PHYSICAL PROPERTIES OF EMULSION STABILIZED BY κ-CASEIN BEFORE AND AFTER TREATMENT WITH CHYMOSIN

A Thesis

by

ANITA GERUNG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2005

Major Subject: Food Science and Technology

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Approved by:

Chair of Committee,	Ronald L. Richter
Committee Members,	Rhonda K. Miller
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Chair of Food Science and Technology Faculty,	Rhonda K. Miller

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ABSTRACT

Physical Properties of Emulsion Stabilized by Κ-casein Before and After Treatment with Chymosin. (December 2005)

Anita Gerung, B.S., National Taiwan Ocean University

Chair of Advisory Committee: Dr. Ronald. L. Richter

In order to determine the effect of lipid concentration on the properties of κ-casein stabilized emulsions, butteroil was added to solutions that contained 0.3% κ-casein to achieve milk fat concentrations of 3, 10, and 20%. These mixtures were adjusted to pH 6.5 and heated to 65 ˚C. They were then homogenized at 20 and 100 MPa and particle size was measured; viscosity and yield stress were measured before and 30 minutes after the addition of chymosin. These experiments were repeated twice. Homogenization of the emulsions at 100 MPa produced smaller particles than homogenization at 20 MPa. Emulsions with 20% milk fat showed the largest particle size. Before treated with chymosin, these emulsions had the greatest viscosity and yield stress, however the differences with the other lipid concentrations were greater after chymosin treatment. A gel with yield stress less than 10 Pa occurred in emulsions with 3 or 10% milk fat. The emulsion with 20% milk fat after chymosin treatment provided the best possibility for the formation of a gel because it had the highest viscosity and yield stress.

The effect of protein concentration on the properties of the emulsions was determined in emulsions that contained 20% milk fat and 0.5, 0.7, and 1.0%. These

emulsions were prepared as previously described. Emulsions homogenized at 100 MPa had smaller particles than emulsions homogenized at 20 MPa. An increase in protein concentration caused the particle size to decrease. Emulsions homogenized at 100 MPa were more stable than emulsions homogenized at 20 MPa and the emulsion with 1.0% κ-casein was the most stable emulsion. The protein load of κ-casein stabilized emulsions ranged from 3 to 6 mg/m^2. The viscosity and yield stress prior to chymosin treatment showed no properties of gelation. After treated with chymosin, these emulsions produced a weak gel with yield stress values that ranged from 14 to 16 Pa.

DEDICATION

To my parents, Benny Gerung and Djuniwati, whom I loved and owed so much. All of their efforts and hard work enabled me to be as I am today. Their love, faith, support, guidance, and encouragement allowed me to have meaningful life.

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CHAPTER I

INTRODUCTION

Emulsification is an important process in the food industry. There are two common types of emulsions, oil in water (o/w) and water in oil (w/o). Separation of the emulsified particles from the bulk phase is the biggest concern associated with liquid emulsions. Lipid and water emulsions tend to separate because of the differences between their hydrophobic and hydrophilic characteristic, the emulsified particle size and their density. This destabilization limits the value of the food in relation to shelf stability. In order to stabilize emulsions, an emulsifier is important to manage the differences between hydrophobic lipid and hydrophilic water. Emulsifiers alter the properties at the interface of the particles and the bulk phase which result in attraction and repulsion between two particles to prevent destabilization. The emulsifiers used to provide stabilization can also participate in the structure, texture, and functionality of the emulsion by interaction with other components intramolecularly and intermolecularly. These interactions frequently change the viscosity and rheology of the emulsion. The effect on the viscosity of an emulsion is influenced by the concentration of the emulsifier in the emulsion and on the surface of the particles in the emulsion. The surface area of the particles limits the amount of emulsifier that can attach and spread at the interface of the surface.

This thesis follows the style of Journal of Food Science.

Milk is an oil in water emulsion. The stabilization of lipid particles in homogenized milk depends on the movement of casein micelles from the milk serum to the lipid interface. Casein micelles have a spherical shape constructed of four caseins; α_{s1}-casein, α_{s2} –casein, β-casein, and κ-casein. Κ-casein surrounds the α-casein and β-casein and exists at the surface of a casein micelle. Its charge and structure enable steric and electrostatic repulsion that keeps the casein micelle in suspension. The existence of κ-casein on the surface of the casein micelle is believed to be the major contributor to provide colloidal stability to milk.

Coagulation of the casein in milk can be caused by acidification of the milk to pH 4.6, or by the addition of chymosin. Chymosin is a proteolytic enzyme that has specific proteolytic activity toward κ-casein. It cleaves the Phe_{105}-Met_{106} bond in κ-casein which triggers the enzymatic coagulation of milk for curd production in cheese manufacture.

The possibility that κ-casein can function as an emulsifier stimulated the initiation of this study. Κ-casein has a unique structure that should cause it to associate with water and lipid at an oil and water interface. If it can mediate the differences between lipid and water phases to produce an emulsion, it might be possible to control gelation of the emulsion by treating it with chymosin.

The objectives of this study were:

1. To determine the ability of κ-casein to form an emulsion.
2. To determine the effect of homogenization pressure on the properties of the emulsions prepared with κ-casein.
3. To determine the effect of the addition of chymosin on the rheological properties of the emulsions stabilized with κ-casein.
4. To determine if gelation can be initiated in emulsions stabilized with κ-casein.

CHAPTER II

LITERATURE REVIEW

2.1 Milk proteins

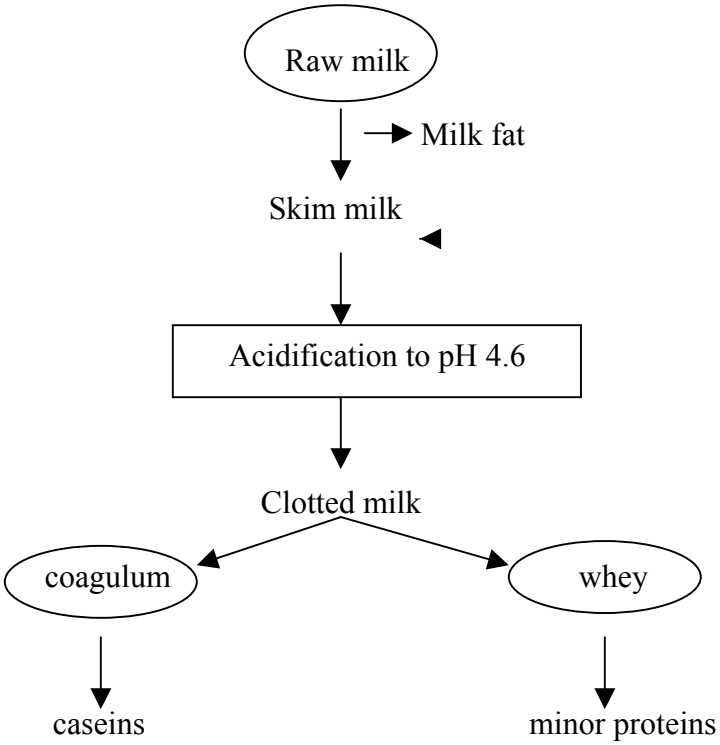

Fig. 1- Separation of caseins and whey proteins

Milk proteins represent about 3.25% of the composition of milk (Walstra and others 1999). They are expressed as the caseins (80%) which have a flexible, disordered protein structure, and the whey proteins (20%) which are more compact and have a

globular structure (Dickinson 2001, Dalgleish 1996). Fig. 1 is a diagram showing the separation of casein from milk by acidifying the milk to pH 4.6 (Walstra and others 1999, Holt and Horne 1996). The four monomeric types of caseins are α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein with a molar ratio of 4 : 1: 4: 1.6, respectively (Walstra and others 1999). Whey proteins are those proteins that remain soluble at pH 4.6 and consist of many minor proteins and the major whey proteins represented by α-lactalbumin, β-lactoglobulin, Bovine Serum Albumin, and the Immunoglobulins (Ig-G, Ig-A, Ig M).

The caseins exist in milk as micelles and are connected by calcium ions through ester-bound phosphate (Walstra and Jenness 1984) which cannot be found in whey proteins. Caseins contain proline that contributes to little or no ordered structure which causes the caseins to resist heat denaturation, unlike whey proteins; which are easily denatured by heat. However, caseins contain less cysteine compare to whey proteins which helps form a thinner film layer on the lipid surface in emulsions and cause the unstability of the caseins. Compared to whey proteins which have equality in hydrophobic and hydrophilic residues, caseins have strong hydrophobic regions which play a role in the stability of the micelle in milk.

2.1.1 Chemical properties of the caseins

α_{s1} -casein is a loose, flexible polypeptide chain that has two predominate hydrophobic regions which represent residues 1 through 44 and residues 90 through 199. These are separated by a highly polar zone where the eight phosphate groups are located.

(Walstra and Jenness 1984). This polarity gives α_{s1} -casein the greatest molecular charge among α_{s1}, β and κ-casein which have molecular charges of -20e, -12e, and -4e at neutral pH, respectively (Dickinson and others 1987, 1983). Therefore, α_{s1} –casein has the fastest electrophoretic mobility at this pH. The molecular weight of the α_{s1} –casein monomer is about 23,000 Dalton (Walstra and others 1999).The eight phosphoserine residues which make it sensitivity to Ca^{2+} (Walstra and Jenness 1984). These phosphoserine residues cause α_{s1} –casein to precipitate at a very low concentration of Ca^{2+}. It also contains 17 proline residues that relates to the bending of the protein chain and inhibits the formation of close-packed, ordered secondary structures.

α_{s2} -casein has a dipolar structure with a negative charge at the N-terminus and a positive charge near the C terminus. It contains 11 phosphoserine residues which bind Ca^{2+} more strongly than α_{s1}-casein (Walstra and others 1999, Walstra and Jenness 1984). This sensitivity to Ca^{2+} is used in methods for the isolation of α_s- casein. Two cysteine residues are present in α_{s2} –casein, and contribute to an α-helix and β- sheet structure. It contains 207 amino acid residues, 10 of which are proline, and has a molecular weight of 25,000 (Walstra and others 1999).

β-casein is a linear amphiphilic polyelectrolyte. It contains 209 residues and has a molecular weight of 24,000. β-casein has little secondary structure since it contains 35 proline residues (Walstra and others 1999). It has a negatively charged N terminal and a very hydrophobic uncharged C terminal. Five charged phosphoserine and proline residues contribute to the thickness of the membrane it creates on lipid globules and they provide stabilization to an emulsion when it is used in an emulsion. The existence of

phosphoserine causes β-casein to easily bind to calcium ions. However, β-casein is less sensitive to calcium precipitation than are α_1-casein and α_2-casein since it has only 5 phosphoserine residues.

The primary structure of carbohydrate free κ-casein consists of 169 amino acid residues and it has a monomer molecular weight of 19,007 Daltons (Eigel and others 1984). There is no secondary structure related to κ-casein since it contains 20 proline residues. The N-terminal of 105 amino acid residues is called para- κ-casein and it contains two cysteine residues. The C-terminal of 64 amino acid residues is called the macropeptide (Walstra and Jenness 1984, Farrell and others 1999). The macropeptide segment has glycosidic residues that contain N-acetylneuraminic acid (NANA) which are located on the surface of the casein micelles and is the binding site for phosphate residues (Dziuba and Minkiewicz 1996). The phosphate groups originate from either the variable number of N-acetyl of neuraminic acid (NANA) residues at SerP-127 or at SerP-149 where there is a carbohydrate free area in the κ-casein molecule (Walstra and Jenness 1984). Up to three phosphate groups can be maintained in the κ-casein molecule (Vreeman and others 1986). These phosphate groups in the macropeptide of the κ-casein molecule contribute negative charges to the molecule and the surface of casein micelles to help stabilize casein micelles in milk by steric and electrostatic repulsion (Dziuba and Minkiewicz 1996, Dickinson and others 1987). Para κ-casein and the macropeptide segment of κ-casein contribute the β-sheet and the α-helix structures in κ-casein. K-casein contains about 10 to 15 % α-helix structures which are located primarily in the

macropeptide fragment and 30 % β-sheet structures which are mostly in the para-κ-casein fragment (Creamer and others 1998).

Gel electrophoresis of κ-casein isolated for this research, reduced with 2-mercaptoethanol, showed a single band representing the κ-casein monomer (Fig. 2). This monomer has a random cross linking pattern generated by three possible combinations of disulfide bonds (11-11, 88-88, and 88-11) which generated a molecular model with an asymmetric arrangement (Rasmussen and others 1992, Kumosinski and others 1993). Disulfide bonds can occur both intramolecularly and intermolecularly. The cysteine residues, Cys 11 and Cys 88, in para-κ-casein can cause unique disulfide bonds to form polymeric chains from monomeric to octamer protein molecules. Oxidation of the sulfhydryl groups of the two cysteine residues will result in disulfide bond formation (Damodaran 1996). The molecular weight of the monomer is about 19,030 Da but polymeric chains have been reported to have molecular weights from 60,000 to 150,000 Da (Vreeman and others 1986, Eigel and others 1984, Walstra and Jenness 1984, Farrell and others 2003). Further association by noncovalent bonding has been shown to produce a polymeric molecular weight of about 650,000 Da (Walstra and Jenness 1984). This polymerization and the presence of only one phosphoserine residue are believed to help stabilize κ-casein against precipitation by Ca^{++} (Walstra and Jenness 1984).

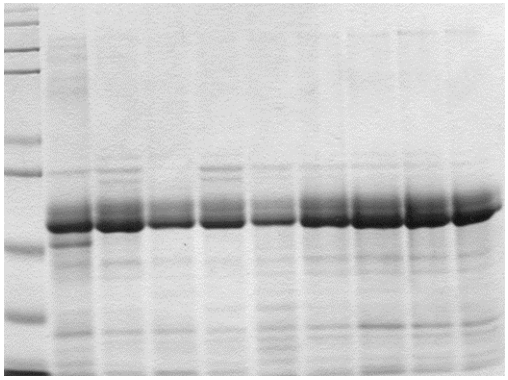

Fig. 2 - Gel electrophoresis of purified κ-casein reduced with 2-mercaptoethanol

2.1.2 Coagulation of milk

Coagulation of the casein in milk results in the formation of a gel which will be processed further to make cheese (Fig 1). The coagulum can be described as a true particle gel network since the casein micelles still maintain their integrity. The gel in milk can be created by acidification and enzymatic treatment. The method of coagulation will alter the formation of the gel.

2.1.2.1 Acid coagulation

The micellar colloidal calcium phosphate will completely dissolve if the pH of milk is reduced below pH 5.0. Horne (1999) reported that most phosphate was released at pH 5.7 and calcium was released at pH 5.3. Walstra (1990) also mentioned that many physical and chemical properties of the casein micelle are changed in the pH range of

5.5 to 5.0. It is understood that calcium phosphate has an important role in maintaining the integrity of casein micelles; however, acidification does not cause the casein micelle to disintegrate. Depletion of the calcium and phosphate cause changes in the nature of protein-protein interactions as pH is lowered. Removing the calcium phosphate will eliminate one form of binding between proteins in the casein micelle, but the other binding interactions, especially hydrophobic or hydrogen bonds, will maintain the integrity of the casein micelle (Horne 1999). The tendency for casein micelles to aggregate increases as the pH is decreased from pH 5.0 to pH 4.6 (Schkoda and others 1999). The number of cationic and anionic sites on casein micelle surfaces is balanced at pH 4.6 which is the isoelectric point of the caseins. At the isoelectric point, the repulsive forces are minimal and a weak attraction exists between the micelles. This phenomenon occurs because the pH of the system approaches the pKa of the polyelectrolyte macropeptide (Glycomacropeptide, GMP) protruding from κ-casein. This reduces the charge density on the surface and the ionic strength of the solution will be increased which decreases the width of the double layer resulting in aggregation of casein particles (Lucey and Singh 2003).

2.1.2.2 Coagulation with enzymatic treatment

Chymosin is a proteolytic enzyme, originally from calf rennet, which is used to hydrolyze κ-casein at the surface of the casein micelle in milk. Chymosin is characterized by two aspartic acid residues (Asp_{34} and Asp_{215}) at the active site which

contribute to an irregular kidney shaped molecule consisting of two lobes with a hydrophobic cleft (Hyslop 2003). The active site is inside the cleft.

The sequence of amino acids in κ-casein critical to its susceptibility to proteolytic activity is located in the sequence of His_{98} to Lys_{112}. This sequence has a β-sheet structure with the whole sequences as (Hyslop 2003):

$$His_{98}\text{-}Pro_{99}\text{-}His_{100}\text{-}Pro_{101}\text{-}His_{102}\text{-}Leu_{103}\text{-}Ser_{104}\text{-}Phe_{105}\text{-}$$

$$Met_{106}\text{-}Ala_{107}\text{-}Ile_{108}\text{-}Pro_{109}\text{-}Pro_{110}\text{-}Lys_{111}\text{-}Lys_{112}$$

The sequence of Ser_{104}-Phe_{105}-Met_{106}-Ala_{107} is needed for chymosin to cleave the Phe_{105}-Met_{106} bond in κ-casein. The residues Leu_{103}-Ser_{104}-Phe_{105}-Met_{106}-Ala_{107}-Ile_{108} of κ-casein are hydrophobic and can accommodate the active site of chymosin (Plowman and Creamer 1995). The positive residues of κ-casein, mostly His_{98}, His_{100}, and His_{102}, will participate in electrostatic interaction with the negative amino acid sequence in chymosin (Plowman and Creamer 1995, Payens and Visser 1981, Payens and Both 1980). De Roos and others (1995) mentioned that these three histidine groups have a key role in the formation of the enzyme-substrate complex that preceeds the cleavage of the Phe-Met bond of κ-casein.

