EFFECT OF ETHANOL ON THE SOLUBILIZATION OF HYDROPHOBIC MOLECULES BY SODIUM CASEINATE

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by

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Abstract

Many drugs and phytochemicals are insoluble in water, and therefore difficult to disperse in aqueous oral formulations. While some can be solubilized with surfactants or ethanol, there is a demand for alternative approaches. Casein has been shown to solubilize hydrophobic small molecules, and this might be an attractive option in some applications. Previous studies have shown that ethanol affects the conformation of the casein micelle, and in this work I consider if ethanol may facilitate the loading of sodium caseinate with lipophilic small molecules (i.e., pyrene and piperine). When ethanol and water are removed, I hypothesize the resultant powder can be readily rehydrated to produce an aqueous suspension of piperine- or pyrene-loaded caseinate with a total concentration in considerable excess to the aqueous solubility limit of the compounds.

A large excess of pyrene or piperine was mixed with 2% (w/v) sodium caseinate in buffer (pH 6.7) and ethanol (0-40%, v/v), stirred for 24 h, and lyophilized to remove ethanol and water. The resultant powder was rehydrated in buffer to achieve a 2% casein suspension. The excess, insoluble pyrene or piperine was removed by gentle centrifugation and the concentration of solubilized fraction (i.e., dissolved in water, plus that which was bound to the the casein) was determined by UV-spectroscopy following extraction into acetonitrile or by fluorescence spectroscopy respectively.
Pyrene is effectively insoluble in water (i.e., not fluorescing), however, in the presence of the caseinate, it was solubilized and showed a characterized fluorescence spectrum in buffer. The solubility of piperine in water was 49 mg/L, but in the presence of 2% casein (i.e., no ethanol used in preparation), the solubility increased to 134 mg/L (i.e., 50 moles of piperine per mole of caseinate). As the ethanol present at the solubilization step increased, the amount of solubilized piperine in the final product increased to a maximum of 342 mg/L (i.e., 175 moles of piperine per mole of caseinate) in 40% ethanol. I hypothesize that both pyrene and piperine are solubilized in water by binding to caseinate and that ethanol both facilitates mass transport to the protein from the insoluble powder by increasing the aqueous solubility of pyrene/piperine, and changes the conformation of the protein to increase binding.

Next, the *in vitro* acid and proteolysis digestibility of the casein-pyrene complexes was tested over 48 hours. During the acid digestion, fluorescence dropped to zero immediately, because caseinate precipitated at the isoelectric point (i.e., 4.6) and pyrene remained the binding with precipitated protein. In the contrast, during the proteolysis, there was a gradual drop of fluorescence without protein precipitation, which meant pyrene were freed then stopped fluorescing. It suggests that the pyrene is able to be released in the gastrointestinal fluid during the enzymatic digestion. Finally, the solubilizing capacity of piperine by various proteins (i.e., whey protein isolate,
caseinate, native casein micelle extracts, and β-lactoglobulin) were compared and caseinate performed the best.
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Chapter 1. Literature review

1.1. Overview

1.1.1. Problems of delivery of hydrophobic bioactive ingredients

One of the largest challenges to improving the nutraceutical profiles in the food system is that many effective health beneficial substances have very poor water solubility (hydrophobic) while most food systems are water-based (hydrophilic) (Pawar and Kumar 2002). To achieve the required bioavailability, hydrophobic health promoting compounds need to be solubilized in the food system (Kimpel and Schmitt 2015) first, then released through digestion. One common method to achieve this goal is to incorporate hydrophobic bioactive ingredients within emulsion-based systems, (e.g., milk, butter, salad dressing). Other than the emulsions, new encapsulation systems based on nanoparticles have been developed to transport hydrophobic compounds (e.g., fatty acids, vitamins, flavonoids, probiotics, pharmaceuticals, antioxidants) in aqueous systems. Nanoparticles can bind or encapsulate active compounds and protect them from the effect of the surrounding environment (i.e. light, moisture, temperature) (Tavares et al. 2014) and deliver to target locations. In this way, the stability, bioavailability and dispersing ability of the poor water-soluble ingredients are enhanced in the food system (Abd El-Salam and El-Shibiny 2012).
1.1.2. Hydrophobic ingredients encapsulation and problem in liquid preparations

The health-beneficial ingredients encapsulation studies are not well taken into account by food manufacturers (Hernández et al. 2014). Tavares et al. (2013) showed that the R&D investment on encapsulation technologies was more than ten times higher in the pharmaceutical industry vs the food industry in 2013 (Tavares et al. 2014). Consequently, huge economic potential profits and social benefits could be sought from functional foods related studies (Kimpel and Schmitt 2015). Researchers are focusing on the optimization of delivery and their objective is to efficiently control and release optimal amount of encapsulated compounds to the targets (Gouin 2004).

Even though the encapsulation is practical and able to increase the solubilization of ingredients, the choice of the encapsulants, excipients or solvents is still in debate, especially in pharmaceutical and nutraceutical preparations for kids and infants. Pediatric drug formulations production is very challenging because the ideal drug should be effective, well tolerated, stable, affordable, and in particularly, have good palatability. Children will refuse anything that does not taste or smell good to them (Pawar and Kumar 2002), however the common solvents used in the liquid formulations to enhance solubility of the active ingredients are not very favorable to children (e.g., ethanol, glycerol, propylene glycol, etc.) (Strickley 2004). But even more than that, there are toxic effects reported of these solvents. Ethanol is considered
toxic to patients who have idiosyncratic reactions to alcohol; large concentration (40%) of glycerol may cause mucositis in stomach and diarrhea and electrolyte disturbances (Pawar and Kumar 2002); and propylene glycol could cause hyperosmolality in premature infants (Tobergte and Curtis 2013). Therefore, finding a natural, readily available, inexpensive, non-toxic, and safe alternative solubilizing material can meet the urgent demands for hydrophobic active ingredients.

1.1.3. Food proteins as delivery carriers

Food proteins, as a typical part in food polymers, are used in formulated food products because of their high nutritional value and structurally properties (Chen, Remondetto, and Subirade 2006). Many food proteins are Generally Recognized as Safe (GRAS) (Kimpel and Schmitt 2015) (e.g., caseins, whey proteins, soy proteins). The amphiphilic property of food proteins is based on their hydrophobic and hydrophilic fractions. The hydrophobic domain of the protein is responsible for binding or encapsulating the ideal compounds based on hydrophobic interaction. Caseins, whey protein isolates, vegetable proteins (i.e. soybean protein (Teng, Luo, and Wang 2012)) and β-lactoglobulin (Kontopidis, Holt, and Sawyer 2004) are protein resources that have been shown to transport and protect bioactive ingredients into food systems (Livney 2010a).
1.1.4. Milk-derived proteins delivery system

Proteins derived from milk are characterized by high nutritional value and good sensory attributes (Kimpel and Schmitt 2015). They are inexpensive, readily available and safe (Livney 2010b). In addition, milk proteins are structurally amphiphilic (constructed of hydrophobic and hydrophilic domains). Because of these properties, milk-derived proteins are largely studied for their potential to deliver hydrophobic ingredients in aqueous food system (Tavares et al. 2014).

