ENZYME TRIGGERED RELEASE OF AROMA MOLECULES

FROM OIL-IN-WATER EMULSIONS

A Thesis in
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by
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The aroma of a food depends on the concentration of certain small, often hydrophobic, molecules in the headspace surrounding it. If these molecules remain bound by the food they will not be perceived. One method to ensure protection and proper delivery of these ingredients to the desired active site is by microencapsulation in an emulsion-based delivery system. This work will demonstrate enzyme-triggered release of a hydrophobic compound from a water-dilutable delivery system. The hydrophobic compound is protected and less active in a supercooled liquid lipid droplet but enzyme treatment triggers droplet crystallization and the compound is released. While the focus of the work is on the release of an aroma, other reactive compounds (e.g. antimicrobials, drugs) could be used in a similar manner.

The effects of trypsin on a 1% casein-stabilized, 10% eicosane emulsion with 0.01 μl/ml added ethyl octanoate (EO) was studied at 28°C. In the absence of trypsin, emulsions were stable over the course of the experiment (t=5 days). In the presence of trypsin, droplet size increased gradually over time (t=3 hours), suggesting protein hydrolysis results in progressive droplet coalescence. During protein digestion, supercooled liquid droplets eventually crystallize (t=18 hours), at which point the headspace concentration of EO increases suddenly. The headspace data are successfully modeled in terms of the partition coefficients with the bulk phases.
The effects of changing other parameters in the system (i.e., added protein, small molecule type and lipid type) are considered. Increased sodium caseinate solution concentrations led to a decrease in headspace ethyl octanoate (EO) concentration. The degree of EO binding by caseinate solution increased as the protein was hydrolyzed. Small molecule types differing in carbon chain length and hydrophobicity (i.e., ethyl octanoate vs. ethyl hexanoate) did not show a significant difference in absolute headspace concentration and time at which this occurred. When tripalmitin and palm oil emulsions were treated with trypsin they did not show the characteristic increase in headspace concentration seen in eicosane dispersions, possibly because the tripalmitin had already crystallized at the start of the experiment while the solid fat content of “solid” palm oil was only 28.7% and not sufficient to cause a change in the binding of EO.
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Chapter 1

LITERATURE REVIEW

This work is concerned with emulsions, solid lipid nanoparticles, their use as encapsulation systems, and their destabilization by proteolytic enzymes.

1.1 Emulsions

Emulsions are dispersions of one immiscible liquid in another and in their most basic form, are composed of only a hydrophobic phase (i.e., oil), a hydrophilic phase (i.e., aqueous) and an amphiphilic emulsifier (Fennema, 1996). These ingredients must be intimately mixed to produce the emulsions. The phase that exists in small, spherical droplets is the dispersed phase within the surrounding continuous phase. A system with oil droplets dispersed in a continuous aqueous phase is an oil-in-water (O/W) emulsion such as mayonnaise, milk and beverages and one with water droplets dispersed in a lipid continuous phase is a water-in-oil (W/O) emulsion such as chocolate and butter. Because of the high interfacial tension, emulsions are thermodynamically unstable and tend to rapidly separate back into the two separate phases. With the use of surfactants such as globular proteins or amphiphilic small molecules however, the two phases can be homogenized and remain stable for long periods of time.
1.1.1 Emulsion Formation

The process of mixing bulk immiscible liquids together is called homogenization and is done using a homogenizer. The intense agitation provided by the shear forces the temporary formation of lipid droplets. One common homogenizer is the high-speed blender that uses rotational force to create coarse, polydisperse emulsions. An advantage with high-speed blenders however is that they are able to quickly and easily create a premix for a further secondary emulsification process. High-pressure homogenizers are very commonly used in the industry and in the laboratory to achieve very fine emulsions (Weiss, 2008). Microfluidizers pump a coarse emulsion through an interaction pressure at high pressures (~30,000 psi) and velocities (~400 ms\(^{-1}\)) through very narrow channels in an interaction chamber where the fluid streams impinge upon one another to generate very fine droplets (Microfluidics, 2011).

1.1.2 Emulsifiers

In the absence of an emulsifier, phase separation happens very rapidly due to the hydrophobic effect as lipid droplets merge and coalesce to reduce the surface of contact with the aqueous phase (Israelachvili, 1992). To create a stable emulsions, an emulsifier is included in the raw materials. Emulsifiers used in foods include proteins, polysaccharides, small molecule surfactants (McClements, 2005). Emulsifiers adsorb at the oil water interface where they reduce the interfacial tension thus reducing the energy required for emulsification and producing finer emulsion droplets during homogenization. They also produce repulsive colloidal forces between the droplets reducing their tendency towards phase separation.
1.1.3  **Emulsion Stability**

Emulsions are thermodynamically unstable structures due to the larger interfacial free energy and will, over time, phase separate. The main mechanisms of emulsion destabilization are coalescence, partial coalescence, flocculation, and gravitational separation.

- **Coalescence** happens when two droplets are in close enough proximity to merge together and form a larger droplet (McClements, 2005). The larger droplet has a decreased surface area and thus the extent and rate of coalescence is the beginning step to complete destabilization. Coalescence happens with the rupture of the lamella separating two adjacent droplets, producing a channel for the inner contents of the particles to interflow and eventually become a single larger droplet (Coupland, 2007). The process of coalescence is irreversible and initial droplet sizes can only be attained again through re-homogenization.

- **Partial Coalescence** may occur when the dispersed phase becomes partially crystallized. The crystals protrude from the droplets and serve as a point of adhesion with an adjacent droplet by puncturing the neighboring lamella. Partially crystalline droplets do not merge to form a single sphere but maintain a double shape from the partial crystallinity of the solid fat network (Walstra, 1996). Partial coalescence can be both a desirable and undesirable effect, advantageous in the production of butter and whipped confections and undesirable for stability and shelf-life (Goff, 1997).

- **Flocculation** is a process of aggregation in which each individual droplet maintains its integrity. Flocculation can result in later coalescence and is always...
associated with an increase in emulsion viscosity. Particles come together to form a for any reason that favors attractive over repulsive interactions for example due to the reduction of electrostatic repulsion, incomplete surface coverage by the surfactant or the presence of a biopolymer that acts as a bridge between two or more droplets (McClements, 1999).

- **Gravitational Separation** occurs if the dispersed phase has a lower density, the droplets will tend to move upward in a process called creaming while if the density of the dispersed phase is higher than the surrounding liquid, then the droplets will move downward in a process called sedimentation (McClements, 2005). Creaming is commonly seen in oil-in-water emulsions while sedimentation is commonly seen in water-in-oil emulsions. The terminal velocity of an isolated, spherical droplet in a Newtonian fluid is given by Stokes’s equation:

\[
V_{Stokes} = \frac{-2 gr^2 \Delta \rho}{9 \eta} \tag{1.1}
\]

where \( r \) is the particle radius, \( \Delta \rho \) is the difference of the density between the continuous and dispersed phases, \( \eta \) is the continuous phase viscosity and \( g \) is the acceleration due to gravity. Gravitational separation is very slow for submicron particles of oil dispersed in water.
While these mechanisms are generally important, in the context of this work it is important to consider how the presence of an active protease can destabilize protein-stabilized emulsions.

1.1.4: Proteolytic Digestion of Emulsions

Proteases are enzymes whose catalytic function is to breakdown proteins by hydrolysis of peptide bonds. The three principle proteases important in digestion are pepsin, chymotrypsin and trypsin. These are all endopeptidases which hydrolyze proteins at non-terminal amino acid sites as opposed to exopeptidases which cleave at terminal sites of a polypeptide chain. In the digestive process, trypsin acts with the other proteinases to break down dietary protein molecules to their component peptides and amino acids.

