**Formation of Thymol Nanoemulsions with Combinations of Casein Hydrolysates and
Sucrose Stearate**

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Abstract

Casein hydrolysates (CHs) have the improved emulsifying activity than caseins, and the emulsion stability can be principally improved by co-adsorbing sucrose stearate (SS). The objective of this work was to fabricate thymol nanoemulsions using the combination of CH and SS. CH produced by 10 min pancreatic hydrolysis was used at 2% and mixed with SS to prepare emulsions by shear homogenization and heating at 90 °C for 20 min. Addition of SS from 0 to 1.0% increased the thymol content in nanoemulsions ($d_{3,2} \approx 50$ nm) from 1 to 3%. Excess SS however increased the emulsion turbidity and instability due to its poor solubility at ambient conditions. Heating improved the solubility of SS, strengthened its hydrophobic interactions with CH, and thus greatly improved the emulsion storage stability and the stability at pH 3.0 and 5.0. Therefore, the combination of CH and SS provides a novel approach to prepare nanoemulsions.

Keywords: casein hydrolysates, sucrose stearate, nanoemulsions, thymol, stability

1. Introduction

Oil-in-water emulsions are frequently studied to deliver lipophilic bioactive components such as beta-carotene (Qian, Decker, Xiao, & McClements, 2012), curcumin (Pan, Zhong, & Baek, 2013), and essential oils (Chen & Zhong, 2015; Luo, Zhang, et al., 2014) in aqueous products. Nanoemulsions have advantages when compared to conventional emulsions. Droplets in nanoemulsions are relatively small (< 200 nm in diameter) and are not as effective in scattering visible light, which provide the unique opportunity of nanoemulsions for use in transparent products (McClements, 2011). The small dimension of droplets also improves the stability against droplet aggregation and gravitational separation (McClements, 2011). Microemulsions are another group of oil-water-surfactant mixtures with structures smaller 100 nm (McClements, 2011). Different from nanoemulsions, microemulsions are thermodynamically stable and isotropic, and the structures of the dispersed phase are not always spherical (McClements, 2011). The ultra-low interfacial tension in microemulsions can enable their formation spontaneously (McClements, 2011). However, the surfactant: oil ratio in microemulsions is typically higher than in nanoemulsions, and the type of surfactants forming food grade microemulsions is limited.

Nanoemulsions have been studied extensively using synthetic surfactants and surface-active biopolymers (Jean-Lue Courthaudon, Dickinson, Matsumura, & Clark, 1991). For food applications, generally-recognized-as-safe (GRAS) emulsifiers such as polysaccharides, proteins, and certain sugar-fatty acid esters are preferred to meet regulatory requirements (McClements, 2011). Whey protein isolate (Chanamai & McClements, 2002), soy protein isolate (Sofos & Allen, 1977), and sodium caseinate (NaCas) (Hu, McClements, & Decker, 2003) are commonly studied as emulsifiers (McClements, 2011). NaCas has excellent emulsification properties due to

its copolymer-like flexible structure (Robson & Dalgleish, 1987). However, with the isoelectric point (pI) being pH 4.6, NaCas has few net charges around pH 5.0 and precipitates due to the weakened electrostatic and steric repulsions, and thus the prepared emulsions can be destabilized (Tuinier, Rolin, & De Kruif, 2002). Additionally, biopolymeric surfactants are not as effective as small molecular surfactants in adsorbing onto new interfaces and reducing interfacial tension during emulsification, and therefore are not as effective in reducing droplet dimension to form nanoemulsions (Jafari, He, & Bhandari, 1997).