Many studies have shown that casein and its related products (i.e. sodium caseinates, β-casein) bind hydrophobic compounds (Kimpel and Schmitt 2015). What is more important, as a very friendly protein source in toddler diets, casein can be used to deliver effective infant drugs (Corzo-Martenez, Mohan, Dunlap, & Harte, 2015) and avoid children’s contact with undesirable, toxic or alcoholic organic solvents.

Factors can affect the structure of casein (e.g. organic solvents, pH, temperature, and pressure) have been investigated by some studies (Ye & Harte, 2013; Tavares et al., 2014). In the later sections, the effect of these factors on casein structure will be reviewed thoroughly. The potential effect of structure changing on casein’s hydrophobic ingredients delivery properties will also be discussed.
1.2. Milk Protein Chemistry

1.2.1. Overview

Bovine milk contains 30 to 36 g/L proteins (Kimpel and Schmitt 2015). The protein content is depending on breed, lactation stage, and health and physiological status of the animal (C. G. De Kruif & Holt, 2003). There are two categories of proteins in milk, caseins and whey proteins. Caseins occupy about 79% of the total protein and whey proteins are 19% of the total protein (Kimpel and Schmitt 2015). Caseins are very heat stable. At pH 6.7, caseins can withstand 100°C for 24 hours whereas whey protein denatures at 90°C for 10 minutes. Caseins are synthesized to meet the amino acid requirement and to transport important nutrients (mostly amino acids and calcium) for neonates (Thompson, Boland, and Singh 2008).

1.2.2. Whey protein and its structure

Whey protein is the general term that describes all the milk proteins other than caseins. Whey proteins consist of a mixture of globular proteins including \(\beta\)-lactoglobulin, \(\alpha\)-lactalbumin, bovine serum albumin, immunoglobulins, and lactoferrin. \(\beta\)-lactoglobulin and \(\alpha\)-lactalbumin are two prevalent small globular proteins found in cow milk. Bovine serum albumin is a larger globular protein found both in milk and blood serum. Lactoferrin, as a member of transferrin family, works
as an anti-microbial component in milk. Whey proteins precipitate at pH 4.8 to 5.3 (Ney 2011). Whey protein concentrate (obtained by filtration) and whey protein isolate (obtained by ion exchange) are two major whey protein products used commercially as food ingredients (Livney 2010b).

1.2.3. Casein and its structure

Caseins are considered intrinsically unstructured proteins in the mammal milk serum (Farrell et al. 2006). The caseins are proline-rich proteins and they precipitate at pH 4.6. Caseins are a mixture of $\alpha_1$-casein, $\alpha_2$-casein, $\beta$-casein, and $\kappa$-casein (Abd El-Salam and El-Shibiny 2012). Caseins are able to self-assemble into natural or artificially simulated micelles (Figure 1.1). Diameters of casein micelles are from 50 nm to 500 nm (average in 150 nm) and the molecular mass is approximately from $10^6$ to $10^9$ Da (Semo et al. 2007) (Livney 2010a).
In casein micelles, $\kappa$-casein is the smallest casein with a low level of phosphorylation and low sensitivity to the calcium. $\kappa$-casein is generally considered as hydrophilic due to the composing of highly hydrophilic glycomacropeptide portion.

While other caseins (i.e., $\alpha_s$-casein, $\alpha_b$-casein, $\beta$-casein) have more phosphorylated residues and higher calcium sensitivity then $\kappa$-casein (McSweeney and Fox 2013).

While there are several models for the structure of the casein micelle, some authors have argued that they are assembled from “submicelles” (Farrell et al. 2006). There are two types of submicelles (Holt et al. 2003):

- $\kappa$-casein rich submicelle (less sensitive to calcium, mostly on the surface of micelles) (Kruif, Huppertz, Urban, & Petukhov, 2012)
- $\kappa$-casein poor submicelle (sensitive to calcium, mostly in the interior of micelles) (Kruif, Huppertz, Urban, & Petukhov, 2012)
The glycomacropeptide portion in κ-casein provides stability of casein micelles to aggregation (McSweeney and Fox 2013). As the strong hydrophilic property of the glycomacropeptide, κ-caseins interacts with water to form a 5-10 nm “hairy layer”, which stabilizes casein micelle structure (Dalgleish, 2011). Other than four types of caseins, casein micelles contain 6% calcium phosphate (Livney 2010b). In recent models, calcium phosphate are colloidal nanocrystals which bind casein aggregates and support the entire micellar structure and β-casein forms some channels to hold water inside the micellar structure.

1.2.4. Food ingredients from caseins

Two readily available casein resources are reviewed in this section: native micellar casein and caseinates (i.e. sodium casemate, calcium caseinate, potassium caseinate) (Dziuba et al. 1999). Even though both of them are named as caseins, the production methods, and the structures are distinct.

Native micellar casein is as an unprocessed or lightly processed product typically extracted from skim milk by membrane filtration (Punidasas and Rizvi 1998), size extrusion or dialysis in order to remove lactose and whey protein components (Patton and Huston 1986). However, the purity is hard to control due to the complex compositions in milk. (Donnelly et al. 1984).

Caseinates are processed casein ingredients (Dziuba et al. 1999). Caseinate
production starts at making casein curd, by acidifying warm skim milk to pH 4.2-4.6 (i.e., isoelectric point of casein). After casein is precipitated, strong alkali (sodium hydroxide or calcium hydroxide) is added to neutralize the pH to a range of 6.8 to 7.6, at which point viscous sodium caseinate or calcium caseinate solutions are formed. The water solubility and viscosity of caseinate solutions are dependent on sodium or calcium content. Finally, spray-drying is used to produce the final caseinate powder from the concentrated caseinate solution (Southward 1998).

Caseinates have similar protein composition as native casein: $\alpha_s$-casein, $\alpha_b$-casein, $\beta$-casein, and $\kappa$-casein, with a weight ratio of 4:1:4:1. Therefore, sodium caseinate can maintain its amphiphilic property ability to behave as an effective emulsifier (Srinivasan, Singh, and Munro 1996).

Qi showed sodium caseinate’s structure differs greatly from that of the native bovine casein micelle in pH 7.0 imidazole buffer. Native casein forms a supramolecular aggregate (i.e., casein micelles) in milk (Figure 1.2), but sodium caseinate without the presence of calcium forms a strand-like complex (Figure 1.3) (P.X. Qi 2009). Even though the native micellar caseins and caseinates form different colloidal particles in water, both of them are have been shown to bind hydrophobic compounds in latter studies (Zimet, Rosenberg, and Livney 2011).
Figure 1.2 (a). Transmission electron micrograph of bovine milk casein micelles.

From (P.X. Qi 2009).
Figure 1.2 (b). Transmission electron micrograph of sodium caseinate in pH 7.0 imidazole buffer from (P.X. Qi 2009).
1.3. Interactions Between Caseins and Small Molecules

1.3.1. Mechanisms of interactions between caseins and small molecules

An important biological function of casein is to transport essential nutrients, proteins, and calcium to the neonate; Moreover, studies showed that caseins are, in effect, a natural delivery system out of milk matrix (Semo et al. 2007).

Caseins are able to entrap substances by the formation of supra-molecular structures, emulsions, and gels (Tavares et al. 2014).