As the precursor of trypsin, trypsinogen is secreted by the pancreas in an inactive form to prevent auto-digestive activity of the active enzyme. The conversion of the inactive form (zymogen) to the active trypsin is triggered by an enterokinase in the small intestine. Trypsin has a molecular weight of 23.3 kDa with an optimal operating pH of 7.4-8.5 and an optimal operating temperature of about 37°C, namely conditions of the small intestine (Voet and Voet, 2010). Trypsin is a pancreatic serine endoprotease which specifically hydrolyzes peptide bonds at the carboxyl end of arginyl and lysyl residues (Sigma, 2012). The catalytic mechanism lies within the active site known as the catalytic triad, consisting of a histidine, serine and arginine residue. This active site modulates the enzyme conformation to change the electrostatic environment, inducing an interaction
Trypsin is inactivated at high pH (11) and high temperatures (> 80°C) (Venkataresh and Sundaram, 1998).

While the enzymatic hydrolysis of proteins has been widely studied (Mun, Decker, McClements, 2007; Abdalla, Klein and Mader, 2008; Sandra, Decker and McClements, 2008; Hui, Lim, Park, Joo, 2009), relatively few studies have focused on the effects of proteolysis on emulsion stability.

Agboola and Dalgleish (1996) studied the trypsinolysis of casein, both in solution and adsorbed to lipid droplet surfaces (i.e., in an emulsion). In the emulsion system trypsinolysis of interfacial casein resulted in an increase in mean particle diameter as protein hydrolysis led to aggregation and coalescence. The rate of hydrolysis of the proteins on the surface of the emulsion droplets were highest within the first few minutes after which the rate gradually fell (Agboola, Dalgleish, 1996). These workers argued that the most easily accessible bonds are the first to be hydrolyzed, giving the high initial rate of hydrolysis, and further enzymatic action is only possible after changes in protein conformation expose remaining trypsin active sites (Agboola and Dalgleish, 1996). These observations were affirmed in other studies (Leaver and Dalgleish, 1990; Dalgleish, 1990).

1.2 Crystallization of Emulsified Lipids

The rate and mechanism of lipid crystallization depends on whether the fat is in emulsified or bulk form (Boode and Walstra, 1993). Regardless, the process of
crystallization can be divided into several main stages consisting of supercooling (or supersaturation) of the liquid lipid, nucleation and crystal growth.

- **Supercooling** When a liquid is brought down to a temperature below its melting point and exists in a metastable liquid state, it is known as supercooled. This persistence in the liquid state is due to an activation energy that must first be overcome in order for crystallization to occur (McClements, 2005). The degree of supercooling is defined as $\Delta T = T - T_{mp}$ where $T$ is the temperature of the material and $T_{mp}$ is the temperature at the melting point (McClements, 1999). The $\Delta T$ or temperature at which crystallization first begins depends on factors such as the presence of impurities, cooling rate and the composition of the oil (McClements, 2005).

- **Nucleation** is the first step towards crystallization and in bulk lipids, is a relatively quick process due to the presence of impurities that act as catalysts to the crystallization process. This is known as heterogeneous nucleation. While nucleation in bulk oils is initiated by the presence of impurities in the continuous volume with rapid propagation throughout, oil in fine dispersed emulsions is divided into a very large number of fine droplets, so that the probability of finding a nucleation event in a single droplet is extremely small (Skoda and Van den Tempel, 1963; Awad, 2004). Liquids in fine droplets often show much deeper degrees of supercooling as they must nucleate via a homogeneous rather than heterogeneous mechanism (Helgason, Awad, Kristbergsson, McClements, Weiss, 2008). The droplets are also isolated from each other, so that nucleation in one droplet does not affect others (Skoda and
Van den Tempel, 1963; Coupland, 2002; Awad, 2004). However, as particle size increases, the ratio of nucleation catalysts to droplets increases and a smaller portion of the lipid is expected to be metastable as a liquid significantly below its melting point.

This process is known as homogenous nucleation since the nucleus arises spontaneously from the homogenous liquid and is predominantly the type of event expected to occur in a supercooled liquid oil.

- **Crystallization** Below the melting point, the formation of a crystal can only occur with the presence of a stable nuclei, usually comprised of several oil molecules that have become associated through random molecular collisions (Hartel, 2001). This small crystalline cluster, the crystal embryo, is thermodynamically favored so there is a decrease in free energy proportional to the volume of the nucleus formed (McClements, 2005). The larger the crystal, the lower the free energy of the system as more molecules are in a preferred lower chemical potential state (Coupland, 2002). With the new interface between a solid and liquid phase however, there is also a need for the input of free energy to overcome this interfacial tension, the amount of which is proportional to the surface area of the nucleus formed (McClements, 2005). Thus the net free energy change ($\Delta G$) for crystal formation is a function of its volume and surface area, as described as:
\[ \Delta G = (k_{\text{surface}} r^2) - (k_{\text{volume}} r^3) \] (1.2)

where \( k_{\text{surf}} \) and \( k_{\text{vol}} \) are constants of the crystal surface and volume.

Functioning under these thermodynamic parameters, a crystal formation event is dependent on the size of the initial crystal embryo, thus changing the free energy of the system. Crystals smaller than the critical radius necessary for crystal formation will reduce their free energy by reducing its size while those greater than the critical radius will reduce their free energy by growing larger (Hartel, 2001; Perepezko, Hockel, Pais, 2002).

Once a stable nuclei has established a starting point for nucleation, crystal growth is an accretion process of mass transfer whereby more and more liquid molecules add to the solid-liquid interface (Hindle, Povey, Smith, 2000; Widlak, Hartel, Narine, 2001). Mass transport is dependent on molecular mobility, which is a function of temperature and concentration (Coupland, 2007). At lower temperature and higher concentrations, the rate of crystal growth will be kinetically limited (Coupland, 2002). The crystallization of emulsified lipids can result in either phase separation (due to partial coalescence or incomplete surfactant coverage of the newly formed solid particle) or the formation of a stable suspension of crystalline droplets, i.e., solid lipid nanoparticles.
1.2.1 Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNs) are fine oil-in-water emulsions where the dispersed phase has crystallized. SLN have been used for drug delivery as a strategy to bypass the low bioavailability of lipophilic bioactive ingredients, to improve ease of bioactive handling and utilization; to facilitate its incorporation within a product, to control the rate or location of its release, or to protect it from chemical degradation (McClements, 2011; McClements 2012). The loading capacity of the lipid for the drug is also determined by the solubility and miscibility of the drug in the lipid melt as well as the polymorphic state of the lipid material (Muller, Mader, Gohla, 2000). The solid lipid has been claimed to protect chemically labile ingredients against chemical decomposition and the possibility to modulate drug release (Muller, Mader, Gohla, 2000). Because of the fine particle sizes (0.1-100 µm), the dispersion is physically stable over long periods of time (over several weeks) or until destabilization is triggered by an external stimulus.

1.3 Interactions of aroma with food components

In a complex food product with many phases, the microstructure plays an extremely important role in the perception of freshness and healthiness which are usually indicated by various volatile molecules. Microstructure is defined as the composition of the food matrix reduced to its most basic units (Parada, 2007). Depending on the matrix (composition, concentration, structure and protein conformations) these volatile compounds may be entrapped in various parts of this continuous matrix and affect
consumer perception (Seuvre, Espinosa-Diaz, Voilley, 2000; Kinsella, 1990; De Roos, 2000; Guichard, 2002)

1.3.1 Interactions with emulsions

Flavor and fragrance are some of the most direct determinants of food quality. More important than the concentration of flavor is its relative distribution within the matrix of the food system, which determines the amount that is released into the headspace where it can be perceived. Voilley, Espinosa, Druaux, Landy (2000) and Relkin, Fabre, Guichard (2004) showed that volatility of an aroma compound decreased from pure water, to protein solution to an emulsion, theorizing that the presence of protein and oil depressed headspace concentrations. Giroux, Perreault, Britten (2007) characterized the flavor release profiles of oil-in-water emulsions, looking at such effects as varying protein concentration, oil concentration and aroma concentration and found that oil concentration had a greater influence on flavor release than the other variables. In further studies, Roberts and Pollien (2000) also had similar findings, with a large reduction in volatility due to proteins only in the absence of fat.