Larsson and Andren (1999) mentioned that chymosin was highly adsorbed onto κ-casein between pH 6 to pH 7.2. The chymosin cleavage site of κ-casein is accessible to the enzyme at about pH 6.5 (Creamer and others 1998). Below pH 6, chymosin has more proteolytic activity toward α_s-casein and β-casein. Chymosin hydrolyzes the Phe_{105}-Met_{106} bond of κ-casein into hydrophobic para-κ-casein (residues 1-105) and the hydrophilic glycomacropeptide (residues Met_{106}- Val_{169}). Proteolysis of this bond is

1000 times faster than the action on any other peptide bond (Waugh 1958). The process can be shown as:

K-casein + chymosin → para-κ-casein + GMP + chymosin

As the negatively charged glycomacropeptide segment of κ-casein is released into the serum, it causes an imbalance in the intermolecular forces in the milk system by increasing the hydrophobic and positive charge on the surface of the casein micelle. Hydrophobic κ-casein does not have the ability to protect the calcium insoluble caseins and coagulation between particles is induced. Subsequent gelation will occur when 80-90% of the κ-casein is hydrolyzed (Walstra and Jenness 1984). The interaction between chymosin and para-κ-casein is definitely not a hydrophobic interaction (De Roos and others 1995). Larsson and Andren (1999) mentioned that the chymosin-catalyzed hydrolysis of κ-casein to para-κ-casein (pI > 7) results in positive net charge on the para κ-casein while chymosin (pI ≈ 4.6) has a negative net charge. These differences in pI result in a wide pH interval for chymosin adsorption to κ-casein (Larsson and Andren 1997).

Affinity between substrate and enzyme is important (*Km*) in enzymatic activity. Enzymes will be more active at lower values of *Km*. It has been shown that the *Km* for isolated κ-casein is lower than the *Km* for intact whole casein. The *Km* for isolated κ-casein ranges from $3.3x10^{-5}$ to $6.6x10^{-5}$ M while *Km* for intact whole casein ranges from $1x10^{-4}$ to $5x10^{-4}$ which means that chymosin has more affinity toward isolated κ-casein than intact casein (Vreeman and others 1986).

In order to hydrolyze the κ-casein on the surface of the particle, chymosin has to be able to penetrate the outer layer of κ-casein in order to form an enzyme-substrate complex. The binding of chymosin to casein in an emulsion would depend on the environment in an emulsion. A low pH in an emulsion should result in greater association between chymosin and casein compared to a high pH because of electrostatic interactions between chymosin and κ-casein. De Roos and others (1995) mentioned that chymosin has a small net negative charge (pI = 4.7) at pH 5.0 which will increase as the pH is increased. At pH values above 6, the positively charged histidine groups become more and more negative which inhibits the association of chymosin and casein.

The ionic strength (*I*) of the solution will also influence the reaction between chymosin and κ-casein. There are two opposing effects of ionic strength to chymosin and casein binding which can be explained by ionic shielding. An increase in the ionic strength of a system will facilitate the complex formation between chymosin and κ-casein since both have a net negative charge. However, an increase in the ionic strength can also inhibit the complex formation between chymosin and κ-casein because the positively charged groups near the active site of κ-casein must complex with negatively charged counterparts on the active site of chymosin. This double phenomenon could be influenced by the thickness of the protein layer. However, it has been reported that when the ionic strength was increased, chymosin association with casein became weaker and the rate of proteolysis decreased (De Roos and others 1995).

The rate of proteolytic activity by chymosin is very dependent on the substrate concentration at the surface of the particles. In milk, a higher substrate concentration

caused an increase in the chymosin mediated clotting time (Payens 1984). However, Dickinson and others (1987) mentioned that κ-casein in a κ-casein stabilized emulsion was hydrolyzed faster if there was excess κ-casein in the emulsion. The presence of protein in the solution resulted in hydrophobic para-κ-casein in the solution which caused some displacement of the κ-casein adsorbed on the colloidal particles (Dickinson and others 1987).

Calcium assists coagulation by acting as a bridge between micelles. Calcium ions binds to the hydrophilic and negative charged macropeptide fragment of κ-casein on the κ-casein stabilized micelle to reduce the electric repulsion and induce the coagulation. However, the addition of $CaCl_2$ does not influence the rate of proteolysis (De Kruif and Holt 2003).

2.2 Emulsions

2.2.1 Emulsion stability

Emulsions are created from water and lipid. They are inherently unstable and tend toward instability. Emulsions can deteriorate by creaming, flocculation, Ostwald ripening, or partial coalescence which leads to coalescence.

Creaming occurs when the density between the liquid (continuous phase) and the lipid (dispersed phase) is different. In creaming, there is buoyancy acting on the particles. Stoke's equation is usually used to define the velocity of the creaming rate. Stoke's equation can be seen as (Walstra 1996):

$$V = d^2 (\rho_d - \rho_c) g /18 \eta$$

where, V = particle velocity

d = particle diameter

ρ_d = discrete phase density

ρ_c = continuous phase density

g = the force of gravity = 9.81 m/s^2

η = continuous phase viscosity

An understanding of the creaming rate helps to understand the stability of the emulsions. Creaming is related to the homogeneity of the particles, the size of the particles, and the viscosity of the system. The creaming rate will be reduced when the particles are homogeneous, small, the density difference between the particles is small, and the system is viscous. An emulsion with more small particles compared to large particles of the same material, will result in higher viscosity which will reduce the creaming rate.

Flocculation is the aggregation of two or more droplets which retain their original structural integrity. It is usually caused by bridges between two fat droplets that are covered by protein (Dickinson 2001). Ostwald ripening is the growth of larger droplets from smaller droplets without coalescence and is related to the solubility gradient between the small and large droplets (Rousseau 2000). It is most likely to be a problem with water in oil emulsions. Partial coalescence is used to describe the association of two droplets when there is penetration of a solid fat crystal from one droplet into a region of liquid oil in another droplet. Emulsified oil droplets originally

contain a network of fat crystals that cannot fully coalesce (Walstra 1996). When the film between these crystals is ruptured, they will form an irregular clump. The partially crystalline droplets merge to form a single irregular shape (McClements 1998). Rupture of the film can be triggered by crystals protruding from the droplets which cause the film to break. Partial coalescence can be affected by shear rate, volume fraction of droplets, fat crystallization, droplet diameter, and surfactant type and concentration (Walstra 1996).

2.2.2 The characteristics of emulsifiers

Lipid and water have characteristics that promote instability in an emulsion. Water molecules tend to associate in clathrate-like structures around hydrophobic molecules in aqueous solutions which cause a large interfacial energy. When two hydrophobic molecules are brought together, the total contact area with the aqueous solution is decreased and the hydrophobic force is increased which causes separation between water and lipid. An emulsifier in needed to minimize this interfacial force by adsorbing onto lipid surface and to associate with the hydrophilic environment. This decreases the interfacial tension between lipid and water as small as possible in order to get stabilization. On the other hand, the presence of the emulsifier increases the viscoelastic properties of the interface that contribute to the inhibition of coalescence.

The stability of an emulsion depends on the properties of the emulsifier at the interface of the particle. There are many colloidal forces important for emulsion stability.

The attraction of particles in an emulsion is affected by London- van der waals interactions which relates to the Gibbs free energy potential. The Gibbs-Marangoni effect helps stabilize emulsions by contributing an interfacial tension between emulsified particles which cause a stream of liquid along the interface to provide repulsion between particles. Becher (1965) mentioned that the Gibbs free energy of the emulsifier in the adsorbed state must be lower than in the unadsorbed state for the emulsifier to be surface active at the interface.

A charged polymer surfactant on the lipid interface will provide steric or electrostatic stabilization by preventing particles from close association. Electrostatic and steric repulsion are influenced by the environment in the solution, which includes ionic strength, pH, and polymer coverage and solubility. Protein as an emulsifier provides low surface charge density which provides low zeta potential which contributes to emulsion stability by controlling the range of the electrostatic and steric repulsion (Mohan and Narsimhan 1997, Bergenstahl and Claesson 1997). The DLVO (Deryagin-Landau, Verwey-Overbeek) theory describes the magnitude of the stabilizing energy barrier between two charged particles which relate to the sum of the electrostatic double-layer repulsion and the van der waals attraction. When the stabilizing energy is negative for all distances the particles will aggregate. If the free energy is balanced or at a maximum, two charge particles will be able to have infinite distance where repulsion between them might be present (Walstra 1996). The ability of an emulsifier to be adsorbed onto the surface of a particle is influenced by the electrostatic forces between emulsifier and the surface. Bergenstahl and Claesson (1997) mentioned that under low

ionic strength conditions, due to the strong electrostatic attraction, highly charged polyelectrolytes adsorb in a very flat conformation on oppositely highly charged surfaces. On the contrary, electrostatic forces are decreased within high ionic strength conditions or by reducing the charge density of the polymer. The adsorbed layer will be a more extended structure under these conditions.

Forces between protein-coated surfaces also influence emulsion stability. The forces involved are not only Van der waals interactions, but also hydrophobic and ionic interactions that are combined with an entropy gain caused by conformational changes of the protein during adsorption to the lipid surface.

As a surface active reagent, an emulsifier has three attributes. First is the ability to rapidly adsorb to an interface. Flexibility of the protein is very important in this case. Secondly, it must be able to rapidly unfold and reorient at an interface. The non-polar side chains of the emulsifier easily move from the unfavorable environment of the aqueous solution to a more favorable lipophilic environment and are adsorbed to the non-polar environment. Finally it should interact with neighboring molecules and form a strong cohesive, viscoelastic film that can withstand thermal and mechanical motions. Milk protein has the tendency to partially unfold during the formation of a film layer to cover the non-polar surface (Dickinson 1992). The hydrophilic head of an emulsifier is the polar fragment and is generally composed of hydroxyl or carboxyl groups and is soluble in the aqueous phase of an emulsion. The lipophilic group is the non polar hydrophobic area which usually has one or more hydrocarbon chains which are soluble in the oil phase (Hernandez 2001).

2.2.3 Types of emulsifier

Two classifications of emulsifiers are large molecule surfactants and small molecule surfactants. Small molecule surfactants such as monoglyceride, diglyceride and lecithin are emulsifiers that can be used in non-ionic or ionic conditions. Large molecule surfactants such as milk protein are also widely used. The amphiphilic nature of milk protein facilitates the orientation of the hydrophobic residues into the oil phase with the hydrophilic residue in the aqueous phase.

The two types of milk protein, whey proteins and caseins, have different molecular structures which will influence the behavior of emulsions. Whey proteins generally have a globular shape but caseins have an irregular shape which provides more flexibility. The flexibility of milk proteins allows them to interact at the surface of the lipid by spreading over the surface and by protruding into the aqueous phase. Caseins act differently from globular proteins at the lipid and aqueous interfacial surface. The caseins provide a more viscoelastic interfacial film that promotes stabilization compared to globular proteins. Kinsella (1984) mentioned that individual, flexible caseins are better at lowering surface tension and stabilize more surface area than globular proteins at the same concentration. Globular protein tends to form a 3-D structure in solution (Dalgleish 1996). After being adsorbed to oil and water interfaces, globular proteins change their structure from their native state to a less regular structure and become denatured. Conformational changes of the protein occur for some proteins at the interface when hydrophobic residues orient toward the nonpolar phase and hydrophilic residues protrude into the polar aqueous phase (Mulvihill and Fox 1989). The caseins

provide a film thickness of 12 nm after being adsorbed from the solution to the interface of lipids, while globular proteins provided a film thickness of only 2 nm (Dalgleish 1996). Holt and Horne (1996) mentioned that the 12 nm film thickness was formed by the macropeptide portion of κ-casein which constitutes about 10% of the total κ-casein.

Full coverage of the fat globule is very important in order to stabilize oil in water emulsions. When there is insufficient protein emulsifier to spread over the oil-water interface during emulsification, coalescence can be a problem. Protein load can be used to understand the spreadability of protein on the surface of a particle. The maximum protein load for caseins on the micellar surface is about 3 mg/m^2. However, the load of κ-casein spread over the interface of the micelle is only about 1 $mg.m^{-2}$. The rest of the micellar surface must be occupied by other proteins (Dalgleish 1998). It means that only one third of the micelle surface is covered with κ-casein. The particle size of the droplets influences the amount of κ-casein needed in the emulsions. Higher homogenization pressures of milk protein and lipid produced emulsions with smaller particles and increased the protein load (Cano-Ruiz and Richter 1997, Sharma and others 1996). Low protein coverage of the lipid surface causes the emulsion to have loose, packed layers and have less adhesion of the protein on the surface of the emulsion (Dickinson and others 1983, Walstra 1996, Rousseau 2000). Therefore, the amount of κ-casein that should be used to create an emulsion is very important in order to ensure coverage of the lipid droplets and produce stabile emulsions. Too much of a large polymer emulsifier like a protein, can affect emulsion stability by causing flocculation depletion where large particles (> 100 nm) flocculate and there is phase separation from smaller particles (<

100 nm). Rousseau (2000) mentioned that the existence of excess protein will cause a tight monolayer at the interface which leads to strong adhesion of the membrane.

It is well known that fat globule size and the distribution of the globule size are key aspects of emulsion formation and stabilization. The size of the droplets and the initial distribution of the droplets can be manipulated by using different shearing or stirring rates during homogenization. The two major mechanisms initiated during homogenization to produce an emulsion are intense turbulence and shear flow fields (Mohan and Narsimhan 1997, Walstra 1983). Turbulence is the most predominant force in the emulsification process which disrupts the dispersed phase into small particles. Smaller fat droplets can be produced by increasing the turbulence (higher homogenization pressure) and larger fat droplets can be produced by reducing the turbulence (low homogenization pressure). Reducing the droplets size will improve the shelf life of the products by reducing the creaming rate and by increasing the surface area of the emulsifier to improve the coating ability or penetration action (Floury J and others 2000). Native milk fat globules whose original size range from 1-10 μm were broken into globules 1 μm in diameter after being exposed to high homogenization pressure (Dalgleish 1996). Commercial homogenization of milk is usually conducted at a minimum pressure of 20MPa.

Two additional factors which influence the size of droplets in an emulsion are interfacial tension and power density (rate of energy diffusion per unit volume) (Dickinson 1997). This can be explained with the Young-Laplace equation (Walstra 1996):

$$pL = 2\gamma / r$$

pL is the Laplace pressure, γ is the interfacial tension and r is the radius of the particles. In order to continually reduce the size of small particles, lower interfacial tension is demanded or higher pressure is needed. In terms of power density, increased power density is needed to produce smaller droplets.

2.2.4 Κ-casein: a stabilizer and an emulsifier

About 95% of the casein in milk exists as micelles containing calcium and phosphate known as colloidal calcium phosphate (CCP) (Farrell and others 2003). In the micelle core, the casein undergoes indefinite self-association in the presence of multivalent cations (Holt and Horne 1996). Hydrophobic bonding, hydrogen bonding, and ionic interactions play a role in maintaining the integrity of casein micelle (Lucey and Singh 2003, Farrell and others 1999). It is believed that the stability of casein in milk is due to the arrangement of the individual caseins in the casein micelles. The sensitivity of the individual caseins to calcium from the most sensitive to the least sensitive is $\alpha_s \geq \beta > \kappa$ (Dalgleish and others 1985). The calcium insensitive casein (κ-casein) protects the calcium sensitive caseins (α- and β-casein) from aggregation by calcium ions by covering the surface of the casein micelles. Walstra (1999) suggested a model where the spherical casein micelle does not have a smooth surface. The chain of the C-terminal end of κ-casein protrudes from the micelle surface to form a 'hairy' layer which is responsible for the stability of the micelles against flocculation by providing steric and electrostatic repulsion.

Κ-casein has been described as the filler between the calcium-sensitive caseins and milk serum. Κ-casein can be involved in intramolecular and intermolecular reactions. The unique disulfide bonds in κ-casein can provide intermolecular crosslinked polymers with the micelle core. Holt and Horne (1996) mentioned that κ-casein reacts with β-casein in the micelle core to create high voluminosity. On the other hand, it is partially solubilized in the milk serum by hydrophilic tails (C terminal) that project into the serum (Creamer and others 1998). The macropeptide segment of κ-casein has been found to be the only mobile material that emerges from the casein micelle (Dalgleish 1998). This segment contributes to the thickness of the hydrodynamic radius of the micelle and the thickness influences the behaviour of particle interactions. Payens and Both (1980) mentioned that if the electrical double layer exceeds the radius of the interacting particles, a high ionic strength solution will result in attraction between the particles and a decrease in the ionic strength of a solution will result in increased repulsion between the particles. The macropeptide segment of κ-casein contributes a thickness of about 5 nm to the diameter of micelles in milk (Dalgleish 1998) and approximately 10 nm to the diameter of micelles in concentrated milk (Horne and Davidson 1993). Similar values have been found in emulsions (Dalgleish 1996, 1998). Κ-casein adsorbed to polystyrene latex particles produced a layer thickness of about 8 to 9 nm (Dalgleish 1996). However, the thickness was decreased by the addition of calcium ions onto the adsorbed layer of κ-casein.