1.3.2. Hydrophobic small molecules interaction with caseins

Hydrophobic interaction is the major mechanism for the casein system to bind and deliver poor water-soluble compounds. As mentioned before, the native casein micelles are supported by hydrophobic caseins and calcium phosphates internally and are stabilized by the "hairy layer" of κ-casein externally. In the presence of hydrophobic compounds (e.g., fatty acids, vitamins, nutraceuticals, lipophilic flavors) in the aqueous system, caseins are able to associate with the compounds to form non-covalent bindings (Dalgleish & Corredig, 2012) based on the hydrophobic interaction. Because water is a polar solvent, the exposure of apolar groups in hydrophobic compounds to water is thermodynamically unfavorable. Thus, the apolar
group in water insoluble compounds can thermodynamically bind the hydrophobic domains in caseins to repel the contact with water. Since the caseins also have hydrophilic domains which are favorable to interact with water, the bound hydrophobic compounds are finally protected by caseins and solubilized in water. With different structures in water system, both casein micelles and sodium caseinates were demonstrated to be able to solubilize hydrophobic small molecules through the hydrophobic interaction (Zimet, Rosenberg, and Livney 2011).

1.3.3. Enhancement of the interactions

Interactions between casein and hydrophobic compounds are sensitive to environmental conditions and processing methods. Many studies have investigated the effect of the addition of salts, pH, temperature, hydrostatic pressure, the presence of organic solvents, homogenization, and ultrasound on caseins’ binding activities and the structures of caseins.

1.3.3.1. Effect of the presence of calcium

In previous studies, calcium was found to work as supporting agents in casein micelle structure (Dalgleish 1998). As reviewed before, different types of caseins form different structures in water. A study found the formation of supra-structure or nanoparticles are actually dependent on the presence of calcium phosphate (Zimet,
In a recent study, two mechanisms were demonstrated to solubilize DHA (ω-3 fatty acid): sodium caseinate and DHA with presence of calcium phosphate (DHA-loaded re-assembled casein micelles, r-CM) and sodium caseinate and DHA without sodium phosphate (DHA-loaded casein nanoparticles, CNP) (Zimet, Rosenberg, and Livney 2011). The researchers showed that the r-CM are formed with a size of 50 to 60 nm diameters and the CNP are formed with a size of 288.9 nm diameter, suggesting that the presence of calcium phosphate did have an effect on the structure of casein present in water. Nevertheless, both r-CM and CNP were reported to bind DHA with a high binding ratio of 3-4 DHA molecules per protein. In addition, both systems showed a remarkable protection of DHA from oxidation and a high colloidal shelf-life stability at 4°C.

1.3.3.2. Effect of high temperature

Many studies have demonstrated the effect of high temperature on the hydrophobic interaction between caseins and hydrophobic substances.

Zhong’s group applied high temperature (60°C) and high ethanol content to facilitate the solubilization of curcumin by casein micelles. After the processing, high temperature exposed the hydrophobic sites in casein micelles to interact with
insoluble curcumin molecules in buffer. The dispersion was then high temperature spray-dried and a remarkably high amount of solubilized curcumin (134 µg/mL, 0.25 g curcumin per 1 g casein) was observed in the final colloidal dispersion (400 times higher than aqueous solubility) (Pan and Zhong 2015).

In another curcumin solubilization enhancement study, critical micelle concentration for β-casein was found to decrease from 25 nmol/L to 8 nmol/L as temperature increased from 25°C to 37°C (Esmaili, Ghaffari, Moosavi-Movahedi, Atri, Sharifizadeh, Farhadi, Yousefi, Chobert, Haertle, et al. 2011). The authors argued that once the temperature increased, entropy in the system was increased, which enhanced the hydrophobic interaction to self-associate casein micelles in the system. Consequently, more hydrophobic domains were exposed during heating process. This phenomenon was also found by another study that more α-tocopherol was solubilized by casein when the temperature increased from 14°C to 34°C (Chevalier-Lucia et al. 2011). It is concluded that heating could enhance the absorption of α-tocopherol into casein micelles because hydrophobic interactions were increased by high temperature.

1.3.3.3. Effect of high-pressure homogenization

High-pressure homogenization (HPH) is another very important step in the formation of casein nanocapsules in many studies. Studies suggested that applying
HPH to casein micelles can disassociate the casein micelles initially and increase its binding ability to hydrophobic substances during re-association.

The Harte group tested the effect of HPH from 0 MPa to 300 MPa on the interaction between casein micelles and triclosan (TCS), a hydrophobic bioactive compounds (Roach, Dunlap, and Harte 2009). It was found that about 80% of TCS was associated with caseins without any HPH processing, and the TCS association increased by 30% once 300 MPa HPH was applied.

Another study found that at 34°C, binding efficiency of α-tocopherol to casein micelles increased 1.5 times from 0 MPa to 250 MPa; while at 14°C, the binding efficiency increased 3 times throughout 0 MPa to 300 MPa, particularly (Chevalier-Lucia et al. 2011). It is concluded that the HPH process changed the assembling ability of casein micelles and the disassociation/re-association under HPH can allow caseins to entrap more α-tocopherol into a more compact structure.

1.3.3.4. Effect of hydrostatic pressure

High-pressure processing is also reported to be a factor in hydrophobic compounds binding by caseins. Similar to HPH, the change of protein structure can result in an enhancement of binding.
Rahimi conducted different levels of hydrostatic pressure (0 MPA to 600 MPA) in skim milk by using vacuum sealing system to facilitate the interaction between curcumin and casein micelles (Rahimi Yazdi et al. 2013). The increase of the amount of bound curcumin in skim milk was observed with the pressure increasing. The reasons author provided are that high hydrostatic pressure can modify the structure of proteins and change the constitution of hydrophobic domains. As a result, the hydrophobicity of milk proteins was increased. However, after the pressure treatment, a significant amount of bound curcumin was released during storage because the milk proteins rearranged to native structures.

1.3.3.5. Effect of pH

Casein aggregates at its isoelectric point (pH 4.6) and this phenomenon might affect the interaction between of casein micelles and hydrophobic compounds.

A study found an increase in particle size of skim milk (from 306 nm to 421 nm) as the pH increased from 6.7 to 8.0 by the addition of alkali in milk (Ghasemi and Abbasi 2013). A reversible particle size reduction was also found with decrease in pH. After mixing the fish or vegetable oils with skim milk, Ghasemi found the binding ability of casein was increased when pH was increased to 8.0. The authors pointed that with the pH increase, more negative charges were gained on casein to create stronger electrostatic repulsions. As a result, a larger and looser structure was formed.
More interior hydrophobic areas were exposed to interact with oil, then the pH was decreased back to 6.7 and the incorporated oil was entrapped in the casein micelles. Consequently, casein binding efficiency was enhanced by pH.

1.3.3.6. Effect of ultrasound

The effect of ultrasound processing on casein micelles binding was also investigated (Ghasemi and Abbasi 2013). A combination of ultrasound and pH was conducted to enhance the interaction between casein micelles and fish or vegetable oils. The result suggested that once the casein structure became loose (due to the increased pH), ultrasound was able to break non-peptide bonds to expose more hydrophobic sites in the casein micelles. Since the oil particles were reduced by sonication (easier to interact with casein micelles), higher binding efficiency between oil and casein micelles was observed.

1.3.3.7. Effect of organic solvents

Many studies used ethanol to disperse more hydrophobic compounds in the solvent phase to interact with casein micelles. However, few studies considered the presence of ethanol could also enhance the hydrophobic binding ability of caseins.