The partitioning of flavor molecules within a food system will vary according to the specific affinities of each molecule for a particular phase. To a first approximation, the equilibrium distribution between phases in an emulsion is a function of the bulk partition coefficients (Buttery, Guadagni and Ling, 1973):
\[ \frac{1}{K_{ge}} = \frac{\phi_o}{K_{go}} + \frac{1-\phi_o}{K_{ga}} \tag{1.3} \]

where \( \Phi_o \) is the volume fraction of oil in the emulsion, \( K_{go} \) and \( K_{ga} \) are the gas-oil and gas-water partition coefficients and \( K_{ge} \) is the overall gas-emulsion partition coefficient. A larger \( K_{ge} \) indicates a lower affinity of the aroma molecules for the emulsion and a greater concentration in the gas phase. This approach has been successfully used to model the headspace volatile concentration in equilibrium with a food sample (Harrison, Hills, Bakker and Clothier, 1997; McNulty and Karel, 1973; Buttery, Guadagni and Ling, 1973).

Equation 1.3 gave a good prediction of the concentration of fatty acid ethyl esters in the headspace above liquid oil-in-water emulsions but not for the corresponding solid-fat-in-water dispersions. To account for the presence of solid fat, Ghosh, Peterson, Coupland (2006) recently made a modification to Equation 1.3 to obtain the following:

\[ \frac{1}{K_{ge}} = \left( \frac{\phi_o(1-\phi_{sfc})}{K_{go}} \right) + \left( \frac{1-\phi_o}{K_{gw}} \right) \tag{1.4} \]

The liquid droplet emulsions were able to bind the aroma molecules more effectively than solid droplet emulsions (smaller \( K_{ge} \)). In the liquid droplets \( K_{ge} \) was not affected by particle sizes while in solid droplets, fine droplets were more effective at binding the aroma molecules. They explained this by the theory that although there is no interaction between solid droplets and the aroma compound, a surface binding
mechanism was employed in which the volatile adsorbs at the oil-water interface. As solid fat content increases, the third term (crystalline fraction) in Equation 1.4 increases while the second term (oil fraction) goes to zero to give an overall larger $K_{ge} (5.61 \times 10^{-2})$ than the $K_{ge}$ for liquid droplets ($1.06 \times 10^{-3}$). Furthermore, Ghosh, Peterson, Coupland (2006) showed that these differences in aroma binding capacity was reversible on repeated remelting and recrystallization, indicating that the differences were thermodynamic in nature.

1.3.2. Interactions with proteins

In the area of protein–flavor interactions, many studies have been conducted mainly with milk proteins (Andriot, Harrison, Fournier, Guichard, 2000; Guichard and Langourieux, 2000; Guichard, 2005; Jasinski and Kilara, 1985; Charles, Bernal, Guichard, 1996; Maier, 1975). Studies on milk proteins are of great importance since they are utilized in numerous food products, including dairy products, bakery and confectionary products (Kuhn, Considine, Singh, 2006). Interactions between proteins and flavors have been known to influence the perceived flavor of a food product binding up volatile compounds and inhibiting their release into headspace where it can be perceived (Overbosch, Afterof, Haring 1991; Druaux and Voilley, 1997; Guichard and Langorieux, 2000; Guichard 2005; Meynier, Lecoq, Genot, 2004). These type of protein-flavor interactions are mostly hydrophobic and reversible (Tromelin, Andriot, Guichard, 2006; O’Neill and Kinsella, 1987; Pelletier, Sostmann, Guichard, 1998).
Chapter 2

STATEMENT OF THE PROBLEM

I note three key points from my literature review:

1. Lipid droplets are stable to crystallization for long periods below their melting point provided the droplet size is small
2. Protein stabilized emulsions are destabilized following proteolytic digestion
3. Liquid droplets bind hydrophobic compounds more than crystalline fat droplets

Based on these three points I hypothesize that protein-stabilized supercooled liquid emulsion droplets will remain stable until digested by a protease. Proteolytic digestion of the emulsion will result in droplet coalescence, crystallization and the expulsion of hydrophobic small molecules from the lipid phase.

The model system to test this hypothesis consists of an alkane for the lipid phase since eicosane has been previously used, is inert and as it is a pure compound, will crystallize rapidly and completely following nucleation at temperatures below its melting point. Sodium caseinate was selected as the protein emulsifier as it is susceptible to digestion by trypsin (Guo, Fox, Flynn, Kinstedt 1995). Trypsin was chosen as the protease as it is an important digestion protein and has been shown to hydrolyze sodium caseinate at a reasonable rate (Agboola and Dalgleish, 1996). Finally, ethyl octanoate
(EO) was used as a model hydrophobic molecule of choice as it was previously used by Ghosh, Peterson, Coupland (2008) in a similar study.

This work will demonstrate enzyme-triggered release of a hydrophobic compound from a water-dilutable delivery system. The hydrophobic compound is protected and less active in the liquid droplet but after enzyme treatment is released where it is more reactive. While the focus of the work is on the release of an aroma, other reactive compounds (e.g. antimicrobials, drugs) could be used in a similar manner.
Chapter 3

Effect of Trypsin on the Stability and Induced Crystallization of Sodium Caseinate Stabilized Eicosane Emulsions with Encapsulated Ethyl Octanoate

Abstract

The effects of trypsin on a 1% protein-stabilized, 10% eicosane emulsion with 0.01 µl/ml ethyl octanoate (EO) was studied at 28°C. In the absence of trypsin, emulsions were stable over the course of the experiment (t=5 days). In the presence of trypsin, there was a lag time before droplet sizes increased gradually over time (t=3 hours) suggesting protein hydrolysis results in progressive droplet coalescence. During protein digestion, supercooled liquid droplets eventually crystallized at a much later time (t=18 hours). Only at the final stages of crystallization was there release of aroma into headspace and concentrations of EO increased suddenly. The amount of EO released from liquid droplets at the final stages of crystallization was equivalent to the constant headspace concentration of EO from solid droplets. The headspace data are successfully modeled in terms of the partition coefficients with the bulk phases.
3.1 Introduction

Emulsions are mixtures of two immiscible liquids in which one is dispersed as droplets in the other. The phase that exists in small, spherical droplets is the dispersed phase within the surrounding continuous phase. Because of the high interfacial tension, emulsions are thermodynamically unstable and tend to rapidly separate back into the two separate phases. With the use of surfactants such as globular proteins or amphiphilic small molecules however, the two phases can remain stable for long periods of time provided that particle sizes are small. Fine emulsions (d=0.1 to 1 µm) are sometimes used to encapsulate small molecules in foods (e.g., aromas, micronutrients) and other applications.

Ghosh, Peterson, Coupland (2006) studied the effects of droplet crystallization and melting on the aroma release properties of model oil-in-water emulsions. They were able to show that the headspace concentration of hydrophobic volatile molecules at equilibrium with an emulsion was greater when the droplets were solid compared to when the droplets were liquid. These workers argued the hydrophobic solute was excluded from the crystalline droplet and was forced out into the headspace. In the Ghosh study, the phase transition needed to trigger headspace release was controlled by changing the temperature. In the present work I consider the use of a proteolytic emulsion to trigger droplet crystallization by destabilizing a supercooled liquid emulsion. I hypothesize that the headspace concentration of volatile molecules at equilibrium with
supercooled liquid droplets will increase during trypsinolysis as the emulsion is destabilized and the oil crystallizes.

3.2 Materials and Methods

3.2.1 Materials

Eicosane, 99% was purchased from Fisher Scientific (Springfield, NJ), sodium caseinate was purchased from Sigma Aldrich (St. Louis, MO), 0.25% trypsin in EDTA solution was purchased from Cellgro (Manassas, VA) and ethyl octanoate, 95% was purchased from Sigma Aldrich (St. Louis, MO).