The effect of the addition of calcium on the charge at the protein surface is due not only to direct ion-binding on the protein, but also to a general divalent counterion

phenomenon (Dalgleish and others 1985). It influences the particle mobility, electrokinetic potential, and the surface charge density. Calcium ions reduce the electrokinetic potential (zeta potential) and the net charge of protein coated surfaces toward zero and increases the ionic strength of the solution. Reducing the charge on the particle surface decreases the thickness of the protein layer which then will induce aggregation between particles. Consequently, the concentration of calcium in a system affects the electrophoretic mobility of the caseins. The order of particle mobility from fastest to slowest at low concentrations of $CaCl_2$ is $\alpha_s > \beta \geq \kappa$. However, at high concentrations of $CaCl_2$, the particle mobility is $\kappa > \alpha_s \geq \beta$ (Dalgleish and others 1985).

2.3 Rheology measurement

Rheology measurements needed to define the functional properties of an emulsion are viscosity, yield stress, flow behavior, and gel strength. These measurements include force application or stress, strain measurement, and time scale. Protein emulsification will change the viscosity of the emulsion which will influence the flow behavior of the emulsion. The application of stress to an object will show the viscoelastic behavior of the object. The viscoelastic behavior of a material depends on the reversible and irreversible properties of the object and the length of time the object is exposed to the stress. Viscoelasticity behavior can be used to predict the yield stress and gel strength of the material as well as to explain the changes in the structure, formation, and the characteristic of the emulsion.

2.3.1 Viscoelastic behavior

Viscoelastic behavior is important to help understand the hydrocolloidal characteristic of an emulsion. Every fluid like or colloidal emulsion possesses unique viscoelastic behavior which contributes to its functionality in food. The viscoelastic behavior of the emulsion explains how particles behave in the emulsion. Viscoelastic behavior depends on the protein concentration in the emulsion, the surface area of the particles, and the mechanical and physical processes used during emulsification. Different emulsification processes produce different interfacial interactions between emulsified particles which contributes to different viscoelastic behavior. Viscoelastic behavior could be solid-like or fluid-like, which is defined by the flow behavior of the object. Generally, there are two types of flow behavior, Newtonian and non-Newtonian. Shear rate and shear stress measurements are necessary to determine the flow behavior of the emulsion. Newtonian fluid behavior is the simplest type of fluid behavior where shear stress is directly proportional to shear rate (Steffe 1996). The relationship is shown as (Steffe 1996):

$$\sigma = \mu \dot{\gamma}$$

Where σ is shear stress, $\dot{\gamma}$ is shear rate, and μ is constant. A Newtonian fluid will have a linear relationship between the shear rate and shear stress with zero intercept. Newtonian behavior shows no time dependency which means that there is no change in viscosity within constant shear rate. When this relationship has not been satisfied, non-

Newtonian fluid behavior has occurred. Non-Newtonian fluid behavior has the relationship as (Steffe 1996):

$$\sigma = f(\dot{\gamma})$$

Where there is no constant relationship between shear rate and shear stress. Non-Newtonian fluid behavior is nonlinear, which means that the viscosity changes with changes in shear rate. A decrease in viscosity as shear rate is increased is shear thinning or pseudoplastic behavior. These changes in viscosity are caused by the alteration in the structure in the material within time. If the viscosity of a fluid decreased within a period of time, the fluid exhibited thixotropic behavior; on the other hand, if the viscosity increased within a period of time, it would have displayed rheopectic behavior. There is a non-zero intercept in non-Newtonian fluid behavior which can be measured as the yield stress in the emulsion. Force must be applied to initiate flow in systems that have yield stress. Yield stress represents the minimum shear stress required to initiate flow. There are two types of yield stress, Herschel-Buckley behavior and Bingham Plastic behavior. Bingham Plastic systems are a solid under static conditions, but once the yield stress value is exceeded and the flow begins, the fluid displays Newtonian flow characteristics. Herschel-Buckley systems will never show the Newtonian flow characteristic even after the flow has been started.

2.3.2 Interfacial rheology of protein stabilized emulsion

The stability of protein stabilized emulsions depends on the characteristics of the proteins adsorbed onto the surface of the particles. The conformation of proteins is

usually changed when they are adsorbed onto an available lipid interface. Intermolecular interaction within the adsorbed protein molecules can occur and might contribute to interfacial rheology of the particles in the emulsion. The two types of surface rheology are shear deformation and dilatational deformation (Dickinson 2001, Walstra 1996). Shear deformation can be measured when the surface area and the concentration of surfactant in the surface area remain constant. Dilatational deformation is related to changes which occur when the surface is enlarged and the concentration of surfactant for the surface remains constant. It is expressed as the surface dilatational modulus, defined as (Walstra 1996):

$$E_{sd} = d\gamma / d \ln A$$

Where E_{sd} is finite for all surfactants, γ is interfacial tension, and A is interfacial area. An increase in surface area results in decreased surface tension which causes surfactant to rapidly diffuse to the enlarged surface. This will increase the protein load and cause the interfacial tension to decrease. Surface dilatational rheology is not as sensitive to protein type and molecular structure as surface shear rheology. However, surface rheological parameters are not easily measured. It depends on the pH, ionic strength, temperature, enzyme, and solvent quality (Walstra 1996).

CHAPTER III

MATERIALS AND METHODS

3.1 K-casein fractionation

K-casein was isolated using the sulfuric acid method described by Zittle and Custer (1963). Acid-precipitated casein isolated from raw skim milk (Oak Farms Dairy, Houston, TX) was freeze dried. Dried acid casein (350 g) was dissolved in one liter of 6.6 M urea (EMD chemicals Inc, Gibbstown, NJ). This solution was acidified with 200 ml of 7 N H_2SO_4 (EM Industries Inc, Gibbstown, NJ). Distilled water (2000 ml) was added after acidification of the casein solution. The pH of the mixture was 1.3 to 1.5. This mixture was undisturbed for two hours to allow the flocculent to precipitate. The precipitate and liquid phase was separated by filtering it through 11 cm filter paper (Qualitative 415, VWR Scientific products, West Chester, PA). K-casein was precipitated from the filtrate by the addition of 132 g (1M) of ammonium sulfate to each liter of filtrate. The precipitate was collected by centrifugation (MR18-12, Jouan Inc, Winchester, VA) at 900x*g* for 15 min at room temperature. The precipitate was suspended in distilled water and dissolved by the addition of 1 N NaOH (EMD chemicals Inc, Gibbstown, NJ) to achieve a final pH of 7.5. The solution was dialyzed overnight and freeze dried. The yields of κ-casein were about 7 to 12% of the acid casein. Isolates of κ-casein were further purified by mixing one volume of a 1% solution of finished, freeze dried κ-casein at pH 7.0 with 2 volumes of ethanol. Ammonium acetate (1 M) in 75% ethanol was added to the mixture until a typical, sticky precipitate

of κ-casein was obtained. The precipitate was dissolved in water by the addition of NaOH (1 M) to bring the pH to 7.5. The dissolved κ-casein was dialyzed and freeze-dried.

3.2 Protein purity

Gel electrophoresis was conducted using a Novex X Cell II Mini-Cell (Novex Co. San Diego, CA, USA). Sample buffer was prepared by mixing 125 µl of NuPAGE SDS sample buffer (Invitrogen, Carlsbad, CA, USA), β-Mercaptoethanol (50 µl) (Sigma chemical Co. St.Louis, MO, USA) and distilled water (75 µl). Running buffer was prepared by mixing 50 ml of NuPAGE MOPS SDS Running Buffer (Invitrogen, Carlsbad, CA, USA) with distilled water (950 ml). Antioxidant (500 µl) (NuPAGE, Invitrogen, Carlsbad, CA, USA) was added to 200 ml of the running buffer for the inner chamber of the electrophoresis cell. Gels (NuPAGE 12% Bis-Tris 1 mm x 10 well) were purchased from Invitrogen (Carlsbad, CA, USA). In order to load the gels with 0.1 to 0.5 µg of protein per band, samples were diluted to achieve a concentration of 1000 µg/ml. Sample and sample buffer were mixed 1:1 and heated at 70 ˚C for 10 min. Electrophoresis was conducted with an initial current of 100-115 mA/gel and a final current of 60-70 mA/gel. Gels were stained for 20-25 min using PhastGel Blue R (Pharmacia Biotech, Uppsala, Sweeden). Gels were destained for at least 2 hours or overnight in destaining solution I followed by destaining for 30 minutes in destaining solution II. SigmaGel from Jandel Scientific software (San Rafael, CA, USA) was used to determine the purity of the κ-casein. The purity of the protein was about 80%.

3.3 Emulsion preparation

3.3.1 Emulsion 1

Butteroil (Level Valley Creamery, Inc., West Bend, WI, USA) was added to solutions containing 0.3% κ-casein to achieve concentrations of 3, 10 and 20% milk fat. These mixtures were adjusted to pH 6.5 and heated in an 80 ˚C water bath to 65 ˚C. The mixtures were homogenized with a APV Rannie 2000 laboratory homogenizer (Albertslund, Denmark) at 20 and 100 MPa.

3.3.2 Emulsion 2

Butteroil (Level Valley Creamery, Inc., West Bend, WI, USA) was added to solutions containing 0.5, 0.7, and 1.0% κ-casein to achieve a concentration of 20% butteroil. These mixtures were then adjusted to pH 6.5 and heated in an 80 ˚C water bath to 65 ˚C. The mixtures were homogenized with a APV Rannie 2000 laboratory homogenizer (Albertslund, Denmark) at 20 and 100 MPa.

3.4 Chymosin treatment

Chymosin (Chr Hansen, Milwaukee, WI, USA) diluted 20X with distilled water which contained 1 M $CaCl_2$ (EM Industries, Inc., Gibbstown, New Jersey, USA) was added to the emulsions after homogenization. The chymosin (42 µl) solution was added to 32 ml of sample to simulate coagulation procedures used for Cheddar cheese manufacture. The treated emulsions were incubated for 30 minutes at 30 ˚C prior to viscosity and flow behavior analysis.

3.5 Particle size analysis

The particle size distribution in the emulsions was measured immediately after homogenization using a Beckman-Coulter LS 230 light scattering instrument (Coulter Corporation, Miami, FL, USA). The diameter (dvs), surface area, and distribution properties of the particles were determined. Particle size was expressed as $d_{4,3}$ (volume weighted mean diameter), and $d_{3,2}$ (surface weighted mean diameter). In this study, $d_{4,3}$ was used to measure the distribution of particle size since it is more sensitive to changes in the number of large particles which might appear in less stable emulsions (Segall and Goff 1999).

3.6 Rheology analysis

Viscosity was measured at room temperature using a Brookfield RV DVIII (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA) viscometer. Flow behavior was analyzed by increasing the shear rate from 0 to 100 s^{-1} which was then decreased from 100 s^{-1} to 0 s^{-1}. The ULA (Ultra-Low Adaptor) spindle was used to measure viscosity in emulsions with low viscosity.

The vane method was used to measure emulsions with high viscosity and yield stress. Yield stress was measured with a constant low shear rate (0.1 rpm) for 100 seconds. The yield stress was measured in an undisturbed emulsion. Viscosities were measured in previously mixed emulsions. The maximum torque of the viscometer used in this measurement was 7,187 dyne cm (Steffe 1996). This number was used to calculate the torque from the percentage of torque obtained from the measurement. The

dimension of the vane and vessel in the vane method must meet: $1.5 \leq h/d \leq 4$, $Z_2/d \geq 0.5$, $Z_1 = 0.0$ or $Z_1/d \geq 1.0$, where h is the height of the vane, d is the diameter of the vane, Z_1 is the distance between the top of the vane to the top of the solution, Z_2 is the distance between the bottom of the vane to the bottom of the solution.

3.7 Emulsion stability index / creaming index

The stability index was calculated as:

$$ESI = Fs/Fo \times 100$$

where:

Fo = fat concentration in the original emulsion

Fs = the concentration of fat in the serum after centrifugation at 1,400x*g* for 30 min at room temperature (Beckman Instruments, Inc. Palo Alto, CA, USA)

3.8 Fat analysis

The majonnier method (AOAC Method 989.05) was used to determine lipid concentration in the emulsions and for protein load calculations.

3.9 Protein load

Emulsions (25 µl) were centrifuged in a Beckman Coulter L8-80M Ultracentrifuge (Beckman Instruments, Inc. Palo Alto, CA, USA) using a SW 28 rotor (Beckman) at 18,000 x *g* for 20 min at 20 ˚C (Sharma and others 1996). The cream layer and the serum were separated carefully. The protein concentration in 500 mg of the fat

layer was determined using a Vario Max CN nitrogen analyzer (Elementar Americas, Inc, Mt.Laurel, New Jersey, USA). The nitrogen concentration of the fat globules was calculated using a nitrogen conversion factor of 6.38.

Then the protein load was calculated by using the equation:

$$\Gamma = (p * \rho_f) / (A * v)$$

where :

Γ = protein load (mg/m^2)

P = protein content of the cream layer (mg/g)

P_f = density of milk fat (0.916g/ml at 20˚C)

A = surface area (m^2) of 1 ml of fat

V = fat fraction of the cream layer (g/g)

3.10 Microscopy analysis

The microstructure of the emulsions was observed using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany). A 100x/1.3 oil immersion objective or a 10x/0.30 oil immersion objective were used for low magnification images. The images were obtained using differential interference contrast (Nomarski) optics. Images were recorded with a Photometrics (Tucson, AZ, USA) Coolsnap cf camera controlled by MetaVue software v.5 (Universal Imaging Corporation, Downingtown, PA, USA).

3.11 Statistical analysis

Statistical analysis was conducted using SPSS (MapInfo Corporation, Troy, NY). An ANOVA with a full factorial design was used to analyze particle size, viscosity, yield stress, emulsion stability index and protein load. All statistical analysis was done by using a 95% confidence interval. Multiple comparisons of means were done using Bonferroni mean separation. Transformations were made for some analysis in order to get equality in variances. Data from emulsions with 0.3% protein in 3, 10, and 20% milk fat were analyzed using a square root transformation of the data for yield stress measurements and an exponential transformation for viscosity measurements without chymosin treatment. A square root transformation of the data was used for particle size, yield stress, and viscosity measurements for emulsions with 20% milk fat in 0.5, 0.7, and 1.0% κ-casein after they were treated with chymosin.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Emulsions with 0.3% κ-casein and 3, 10, and 20% milk fat.

4.1.1 Particle size analysis

Probability values for the effects of homogenization pressure and fat concentration on the size of particles in the emulsions are presented in Table 1. The probability value for the interaction is not shown because it was not significant (p=0.109). Homogenization pressure and milk fat concentration influenced the mean particle size. The mean particles size for samples homogenized at 20 MPa was 1.630 μm which decreased to 0.31 μm when the samples were homogenized at 100 MPa (Table 2). The decrease in mean particle size with increased homogenization pressure was also reflected in the particle size distribution (Fig. 3). An increase in the homogenization pressure caused an increase in the percentage of smaller particles in each emulsion regardless of the lipid concentration (Fig. 4). All of the emulsions had the majority of the particles in the size range of 1 to 5 μm when they were homogenized at 20 MPa. However, after homogenization at 100 MPa, differences in the distribution patterns were more evident between fat concentrations. The majority of the particles in samples with 3% fat ranged from 0.04 to 0.15 μm in diameter. Emulsions that contained 10% fat had some particles in that range but most of the particles in this emulsion ranged from 0.1 to 0.5 μm in diameter. The emulsion that contained 20% fat had the majority of particles in the range from 0.2 to 1 μm in diameter. The $d_{4,3}$ mean for emulsions that contained 3, 10 and 20%

milk fat were 0.861, 0.856, and 1.197 μm (Table 2). The difference in the mean particle size between the emulsions that contained 3 and 10% fat (Table 2) was not significant ($p>0.05$). However, the mean particle size was different between emulsions with 3% and 20% milk fat and those with 10% and 20% milk fat ($p=0.000$). The particle size increased as the fat concentration increased at each homogenization pressure (Table 2, Fig. 5). Homogenization at 100 MPa compared to 20 MPa caused the mean volume diameter of individual lipid particles to decrease and this increased the number of smaller particles and the surface area of the fat globules that needed to be covered to create an emulsion. The ratio of protein to lipid is very important in order to provide sufficient protein to cover the increased surface area. An increase in the lipid concentration in conjunction with the increased surface area created by homogenization would provide more opportunity for lipid-lipid interactions than protein-lipid interactions which would favor an increased particle size associated with an increased concentration of milk fat.