In milk, apparent average hydrodynamic radius of casein micelles decreased from
93 nm (in the absence of ethanol) to 83 nm (in the presence of 20% ethanol) and then increased dramatically at higher ethanol levels (Hornes 1984). The hydrodynamic radius decrease was believed due to the collapse of exterior hairy layer formed by κ-casein. The hairy layer is for stabilizing the casein micelle structure in the dispersion, while the addition of ethanol changed the electrostatic repulsion between polyelectrolyte hairs and caused a disassociation or splitting of micelles (McSweeney and Fox 2013). As a result, the hydrodynamic radius of casein micelles was changed.

An increase of pH in a sodium caseinate dispersion was found with the addition of ethanol (O’ Kennedy 2001). As described in previous sections, even though is not capable of forming micelle structures, sodium caseinates are still composed of four type of caseins and form a strand-like aggregated complex. With the addition of ethanol, the dielectric constant of solvent was decreased, and pKa of the α-carboxylic acid groups in caseins was upward shifted. Consequently, the terminal carboxylic acid became less associated and pH was increased (Radford, Dickinson, and Golding 2004). In another study, Mezdour measured the particle size distribution of sodium caseinate in ethanol and water solutions with a helium-neon laser. The modal volume particle size of sodium caseinate in water was about 200 nm and decreased to 64 nm in the presence of 60% ethanol (Mezdour and Korolczuk 2010). The particle size reduction was believed due to the disassociation of caseinate aggregates. Since the dielectric constant and the pH of solvent were changed in the presence of ethanol, the
electrostatic repulsion within casein fractions was increased and sodium caseinates particles disassociated into sub-particles. As a result, the particle size decreased.

The disassociation of caseins particles could result in an exposure of hidden hydrophobic sites in caseins. Consequently, a higher amount of lipophilic compounds could be hydrophobically bound with caseins and became solubilized. A study combined high ethanol content (40%) and high temperature (60°C) to enhance curcumin’s dispersibility in water (Pan and Zhong 2015) and another study combined high content of ethanol and HPH (Roach, Dunlap, and Harte 2009) to enhance the triclosan solubility.

However, there are no studies to explicitly studied the effect of ethanol on interaction between hydrophobic compounds with sodium caseinates.

1.4. References


Dziuba, Jerzy, Andrzej Babuchowski, Michal Smoczynski, and Zbigniew Smietana.


Hernández, Héctor (European Commission), Alexander (European Commission) Tübke, Fernando (European Commission) Hervás, Antonio (European Commission) Vezzani, Mafini (European Commission) Dosso, Sara (European Commission) Amoroso, and Nicola (European Commission) Grassano. 2014.


Chapter 2. Statement of Problems and Overview of Goals

2.1. Problems and Objectives

About 40% of pharmaceuticals in drug preparations and nutrients in supplements are hydrophobic (Savjani, Gajjar, and Savjani 2012). Bioactive compounds including vitamins (e.g., Vitamin A, Vitamin D, Vitamin E), essential oils (e.g., ω-3 fatty acids), pharmaceuticals (e.g., ritonavir, triclosan, ibuprofen) polyphenols, and alkaloids (e.g., piperine) exhibit very low solubility in aqueous systems, and some of them showed low bioavailability. Hydrophobic compounds are hard to deliver in liquid aqueous foods or pharmaceutical preparations and must often be solubilized using a co-solvent (e.g., ethanol) or dispersed using surfactants. Especially for infant and children oral drugs, ethanol and glycerol are two commonly used in liquid preparations to enhance the solubility of active ingredients, which are both reported to be toxic at large doses (Pawar and Kumar 2002). Casein is an inexpensive, readily available, natural and GRAS milk-derived protein. In many studies, it has been reported to be able to solubilize and deliver hydrophobic compounds (Livney 2010a). Therefore, identifying a milk-derived protein based delivery system could meet the demand for health-beneficial ingredients.
A casein-based system could be an acceptable substitution to organic solvent or surfactant-based pharmaceutical delivery systems. However, many factors that may influence the binding efficiency, such as pH, temperature, pressure and presence of organic solvents, have not yet been adequately studied. Previous studies showed the addition of ethanol to milk could cause a conformation transition of casein particles in which hydrophobic sites might be exposed to absorb more bioactive compounds.

The ultimate aim of this work is to produce a caseinate powder which can be readily rehydrated in water to produce a suspension with a total concentration of hydrophobic compounds in considerable excess to their aqueous solubility. The objectives of this research were, (i) to investigate the mechanism of caseinate binding of hydrophobic compounds (i.e., pyrene and piperine), (ii) to monitor the release of pyrene during the in vitro acid and proteolysis digestibility of the casein-pyrene complexes, (iii) to test the effect of ethanol on the piperine solubilization by caseinate and to monitor the interaction between sodium caseinate and piperine as a function of time, (iv) to investigate the mechanisms of the effect of ethanol, (v) to compare the solubilization enhancing performance of sodium caseinate with other milk proteins. I believed that both pyrene and piperine are able to hydrophobically interact with caseinate and there are two mechanisms for the ethanol to enhance the interaction between caseinate and piperine, (1) ethanol facilitates mass transport to the protein from the insoluble powder by increasing the solvent solubility of pyrene/piperine, and
(2) ethanol changes the conformation of the protein to increase the interaction. I hypothesized that sodium caseinate can solubilize more piperine/pyrene in the aqueous system and ethanol can facilitate the solubilization through both mechanisms.

Chapter 3 is the bulk of the experimental works and it is formatted as a research paper, thus there is some repetition in introduction and methods with the literature review chapter.

2.2. References


Chapter 3. Effect of Ethanol on the Solubilization on Hydrophobic Molecules by Sodium Caseinate

3.1. Introduction

Casein is an inexpensive, readily available, non-toxic, safe and natural bovine milk protein (Saiz-Abajo et al. 2013). It is a biodegradable and GRAS ingredient (Semo et al. 2007). Casein consists four principal proteins: α1, α2, β and κ casein. The former three proteins (i.e., α1, α2, β) are relatively hydrophobic whereas the latter (i.e., κ-casein) is a glycoprotein with a hydrophilic oligosaccharide. About 95% of casein in milk is present as colloidal particles known as casein micelles (Saiz-Abajo et al. 2013), with an average radius of 150 nm and a molecular mass in $10^6$ to $3*10^9$ Da (Livney 2010a). Some authors argue the casein micelle is itself assembled from sub-micelles with interior water channels. In this model, calcium phosphate was proposed to be the linkage to connect the submicelles (Douglas G Dalgleish and Corredig 2012). However, the precise structural nature of casein is still the subject of debate.

There are two types of commercially available casein-based ingredients: micellar casein and caseinate salts (e.g., sodium caseinate). Micellar caseins are intact casein...
micelles that are extracted from milk, often by dialysis. Caseinate, on the other hand, is a chemical preparation obtained through acidification and subsequent alkalization of native casein. As such, caseinate salts are not able to form micelles due to the absence of adequate calcium (Phoebe X Qi 2007), which is essential for native casein’s micellar structure. Casein ingredients are used in foods to deliver certain functional properties such as stabilizing emulsions, forming gels and interacting with other biopolymers (Livney 2010a).