3.2.2 Methods

The oil-in-water emulsions were prepared by adding n-eicosane (10% w/w) to sodium caseinate (1% w/w) in phosphate buffered saline solution (pH 7, 0.1 M) containing sodium azide (0.01% w/w) as an antimicrobial agent (Sigma Chemical Company, St. Louis, MO). An emulsion premix was prepared with a high-speed blender (Brinkmann Polytron, Brinkmann Instruments Inc., Westbury, NY) for 1 minute. This coarse emulsion was then allowed to circulate through a microfluidizer (Microfluidics M110Y, Newton, MA) for 2 minutes at 15,000 psi. The emulsions as well as all equipment used in manufacture were maintained at >40°C to ensure the lipid remained liquid for homogenization. After homogenization, samples were either kept at 28°C to produce liquid droplets or cooled to 10°C and rewarmed to 28°C to produce solid droplets. Note that the appearance of the emulsions (i.e., low viscosity, white opaque liquids) was apparently unchanged following a phase transition in the dispersed phase.
Sometimes in the discussion emulsions will be referred to as “liquid” or “solid” for convenience but this only reflects changes in the dispersed phase. Ethyl octanoate (EO, 0.01 µl/ml) was added to some samples and emulsions were treated with either 1 ml of trypsin (250 AU/mg) or an equivalent volume of buffer and incubated at 28°C.

Triplicate samples were taken from a stock solid or liquid emulsion with added EO for treatment with enzyme (or buffer). In some cases, due to experimental logistics, similar separate emulsions were used for different analyses.

3.2.3 Static Light Scattering

Static light scattering (Horiba LA 920, Irvine, CA) was used to measure mean particle size and distribution using a relative refractive index of 1.44. In some studies a Malvern Mastersizer 2000 (Malvern, UK) was used to determine particle size based on the same light scattering principle.

3.2.4 Differential Scanning Calorimetry

The crystallization and melting profile of bulk and emulsified eicosane was analyzed with a differential scanning calorimeter (VP DSC, MicroCal, Piscataway, NJ). Samples were diluted to 0.1% solid fat content and aliquots (~500 µL) were heated in the DSC from 28°C to 45°C at 90°C hour⁻¹. Deionized water was used as the reference for the emulsion samples.
3.2.5 Isothermal Calorimetry

The heat released on digestion and crystallization was measured by isothermal calorimetry (µ-DSCIII, SETARAM, Calluire, France). Samples (total volume of 1 ml) were treated with 0.2 ml enzyme/ml and held at 28°C in the instrument over 30 hours. Phosphate buffered saline was used as the reference.

3.2.6 Gas Chromatography

The headspace concentration of EO was measured after equilibration of samples. Aliquots of headspace gas (1 mL) was withdrawn by an autosampler (Gerstel, Baltimore, MD) and injected into a gas chromatograph (Agilent 7890, Agilent Technologies, Palo Alto, CA) equipped with a DB-5 capillary column (30m x 0.32mm id with a 1 µm film thickness) and a flame ionization detector. The operating conditions were as follows: sample was injected in splitless mode (purge value on at 1 min), inlet temperature was 200°C, detector was 250°C, oven program was 30°C for 1 min, then increased at 35°C min⁻¹ from 30 to 200°C and held for 2 min, carrier constant flow rate 2.0 mL min⁻¹ (He). Static headspace concentrations were determined from peak areas using a standard calibration curve ($r^2=0.99$). All measurements are expressed as the mean and standard deviation of at least two full experimental replicates.

3.2.7 Statistical Analysis

Statistical analysis was performed using 2-Sample t-tests in Excel software (Microsoft Corporation, Redmond, WA) with significance at $\alpha=0.05$. 
3.3 Results and Discussion

The emulsions had a unimodal distribution with a mean particle diameter of approximately 0.2 µm (Figure 3.1).

![Graph showing initial particle size distribution](image)

Figure 3.1: Initial particle size distribution of 1% caseinate-stabilized, 10% eicosane emulsion.

In the absence of enzyme, both liquid and solid emulsions were stable over the course of the experiment with no visible phase separation (Figure 3.2) or changes in droplet size (Figure 3.3). However in the presence of trypsin there was an increase in droplet size following a short lag period (Figure 3.3). This is presumably because the caseinate stabilizing the lipid droplets is being hydrolyzed by enzymatic action leading to droplet flocculation and eventual coalescence. The mean diameter of the liquid droplets is significantly greater than that of the solid droplets after 6 hours of digestion. This may be
because the liquid droplets can more easily merge and coalesce after the trypsin hydrolyzes the protein emulsifier while solid droplets only flocculate. Particle size could only be reliably measured initially (first 6 hours) after which point the emulsions visibly phase separated and representative sampling became impossible (Figure 3.2). The large pieces of aggregated fat formed on extensive hydrolysis of the supercooled liquid eicosane emulsions were solid and apparently crystalline suggesting that the liquid droplets crystallized as a result of the process.

Figure 3.2: Photograph of solid and liquid droplet 1% caseinate-stabilized 10% eicosane emulsions without trypsin treatment (A) and with trypsin treatment (B). All samples were incubated at 28˚C for 30 hours.

Two sets of particle size experiments were performed with two different instruments (Malvern Masterzier vs. Horiba). In both sets of data there was a lag time and then an increase in mean particle size as hydrolysis destabilized the dispersion. While
the two sets of data are largely consistent with one another, there were some differences observed in final droplet sizes after the characteristic destabilization following three hours of enzymatic digestion (Figure 3.3). These differences may be due to the different instruments used (Mastersizer vs. Horiba) or the inherent difficulty in sampling an unstable system.

Figure 3.3: Mean droplet diameter ($D_{32}$) in liquid eicosane emulsions with ($\bullet$) and without ($\bigcirc$) enzyme and solid eicosane emulsions with ($■$) and without ($□$) enzyme at 28°C. Samples were analyzed using static light scattering performed with either a Horiba or a Malvern instrument. Error bars indicate standard deviation and letters indicate significant difference at $p=0.05$ between the solid and liquid emulsions at a given time point and measured by a given instrument.

Differential scanning calorimetry (DSC) is a thermoanalytical technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. When the sample undergoes a
physical transformation such as a phase transition, the amount of heat necessary to maintain the sample at the same temperature as the reference will fluctuate.

The change in solid fat content in emulsions was monitored as a function of time by differential scanning calorimetry. Samples at 28°C were diluted in buffer to 0.1% fat content and injected into the microcalorimeter where they were heated from 28°C to 45°C at 90°C/hour against a similar reference cell filled with deionized water. In all eicosane emulsions incubated in the absence of trypsin, there was no peak over the course of the experiment proving the supercooled liquid eicosane droplets were stable under these conditions (data not reported). However, for the eicosane emulsions treated with trypsin, a peak appeared (peak maximum: 37°C) and increased in size with incubation time (Figure 3.4a). The peak maximum temperature corresponded to the melting point of crystalline eicosane (36.5°C Haynes, 2011) that had formed as a result of the incubation with trypsin. The area under the peak (i.e., enthalpy of fusion or crystallization of the solid fat) is proportional to the amount of solid fat present. To provide a reference of a fully crystalline sample an aliquot of eicosane emulsion was cooled to well below its crystallization point (24 °C). The area of the melting curve of this sample was then taken to be the maximum melting enthalpy of this particular emulsion system and the percentage crystallinity due to hydrolysis of the enzyme-treated samples was measured relative to this value. Peak areas were calculated with using a straight baseline with the analysis software provided. The percentage crystallinity in the emulsion increased with incubation time for the first three hours of the process (Figure 3.4b) after which extensive phase separation made representative sampling impossible (Figure 3.2).
Figure 3.4: (a) Heating thermogram (90°C/hour) of supercooled 10% eicosane liquid droplet emulsions stabilized with 1% caseinate-stabilized after different incubation times with trypsin. Fully crystalline sample was prepared by cooling the sample prior to analysis. (b) Percentage crystallinity as a function of incubation time for similar samples.
An alternative approach to solid fat content determination is isothermal calorimetry, which allows a single sample to be analyzed at one temperature over an extended period, thus avoiding the sampling problem mentioned above. The isothermal calorimetric experiments were conducted at 28°C and PBS was used as the reference in all measurements to ensure that no reactions would occur in the reference sample.

