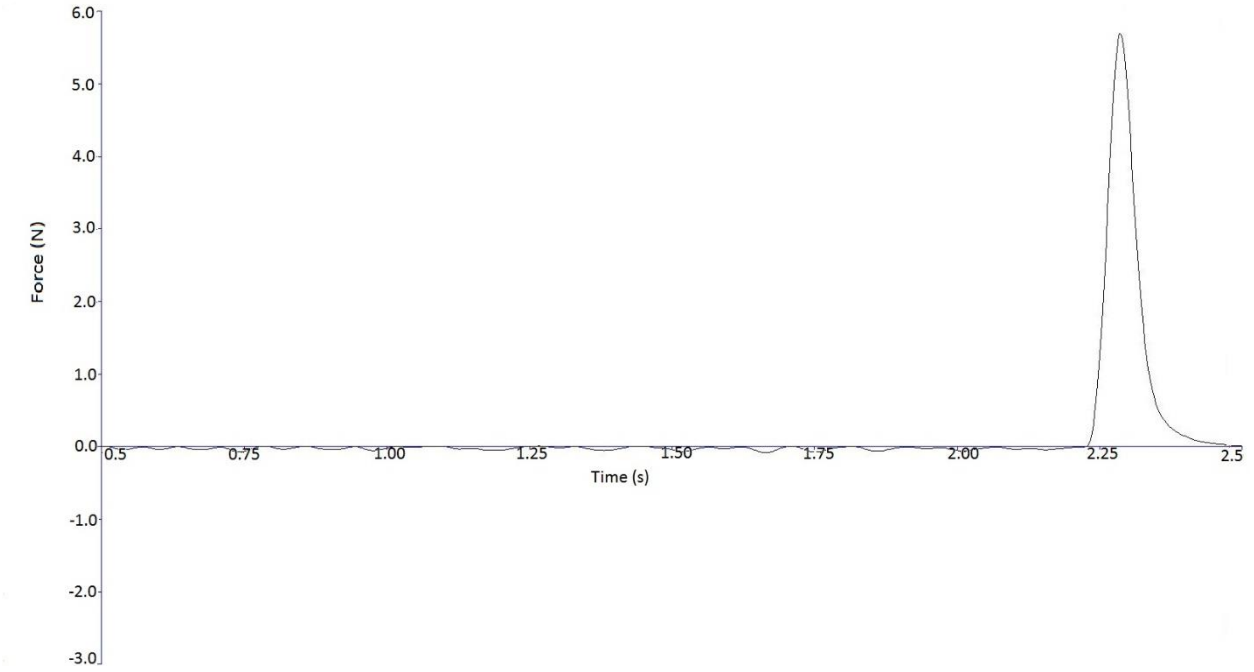
Appendix 5. First transition measured using MDSC found in pure whey at a concentration of 20% (w/v) water.



Appendix 6. Second transition measured using MDSC found in pure whey at a concentration of 20% (w/v) water.



Appendix 7. Stickiness of 100:0 CN-C:whey coating measured by a probe-tack test. Stickiness is the maximum peak force experienced prior to cohesive failure.



Appendix 8. Stickiness of 0:100 casein:whey coating measured by a probe-tack test. Stickiness is the maximum peak force experienced prior to cohesive failure.