Many bioactive compounds have high hydrophobicity and low water solubility, which results in low dissolution rates in gastrointestinal fluid and, thus, low bioavailability. A recent study demonstrated the low bioavailability of ritonavir, a hydrophobic antiretroviral drug (Corzo-Martinez et al. 2015). These water insoluble compounds are generally speaking challenging to deliver in liquid foods or pharmaceutical preparations, and must often be solubilized by ethanol or with the use of surfactants (Strickley 2004). Ethanolic and surfactants preparations are not desirable in the case of pharmaceutical delivery systems for infants; therefore, alternative strategies for solubilizing hydrophobic bioactive compounds are greatly needed in pediatric pharmacies.

Recently, casein has been shown to enhance the aqueous solubility of bioactive hydrophobic compounds, including docosahexaenoic acid (Zimet, Rosenberg, and
Livney 2011), curcumin (Esmaili, Ghaffari, Moosavi-Movahedi, Atri, Sharifizadeh, Farhadi, Yousefi, Chobert, Haertlé, et al. 2011), vitamin A (Mohan, Jurat-Fuentes, and Harte 2013), vitamin D (Semo et al. 2007), and ritonavir (Corzo-Martinez, Mohan, Dunlap, & Harte, 2015). In some studies, caseins are assembled as micelles by adding calcium and dipotassium phosphate (Saiz-Abajo et al. 2013; Semo et al. 2007), while in other work (Zimet, Rosenberg, and Livney 2011), non-micellar caseinate has been used to solubilize ω-3 fatty acids in aqueous system and/or to protect chemically labile molecules (e.g., docosahexaenoic acid) from oxidation reactions. There have been many studies investigating the effect of extrinsic factors such as pH (Ghasemi and Abbasi 2013) and temperature (Esmaili, Ghaffari, Moosavi-Movahedi, Atri, Sharifizadeh, Farhadi, Yousefi, Chobert, Haertlé, et al. 2011; Pan and Zhong 2015), or processing methods such as high pressure homogenization (Roach, Dunlap, and Harte 2009), high hydrostatic pressure (Rahimi Yazdi et al. 2013), ultrasound (Ghasemi and Abbasi 2013) on casein’s ability to solubilize hydrophobic molecules. Some researchers have considered the role of ethanol in enhancing casein’s solubilization capacity; for example, a combination of 50% ethanol and high-pressure homogenization enhanced the solubility of triclosan in skim milk up to 108% (Roach, Dunlap, and Harte 2009) and high temperature combined with 40% ethanol increased the amount of solubilized curcumin to 137 µg/mL in casein solution, which is 40 times greater than the aqueous solubility of curcumin (Pan and Zhong 2015), yet the underlying mechanism for the change in solubilization is not well understood.
There are two possible mechanisms of the addition of organic solvents to increase the solubility of organic compounds by caseinate have been proposed. According to the first proposed mechanism, water miscible organic solvents (e.g., ethanol, acetone) can increase the solvent solubility of hydrophobic compounds to facilitate their hydrophobic interaction with casein in the system (Mohan, Jurat-Fuentes, and Harte 2013; Shapira, Assaraf, and Livney 2010; Zimet, Rosenberg, and Livney 2011). According to the second mechanism, organic solvents may actually change the conformation of proteins (Coupland 2014) to increase solubilization (e.g., allowing the protein’s hydrophobic domains to interact with the small hydrophobic molecule).

A previous study reported that a wide ranges of particles size of the casein micelles were obtained after the disassociation by ethanol (Ye and Harte 2013). Other studies showed that lower levels (16-20%) and higher levels (20-40%) of ethanol in milk decreased and increased the hydrodynamic radius of casein, respectively, by collapsing the hair layers of the casein micelles (Horne and Davidson 1986; Horne 1984), the presence of ethanol was able to split sodium caseinate to smaller sub-particles by changing the electrostatic repulsion between casein fractions (Mezdour and Korolczuk 2010), resulting in a conformation change of the casein and exposure of hydrophobic sites. It was argued that this disassociation might be able to enhance the interaction between hydrophobic compounds and the caseinate in order to facilitate their solubilization within the aqueous phase.
Two hydrophobic probes are used in my study, pyrene is an extremely hydrophobic compound (aqueous solubility, 0.7 µM, 0.07 mg/L, (Liu and Guo 2008)) with strong fluorescing ability if it is dispersing in the system. Pyrene seems suitable for the investigation of caseinate solubilization mechanism; and piperine is a hydrophobic alkaloid with some bioactivity that is extracted from black peppercorns (Piper nigrum) (Bhardwaj 2002). Piperine has low aqueous solubility (40 mg/L, (Vasavirama and Upender 2014)) and maximum UV absorption at 335 nm. Piperine represents a food grade hydrophobic compound that is potentially suitable for solubilizing by caseinate. The purpose of the present study was to demonstrate solubilization of two model lipophilic molecules (piperine and pyrene) using sodium caseinate in order to overcome their low aqueous solubility. Furthermore, I demonstrate that the presence of ethanol increases the loading capacity of sodium caseinate with piperine. When ethanol and water are removed, the resultant caseinate and piperine/pyrene powder can be readily rehydrated to produce an aqueous suspension with a total piperine/pyrene concentration in considerable excess to its aqueous solubility.
3.2. Material and Methods

3.2.1. Materials

Sodium caseinate (casein sodium salt from bovine milk, >98.0%), trypsin (from porcine pancreas) and piperine (>97%) were purchased from Sigma-Aldrich (St. Louis, MO). Imidazole, sodium phosphate dibasic anhydrous and sodium phosphate monobasic monohydrate were purchased from Fisher Scientific (Waltham, MA). Pyrene was kindly donated by Prof. Harry Allcock and Dr. Zhongxing Li (Department of Chemistry, The Pennsylvania State University) and was purchased from Sigma-Aldrich (St. Louis, MO). Ethyl alcohol (200 proof) was purchased from Pharmco Products Inc. (Brookfield, CT). BiPRO whey protein isolate and bovine β-lactoglobulin were purchased from Davisco (Le Sueur, MN). Native micellar casein was kindly prepared by Ms. Manpreet Cheema (Department of Food Science, The Pennsylvania State University) according to previously reported methods (Cheema et al. 2015).

3.2.2. Methods

3.2.2.1. Protein solutions preparation

Proteins (i.e., sodium caseinate, β-lactoglobulin, whey protein isolates, native casein powder) were prepared by dissolution in imidazole buffer (pH 6.8, 20 mM)/
phosphate buffer (pH 6.8, 50 mM) and then mixed with varying concentrations of ethanol for 24 hours at room temperature (20°C) to achieve a final concentration of 2% w/v protein and 0-40% v/v ethanol.

Protein content was determined by the Bradford and Dumas methods. Bradford reagent was prepared by dissolving Coomassie Brilliant Blue G-250 (100 mg) in 95% ethanol (50 ml), followed by the addition of 85% phosphoric acid (100 mL). The reagent solution was then brought to volume (1 L) with water. When dissolved, the reagent was filtered and stored in the dark at -4 °C. Protein samples (100 µl) were mixed with 5 mL reagent and incubated for 5 min. The absorbance was measured at 595 nm and the protein concentration was calculated based on an external standard curve prepared from sodium caseinate. For protein analysis by the Dumas method, nitrogen content was measured by combustion of the protein powder (20 mg) in a LECO CHNS-932 Analyzer (Saint Joseph, MI).