When caseinate solution was incubated with trypsin, there was a lag time before a small peak was seen beginning at 5 hours and ending at 17 hours. This is most likely due to enzymatic digestion of casein by trypsin and results from the heat released due to hydrolytic bond cleavage.

When supercooled liquid eicosane emulsion samples were incubated with trypsin, there was a much larger exothermic release which began before measurements could be made. The reaction began so quickly that preliminary baselines could not be established. The rate of reaction reached a maximum at 3 hours, after which it declined and continued at a decreasing rate up to 40 hours. The total enthalpy for supercooled liquid eicosane emulsion crystallization was much larger than that seen in caseinate solution. It can be seen then, that enzymatic hydrolysis of caseinate on droplet surfaces in an emulsion system started very quickly upon trypsin addition and proceeded over an extended period (Figure 3.5).
From the DSC measurements, droplet crystallinity reached 80% at 3 hours (Figure 3.4b) after which no additional measurements could be made due to limitations in sampling. Although isothermal calorimetry could not be used to measure the early stages of the reaction, it is clear that crystallization actually continues over a much longer time.

In all emulsion samples incubated without an enzyme, the dispersion was stable and droplets remained liquid or solid with no change in mean droplet sizes. In the presence of trypsin, destabilization reaction happens rapidly at the start (seen in isothermal calorimetry and particle size experiments) and then decreases as the reaction...
rate slows down (seen in isothermal calorimetry experiments) finally reaching equilibrium after about 40 hours. The effect of these changes on the headspace concentration of EO in equilibrium with the emulsions was measured by gas chromatography.

After 1 hour equilibration, both solid and liquid droplet emulsions in the absence of enzyme had a constant headspace concentration of EO (Figure 3.6). The headspace concentration for the solid droplet emulsions was higher than that for the liquid droplet emulsions. With enzymatic hydrolysis of solid eicosane droplet emulsions, there was no significant effect seen in headspace concentration. However, with enzymatic hydrolysis of supercooled liquid eicosane droplet emulsions, there was an increase in EO headspace concentration after a critical time to approximately the value seen in equilibrium with solid eicosane droplets. Similar experiments with tetradecane emulsions (i.e., a liquid oil that will not crystallize on destabilization. Data not reported) also showed no change in headspace EO suggesting the change seen in the eicosane required destabilization to induce crystallization of the lipid. It is notable that the dramatic increase in headspace concentration after enzymatic digestion of the supercooled liquid eicosane occurs after approximately 12 hours while visible phase separation happens much earlier (Figure 3.2) since destabilization of the system is a much faster reaction than crystallization.
Figure 3.6: Headspace ethyl octanoate in liquid emulsions with trypsin (●), liquid emulsions without trypsin (⊙), solid emulsions with trypsin (■) and solid emulsions without trypsin (□). All samples were 1% caseinate-stabilized, 10% eicosane emulsions kept at 28°C. Horizontal lines indicate headspace concentration as predicted by Equation 3.1.

The Buttery equation is often used to model headspace concentration of a volatile compound in equilibrium with an emulsion in terms of its partition coefficients with each separate phase and the phase volume ratios (Buttery, Guadagni, Ling, 1973):

\[
\frac{1}{K_{ge}} = \frac{\phi_o}{K_{go}} + \frac{(1-\phi_o)}{K_{gw}} \tag{3.1}
\]
where the gas-oil and gas-water partition coefficients, \((K_{\text{go}}\) and \(K_{\text{gw}}\) respectively), are determined experimentally and \(\Phi_o\) is the mass fraction of oil.

In order to determine bulk \(K_{\text{go}}\) and \(K_{\text{gw}}\), aliquots of EO were added to hexadecane or water and allowed to equilibrate for 1 hour at 28°C before headspace concentration was measured (further equilibration time, up to 24 hours, made no difference). Hexadecane was used in place of eicosane to determine \(K_{\text{go}}\) because bulk eicosane is a solid at this temperature and because its chemical structure is similar to that of eicosane and thus provides a similar solvent for EO. For ethyl octanoate, \(K_{\text{go}}\) was \(9 \times 10^{-5}\) and \(K_{\text{gw}}\) was \(5.61 \times 10^{-2}\) (Table 1). While \(K_{\text{gw}}\) was approximately twice as large as the value reported in the literature, \(K_{\text{go}}\) was approximately 50 times larger (Ghosh, Peterson, Coupland, 2008). The difference in the gas-oil partition coefficient may be due to differences in the experimental temperature (28°C in this work versus 30°C in the literature). Using values from Table 3.1 in Equation 3.1, the predicted headspace concentration for liquid oil droplets was 0.0126 µl/ml, in good agreement with the measured value (0.0102 µl/ml).

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<th>This work</th>
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<tr>
<td>(K_{\text{go}})</td>
<td>(9.00 \times 10^{-5})</td>
<td>(2.00 \times 10^{-6})</td>
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<tr>
<td>(K_{\text{gw}})</td>
<td>(5.61 \times 10^{-2})</td>
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In order to determine the value of $K_{ge}$ for solid eicosane droplets, we followed the approach of Ghosh, Peterson, Coupland (2006) and assumed that there is no interaction between EO or with crystalline eicosane droplets. The Buttery equation (3-1) was then modified to obtain the following:

$$\frac{1}{K_{ge}} = \left( \frac{\phi_o(1-\phi_{sfc})}{K_{go}} \right) + \left( \frac{1-\phi_o}{K_{gw}} \right)$$  \hspace{1cm} (3-2)

where $\phi_{sfc}$ is the proportion of total lipid in the emulsion that is solid. The overall gas-emulsion partition coefficient, $K_{ge}$ can then be calculated for any value of $\phi_{sfc}$.

At least qualitatively, Equation 3-2 provides a reasonable explanation for the sudden release of EO from supercooled liquid eicosane droplets following enzyme-induced crystallization. Low levels of droplet crystallization cause only moderate increases in headspace concentration but as solid fat content approaches 100%, the headspace concentration rapidly increases (Figure 3.7). Presumably the sudden increase seen experimentally is due to fat crystallization reaching some critical level so that the aroma molecule can no longer be effectively solubilized in the few remaining liquid droplets. However, the qualitative predictors of headspace concentration for crystalline droplets do not fit well with the data.
When SFC is taken to be 100% (completely solid droplets) in Equation 3-2, the predicted concentration of headspace EO is 0.447 µl/ml, about an order of magnitude greater than that seen experimentally (0.04 µl/ml, Figure 3.6). Thus, while the model is a good prediction for interaction of aroma with liquid droplets, it is an unreliable predictor for solid droplets. There are several possibilities accounting for the differences between theoretical and experimental data for crystalline fat.

One possible mechanism is the co-crystallization of EO with the eicosane (Figure 3.8a). However, the chemical structure of EO is different from the alkane so co-crystallization is unlikely. Eicosane crystallizes in tightly arranged aliphatic chains organized in a triclinic crystal structure (Ueno, Hamada and Sato, 2003) with few defects.
that might accommodate aroma molecules in the crystal lattice (Ghosh, Peterson, Coupland, 2008). Furthermore, the melting profiles of emulsions with and without aroma are similar to one another suggesting no differences in crystal composition (Figure 3.9).