To improve functionalities of proteins, hydrolysis of proteins to smaller molecules is a common approach (Mahmoud, Malone, & Cordle, 1992). The reduction of molecular weight improves the flexibility and solubility of proteins (Luo, Pan, & Zhong, 2014; O'Regan & Mulvihill, 2010), as well as antioxidant properties (Taghvaei et al., 2014). Hydrolysis of caseins using proteases is the most commonly studied method to improve functional properties such as inhibition of angiotensin I-converting enzyme and antihypertensive properties (Miguel, Contreras, Recio, & Aleixandre, 2009), hypersensitiveness (Sampson, Bernhisel-Broadbent, Yang, & Scanlon, 1991), antioxidant activity (Gómez-Ruiz, López-Expósito, Pihlanto, Ramos, & Recio, 2008), and antimicrobial activity (Hayes, Ross, Fitzgerald, Hill, & Stanton, 2006). Therefore, it is of great interest to prepare nanoemulsions using casein hydrolysates (CHs) as not only an emulsifier but also a functional ingredient.

However, there are a few limitations of using CHs to prepare nanoemulsions. The stabilization of emulsions via the steric repulsion mechanism of NaCas is weakened, and the stability of the prepared emulsions around pH 4.6 can still be poor (Luo, Pan, & Zhong, 2014). The elasticity of interfacial films and therefore the stability of emulsions can also be reduced when the molar mass of proteins/peptides decreases (Dwyer, He, James, Nelson, & Middelberg,

2013). To overcome these limitations, nonionic emulsifiers may be used to co-emulsify oils with CHs, because the nonionic head groups provide steric hindrance to stabilize emulsions and are insensitive to pH and ionic strength (Anarjan & Tan, 2013b).

The objective of the present work was to fabricate thymol nanoemulsions using CHs and sucrose stearate (SS). SS is produced by esterification of sucrose with edible stearic acid (Fanun, 2008) and is GRAS (Hasenhuettl, 2008). Because there are eight hydroxyl groups available on one sucrose molecule, different amounts of stearate molecules can be grafted on sucrose to form mono-, di-, tri-, or polyesters to obtain a wide range of hydrophile-lipophile-balance (HLB) values (Glatter et al., 2001). Furthermore, because the conformation of stearate is a function of temperature (Fanun, 2008), temperature can be used as an additional factor to fabricate emulsions. Thymol, the major component in the essential oil extracted from *Carum copticum* or *Thymus vulgaris* (Deb, Parimala, Saravana Devi, & Chakraborty, 2011), was chosen as a model lipophilic compound because it is classified as GRAS by the U.S. Food and Drug Administration (Weiss, Gaysinsky, Davidson, & McClements, 2009). The excellent antioxidation (Aeschbach et al., 1994) and antimicrobial activities (Guarda, Rubilar, Miltz, & Galotto, 2011) of thymol have been widely reported. The solubility of thymol in water is 0.75 mg/mL at 21 °C, and nanoemulsification of thymol by NaCas can improve its antimicrobial activity in complex food matrices such as milk (Pan et al., 2014).

2. Materials and methods

2.1. Materials

NaCas, thymol (>99% purity), and pancreatin (catalogue number P1750) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Propylene glycol (PG) was purchased from

Fisher Scientific (Pittsburgh, PA, USA). The commercial SS product (S1170) donated by Mitsubishi-Kagaku Foods Corporation. (Tokyo, Japan) contained 55% monoesters and 45% di-/tri-/poly-esters, according to the manufacturer.

2.2. Preparation of casein hydrolysates

NaCas was dissolved at 5% w/v in 200.000 mL of 0.010 M sodium phosphate-buffered saline (PBS), and pancreatin was dissolved at an enzyme: substrate mass ratio of 1:1000 (Mahmoud et al., 1992). The mixture pH was adjusted to 8.0 before incubation in a shaking water bath (C 76 classic series, New Brunswick Scientific Co., Inc., Edison, NJ, USA) operating at 120 rpm and 37 °C. Fifty milliliters of samples were withdrawn after hydrolysis for 10 min, 1 h, 4 h, and 20 h without pH adjustment. The withdrawn samples were heated at 95 °C for 10 min to inactivate the enzyme. After cooling in an ice/water bath, the samples were centrifuged (SORVALL RC5B Plus centrifuge, DuPont, Wilmington, DE, USA) at 5000 g for 20 min to remove insoluble contents. The supernatants were freeze-dried (VirTis AdVantage Plus EL-85 benchtop freeze dryer, SP Scientific Inc., Gardiner, NY, USA), and the powder was collected and stored at -20 °C before further use.