The turbidity of sodium caseinate solutions were measured at 287 nm (Genesys 10S UV-Vis, Daly City, CA).

3.2.2.2. Pyrene caseinate solution preparation and characterization

Pyrene powder was dissolved in ethanol and then mixed with sodium caseinate solution for 24 hours to achieve a final concentration of 2 µM (0.405 mg/L) pyrene
3.2.3. Piperine caseinate solution preparation and characterization

Sodium caseinate powder, a large excess of piperine powder (0.1 g in 5 mL, i.e., 444 times higher than the piperine solubility in water) and ethanol were mixed for 0-24 hours at room temperature to achieve a final concentration of 2%, w/v protein and 0-40% ethanol. The sample was then freeze dried for 72 hours, then rehydrated with imidazole buffer (pH 6.8, 20 mM) for 24 hours. Excess, insoluble piperine was then separated by gentle centrifugation (20 min, 2000 rpm, 20°C).

Piperine from the supernatant was extracted by according to previously described methodology (Jedziniak, Szprengier-Juszkiewcz, and Olejnik 2009). In brief, 5 mL sample, 5 mL acetonitrile, and 1 g sodium chloride were vortexed for 30 seconds and then centrifuged (4000 rpm, 20°C, 10 min). Piperine was extracted in acetonitrile and

and 2%, w/v protein. The mixture was freeze dried (FreeZone 2.5, Lachconco, Kansas City, MO) for 72 hours and subsequently rehydrated with phosphate buffer (pH 6.8, 50 mM) for 24 hours. Insoluble pyrene was removed by gentle centrifugation (20 min, 2000 rpm, 20 °C). The solubilized pyrene present in the supernatant was characterized through fluorescence spectroscopy (Fluorolog-3, Horiba Scientific, Edison, NJ). During the measurement, the excitation and emission pass filters were fixed at 0.5 and 0.5 nm, respectively, the excitation wavelength was set at 338 nm, and the emission spectra were collected from 350 nm to 550 nm.
determined through UV-Vis spectroscopy (Genesys 10S UV-Vis, Daly City, CA). Absorbance spectra were collected from 200 nm to 600 nm, and 338 nm was selected as the maximum absorption. The concentration of piperine (mg/L) was then calculated through Beer’s Law equation and using the molar extinction coefficient of 25472 L•mole\(^{-1}\)•cm\(^{-1}\), determined via a calibration curve.

3.2.4. Caseinate in vitro digestion study

Pyrene caseinate solution (2 µM pyrene and 2%, w/v caseinate) was mixed with hydrochloric acid (12 M) to achieve a solution pH of 3.5 or trypsin powder to a final concentration of 5 mg/mL. Then the samples were incubated at room temperature for 0-48 hours without stirring. The absorbance of supernatant was measured after a gentle centrifugation (2000 rpm, 5 min, 20°C).

3.2.5. Statistical analysis

Analysis of variance for variables were done with three replicates using Minitab (State College, PA). Tukey’s t-test was used to determine significant difference between experimental means. Differences were considered significant when p<0.05.
3.3. Results and Discussion

3.3.1. Interaction between sodium caseinate and pyrene

Pyrene was initially studied to investigate the solubilization of hydrophobic compounds by sodium caseinate.

Ethanolic pyrene stock solution was added to an aqueous sodium caseinate solution to a final concentration of 2% v/v, ethanol, 2 μM pyrene and 0-4% protein. After equilibration, the samples were freeze dried to remove ethanol and water, and then were rehydrated using phosphate buffer. Similar concentrations of caseinate in buffer and pyrene stock solution in buffer were prepared as controls.

Because pyrene is effectively insoluble in buffer (aqueous solubility, 0.7 μM, 0.07 mg/L, (Liu and Guo 2008)), in the absence of caseinate, the fluorescence associated with this molecule might be below the instrumental detection limit and was hardly observed in Figure 3.1. The caseinate in buffer (without pyrene) gave diffuse and weak fluorescence, presumably due to the presence of aromatic amino acid residues. However, the pyrene plus caseinate treatment resulted in a strong fluorescence spectrum that is characteristic of pyrene in organic solution (Tian, Chen, and Allcock 2014). This indicates that the pyrene was able to interact with the sodium caseinate and, thus, was effectively solubilized. When ethanolic pyrene stock was
added to water, the ethanol was diluted and pyrene became insoluble (Vitale and Katz 2003). In the absence of caseinate, pyrene precipitated and no fluorescence was observed. However, in the presence of caseinate, the insolubilized pyrene was presumably absorbed by the hydrophobic sites in the sodium caseinate and remained solubilized (as evidenced by its fluorescence) in the aqueous system.
Figure 3.1. Fluorescence emission spectrum (excitation wavelength 338 nm) of pyrene in phosphate buffer with sodium caseinate (2%), pyrene in phosphate buffer, and sodium caseinate (2%) in phosphate buffer.
Similar experiments were conducted in which caseinate concentration was varied (Figure 3.2 (a)). In fluorescence studies, $I_1/I_3$ (as measured by calculating ratio of intensity at 373 nm and intensity at 393 nm) was calculated to represent the dispersibility of pyrene. In Figure 3.2 (b), it was observed that $I_1/I_3$ was proportional to caseinate concentration. This suggests that while the total amount of pyrene was constant, the amount of solubilized pyrene was dependent on protein concentration. The remainder of the added pyrene presumably precipitated without interacting with sodium caseinate and was not fluorescent. There were no obvious changes in the slope of fluorescence intensity ratio as a function of protein content, which suggests the binding properties of caseinate is not dependent on the protein concentration. Other authors have used a change in the slope to determine the critical micellar concentration (CMC) of polymer or surfactant systems (Liu and Guo 2009); however, the absence of a change in slope in the this experiment suggests that no micellization is occurring.

The present study shows that the caseinate is capable of solubilizing hydrophobic small molecules added as an ethanolic solution. Rather than simply precipitating when diluted in water, pyrene presumably binds to the hydrophobic domains in caseinate and remains solubilized in the aqueous system, even after the ethanol is removed.
Figure 3.2 (a). Fluorescence emission spectrum at an excitation wavelength of 338 nm of pyrene in phosphate buffer (20 mM, pH 6.8) in the presence of 0-4% sodium caseinate (sodium caseinate spectrum was subtracted as a blank).
Figure 3.2 (b). Plot of the ratio ($I_1/I_3$) of fluorescence intensity at peak 373 nm and fluorescence intensity at peak 393 nm against sodium caseinate concentrations, linear regression analysis was conducted, $R^2 = 0.913$. 

\[ \frac{I_1}{I_3} \]
3.3.2. *Digestion of pyrene-caseinate complexes*

Having established caseinate can be used to solubilize hydrophobic pyrene as a model for hydrophobic drugs/nutrients, the next step in a pharmaceutical/nutraceutical study would be to determine if the drug/nutrients remain bioavailable despite their association with the carrier (i.e., casein). A full bioavailability study is beyond the scope of this project, but instead I studied the release kinetics of pyrene during different forms of simulated *in vitro* digestion which included either acid catalyzed and enzymatic digestion.