Figure 3.8: Highly schematic diagram illustrating possible modes of aroma-solid droplet interaction. (a) Co-crystallization of EO within a solid eicosane droplet (b) pockets of supercooled melt with entrapped EO within a solid eicosane droplet (c) surface adsorption of EO with a solid eicosane droplet d) entrapment of EO within the inter-droplet space of a floc. Black rectangles represent aroma compound and white rectangles represent lipid crystal.
Figure 3.9. Differential scanning calorimetry trace of 1% caseinate-stabilized 10% eicosane solid droplet emulsions with (top) and without (bottom) encapsulated ethyl octanoate. Samples were kept at 28°C prior to analysis then cooled to 10°C and were heated at 90°C/hour.

A second possible mechanism for EO incorporation in solid droplets is within pockets of liquid inside the crystalline eicosane droplet (Figure 3.8b). Upon droplet crystallization the solid and liquid phases reach an equilibrium in which free energy is equivalent (Sato, 2001). Various workers have suggested that crystallization of alkanes begin at the surface and progresses toward the center, retaining a liquid core (Coupland, 2002). In an experiment to test this model, EO was added to either supercooled liquid droplets which were then subsequently crystallized and the headspace concentration was compared to that of similar emulsions where the EO was added directly to solid eicosane droplets. If this model was valid, the EO would be partially bound up within the temperature-cycled droplets (first liquid, then solid) and thus have a lower headspace
concentration. However, the headspace concentration when the EO was added before crystallization (0.026 µl/ml ± 0.0001) was not significantly different from the value when EO was added after crystallization (0.009 µl/ml ± 0.0009) after 24 hours at α=0.05, suggesting this model is not valid.

A third mechanism of entrapment is adsorption of EO to droplet surfaces (Figure 3.8c). Ghosh, Peterson, Coupland (2006) showed that $K_{ge}$ decreased with volume fraction of solid droplets and while this effect is smaller than that seen for binding by liquid oil, it establishes the presence of an interaction between solid fat droplets and aroma molecules. Furthermore, $K_{ge}$ decreased with decreasing particle size of solid droplets, suggesting a surface adsorption mechanism. Yucel (2011) used electron paramagnetic resonance to show that a hydrophobic compound in a solid lipid nanoparticle dispersion accumulated at the droplet interface and not in the crystal lipid droplet core. However the magnitude of solid-aroma interactions is often much less than liquid-aroma interactions and may not be significant in this case.

To take surface binding into account, Ghosh, Peterson, Coupland (2008) added an additional term to Equation 3-2 to give the following equation:

$$\frac{1}{K_{ge}} = \frac{\Phi_o}{K_{go}} + \frac{(1-\Phi_o)}{K_{gw}} + \frac{K_{lw} \cdot A_o}{K_{gw}} \quad (3-3)$$
where $K_{lw}$ is the apparent surface binding coefficient and $A_s$ is the interfacial area per unit volume of emulsion. However, using $K_{lw} = 4.5 \times 10^{-6}$ from Ghosh’s work in Equation 2 to approximate surface binding, $K_{ge}$ was $1.066 \times 10^{-3}$ in comparison to $1.061 \times 10^{-3}$ without the $K_{lw}$ term. Indeed, Ghosh, Peterson, Coupland (2006) also found this term to have an almost inappreciable effect in the model under similar conditions as the effect of surface binding is much smaller than the effect of partitioning into liquid oil. In this work, the surface binding effect proposed by Ghosh, Peterson, Coupland (2006) appears insignificant but the possibility remains that the constants used are not correct and this effect is in fact responsible for the discrepancies seen here.

A fourth mechanism of interpretation is that as the supercooled liquid eicosane droplets destabilize and coalesce into flocs, aroma may then be trapped in the interdroplet space, also inhibiting headspace release (Figure 3.8d). However, there is no significant difference in EO headspace concentration between stable and unstable solid emulsions (i.e., those incubated with and without enzyme) so this model is also unlikely (Figure 3.6).

A fifth and final mechanism of entrapment involves the binding of EO by caseinate and its enzymatic digestion products. Guichard and Langourieux (2000) have shown the binding of aroma compounds by dairy proteins through reversible non-covalent interactions (hydrogen bonding) or irreversible covalent bonds. The association
of bovine casein with small hydrophobic molecules involves an inclusion mechanism within the hydrophobic interior of this micelle-like structure (Farrell, Qi, Brown, Cooke, Tunick, Wickham, 2002). Sahu, Kasoju, Bora (2008) has shown the presence of channels that interconnect internal cavities within the casein micelles. These internal cavities may have the ability to form complexes and accommodate a foreign compound such as an aroma. Moreover, the influence of enzymatic digestion on any protein-aroma binding has not been studied.

To study the effect of protein-aroma interactions during enzymatic digestion, EO was added to a 1% caseinate solution and headspace concentration measured over 30 hours (Figure 3.10). There was no change in headspace concentration suggesting that in this system, digestion of the protein did not affect the binding of EO. Furthermore the average value for \( K_{gs} \) (i.e., gas-protein solution partition coefficient) was less than the \( K_{gw} \) (i.e., 0.0043 and 0.0561 respectively) suggesting some protein-aroma interaction.

The headspace concentration of EO above 10% liquid and solid dispersions of eicosane was calculated using \( K_{gs} \) for 1% protein in place of \( K_{gw} \) in Equations 3.1 and 3.2 respectively. The calculated value for liquid droplets changed only slightly due to the substitution (0.010 µl/ml vs 0.013 µl/ml) since in liquid emulsions the binding is dominated by the liquid oil. However in the solid fat the calculated value was much lower due to the substitution (0.05 µl/ml vs 0.45 µl/ml) and much closer to the experimental value (0.04 µl/ml).
Figure 3.10. Apparent gas-solution partition coefficient for EO with a 1% caseinate solution (12 µl of 0.01 µl/ml concentration of EO in equilibrium with 1% caseinate solution during digestion with trypsin.

3.4 Conclusions

From this work I had set out to initially produce and define a suitable emulsion based carrier system that will remain stable until digested by a protease. I hypothesized that proteolytic digestion of the emulsion will result in droplet coalescence, crystallization and the eventual expulsion of the hydrophobic small molecule from the lipid phase.
From this work I conclude:

(i) The interaction of volatile molecules can be described in terms of a three phase partitioning equation (Equation 3-1)

(ii) Protein stabilized eicosane emulsions are sufficiently stable to act as a carrier for volatile molecules until digested with enzyme

(iii) There is an initial lag time during enzymatic hydrolysis before the emulsion is destabilized inducing coalescence and eventual crystallization

(iv) Non polar volatiles interact better with supercooled liquid eicosane emulsion droplets than solid eicosane emulsion droplets

(v) Crystallization of supercooled liquid eicosane droplets reaches a critical level before encapsulated EO is released into headspace

(vi) Protein can bind aroma and can significantly affect headspace concentrations, particularly for solid droplets
Effect of Other Variables on Enzymatic Digestion of Caseinate Stabilized Emulsions

Abstract

In Chapter 3 I examined the effect of trypsin on the stability and crystallization of an emulsion containing added ethyl octanoate (EO). In this section I consider the effects of changing other parameters in the system (i.e., added protein, small molecule type and lipid type). Increased sodium caseinate solution concentrations led to a decrease in headspace EO concentration. The degree of EO binding by caseinate solution increased as the protein was hydrolyzed. Small molecule types differing in carbon chain length and hydrophobicity (i.e., EO vs. ethyl hexanoate) did not show a significant difference in absolute headspace concentration and time at which this occurred. When tripalmitin and palm oil emulsions were treated with trypsin they did not show the characteristic increase in headspace concentration seen in eicosane dispersions. Tripalmitin had solidified at the beginning of the experiment and the solid fat content of palm oil at 28.7% was insufficient to cause a change in EO binding. All statistical analyses were done as described in Chapter 3.

4.1: Interaction of EO with Caseinate Solutions during Trypsinolysis

The interactions of EO with caseinate was shown to affect the headspace concentration in equilibrium with emulsions and in particular liquid particles (Figure
3.6). Surprisingly there was no change in the degree of interaction during digestion by trypsin despite the obvious changes in protein structure. In this section I will consider more fully the effects of protein concentration on the interaction during trypsinolysis.