Table 1 - Probability values for the effects of fat concentration and homogenization pressure on mean particle size

Source ($R^2 = 0.987$)	P Values
Fat	0.000
HP	0.000

HP = Homogenization Pressures

Table 2 - Effect of homogenization pressure and milk fat concentration on the mean particle size ($d_{4,3}$)

Fat	Homogenization Pressure		Mean value (μm)
	20 MPa	100 MPa	
	---------- μm ----------		
3%	1.546	0.176	0.861[a]
10%	1.464	0.248	0.856[a]
20%	1.881	0.513	1.197[b]
$\bar{x}$	1.630[A]	0.312[B]	

a,b means in the same column followed by a different letter are significantly different. .

A,B means in the same row followed by a different letter are significantly different.

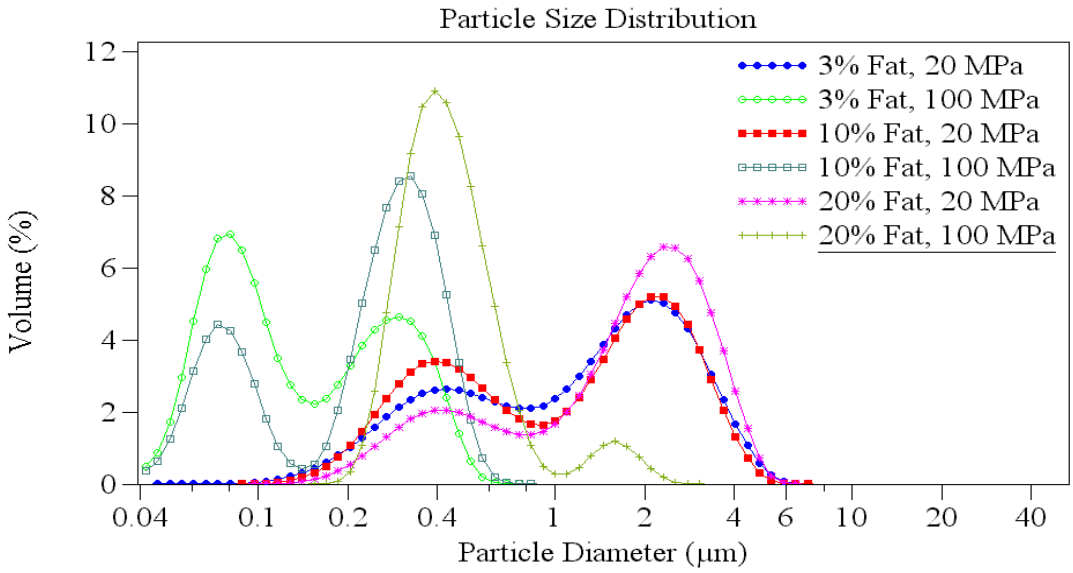

Fig. 3 - Particle size distribution in emulsions that contained 0.3% κ-casein

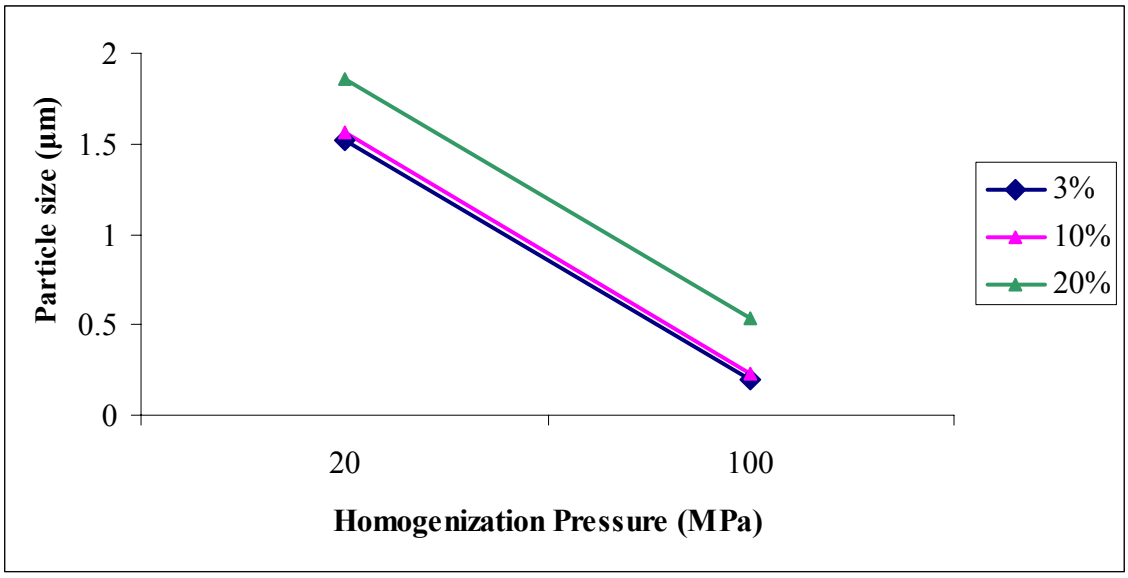

Fig. 4 - Effect of homogenization pressure on the mean particle size in emulsions that contained 3, 10, and 20% fat and 0.3% κ-casein

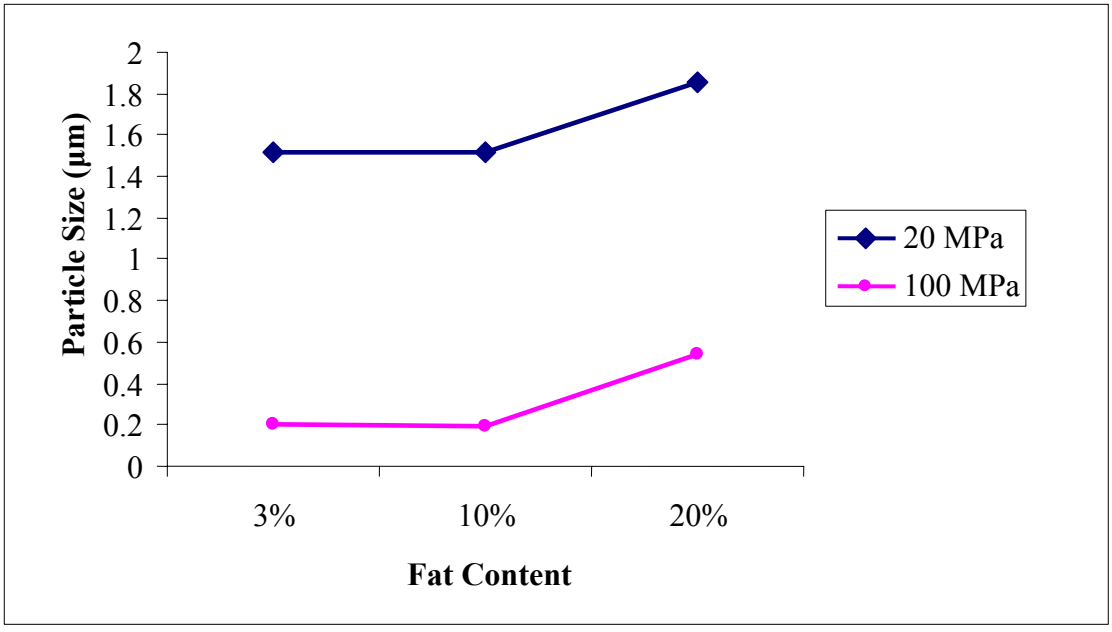

Fig. 5 - Mean particle size of particles in emulsions homogenized at 20 and 100 MPa

The emulsions homogenized at 20 MPa had a bimodal distribution with a main peak af particles with a diameter of approximately 2 to 3 μm and a smaller peak which indicated diameters of about 0.3 to 0.5 μm (Fig. 6). The highest percentage of particles in the range of 2 to 3 μm diameter was associated with the sample that contained 20% milk fat. The inverse was true for particles with a diameter that ranged from 0.3 to 0.5 μm. However, emulsions with 10% milk fat had a higher percentage of particles with a diameter of 0.3 to 0.5 μm than the emulsions with 3% milk fat. This difference did not affect the mean particle diameter (Table 2) and doesn't influence the fact that the emulsion with 20% milk fat had the least particles in this size range. The higher percentage of particles with diameters of 1 to 6 μm in the sample with 20% milk fat might be caused by coalescence of fat globules during homogenization. Mohan and Narsimhan (1997) mentioned that the rate of drop coalescence was related to droplet collisions caused by turbulence shear. The particle size got larger with increased fat concentrations which could have been caused by coalescence between the particles at the higher shear values.

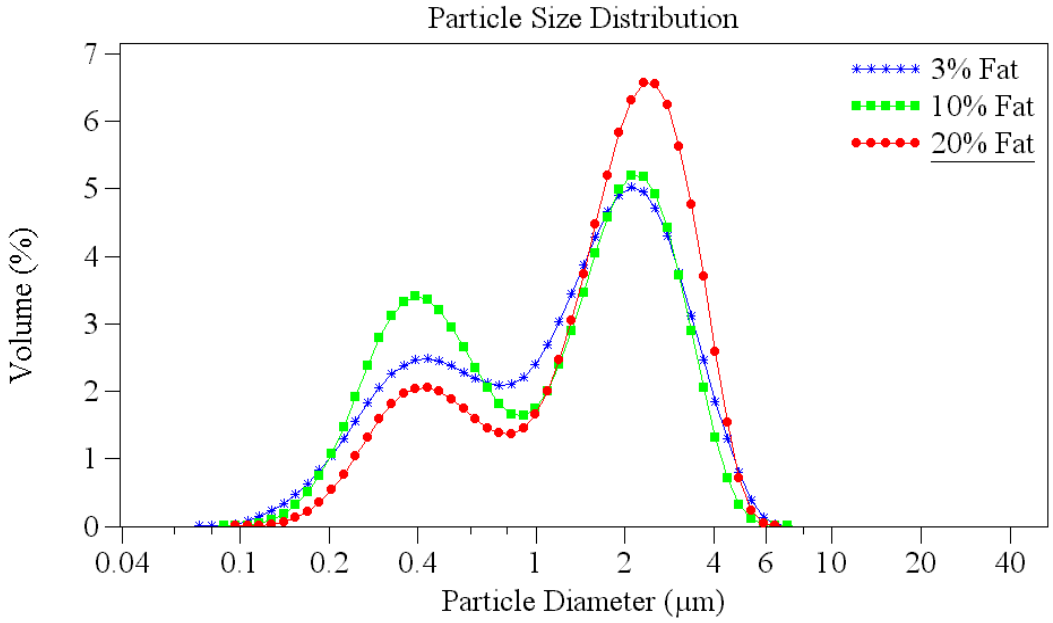

Fig. 6 - Effect of fat content on the particle size distribution in emulsions that contained 0.3% κ-casein and were homogenized at 20 MPa

The emulsions homogenized at 100 MPa showed much smaller particle size than emulsions homogenized at 20 MPa. Nevertheless, the largest particles were still associated with emulsions that contained 20% fat after homogenization at 100 MPa. However, the distribution of particle size was more unimodal for the sample with 20% fat than for the emulsions that contained 3 and 10% fat which had distinct bimodal particle size distribution patterns (Fig. 7). The highest percentage of particles in the sample with 3% fat had diameters that were approximately 25% of the diameter of the highest percentage of particles that contained 20% fat. This might be caused by the

increased turbulence which would have resulted from homogenization at 100 MPa in conjunction with more fat and a lower protein to lipid ratio.

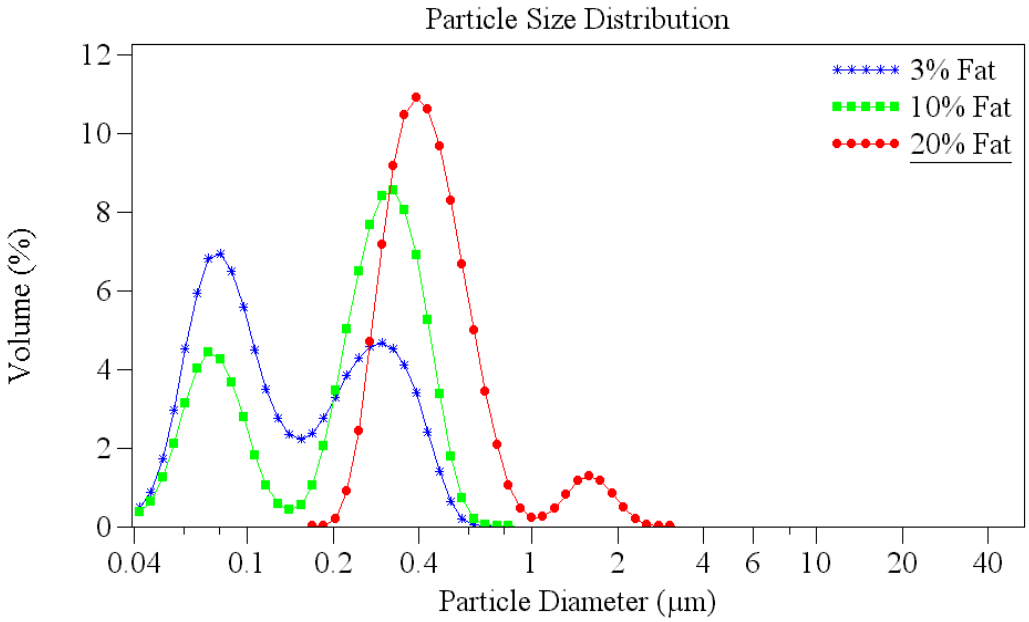

Fig. 7 - Effects of fat concentration on the particle size distribution in emulsions that contained 0.3% κ-casein and were homogenized at 100 MPa

4.1.2 Viscosity analysis

4.1.2.1 Viscosity prior to chymosin treatment

Probability values for the effects of fat, homogenization pressure, chymosin treatment, and their interactions on the viscosity of the emulsions are presented in Table

3. Homogenization pressure, fat concentration, treatments, and their interaction had a significant effect on the viscosity of the sample ($p<0.05$). The mean values for the viscosity of the emulsions prior to treatments with chymosin were 1.48 and 1.72 cP after they were homogenized at 20 and 100 MPa, respectively (Table 4). The higher viscosity after homogenization at 100 MPa ($p<0.05$) can be explained by the increased number of smaller particles which resulted in more surface area that had to be covered with protein. This would cause more intermolecular interactions between proteins on adjacent particles which would have increased the viscosity.

The viscosity of the emulsions was also affected by milk fat concentration ($p=0.000$). The means value for the viscosity of the emulsions that contained 3, 10, and 20% milk fat were 1.22, 1.45, and 2.12 cP, respectively (Table 4). Emulsions that contained higher fat concentration showed higher viscosity (Fig. 8). The increased viscosity related to the increased fat content can be explained by more fat particles in the emulsion. Emulsions with a higher milk fat content will have more fat particles in them and that will increase the viscosity because the fat would represent a higher percentage of the bulk phase in the emulsions. Statistical analysis revealed an ordinal interaction between homogenization pressure and fat content (Fig. 8). The emulsion that contained 20% milk fat had significantly greater viscosity than the other emulsions and would be the best candidate for the possible formation of a gel.

Table 3 - Probability values for the effect of fat, homogenization pressure, and chymosin treatment on the viscosity of emulsions

Source ($R^2 = 0.974$)	P Values
Fat	0.000
HP	0.011
Treatment	0.000
Fat*HP	0.018
Fat*treatment	0.000
HP*treatment	0.020
Fat*HP*treatment	0.017

HP = Homogenization Pressures

Treatment = with and without chymosin

Table 4 - Comparison of the mean viscosity for the effect of homogenization pressure, milk fat concentration, and chymosin treatment

Fat	No Chymosin treatment Homogenization Pressure			Chymosin treatment Homogenization Pressure		
	20 MPa	100 MPa	$\bar{x}$	20 MPa	100 MPa	$\bar{x}$
	---------- cP ----------			---------- cP ----------		
3%	1.17	1.27	1.22[a]	3.00	4.08	3.54[d]
10%	1.34	1.57	1.45[b]	11.42	24.95	18.18[e]
20%	1.93	2.32	2.12[c]	50.88	51.47	51.17[f]
$\bar{x}_1$	1.48[A]	1.72[B]		21.77[C]	26.83[D]	
$\bar{x}_2$			1.60[E]			24.30[F]

a,b,c,d,e,f means in the same column followed by a different letter are significantly different.

A,B,C,D,E,F means in the same row between treatments followed by a different letter are significantly different.