In the absence of digestion, pyrene fluorescence intensity remained constant for the duration of the experiment (Figure 3.3). However, during digestion with hydrochloric acid, a dramatic decrease in pyrene fluorescence intensity was observed in the few seconds after the acidification (Figure 3.3). In the acidified environment (pH=3.5, i.e., < isoelectric point of caseinate, 4.6), caseinate precipitated due to the loss of net electrostatic charge. After centrifugation, pyrene either remained hydrophobically bound to the aggregated caseinate or was released from protein but precipitated, thus, there was no fluorescence measured in the supernatant phase (Figure 3.4). Since the protein structure was not destroyed during the aggregation, and because there is no charge on pyrene, we prefer the first explanation – the pyrene remains bound to the precipitated protein.
In contrast, during enzymatic digestion, a rather gradual decrease of pyrene fluorescence intensity was observed over 36 hours, and there was no obvious precipitation of the protein. Presumably as the caseinate was digested, the pyrene was released, precipitated and therefore no longer fluoresced.

This study suggests that caseinate solubilized hydrophobic compounds are released through processes related to in vivo digestion (i.e. enzymatic hydrolysis). However, following acidic digestion, compounds remain insoluble in the precipitated caseinate. Further bioavailability studies are still needed, however, I expect that hydrophobic compounds will remain comparably bioavailable as directly added undissolved hydrophobic compounds after their release in the small intestine.
**Figure 3.3.** Fluorescence intensity of caseinate-pyrene complexes after acidification (blue), proteolysis (red) and control (green) as a function of time.
3.3.3. Interaction between sodium caseinate and piperine

In the second study, I considered the effect of ethanol on solubilization of the hydrophobic molecule piperine. An excess of piperine powder was added to an aqueous/ethanolic sodium caseinate solution to achieve a final concentration of 0-40 vol%, ethanol and 0-4 wt% protein. After equilibration and centrifugation (2000 rpm, 20 min), the supernatant saturated with piperine was freeze dried and then rehydrated with imidazole buffer. The solubilized piperine was then determined by UV spectroscopy.

In the first study, the solubilizing capacity of piperine by caseinate was studied without using ethanol in the preparation stage. The absorbance spectra of acetonitrile extracts of the supernatants in an aqueous solubility assay are shown in Figure 3.4. In the absence of caseinate, there was a weak absorbance peak at 335 nm corresponding to the low aqueous solubility of piperine. However, in the presence of caseinate, much higher absorbance was observed, which indicates that piperine was also able to interact with the caseinate and solubilized in the aqueous system in a similar manner to pyrene in the previous experiment. The difference between the two curves corresponds to the piperine interacting with the protein.
Figure 3.4. UV spectrum of saturated piperine in 20 mM imidazole buffer, pH 7 with 2% casein (red line) and without caseinate (blue line).
Having established that caseinate can solubilize piperine, further experiments were conducted to investigate the effect of ethanol on the process. In the absence of caseinate, the solubility of piperine was low (ca. 49 mg/L) and was independent of the ethanol present in the preparation step. This is unsurprising as the ethanol was removed prior to solubilization and the solubility measured is close to reported literature values in water. In the presence of ethanol, the amount of solubilized piperine was higher than that in the absence of ethanol, and increased approximately linearly as a function of ethanol content. The amount of piperine associated with the caseinate (i.e., the difference between the two curves in Figure 3.5 (a)) increased with ethanol content. Expressing this difference in terms of moles of piperine per mole of casein (Figure 3.5 (b)), the loading capacity of caseinate was calculated by dividing the amount of solubilized piperine by the sodium caseinate molar concentration (MW of sodium caseinate was taken as 20 kDa (Dewan and Bloomfield 1973)). The result reveals that one molecule of caseinate binds 50 molecules of piperine in the absence of ethanol, and increases to 175 molecules of piperine when 40% ethanol was used during the sample preparation. This experiment suggests that the presence of ethanol is able to enhance the binding between piperine and caseinate and result in an increase of piperine solubilization in the aqueous system.

Similar experiments were conducted to measure the amount of solubilized piperine as a function of caseinate concentration in the presence of varied amount of
ethanol (Figure 3.6 (a)). While the total amount of piperine is constant; the amount of solubilized piperine increased with both caseinate and ethanol concentration. The result is consistent with our observations from the previously described pyrene study (Figure 3.2 (b)). In the absence of ethanol and protein, piperine solubility remained as low as its aqueous solubility shown in Figure 3.5 (a). While the ethanol concentration is constant, in the presence of caseinate, the amount of hydrophobic domains increased, hence more piperine was solubilized. Alternatively, while the caseinate concentration is constant, the presence of ethanol enhanced the solubilization of piperine. To investigate the effect of both factors, the loading capacity (i.e., piperine bound per unit mass of caseinate) was calculated as a function of ethanol concentration (Figure 3.6 (b)). The loading capacity of casienate in the absence of ethanol was shown as the intercepts, then it increased linearly with the ethanol concentration. While at the same ethanol content, the different caseinate concentrations held the similar amount of piperine per mole of caseinate, which suggests that the loading capacity of caseinate is not effectively dependent on the protein concentration but the ethanol concentration. Nevertheless, even though the binding property of caseinate is not changed, higher solubilization of piperine (i.e., more binding) could still be achieved by increasing the protein concentration in the solvent phase.

As noted in the introduction, ethanol may (i) change the conformation of
caseinate to increase the hydrophobic binding with piperine, or (ii) facilitate the mass transport of insolubilized piperine to caseinate by increasing the solvent solubility of piperine or (iii) both.

To distinguish these three hypotheses, similar experiments were conducted in the absence of piperine (i.e., hydration of caseinate in ethanolic buffer, freeze drying, then rehydration in buffer). In addition, the solubility of piperine in ethanolic buffer (i.e., in the absence of caseinate) was measured. The protein content of the caseinate solutions was the same before and after freeze drying (Figure 3.7), which shows that no protein was lost or rendered insoluble during the process.

The optical properties of the caseinate were measured before and after freeze-drying and rehydration (Figure 3.8). In the presence of ethanol, the turbidity of the caseinate solutions was proportional to the ethanol concentration, which suggests that the caseinate conformation is changed during the addition of ethanol (Figure 3.8). Similar concentrations of ethanol have been shown to disassociate casein micelles into different levels (Hornes 1984; Mezdour and Korolczuk 2010; Ye and Harte 2013) and a similar change in caseinate conformation could have led to the change in turbidity here. If the caseinate has a different conformation in the presence of ethanol it may be easier to load with piperine (i.e., supporting the first hypothesis). However, after freeze drying and rehydration, the absorbance of the caseinate samples is the
same as that of the caseinate originally dispersed in buffer and independent of ethanol concentration. This suggests that the changed caseinate molecules shrink back to their previous conformation on rehydration and are not permanently altered. Therefore, this demonstrates the first of the hypotheses that the conformation of caseinate is changed due to the addition of ethanol in preparation stage. Secondly, the solubility of piperine in buffer (i.e., in the absence of caseinate) was shown to increase with the addition of ethanol (Figure 3.9). Presumably during the loading stage of the process, the piperine crystals must dissolve in the solvent phase then diffuse to the caseinate. If the solubility of the piperine in the solvent phase is greater at higher ethanol concentrations, then the rate of loading of the caseinate is greater and the amount bound is higher (Figure 3.5 (b)). This is consistent with the second of the hypotheses described above.