Caseinate solutions (0-10%) were mixed with ethyl octanoate and trypsin and the headspace concentration measured as a function of time at 28°C as described in section 3.2.2. All data was the average of two or more trials.

After an initial equilibration period, the headspace concentration of EO decreased with protein concentration suggesting binding (e.g., compare 6 hour data, Figure 4.1). Additionally, at higher protein concentrations, headspace concentration decreased with respect to time (Figure 4.1). One explanation may be that with progressive trypsinolysis, there was a greater affinity of hydrolyzed enzyme for the aroma substrate. Another explanation is that the binding of aroma by protein is a slow reaction, which becomes stronger over time thus accounting for the decrease in headspace release only after an extended period.
Figure 4.1. Headspace concentration of EO in equilibrium with various concentrations of sodium caseinate solution [1% (■), 5% (▲) and 10% (◆)] as a function of time of incubation with trypsin. 12 µl of EO was added to 1 mL of solution in equilibrium with 9 mL of headspace.

The protein-aroma interaction can be quantified in terms of a partition coefficient \( K_{gp} \) using a modification of the Buttery equation:

\[
\frac{1}{K_{gs}} = \frac{\phi_p}{K_{gp}} + \frac{(1-\phi_p)}{K_{gw}}
\]

(4-1)

where \( K_{gs} \) is the measured gas-solution partition coefficient, \( K_{gw} \) is the gas-water partition coefficient and \( \phi_p \) is the mass fraction of protein in the solution. \( K_{gp} \) can be readily calculated from the best fit of the change in \( K_{gs} \) with protein content at any time interval using a linearized version of Equation 4-1:
When $1/K_{gs}$ is plotted against protein concentration function should be linear at each time point with an intercept $= 1/K_{gw}$ (~18) and a slope of $1/K_{gp} - 1/K_{gw}$. However, in this case the data does not show a linear trend and the intercept is not 18 (Figure 4.2). That is to say the data does not fit with the simple two-phase binding model used and the actual nature of the protein-EO must be more complex and probably concentration dependent.

\[
\frac{1}{K_{gs}} = \frac{1}{K_{gw}} + \phi_p \left( \frac{1}{K_{gp}} - \frac{1}{K_{gw}} \right)
\]  (4-2)

Figure 4.2: $1/K_{gs}$ as a function of time at (-) 6 hours, (x) 12 hours, (+) 18 hours, (○) 24 hours and (□) 30 hours of incubation with trypsin. Linear regressions are shown alongside the data.
4.2: Effect of Added Protein Concentration on the Release of EO from Emulsions during Trypsinolysis

In Chapter 3, I showed aqueous caseinate bound to EO depressed the headspace concentration in emulsions, and more so for SLN. In this section I consider a wider range of protein concentrations and the effects of protein binding developed in Chapter 4.1. I hypothesize that:

- The headspace EO concentration above supercooled liquid emulsions will be lower than the headspace EO concentration above simple sodium caseinate solution since there would be both the solubilizing effect of the liquid lipid droplets in addition to the binding effect of the protein.
- Higher protein concentrations will take longer to digest since there is a higher substrate to enzyme ratio and there will be a longer time for a change in headspace EO.
- Headspace EO concentrations will decrease with increasing protein concentrations in SLN and this effect will be larger than in liquid droplets since the amount of unbound caseinate is more critical when lipid is in a crystalline state.

A supercooled liquid eicosane emulsion (fat content 15%, protein concentration in the initial aqueous phase 1%) was prepared as described in Chapter 3.2.2 and then diluted with the appropriate amount of protein solution to arrive at the same lipid content (10%) but with the different final protein concentrations (1, 5, 10% with respect to the aqueous
phase). The samples were incubated with trypsin as described in Chapter 3.2.2 and the headspace EO concentration was measured as a function of time.

At all protein concentrations, the shape of the kinetic curves were similar to those seen in Figure 3.6 in that the samples had an initial steady headspace concentration which after a critical time increased to a second higher plateau value (Figure 4.4). The concentration in the headspace after this increase at a critical time was significantly different between all protein concentrations.

Initial headspace concentrations of EO in equilibrium with supercooled liquid droplets decreased with protein concentration (Figure 4.4). The difference between the headspace concentrations of higher protein emulsions with their respective simple protein solutions is approximately an order of magnitude, highlighting the binding effect of both protein-aroma interactions as well as the solubilizing capacity of the liquid oil.

Contrary to what was initially hypothesized, the time at which the headspace concentration increased from the low to high plateau value was lower for the high protein samples (5% and 10%) compared to the low protein sample (1%) (Figure 4.4). Similarly, visible phase separation occurred sooner in the high protein samples (Figure 4.5). One possible explanation may be that this faster destabilization is due to depletion flocculation in which excess casein in the continuous phase aggregates as a result of digestion. Indeed, this has been observed by other researchers in similar work (Dickinson and Golding, 1997a; Dickinson and Golding, 1997b; Radford and Dickinson, 2004).
The final headspace concentration of EO decreased with increasing protein concentrations, which suggests binding by the casein (Figure 4.1) as expected.

Figure 4.3: Headspace concentration of ethyl octanoate in 10% supercooled liquid eicosane emulsions at different sodium caseinate concentrations [1% (■), 5% (▲) and 10% (◆)] as a function of time. EO (12 µl) was added to 1 mL of emulsion in equilibrium with 9 mL of headspace. Error bars indicate standard deviation and letters indicate significant difference at p ≥ 0.05 at 30 hour timepoint. All samples were analyzed at 28˚C.
Figure 4.4: Photograph of (a) sodium caseinate solution at the three different protein concentrations in comparison with appearance of emulsions after (b) 1 hour, (c) 2 hours, (d) 3 hours incubation with trypsin

4.3 Effect of Small Molecule Type

The initial experiments were conducted with ethyl octanoate (EO, Chapter 3) but it is interesting to consider how other volatile molecules would behave in this system. Ethyl heptanoate (EH) was selected for comparison as it has similar chemical properties as EO but is both more volatile and less hydrophobic. It was also used in similar studies
by Ghosh, Peterson and Coupland (2006a) in which it was determined that EH and EO differences in $K_{gw}$ ($1.82 \times 10^{-2}$ vs. $2.67 \times 10^{-2}$) and $K_{go}$ were negligible ($6.0 \times 10^{-6}$ vs. $2.0 \times 10^{-6}$).

Supercooled liquid eicosane emulsions (10% lipid stabilized with 1% caseinate) were prepared in the manner described in Chapter 3.2.2 and kept at 28°C. Samples were prepared with 0.1 µl/ml of either EO or EH then trypsin was added and the headspace concentration measured as a function of time. I hypothesize that:

- In liquid oil emulsions there will be a higher headspace concentration of EH than EO since EH is less hydrophobic and more volatile and would partition more readily in the gas phase.
- In SLN there should be no solid lipid-aroma interactions and the headspace concentration for EH with a higher volatility would be significantly higher than the headspace concentration of EO.

In both emulsions, the headspace concentration of volatile had an initial steady state value up to a critical time at which point it increased to a second higher plateau (Figure 4.6). At all initial headspace concentrations before the critical timepoint, the amount of EH was higher than EO. The lower amount of EO in the gas phase can be explained as it is more hydrophobic than EH ($K_{go} = 2.0 \times 10^{-6}$ vs. $6.0 \times 10^{-6}$, Ghosh, Peterson, Coupland, 2006a) and is therefore more strongly bound by oil droplets. Carey, Asquith, Linforth, Taylor (2002) also showed the binding effects of a cloud emulsion increased with the number of carbons (hydrophobicity) of the aroma compound.
Seemingly, aroma type has only a minor effect on digestion time or the subsequent headspace expulsion. A possible reason for this may be interactions between the volatile molecules with the enzyme. However, the difference in this set of aroma molecules used in the present work is very small and thus the generality of any conclusion is limited.