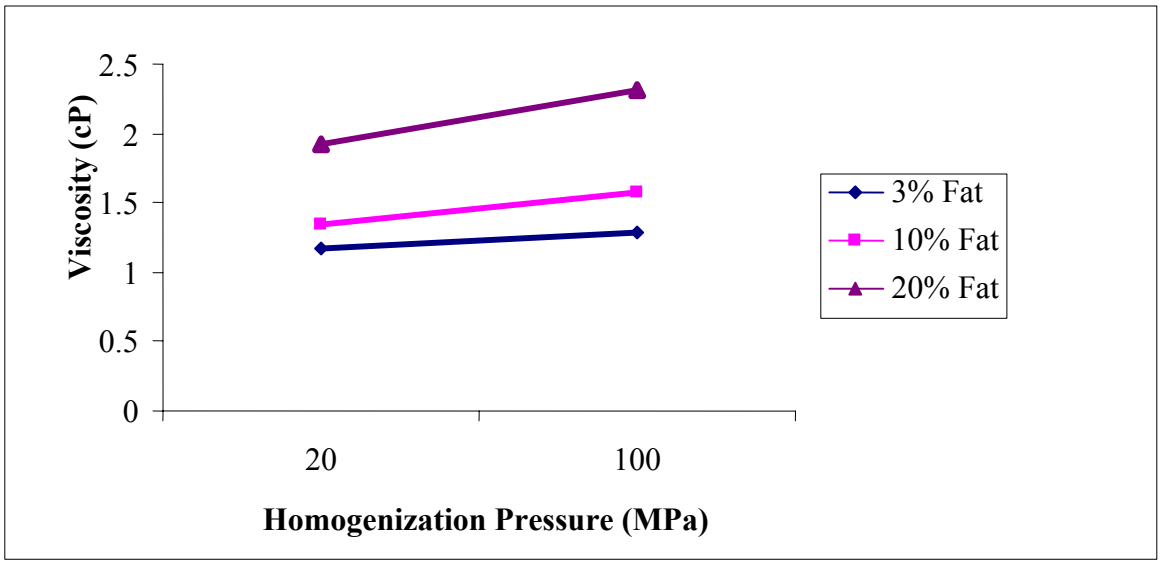

Fig. 8 - Effect of homogenization pressure and fat content on the viscosity of emulsions that contained 0.3% of κ-casein

4.1.2.2 Viscosity after chymosin treatment

Chymosin treatment affected the characteristic of the viscosity of the emulsions. The viscosity of emulsions treated with chymosin were approximately 3X, 13X, and 24X greater than emulsions without chymosin treatment for emulsion with 3, 10 and 20% milk fat, respectively (Table 4). The addition of chymosin to the emulsions homogenized at 20 and 100 MPa caused the viscosity to increase 15X compared to viscosity of similarly homogenized emulsions prior to treatment with chymosin (Table 4, Fig. 9).

Homogenization of the emulsions at 100 MPa compared to homogenization at 20 MPa caused an increase in the percentage of smaller particles in each emulsions and an increase the total surface area. The greater number of small particles would have also caused an increase in the packing density of the particles which would have allowed more contact points between particles for interactions. Chymosin could have promoted interfacial interaction between particles after hydrolysis of the κ-casein.

The viscosity of the emulsions treated with chymosin increased when the concentration of the fat was increased. Mean values for the viscosity of the emulsions with 3, 10, and 20% fat content were 3.54, 18.18, and 51.17 cP, respectively (Table 4). An increase in lipid concentration in conjunction with the increased surface area created by homogenization pressure would provide more binding sites for the hydrolyzed κ-casein to interact at the particle surfaces.

The interaction between homogenization pressure and fat content had a significant effect ($p < 0.05$) on the viscosity which indicated that the emulsions behaved differently at each homogenization pressure (Fig. 10). However, the emulsions with a concentration of 20% fat had the highest viscosity at both homogenization pressures (Fig. 11).

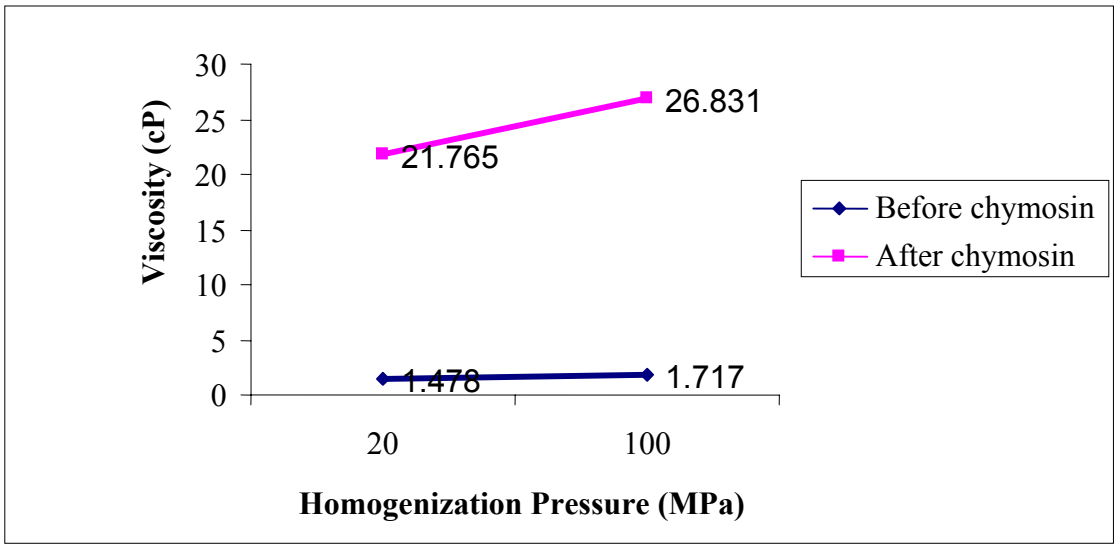

Fig. 9 - Effect of chymosin treatment and homogenization pressure on the viscosity of the emulsions that contained 0.3% κ-casein

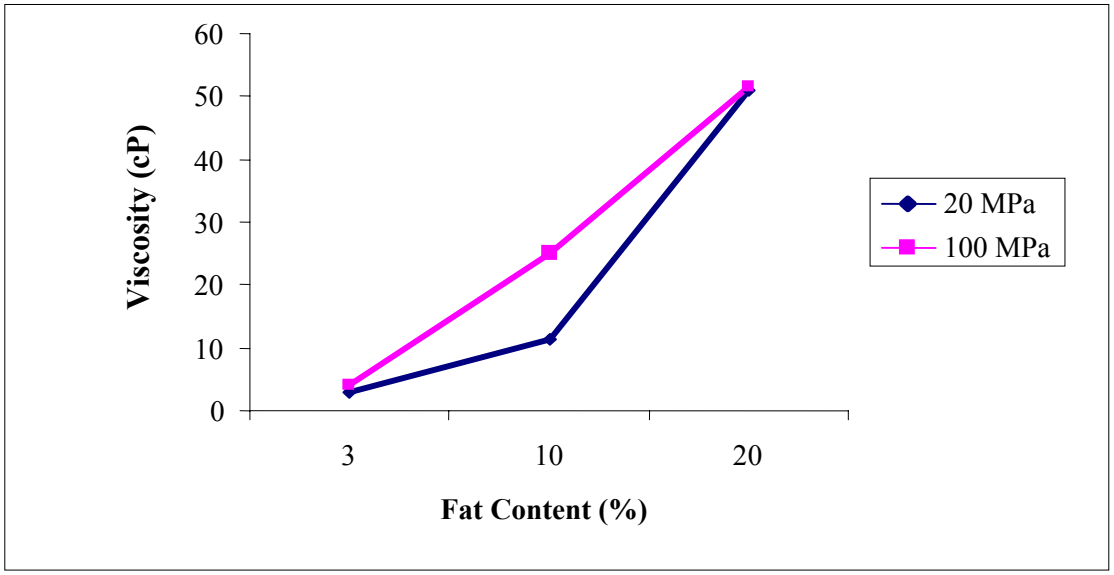

Fig. 10 - Mean viscosity of chymosin treated emulsions with 3, 10, and 20% fat and 0.3% κ-casein after homogenization at 20 and 100 MPa

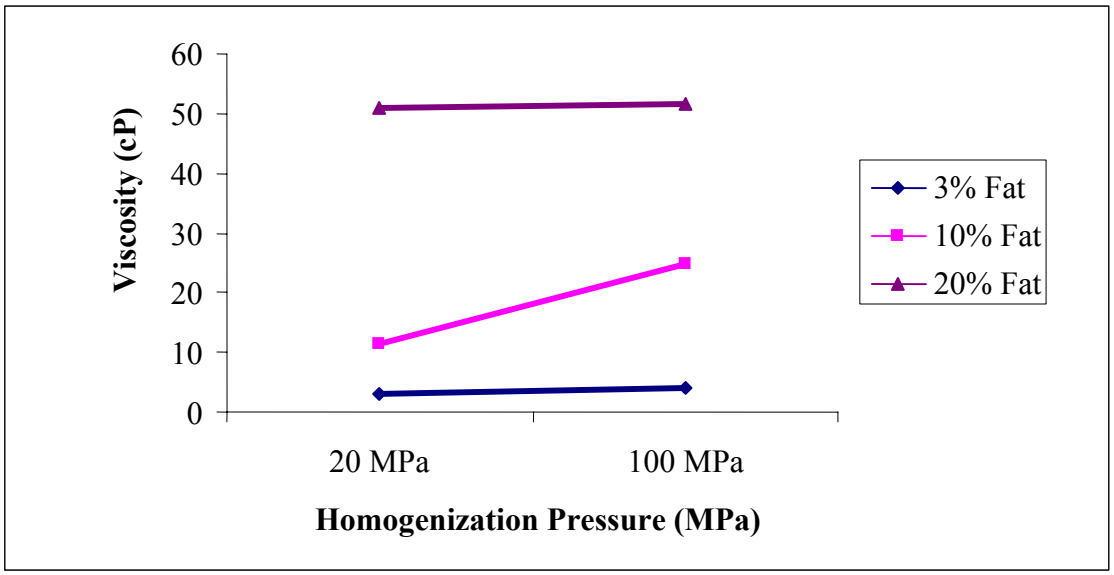

Fig. 11 - Effect of homogenization pressure at 3, 10, and 20% fat on the viscosity of emulsions that contained 0.3% κ-casein after chymosin treatment

4.1.3 Rheology measurement

4.1.3.1 Yield stress prior to chymosin treatment

Yield stress is an important indicator for identifying the formation of a gel. The addition of chymosin to the κ-casein stabilized emulsion will cause proteolysis of the κ-casein. If a gel is formed, the yield stress should increase and be an indicator of gelation. Yield stress is the stress needed to make a system flow. Yield stress measurements permit analysis of the effect of chymosin on κ-casein stabilized emulsion and on the characteristics of any gel that might be formed.

Analysis of the data revealed that fat concentration, homogenization pressure, chymosin treatment, and their interactions except for the interaction between homogenization pressure and chymosin treatment affected the yield stress (Table 5). However, fat concentration was the only factor that caused differences in the means for yield stress ($p < 0.05$) for emulsions not treated with chymosin (Table 6). The yield stress values increased as the fat concentration was increased. The mean yield stress values were 0.06, 0.07, and 0.17 Pa for emulsions that contained 3, 10, and 20%, respectively. The emulsions with 20% fat had the highest yield stress ($p < 0.05$) but the mean yield stress for the samples that contained 3 and 10% milk fat were not different (Table 6). Homogenization pressure had no significant ($p > 0.05$) effect on the yield stress of emulsions without chymosin treatment with mean yield stress values of 0.09 and 0.11 after homogenization at 20 and 100 MPa, respectively (Table 6).

Table 5 - Probability values for the effect of fat, homogenization pressure, and chymosin treatment on yield stress

Source (R^2= 0.972)	P values
Fat	0.000
HP	0.020
Treatment	0.000
Fat*HP	0.025
Fat*Treatment	0.000
HP*Treatment	0.052
Fat*HP*Treatment	0.033

HP = Homogenization Pressures

Treatment = with and without chymosin

Table 6 - Means for the effect of homogenization pressure, milk fat concentration, and chymosin treatment on the yield stress of the emulsions

Fat	No Chymosin treatment			Chymosin treatment		
	Homogenization Pressure			Homogenization Pressure		
	20 MPa	100 MPa	$\bar{x}$	20 MPa	100 MPa	$\bar{x}$
	---------- Pa ----------			---------- Pa ----------		
3%	0.04	0.08	0.06^{a}	0.44	0.72	0.58^{c}
10%	0.05	0.08	0.07^{a}	3.73	11.11	7.42^{d}
20%	0.18	0.17	0.17^{b}	21.43	21.00	21.27^{e}
$\bar{x}_1$	0.09^{A}	0.11^{A}		8.53^{B}	10.94^{C}	
$\bar{x}_2$			0.14^{D}			9.74^{E}

a,b,c,d,e means in the same column followed by a different letter are significantly different.

A,B,C,D,E means in the same row between treatments followed by a different letter are significantly different.

4.1.3.2 Yield stress after chymosin treatment

Treament of emulsions with chymosin affected the yield stress of the emulsions (Table 5). Data for the yield stress before and after treatment of the emulsions with chymosin is presented in Table 6 and Fig. 12. Emulsions that contained 3, 10 and 20% fat and treated with chymosin showed increased yield stress of 9X, 106X, and 125X, respectively, compared to emulsion not treated with chymosin. Emulsions with chymosin treatment showed 100X greater yield stress than emulsion with no chymosin treatment at comparable homogenization pressure. However, the emulsion homogenized at 100 MPa showed 1.3X greater yield stress than emulsion homogenized at 20 MPa (Table 6).

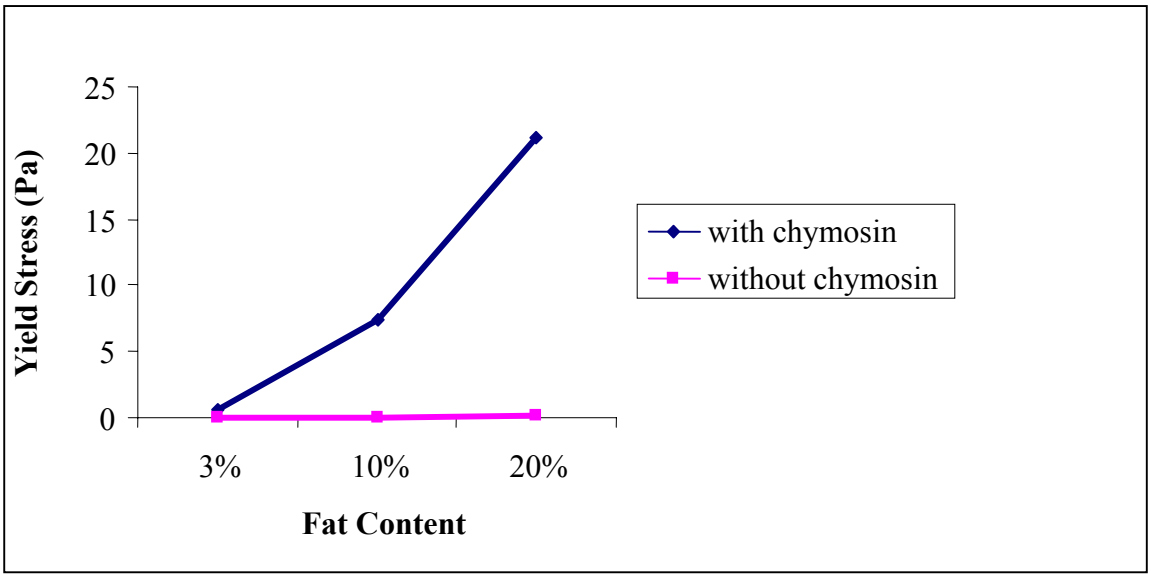

Fig. 12 - Effect of chymosin treatment on the yield stress of emulsions at milk fat concentrations of 3, 10, and 20% and 0.3% κ-casein

The change in yield stress associated with increased concentrations of milk fat was greater for the emulsions treated with chymosin compared to the changes observed for the emulsions that were not treated with chymosin. The fat content affected ($p < 0.05$) the yield stress with means of 0.58, 7.42, and 21.21 Pa for fat concentrations of 3, 10 and 20%, respectively ($p = 0.000$). The interaction between homogenization pressure and fat content was significant ($p < 0.05$). However, observation of the ordinal interaction (Fig. 13) revealed that the emulsions with 20% milk fat produced the highest yield tress and would be the best candidate for the production of a gel from the emulsion.

Homogenization pressure affected the yield stress values for the emulsions treated with chymosin ($p < 0.05$). There was an interaction ($p = 0.05$) between homogenization pressures and chymosin treatment and between homogenization pressure and fat concentration. The mean yield stress values for these samples homogenized at 20 and 100 MPa were 8.53 and 10.94 Pa, respectively. This observation indicated that the chymosin treatment caused some aggregation between particles and that homogenization at 100 MPa produced more and smaller particles that participated in the aggregation. This increased interfacial interaction between particles appeared to have induced gelation. Therefore, the emulsion homogenized at a 100 MPa had a greater yield stress than the emulsion homogenized at 20 MPa.

It can be concluded that the combination of homogenization pressure, fat content, and the addition of chymosin changed the rheological behavior of the

emulsions. Higher homogenization pressure increased the percentage of smaller particles in the emulsions which would have affected the amount of protein required on the lipid surface. The addition of chymosin appeared to generate a protein network between κ-casein stabilized particles which would have increased the viscosity and yield stress and would promote possible gel formation from the emulsions.