Together, these results are consistent with both the hypothesized mechanisms being true. In the ethanolic environment, more hydrophobic compounds are soluble the solvent phase. At the same time, caseinate conformation is expanded, thus presumably more hydrophobic domains are exposed and able to absorb more hydrophobic compounds. Once ethanol is removed, hydrophobic compounds presumably remain the bindings with the dispersed caseinate in the solvent phase. Consequently, aqueous solubility of hydrophobic compounds is enhanced by caseinate under the influence of ethanol.
Figure 3.5 (a). Aqueous concentration of piperine in the presence of 0% (square) or 2% (circle) sodium caseinate as a function of ethanol concentration in the preparation step. All ethanol was removed prior to solubility measurement.
Figure 3.5 (b). Loading capacity of 2% sodium caseinate solution as a function of ethanol concentration in the preparation step. All ethanol was removed prior to the measurement.
Figure 3.6 (a). Aqueous concentration of piperine as a function of caseinate concentration in the presence of 0% (red), 10% (yellow), 20% (blue), and 40% (green) ethanol content in the preparation step.
Figure 3.6 (b). Loading capacity of 2% (red), 3% (green) and 4% (blue) sodium caseinate as a function of ethanol content in preparation step.
Figure 3.7. The protein content of 2% w/v sodium caseinate solution with 40% v/v ethanol before freeze drying (with ethanol) and after freeze drying and rehydrated (without ethanol).
**Figure 3.8.** Turbidity of 2% w/v sodium caseinate solution as a function of ethanol concentration in the preparation step before (circle) and after (square) freeze drying.

The appearance of sodium caseinate were shown above each ethanol concentration.

All samples are diluted ten-fold with imidazole buffer prior to turbidity measurements.
Figure 3.9. Solubility of piperine in imidazole buffer as a function of ethanol concentration.
To further investigate the process of caseinate loading with piperine, a 24-hour kinetic study was conducted in the absence and presence of ethanol (Figure 3.10). The two systems reached equilibrium in ca. 3 hours at significantly different loading capacities. However, the system with ethanol has a steeper slope than the system in the absence of ethanol, which suggests that that the ethanol present increases the piperine loading rate of caseinate. The fact that the loading rate is faster in the presence of ethanol is suggestive of faster mass transport of piperine through the solvent phase during the preparation. On the other hand, two systems reached different equilibriums is because the caseinate conformation is changed by ethanol and the piperine loading is enhanced. Again, both possible mechanisms for the role of ethanol seem to be important.
Figure 3.10. Plot of the loading capacity of 2% sodium caseinate solution in the presence of 20% ethanol (square) and in the absence of ethanol (circle) as a function of time.
3.3.4. *Role of ethanol in the piperine solubilization by other proteins*

Having investigated the effect of ethanol on piperine solubilization by caseinate, similar experiments were conducted to test whether ethanol affected other proteins (i.e., whey protein isolate, β lactoglobulin, and the native casein micelle extracts) in a similar manner. In the present experiment, 2% wt of each protein was used despite the fact that they had different nitrogen contents (acquired from the Dumas method) (Figure 3.12). Thus, in Figure 3.11, protein loading capacity was normalized and calculated based on nitrogen content instead of nominal protein concentration. As was the case with sodium caseinate, the binding capacity of other proteins were seen to be dependent on ethanol concentration. Nevertheless, compared to other proteins, sodium caseinate showed the best performance on the piperine solubilization enhancement.

This experiment suggests that ethanol is able to facilitate piperine solubilization in a variety of proteins, and that this effect is not unique to sodium caseinate.
Figure 3.11. Nitrogen content in 2% sodium caseinate, whey protein isolates, β-lactoglobulin, and native casein micelle extracts solutions.
Figure 3.12. The piperine loading capacity of nitrogen as a function of the ethanol concentration in the presence of 2% sodium caseinate (red), whey protein isolates (green), β-lactoglobulin (blue), and native casein micelle extracts (yellow).
3.4. Conclusions

Sodium caseinate is able to solubilize lipophilic small molecules (e.g., piperine, pyrene) in the aqueous phase, and this is presumably due to hydrophobic interaction. After freeze drying and rehydration, a colloidal sodium caseinate solution with a piperine concentration six times higher than its aqueous solubility was prepared, I demonstrated that the solubilization of these small hydrophobic molecules by caseinate can be increased by increasing ethanol concentration. This is because ethanol can either facilitate the mass transport of piperine to solvent phase and/or change the conformation of sodium caseinate in a way that results in an increase in its loading capacity. The digestibility study suggested that the caseinate bound hydrophobic compounds were able to be released through the digestion (i.e. enzymatic hydrolysis) and I expected the hydrophobic compounds presumably remain bioavailable, however a full set of bioavailability study is still needed. In the comparison study, sodium caseinate showed the best performance of solubilizing hydrophobic compounds compared to other milk proteins.

The sodium caseinate system described here represents a simple approach to increasing hydrophobic compounds dissolution in gastrointestinal fluid while possibly retaining the bioavailability of lipophilic drugs in the small intestine; however, the question of bioavailability is beyond the scope of the present study and should be
explored further. In addition, the system described here can be adapted to produce a range of customer-friendly (e.g., alcohol free) formulations appropriate to disperse insoluble bioactive ingredients and pharmaceutical compounds in a liquid based food system.

3.5. References


Coupland, John N. 2014. “An Introduction to the Physical Chemistry of Food.”


Roach, A., J. Dunlap, and F. Harte. 2009. “Association of Triclosan to Casein Proteins...
through Solvent-Mediated High-Pressure Homogenization.” *Journal of Food Science* 74(2).


Chapter 4. Conclusions

The overall goal of this work was to produce a caseinate based powder which can be readily rehydrated to an aqueous suspension with a piperine concentration in considerable excess to its water solubility limit. At the end of the experiments, I demonstrated that caseinate based system is able to enhance the solubility of hydrophobic compounds (i.e. pyrene and pyrene) in water based food. Sodium caseinate improved the solubilization of piperine to 135 mg/L in buffer (50 mole piperine per mole caseinate) which is approximate 3 times to piperine aqueous solubility.

In the in vitro digestibility study, after the acidification, sodium caseinate approached the isoelectric points, which resulted in an aggregation of both casein and bound compounds. After the proteolysis, the bound compounds are able to be released into the gastrointestinal fluid and presumably remain bioavailable. A full set of bioavailability study is needed in the future to investigate the fate of bound compounds during the digestion and the efficiency of expression bioactive functions.

In addition, the presence of the ethanol in the preparation stage can enhance the piperine hydrophobically binding with caseinate through two mechanisms, (1) facilitating the mass transport of piperine to the caseinate in the aqueous phase and (2)
changing the caseinate conformation to preferably interact with more piperine.

Consequently, after the freeze drying, ethanol was removed from the system, the piperine concentration in the final colloidal caseinate dispersion reached the maximum of 337 mg/L (598% enhancement to piperine aqueous solubility) when 40% ethanol was added in the preparation stage.

The sodium caseinate system described here represents a simple approach to increasing hydrophobic compounds dissolution in gastrointestinal fluid while possibly retaining the bioavailability of lipophilic drugs after digestion; however, the question of bioavailability is beyond the scope of the present study and should be explored further. In addition, the work described here can be adapted to produce a wide range of customer-friendly (e.g., alcohol free) formulations without any off-flavor from surfactants and be appropriate to disperse any insoluble bioactive ingredients and pharmaceutical compounds in a water-based food system.