Figure 4.5: Headspace concentration of 1% caseinate-stabilized 10% supercooled liquid eicosane emulsion with 0.1 µl/ml ethyl octanoate and 0.05ml/ml trypsin (■) and 0.1 µl/ml ethyl heptanoate and 0.05ml/ml trypsin (□)

The final plateau values at 30 hours for both EH and EO were not significantly different from one another (p ≥ 0.05). In this second stage of headspace release, particles have crystallized and are no longer a solubilizing agent for the hydrophobic molecules. In
the solid state, the lipid is governed by the gas-water partition coefficient ($K_{gw}$) while in liquid oils both gas-water and gas-oil partition coefficients ($K_{gw}$ and $K_{go}$) are important. The values of $K_{gw}$ for EO and EH are not significantly different from one another (5.61 x10^{-2} and 1.82 x 10^{-2} respectively, Ghosh, 2008) and there was no difference in the final headspace concentration.

4.4 Preliminary Studies on the Effect of Lipid Type

The work so far has focused on a pure lipid with relatively simple crystallization properties and deep supercoolings. In a real food system however, the fat is usually a heterogeneous mixture of various lipids with a range of melting temperatures and polymorphic structures. To examine how the effects seen with an alkane would affect more realistic food products, other lipids were considered: tripalmitin (a polymorphic pure triglyceride) and palm oil (a polymorphic triglyceride blend). Ghosh, Peterson, Coupland (2006) also used these fats in related studies.

Samples of the bulk fats were heated to 110°C for 15 minutes to melt them completely and approximately 5 g were poured into NMR tubes. Samples were then cooled to 25°C for 30 minutes and reheated to 28°C for 24 hours prior to solid fat content (SFC) determination by NMR (Bruker Biospin Minispec, The Woodlands, TX) according to methodology as described by Bruker Biospin (2011).

Emulsions of these fats were prepared as described in Chapter 3.2.2, cooled to 28°C and the headspace concentration of EO was measured as a function of time during
trypsinolysis. The melting and crystallization of the freshly prepared emulsions (without added trypsin) was measured by differential scanning calorimetry according to the method described in Chapter 3.2.4.

The release from eicosane during trypsinolysis is as reported in Figure 4.7 and is as discussed in Chapter 3. In palm oil samples the headspace concentration was low and constant while in tripalmitin samples, headspace concentration is high and constant but higher than liquid eicosane droplets and lower than solid eicosane droplets.

The onset of crystallization in palm oil (24°C, Figure 4.7b) was below the experimental temperature so it was probably supercooled at the start of the experiment. However even after nucleation, palm oil has a solid fat content of only 28.7% at 28°C (Table 4.1) and the high residual liquid oil is sufficient to prevent a measurable change in headspace EO (see the calculated plot of headspace concentration as a function of SFC in Figure 3.7).

Tripalmitin has a high solid fat content at 28°C (99.5%) similar to that of eicosane (Table 4.1) and would therefore be expected to behave like eicosane at the end of the experiment. However the crystallization onset of emulsified tripalmitin droplets (27°C, Figure 4.7c) is below the experimental temperature and it seems likely that these droplets had crystallized before the experiment started and thus there were no changes in SFC on trypsinolysis.
Figure 4.6: Headspace concentration of ethyl octanoate in 10% supercooled liquid eicosane emulsions stabilized by 1% sodium caseinate (■), 10% tripalmitin emulsions stabilized by 1% sodium caseinate (▲) and 10% palm oil emulsions stabilized by 1% sodium caseinate (●). Data points represent single experimental measurements.

Table 4.1: Solid fat content of sample fats at 28°C. Measurements performed by Lindsay Wolfe.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>SFC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eicosane</td>
<td>100</td>
</tr>
<tr>
<td>Tripalmitin</td>
<td>99.5</td>
</tr>
<tr>
<td>Palm Oil</td>
<td>28.7</td>
</tr>
</tbody>
</table>
(a) Heat Flow (endothermic up)

(b) Heat Flow (endothermic up)

Temperature (°C)
Figure 4.7: Differential scanning calorimetry trace of 1% caseinate-stabilized 10% supercooled liquid (a) eicosane, (b) palm oil, (c) tripalmitin emulsions with encapsulated ethyl octanoate during heating (top traces) and subsequent cooling (bottom traces).

Neither of the food lipids selected showed the expected enzyme triggered release seen in eicosane. To demonstrate this effect it would be necessary to choose (i) a fat with a droplet nucleation temperature significantly below the melting point, (ii) a fat with a high solid fat content at the incubation temperature, (iii) an incubation temperature between the droplet nucleation and melting temperature of the lipid. Some possible candidate lipids include cocoa butter or shea butter.
Chapter 5

Conclusion and Future Work

The goal of this work was to investigate the effect of trypsin-triggered digestion of a supercooled liquid dispersion in the coalescence of the emulsion, crystallization of the lipid and release of a hydrophobic small molecule.

Casein stabilized emulsions were stable until digested by a trypsin which resulted in droplet coalescence and eventual crystallization (Chapter 3). Crystallization occurred much more slowly than phase separation and sudden release of entrapped ethyl octanoate only occurred after the lipid crystallization was nearly complete. The data could be modeled well in terms of bulk air-protein solution and air-oil partition coefficients assuming no interaction with the solid fat.

The second objective of this study was to understand the effects of other variables (change in protein concentration, small molecule type, lipid type) on the enzymatic digestion of the protein-stabilized dispersion. This was accomplished in Chapter 4. With higher protein concentrations there was a respective decrease in headspace concentration, the effect of which increased over time, suggesting more and better binding by the caseinate solution. Calculation of the protein partition coefficient confirmed that protein-aroma interactions were indeed significant. In emulsions with the higher protein
concentrations, destabilization occurred sooner and showed depressed headspace concentrations.

With a different small molecule type there was no significant difference as a more volatile and less hydrophobic molecule (EH) did not have a significantly different headspace concentration than the original model compound (EO). The time of increase for EO also unexpectedly occurred sooner than EH.

The two alternatives of lipid type selected did not show the characteristic enzyme-triggered release as was seen with the model lipid. Tripalmitin, which has a solid fat content profile similar to that of eicosane did not show a sufficiently deep supercooling to remain in the liquid state necessary to entrap aroma molecules. Particles were already solid at the incubation temperature at the start of the experiment. Conversely, palm oil was supercooled at the incubation temperature but containing only a fraction of the solid fat as that in eicosane, also did not show the characteristic enzyme triggered release.

In the present study, a protein stabilized supercooled liquid droplet can be triggered by an enzyme to release the encapsulated small molecule. It was found that liquid oils are an efficient reservoir for aroma compounds compared to solid fat and more volatile compounds would be partitioned in the liquid droplets leading to a lower headspace concentration as compared to solid droplets. Given the requirements necessary for a real fat to be suitably used in a protein stabilized supercooled liquid droplet model system (ie. high solid fat content, deep supercooling) there may only be a limited number
of triglycerides that fit this profile. Additionally, proteins also have the capacity to interact with aroma and this effect was more significant in solid droplet emulsions as compared to liquid droplet emulsions.

Further studies showed that while a smaller and less hydrophobic compound (EH) did not show a significant difference in headspace concentration than a more hydrophobic compound (EO), the latter was seen to increase sooner in headspace concentration. The greater volatility of EH does not seem to have a significant difference on headspace concentration. Thus, it may be a more hydrophobic compound with a sooner release time that may be a good candidate as the model aroma compound.

It can be concluded that in order to see enzyme triggered release of an encapsulated small hydrophobic molecule, several conditions must be satisfied:

(i) a fat with a droplet nucleation temperature significantly below the melting point,
(ii) a fat with a high solid fat content at the incubation temperature
(iii) an incubation temperature between the droplet nucleation and melting temperature of the lipid.
(iv) a hydrophobic molecule with a known release time profile
REFERENCES