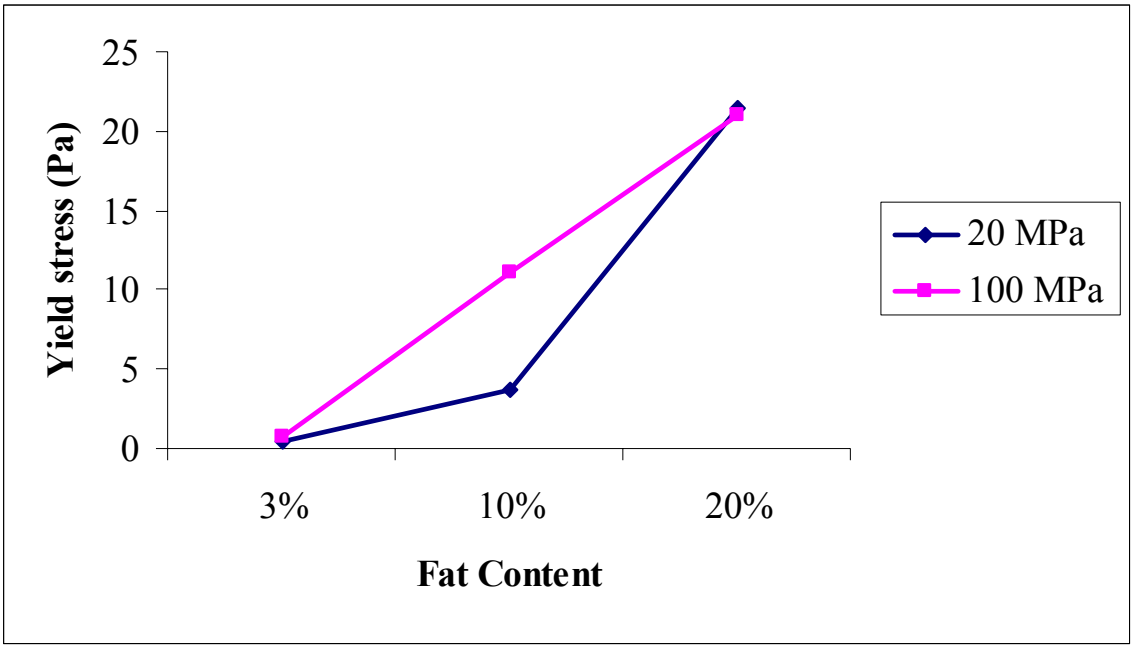

Fig. 13 - Effect of homogenization pressure and fat content on the yield stress of emulsions with 0.3% κ-casein after chymosin treatment

The linearity of flow behavior for the emulsions without chymosin and the emulsions with chymosin treatment was reflected by the rheological measurements (Fig. 14 and 15). Emulsions not treated with chymosin had Newtonian flow behavior and the rheological properties for the emulsions that contained 3 and 10% fat were similar (Fig. 14). Even though the emulsion that contained 20% fat exhibited Newtonian behavior similar to the other emulsions, the viscosity of this emulsion was much greater than the viscosity in emulsions that had a fat concentration of 3 or 10%. Treatment of the emulsions with chymosin changed the flow behavior of the emulsions. The change in rheological behavior increased as the concentration of milk fat increased. Little effect of the chymosin treatment was noted in the emulsions that contained 3% milk fat (Fig. 15). However, the change from Newtonian to Bingham Plastic flow behavior was clearly evident in the emulsions that contained 10 and 20% milk fat. A hysteresis loop was obviously present between the increasing and decreasing behavior curves for emulsion with 10 and 20% milk fat (Fig. 15). The greatest change in observed flow behavior was for the emulsion that contained 20% milk fat and was homogenized at 100 MPa (Fig. 15).

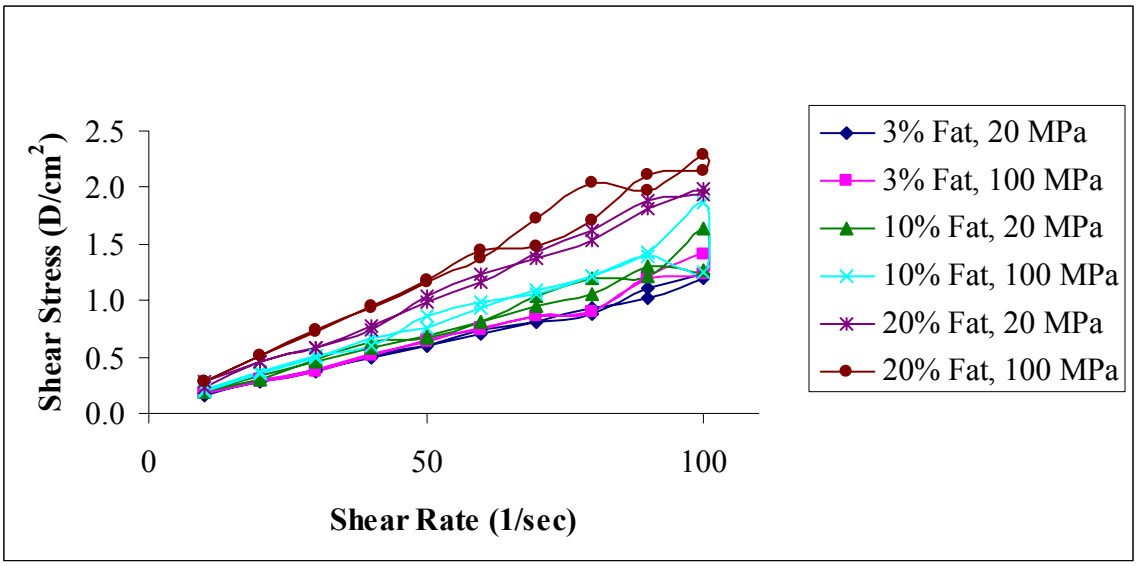

Fig. 14 - Effects of homogenization pressure and fat content on rheological behavior of κ-casein stabilized emulsions without the addition of chymosin

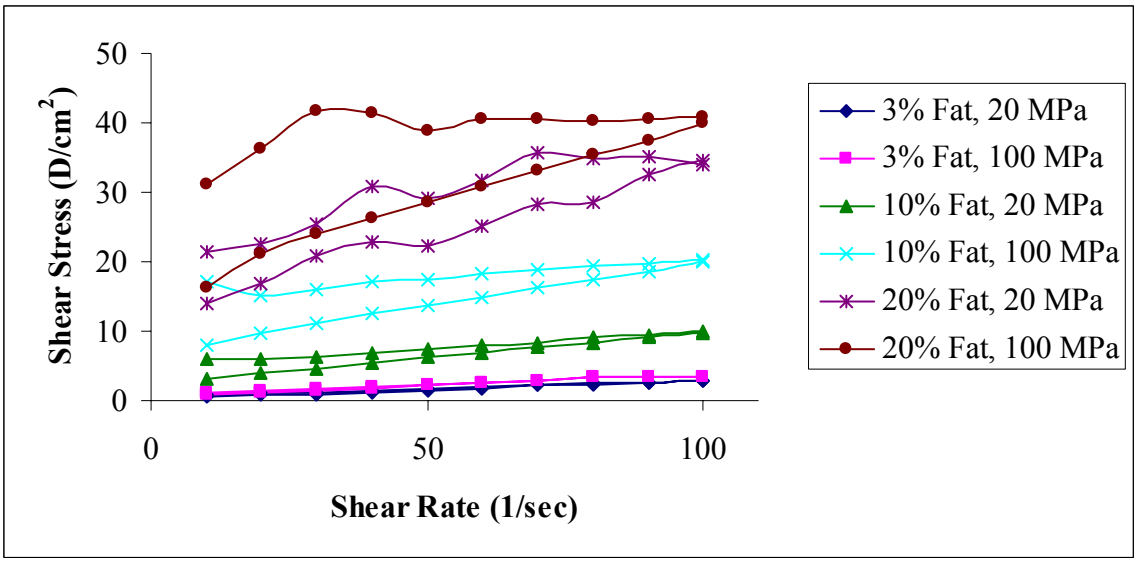

Fig. 15 - Effects of homogenization pressure and fat content on rheological behavior of κ-casein stabilized emulsions with the addition of chymosin

4.2 Emulsions with 20% milk fat and 0.5%, 0.7%, and 1% κ-casein

Data from emulsions that contained 0.3% κ-casein and 3, 10, or 20% fat indicated that emulsions that contained 20% fat would be the best candidate to investigate for the potential creation of gels from κ-casein stabilized emulsions. This emulsion had the highest viscosity and yield stress after treatment with chymosin. In order to investigate the role of κ-casein in possible particle interactions to create gels, these emulsions were made with 20% fat to contain 0.5, 0.7, and 1% κ-casein.

4.2.1 Particle size

Probability values for the effects of homogenization pressure and protein on the particle size in the emulsions are presented in Table 7. The probability value for the interaction between protein and homogenization pressure is not shown because the interaction was not significant ($p= 0.059$). Homogenization affected the particle size and the particle size distribution. The particle size distribution was similar to the particle size distribution in the emulsions that contained 0.3% protein. Higher homogenization pressure produced smaller particles. The mean particle size for samples homogenized at 20 MPa was 2.152 μm which decreased to 1.077 μm when the samples were homogenized at 100 MPa (Table 8).

Differences in the mean particle size due to changes in protein concentration ($p = 0.112$) were not detected even though there was a large decrease in the mean particle size associated with increased protein concentration in the emulsions. The mean value

for the diameter of particles in the emulsions that contained 0.5, 0.7 and 1.0% protein concentration were 1.715, 1.697, and 1.433, respectively.

Table 7 - Probability values for the effect of homogenization pressure and protein on the size of particles in emulsions with 0.5, 0.7, and 1.0% κ-casein

Source ($R^2 = 0.731$)	P Values
Protein (0.5%, 0.7%, 1%)	0.112
HP (20 MPa, 100 MPa)	0.000

HP = homogenization pressure

Table 8 - Effect of homogenization pressure and protein concentration on the mean particle size in emulsions with 0.5, 0.7 and 1.0 % protein

Protein	Homogenization Pressures		Mean Values (μm)
	20 MPa	100 MPa	
	---------- μm ----------		
0.5%	2.271	1.158	1.715[a]
0.7%	2.250	1.143	1.697[a]
1%	1.936	0.929	1.433[a]
$\bar{x}$	2.152[A]	1.077[B]	

a means in the same column followed by the same letter are not significantly different.

A, B are means in the same row followed by different letter are significantly different.

The decreased mean particle size associated with increased homogenization pressure was also reflected in the particle size distribution (Fig. 16). Contrary to the observation from emulsions with 0.3% κ-casein, these emulsions with 20% fat and higher concentrations of protein showed a mostly unimodal distribution of the particle sizes. The emulsions that contained 0.5 and 0.7% κ-casein and homogenized at 20 MPa had a major peak for particles with a diameter that ranged from 1 to 10 μm. However, when the protein concentration was increased to 1.0%, the major concentration of particle size diameters ranged from 0.5 to 5 μm. It is possible that when more protein was available in the emulsions to interact at the lipid interface, this decreased the size of the particles. The emulsions that contained 0.5 and 0.7% κ-casein and homogenized at 100 MPa had a main peak representing particles with diameters of approximately 0.4 to 2 μm. The particle size distribution shifted to smaller particles when the protein concentration was increased to 1.0%. Most of the particles in this emulsion had diameters that ranged from 0.2 to 1 μm.

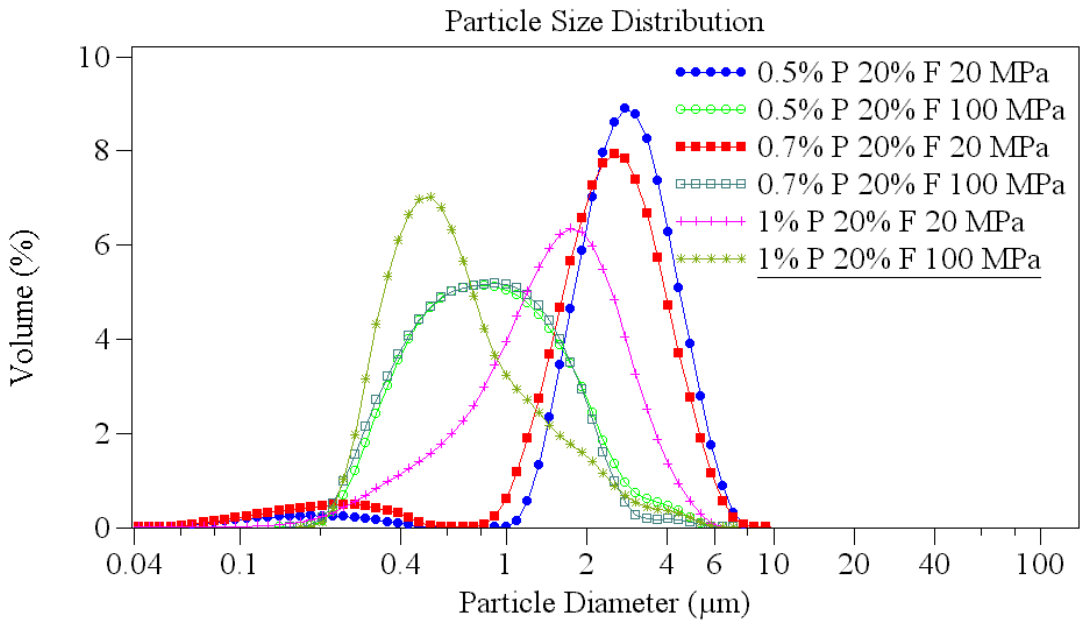

Fig. 16 - Effect of homogenization pressure on the particle size distribution in emulsions with 20% fat and 0.5, 0.7, and 1% protein

4.2.2 Protein load

Protein load is the amount of protein per unit area of the lipid interfaces. It reflects the affinity of the protein for the surface and the molecular structure of the protein. It helps understand the spreadability of the protein on the lipid surface. It not only helps understand the area of fat globules covered with protein, but also helps understand the stability of the emulsion. There are many factors that influence the protein load; homogenization pressure, protein concentration, and the aggregation state of the protein. Homogenization pressure influences the particle size and consequently, the surface area to be emulsified with the available protein. Dalgleish (2004) mentioned that if there is an excess concentration of the surfactant, the particle size is limited by the characteristic of the homogenizer. If there is a low concentration of the surfactant, the surfactant concentration will limit the size of the particles because insufficient emulsification will trigger the coalescence of emulsion droplets. Observation of data in Table 9 revealed that protein concentration did not affect protein load ($p > 0.05$) but homogenization pressure had a significant effect ($p < 0.05$) on the protein load. This indicated that there must have been an excess concentration of protein in the bulk phase. The mean values for the protein load were 5.72 mg/m^2 for the emulsion homogenized at 20 MPa and 3.92 mg/m^2 for the emulsion homogenized at 100 MPa (Table 10). This result revealed that higher homogenization pressure will produce smaller particles which will have a lower protein load and it is true with the inverse. Protein concentration and protein conformation on the lipid interface can influence protein load. Walstra (1996) mentioned that when the protein to lipid surface ratio is low, the protein will unfold at the oil and

water interface and form a stretched polypeptide layer with a protein load of about 1 mg/m^2. However, when the protein concentration on the lipid surface has already reached the maximum amount, a highly water soluble protein will give a protein load of about 3 mg/m^2 (Walstra 1996). Flexibility of the protein also influences the ability of the protein to occupy space at the particle surface. K-casein is one of the flexible and soluble casein that produced a mean protein load value of approximately 4.8 mg/m^2 of lipid surface (Table 10). Protein concentration and the interaction between protein and homogenization pressure had no effect on the protein load. The protein load for emulsions stabilized with K-casein ranged between 3 – 6 mg/m^2.

Table 9 - Probability values for protein concentration, homogenization pressures and their interactions on the protein load

Source (R^2 = 0.424)	P Value
Protein	0.986
HP	0.005
Protein * HP	0.256

HP = Homogenization Pressure

Table 10 - Effect of homogenization pressure on the protein load in emulsions with 0.5, 0.7, and 1.0% κ-casein

Protein	Homogenization Pressures		Mean Value (mg/m^2)
	20 MPa	100 MPa	
	---------- mg/m^2 ----------		
0.5%	6.05	3.61	4.83[a]
0.7%	5.09	4.65	4.87[a]
1%	6.02	3.50	4.76[a]
$\bar{x}$	5.72[A]	3.92[B]	4.82

a means in the same column followed by the same letter are not significantly different.

A,B means in the same row followed by different letter are significantly different.

4.2.3 Emulsion stability

The existence of protein on the lipid surface can provide emulsion stability by stearic or electrostatic repulsion. However, it can also destabilize emulsions if it causes aggregation of particles. The emulsion stability index (ESI) is an important measurement to evaluate the potential for creaming in an emulsion. ESI values close to 100 indicates excellent emulsion stability while lower numbers might indicate a problem with stability.

Factors that influence emulsion stability are particle diameter, density, and viscosity. Homogenization will influence the diameter of particles which will then influence the protein or emulsifier concentration needed in the emulsion. Particles with a large diameter will increase the possibility of creaming and cause a low ESI. This theory is revealed by the probability values in Table 11 and the data in Table 12 where the mean ESI values for the emulsions homogenized at 20 MPa were 28.30 which increased to 71.34 for emulsions homogenized at 100 MPa. Homogenization at 100 MPa increased the number of smaller particles and these would probably give a higher density to the globule in the solution and cause the particles to be more stable in the solution. When the density of the continuous phase is near the density of the discrete phase, it will decrease the creaming rate.

Once the protein adsorbed onto the lipid surfaces, intramolecular and intermolecular interaction among protein molecules would help stabilized particles in the emulsions. Similar intermolecular reaction between the protein on the particles and soluble protein in the bulk phase or between proteins in the bulk phase will influence the viscosity of the emulsion. Higher viscosity will decrease the creaming rate of the emulsion which was revealed in higher ESI values. The viscosity of the emulsion homogenized at 100 MPa was higher than the viscosity of the emulsion homogenized at 20 MPa. This helped explain the ESI of 28.30 for the emulsion homogenized at 20 MPa and the mean ESI of 71.34 for the emulsions homogenized at 100 MPa (Table 12). The ESI of 71 after homogenization at 100 MPa was an acceptable value that indicated extended storage stability for the emulsion but it was not excellent which indicates that

κ-casein was not a good emulsifier except when its concentration was 1.0% and the ESI was 86.

Protein concentration had a significant effect on ESI (Table 11). The effect of protein on the ESI of the emulsions is shown in Figure 17. The emulsions that contained 0.7% protein had the lowest ESI and emulsions with 1.0% protein had the highest ESI at both homogenization pressures. The reason the emulsion with 0.7% protein has the lowest ESI is difficult to explain. However, the ESI of this emulsion was not different from the ESI of the emulsions that contained 0.5% protein but the mean ESI of the emulsion with 0.5% protein was lower than the means of the emulsion that contained 1.0% protein. The probability value for the interaction is not shown in Table 11 because it was not significant ($p = 0.259$).

Table 11 - Probability values for the effect of protein concentration and homogenization pressure on the emulsion stability index

Source ($R^2 = 0.916$)	P value
Protein	0.007
HP	0.000

HP = Homogenization Pressure

Table 12 - Effect of homogenization pressures on the emulsion stability index for emulsions that contained 0.5, 0.7, and 1.0% κ-casein

Protein	Homogenization Pressure		Mean Values
	20 MPa	100 MPa	
0.5%	26.42	69.45	47.94^{ab}
0.7%	15.29	58.33	36.81^{a}
1%	43.19	86.23	64.71^{b}
$\bar{x}$	28.30^{A}	71.34^{B}	

a,b means in the same column followed by different letters are significantly different.

A,B means in the same row followed by different letters are significantly different.

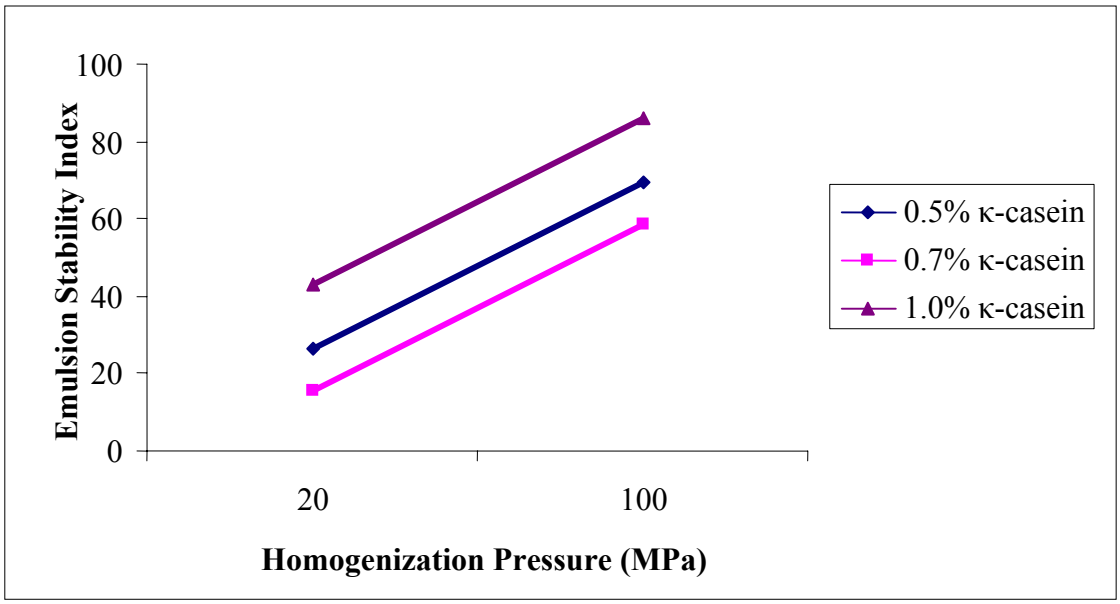

Fig. 17 - Effect of homogenization pressure and κ-casein concentration on the emulsion stability index

4.2.4 Viscosity

4.2.4.1 Viscosity prior to chymosin treatment

Homogenization pressure, protein concentration, treatment with chymosin and all of the interactions except the interaction between homogenization pressure and treatments had a significant effect ($p < 0.05$) on the viscosity of the samples (Table 13). Homogenization pressure had a significant effect on the viscosity of the samples prior to the addition of chymosin. The mean value for the viscosity of the emulsions homogenized at 20 MPa was 2.12 which increased to 2.36 cP after homogenization at 100 MPa (Table 14). The higher viscosity after homogenization at 100 MPa can be explained not only by the increased number of smaller particles which resulted in more surface area that had to be covered with protein, but it could also have been increased because of intermolecular reactions between molecules of κ-casein.

The concentration of protein in the emulsions significantly affected the viscosity ($p < 0.05$). Mean viscosity values for emulsions that contained 0.5, 0.7, and 1.0% protein were 1.87, 2.39, and 2.46, respectively (Table 14). The viscosity of the emulsions with 0.7% protein was not different from the viscosity of emulsion that contained 1.0% protein; however, the emulsion with a concentration of 1.0% protein had the highest viscosity (Fig. 18) and this viscosity was significantly different from the viscosity of the emulsion that contained 0.5% protein.

Table 13 - Probability values for the effects of protein, homogenization pressure and their interactions on the viscosity of the emulsions that contained 0.5, 0.7, and 1.0% κ-casein

Source (R^2 =0.881)	P Values
Protein	0.001
HP	0.000
Treatment	0.000
Protein*HP	0.033
Protein*Treatment	0.000
HP*Treatment	0.132
Protein*HP*Treatment	0.000

HP = Homogenization Pressure

Treatment = without and with chymosin

Table 14 - Comparison of the effect of homogenization pressure, protein concentration, and chymosin treatment on the mean of viscosity of emulsions that contained 0.5, 0.7, and 1.0% κ-casein

Protein	No Chymosin treatment			Chymosin treatment		
	Homogenization Pressure			Homogenization Pressure		
	20 MPa	100 MPa	$\bar{x}$	20 MPa	100 MPa	$\bar{x}$
	---------- cP ----------			---------- cP ----------		
0.5%	1.75	1.99	1.87^{af}	4.35	10.52	7.44^{dg}
0.7%	2.27	2.51	2.39^{bcf}	5.84	4.41	5.13^{eg}
1%	2.34	2.58	2.46^{cf}	6.32	10.23	8.30^{dg}
$\bar{x}_1$	2.12^{A}	2.36^{B}		5.50^{C}	8.39^{D}	
$\bar{x}_2$			2.24^{E}			6.95^{F}

a,b,c,d,e means in the same column followed by a different letter are significantly different.

A,B,C,D,E,F means in the same row followed by a different letter are significantly different.

f,g means in the same row followed by a different letters are significantly different.

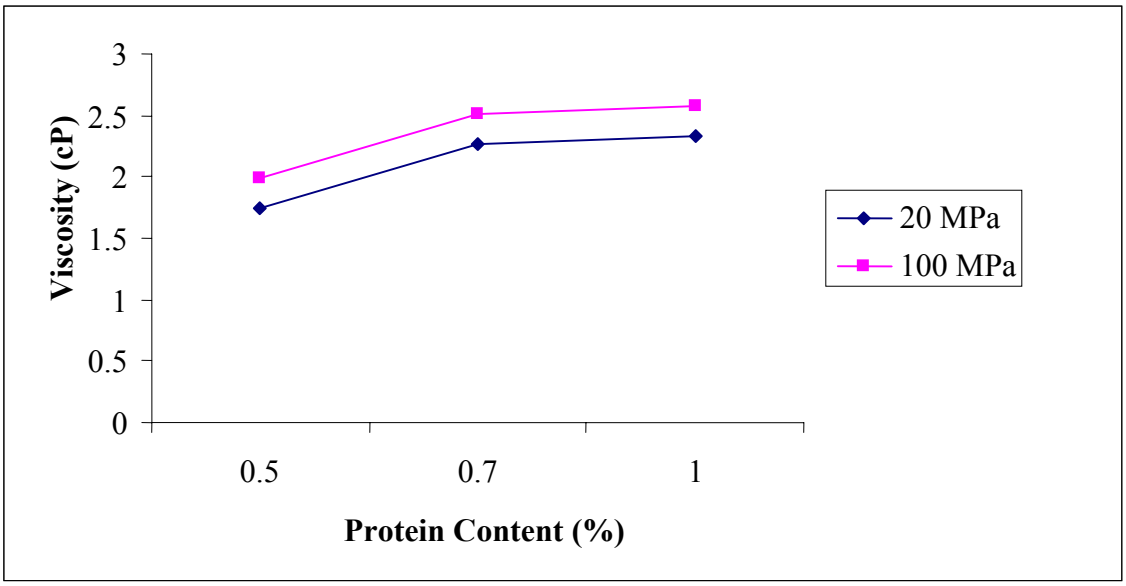

Fig. 18 - Effect of homogenization pressure and protein content on the viscosity of the emulsions that contained 0.5, 0.7, and 1.0% κ-casein before treatment with chymosin

4.2.4.2 Viscosity after treatment with chymosin

The addition of chymosin to the emulsions had a significant effect on the viscosity of the emulsions (Table 13). The viscosity of emulsions treated with chymosin was approximately 3X greater than the viscosity of emulsions not treated with chymosin at both homogenization pressures (Table 14). This was true for each concentration of protein in the emulsions ($p = 0.000$).

The mean viscosity for the emulsions that were treated with chymosin and homogenized at 20 MPa was 5.50 cP which increased to 8.39 cP when the emulsions were homogenized at 100 MPa (Table 14). Chymosin treated emulsions showed 2X greater viscosity for emulsions homogenized at 20 MPa and 4X greater for emulsions

homogenized at 100 MPa compared to emulsions that were not treated with chymosin. The increased viscosity caused by increased homogenization pressure can be explained by the increased number of particles in emulsions homogenized at 100 MPa compared to those homogenized at 20 MPa. The increased viscosity caused by treatment of the emulsions with chymosin was probably caused by intermolecular reactions between hydrolyzed molecules of κ-casein. Chymosin would have hydrolyzed the κ-casein attached to the lipid and the κ-casein in the solution. This can cause the development of a networking structure or particle aggregation which would result in higher viscosity. The viscosity increased when the protein concentration was increased from 0.5 to 1%. However, the viscosity of the emulsion with 0.7% protein was lower than the viscosity of the emulsion that contained 0.5% protein after homogenization at 100 MPa (Fig. 19). This decrease in viscosity did not occur when the emulsion was homogenized at 20 MPa. This is difficult to explain but might have been caused by experimental error in emulsion preparation since this emulsion also had a low ESI.

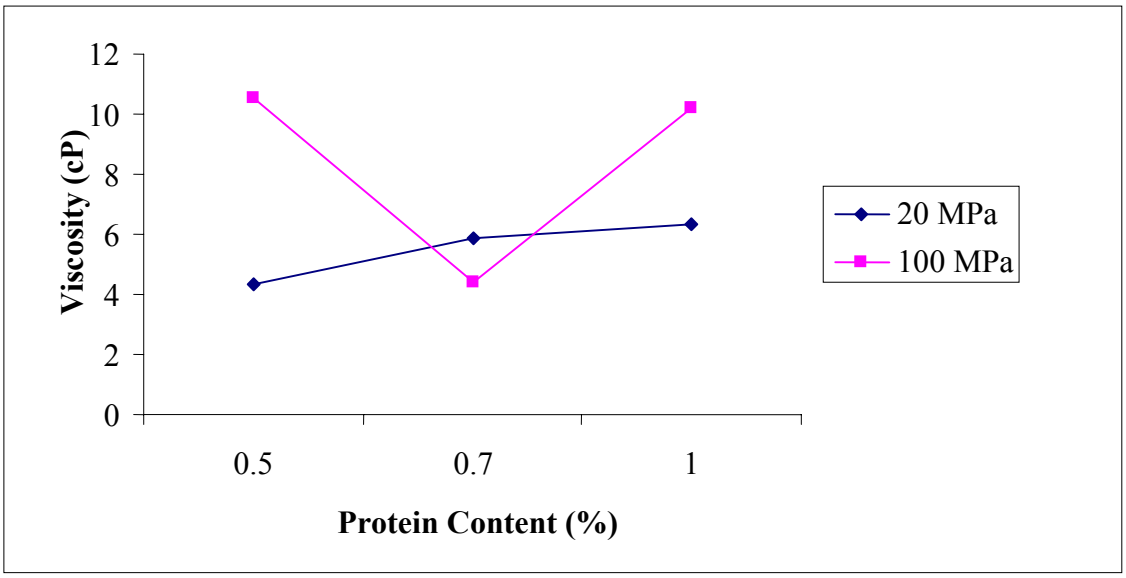

Fig. 19 - Effect of homogenization pressure and protein content on the viscosity of emulsions with 0.5, 0.7, and 1.0 % κ-casein after chymosin treatment

4.2.5 Rheology measurement

4.2.5.1 Yield stress prior to chymosin treatment

Homogenization pressures and chymosin treatment affected the yield stress (Table 15). However, the yield stress values of the emulsions homogenized at 20 and 100 MPa prior to treatment with chymosin were not significantly different with mean values of about 0.1 Pa (Table 16).

The rheological behavior of emulsions that were not treated with chymosin are reflected in Fig. 20. An increase in protein concentration did not cause a difference in the yield stress of the emulsions. However, the emulsions with 0.7% protein showed the highest yield stress after homogenized at both 20 and 100 MPa. This abberation and

unexpected observation is consistent with previous observations that found the lowest ESI values for the emulsion with 0.7% protein and it had the lowest viscosity after homogenization at 20 and 100 MPa.

Table 15 - Probability values for the effects of protein concentration, homogenization pressure, chymosin treatment and their interactions on the yield stress

Source ($R^2 = 0.819$)	P Values
Protein	0.882
HP	0.001
Treatment	0.000
Protein*HP	0.834
Protein*Treatment	0.901
HP*Treatment	0.001
Protein*HP*Treatment	0.816

HP = Homogenization Pressure

Treatment = without and with chymosin

Table 16 - Comparison of the effect of homogenization pressure, protein concentration, and chymosin treatment for yield stress on emulsions with 0.5, 0.7, and 1.0% κ-casein

Protein	No chymosin			Chymosin		
	HP			HP		
	20 MPa	100 MPa	$\bar{x}$	20 MPa	100 MPa	$\bar{x}$
	----------Pa----------			----------Pa----------		
0.5%	0.08	0.06	0.07^{a}	9.09	19.77	14.43^{b}
0.7%	0.13	0.19	0.16^{a}	10.83	21.51	16.17^{b}
1%	0.08	0.05	0.07^{a}	9.92	20.61	15.27^{b}
$\bar{x}_1$	0.10^{A}	0.10^{A}		9.95^{B}	20.63^{C}	
$\bar{x}_2$			0.10^{D}			15.29^{E}

a,b means in the same column and rows followed by a different letter are significantly different.

A,B,C,D,E means in the same rows followed by a different letter are significantly different

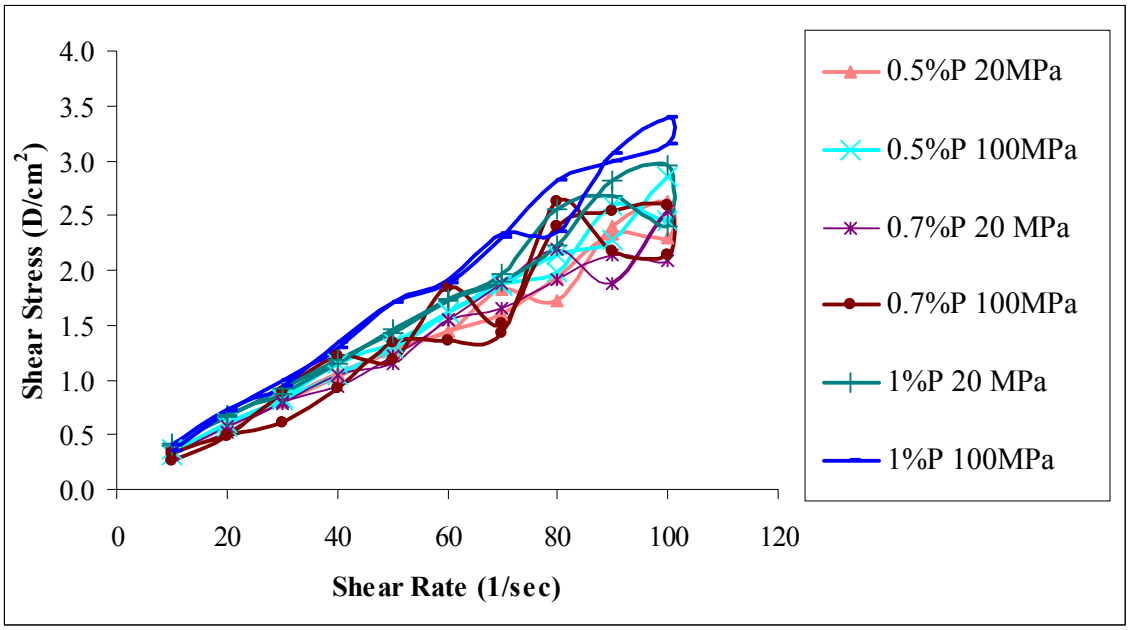

Fig. 20 - Effects of homogenization pressure and protein concentration on hysteresis curves of emulsions stabilized with 0.5, 0.7, and 1.0% κ-casein prior to the addition of chymosin

4.2.5.2 Yield stress with chymosin treatment

Homogenization pressure and chymosin treatment affected yield stress for emulsions that were subjected to hydrolysis by chymosin (Table 15). The mean yield stress for the emulsions homogenized at 20 MPa was 9.95 Pa which was 50% of the yield stress of 20.63 Pa observed for the emulsion homogenized at 100 MPa (Table 16). The increase in yield stress with the increased homogenization pressure was also reflected by observation of the yield stress curves (Fig. 21). The large increase in yield stress after homogenization at 100 MPa was probably caused by the greater number of particles in the emulsion. The increased number of particles would have generated significantly more surface area for interactions and the particles would be much closer together in addition to being able to diffuse more rapidly because of the reduced size. Surface active or bulk phase reactive κ-casein molecules generated by chymosin activity would also have had significantly more opportunity to interact in this system compared to the emulsions homogenized at 20 MPa. This most likely caused the 2X increase in yield stress in the emulsions homogenized at 100 MPa.

Protein concentration did not affect ($p = 0.904$) the yield stress of the emulsions treated with chymosin. However, emulsions with chymosin treatment showed 100X greater yields stress than emulsions without chymosin treatment homogenized at 20 MPa and 200X greater yield stress for emulsions homogenized at 100 MPa. When yield stress was measured and observed at selected rotational speeds (Fig. 22), the greatest yield stress was observed for the emulsion that contained 1.0% protein that was homogenized at 100 MPa and measured at a rotational speed of approximately 0.5 rpm. However,

observation of the mean values for yield stress showed that emulsion with 0.7% protein had the highest yield stress after chymosin treatment (Table 16). The previously noted instability of this emulsion with 0.7% protein could have contributed to this observation. There might have been more protein in the bulk phase to be hydrolyzed by the chymosin which might have resulted in bulk phase networking between protein in the stabilized emulsion and, more likely, protein-protein interaction in the bulk phase.

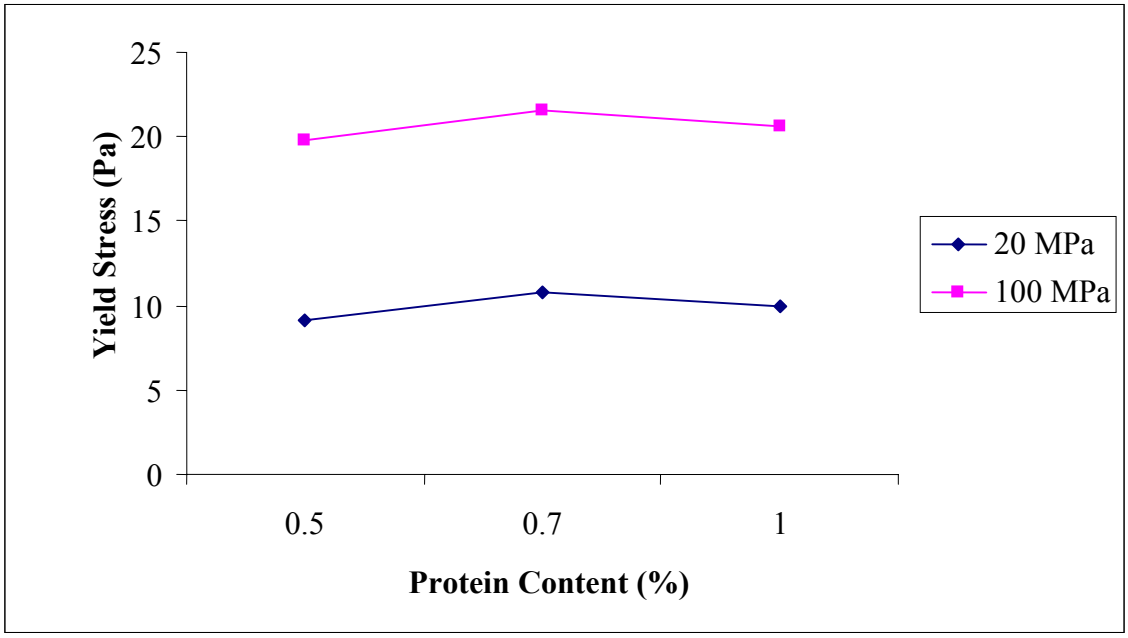

Fig. 21 - Effect of homogenization pressure and κ-casein concentrations of 0.5, 0.7, and 1.0% on the yield stress of emulsions after chymosin treatment

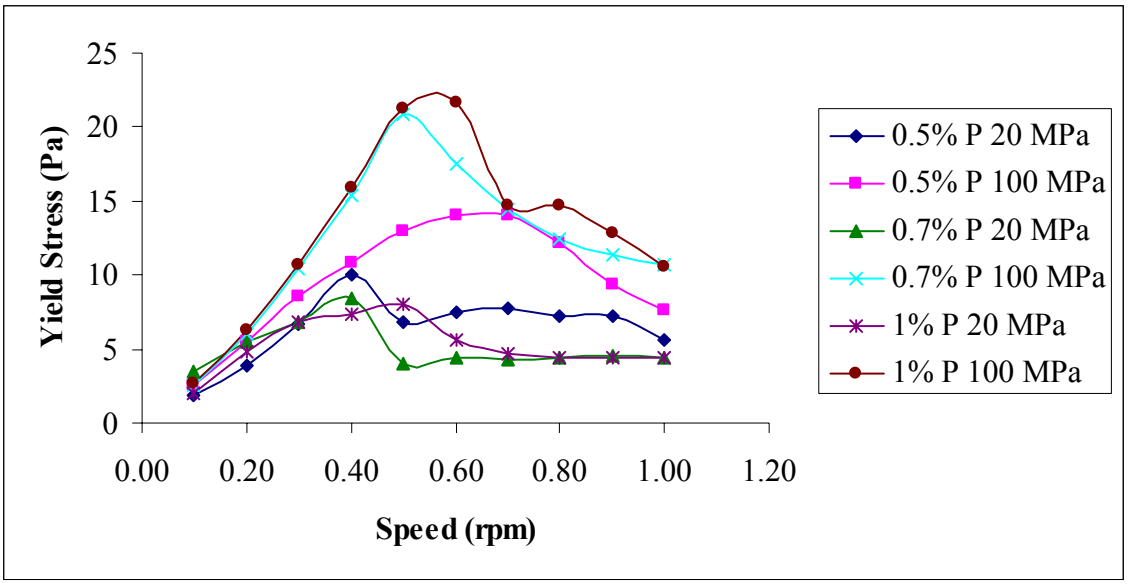

Fig. 22 - Effects of homogenization pressure and κ-casein concentration of 0.5, 0.7, and 1.0% on the yield stress of emulsions treated with chymosin

4.2.6 K-casein stabilized gel

From this research, some basic principles of gelation can be found associated with κ-casein stabilized emulsions. Walstra (1996) mentioned that a typical gel is a material that exhibits a yield stress, has viscoelastic properties and a moderate modulus. After the addition of chymosin, κ-casein stabilized emulsions did exhibit increased viscosity and yield stress for emulsions with different milk fat and protein concentrations. As the protein and milk fat concentrations of the emulsion were increased, the yield stress and viscosity increased. The flow behavior of the emulsion before and after the addition of chymosin revealed in the rheological measurements showed a change in flow behavior from Newtonian to Bingham Plastic. Gels can be formed as more bonds occur between protein stabilized particles in emulsions. Polymerization between proteins might occur randomly and effectively immobilize a large amount of water and cause an increase in viscosity (Walstra 1996). It is also possible that water in the restricted environment of each cell of the gel structure might act as a hydrogen bonding cross-linker between C=O and N-H groups of peptide segments (Damodaran 1996). Network formation of intermolecular cross-links between protein stabilized particles in an emulsion will influence the stability of the gel. The conformation of κ-casein on the lipid surface explains the formation of a gel. It contains cysteine that might form disulfide interchanges and a covalent network. This disulfide formation facilitates polymerization and increases the molecular weights of κ-casein polymers to more than 23,000. Molecules with a molecular weight less than 20,000 can not form a gel (Damodaran 1996).

Chymosin treatment did influence the possibility for gelation in the emulsion by hydrolyzing the κ-casein and to change the balance between hydrophobic interactions and repulsive electrostatic interactions. Damodaran (1996) mentioned that these two forces control the balance between protein-protein and protein-solvent interactions in gelling system. When the protein-protein interaction is predominant, a precipitate or a coagulum would likely form. Figs. 23 and 24 show the differences between the emulsion without chymosin treatment and the emulsion after treatment with chymosin.

Emulsion that contained 0.5% κ-casein after homogenization at 100 MPa with no chymosin treatment had liquid-like flowability. It had homogenious, newly formed lipid droplets surrounded by κ-casein and water as a continuous phase (Fig. 23). Water, lipid, and κ-casein interact to maintain the stability of the emulsion. It is suggested that the bigger droplets formed during or after homogenization were caused by the tendency of the lipid droplets to coalescence since they might not have been completely emulsified by the protein during homogenization. After the addition of chymosin , the emulsified particles aggregated as shown in Fig. 24 and formed a gel-like structure as shown in Fig. 25.

Weak and strong gels can be defined by the yield stress of the gel. Very weak gels usually have yield stress less than 10 Pa (Walstra 1996) and usually appear to be liquid-like. K-casein stabilized emulsions treated with chymosin had the appearance of a gel (Fig. 25). Our emulsions with different concentrations of lipid and emulsions with different concentrations of proteins without chymosin treatment could be categorized as very weak gels as the yield stress was around 0.1 to 0.2 Pa (Table 6 and 17). However,

after the addition of chymosin, the yield stress increased to more than 10 Pa. This was especially apparent in emulsions that contained 20% fat with 0.5, 0.7, and 1.0% κ-casein. Walstra (1996) mentioned that weak gels of acid casein gels had fracture stress of approximately 100 Pa, which is similar to 10 Pa for rennet gels. From these results, the gels made from κ-casein stabilized emulsion and treated with chymosin can be categorized as a weak gel.

Syneresis will always occur in the association with weak gels. The coagulation of the emulsified particles was not fully formed as revealed in Fig. 24. Some particles remained in the bulk phase and water was not intimately well-immobilized within the gel network. A rearrangement of the network of particles occurs as syneresis progresses and this causes deformation of the gel. Walstra (1996) mentioned that syneresis is very common in rennet gels especially above 20˚C which is relevant to the κ-casein stabilized gel in this research. Syneresis was not measured in this study but extensive syneresis was observed associated with the chymosin treated emulsions.

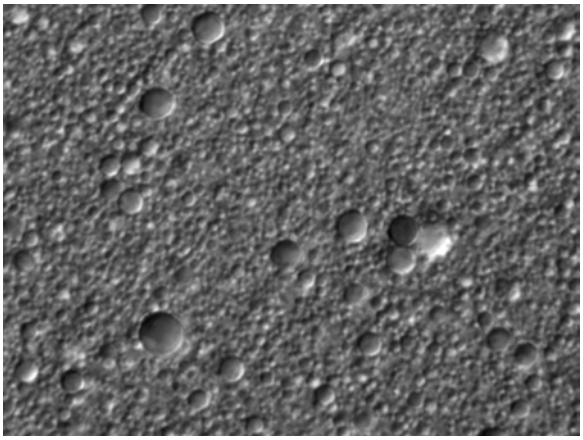

Fig. 23 - Microscopy analysis of emulsion with 0.5% κ-casein and homogenized at 100 MPa before chymosin treatment

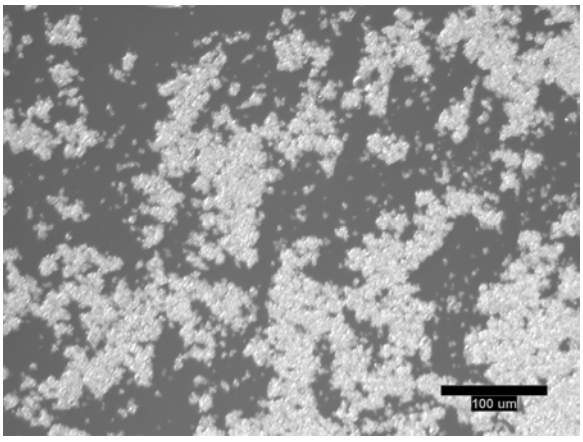

Fig. 24 - Microscopy analysis of emulsions with 0.5% κ-casein and homogenized at 100 MPa after chymosin treatment

Fig. 25 - 0.5% κ-casein stabilized emulsion homogenized at 100 MPa gel after treatment with chymosin

CHAPTER V

SUMMARY AND CONCLUSIONS

The stability and characteristics of emulsions formed with κ-casein were determined. Emulsions were made with 3%, 10%, and 20% of milk fat and 0.3% κ-casein. Homogenization pressures were varied to understand its influence on particle size and the rheological properties of the emulsions. Homogenization at 100 MPa compared to homogenization at 20 MPa caused smaller particles and increased the number of the particles. The concentration of milk fat in emulsions with 0.3% κ-casein changed the viscosity and the yield stress of the emulsions which increased with increased milk fat concentration. Emulsions homogenized at 100 MPa showed higher viscosity and yield stress than emulsion homogenized at 20 MPa. These emulsions had Newtonian behavior as there was no time dependence and they occured as a liquid-like emulsion.

Chymosin was added to the emulsions to understand the functionality of these κ-casein stabilized emulsions and to determine if a gel could be formed from the emulsions. The addition of chymosin to emulsions with milk fat concentrations of 3%, 10%, and 20% caused viscosities 2X, 13X, and 24 X higher than the viscosity in emulsions without chymosin treatment. Similar results occured for the yield stress of emulsions treated with chymosin that had 10X, 100X, and 125X higher yield stress than emulsions without chymosin treatment. An increase in the homogenization pressure from 20 to 100 MPa resulted in higher viscosity and yield stress in the emulsions. The

flow behavior of emulsions after the addition of chymosin was Bingham Plastic and that is a characteristic of viscoelastic behavior in a gel. The emulsions with 10% and 20% milk fat could function as pre-gel emulsions. However, for the purpose of forming a gel in this research, the emulsions with 20% milk fat were the best candidates as they had the maximum viscosity and yield stress values.

Protein concentration affected the properties of emulsions that were made with 0.5, 0.7, and 1.0% κ-casein and 20% milk fat. Homogenization at 100 MPa produced smaller particles compared to homogenization at 20 MPa. The viscosity of the emulsions increased as protein concentration increased and the highest viscosity was observed after homogenization at 100 MPa. The yield stress of the emulsions was not affected by protein concentration or homogenization pressure. Emulsions with 0.5%, 0.7%, and 1.0% κ-casein had Newtonian flow behavior prior to chymosin treatment.

The addition of chymosin to these emulsions caused an increase in the viscosity of approximately 3X compared to the emulsions without chymosin. The yield stress of the emulsions increased 100X to 200X when chymosin was added to the emulsions. The yield stress values of 10 to 20 Pa support the idea that κ-casein stabilized emulsion could be transformed into a solid, soft gel.

The stability of the κ-casein stabilized emulsions was evaluated by the ESI which showed that the ESI increased with increased protein concentration, except for the emulsion with 0.7% protein. Homogenization at 100 MPa produced a higher ESI than homogenization at 20 MPa and should be used to increase the stability of these emulsions. Emulsion with 1.0% protein showed the highest ESI.

The characteristic of κ-casein as an emulsifier was also measured by determining the amount of κ-casein on the lipid surface. The protein load of the κ-casein stabilized emulsion ranged from 4-6 mg/m^2. Emulsions homogenized at 100 MPa had a lower protein load than emulsions homogenized at 20 MPa but had a higher ESI than emulsions with a higher protein load.

It can be concluded that κ-casein can be used as an emulsifier; however, it did not produce a very stable emulsion under most of the experimental conditions. The addition of chymosin into the κ-casein stabilized emulsion showed a possibility for the conversion of this emulsion into a gel.

Results from this study can be extended to additional applications to examine future applications of this unique research. Emulsion stabilized by κ-casein could be mixed with skimmed milk and then converted to gels by chymosin to stimulate coagulation in the manufacture of cheese. Gels made in this method could be compared to gels made from milk. Studies of this nature might expand the uses for milk fractionation products that are currently being developed in the dairy industry.

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