2.3. Preparation of emulsions

Thymol was dissolved in PG at different concentrations. SS was mixed with CH solutions, followed by stirring overnight to ensure complete hydration. The thymol/PG solution was added into the emulsifier dispersion. The acidity was adjusted to pH 6.8. The mixtures were homogenized at 10,000 rpm for 2 min using an Ultra-Turrax high speed homogenizer (model IKA T25 digital, IKA® Werke GmbH & Co., Staufen, Germany) to prepare coarse emulsions. The coarse emulsions were heated at 90 °C for 20 min in a water bath, followed by quenching in an ice/water bath under static conditions. The first variable was studied for conditions of

preparing CHs, by preparing emulsions using fixed concentrations of SS and thymol. The next group of variables were then studied for emulsions prepared with a fixed CH concentration but varying SS and thymol concentrations.

2.4. Turbidity and particle size of emulsions

Sample turbidity was measured for absorbance at 600 nm (Abs_{600}) as an indicator using a UV-Vis spectrophotometer (model Biomate 5, Thermo Electron Corporation, Woburn, MA, USA). Deionized water was used as a blank.

The particle size of samples was measured using a Delas Nano particle analyzer (Beckman Coulter, Fullerton, CA, USA). All samples were diluted to an appropriate concentration in deionized water prior to analysis to meet the required sensitivity of the instrument. The area-volume ($d_{3,2}$) mean diameters were calculated using Eq. (1).

$$d_{3,2} = \frac{\sum_{i=1} n_i d_i^3}{\sum_{i=1} n_i d_i^2} \quad (1)$$

where d_i is the diameter of the i^{th} group of droplets and n_i is the corresponding number of droplets.

2.5. Atomic force microscopy (AFM)

The morphology and dimension of particles were characterized using a Multimode VIII microscope (Bruker Corporation, Billerica, MA, USA). Nanoemulsions were diluted to 50 ppm CH using deionized water. Five microliters of the diluted sample was dropped on a freshly cleaved mica disk and dried under ambient temperature (21 °C) in a chemical fume hood overnight. Images were collected at the tapping mode and analyzed using the instrument software.

2.6. Fluorescence spectroscopy

The interactions between SS and CH were studied by fluorescence spectroscopy using a spectrofluorometer (model RF-1501, Shimadzu Corp., Kyoto, Japan). The solubility of SS in water was limited at room temperature (21 °C), but aqueous dispersions with 0-4 mg/mL SS and 0.5 mg/mL CH adjusted to pH 6.8 were overall transparent after 2-h incubation at both 21 and 90 °C. Furthermore, no difference in fluorescence spectra was observed for samples heated at 90 °C for 2 h or 20 min after cooling down to room temperature in an ice/water bath. Fluorescence spectra were collected at an excitation wavelength of 280 or 295 nm. The slit width for excitation and emission was set at 10 nm.

2.7. Stability of dispersions as affected by pH

The nanoemulsions prepared with 2% w/v CH, 1% w/v SS and 2% w/v thymol by heating at 90 °C for 20 min were acidified through two approaches. The first group of emulsions were heated to 75 °C and adjusted to pH 3.0-6.8 with 1.0 M and 0.5 M HCl at 75 °C. After stirring for 2 min at 75 °C, samples were cooled in an ice water bath. The final pH was verified and readjusted when the temperature reached ambient conditions (21 °C). Another group of nanoemulsions were acidified at room temperature with 1.0 M and 0.5 M HCl. The thymol nanoemulsions prepared with 2% w/v CH only and those prepared with 2% w/v NaCas and 1% w/v SS were also adjusted to pH 3.0-6.8 at 75 °C using the above method. All the acidified samples were diluted to a thymol concentration of 0.1% w/v using 0.01 M PBS adjusted to sample pH, and the stability was evaluated by visual observation of precipitation.

2.8. Zeta (ζ)-potential measurement

The ζ -potential of samples was measured using a model Zetasizer Nano ZS (ZEN3600) instrument (Malvern Instruments Ltd., Malvern, Worcestershire, UK). Emulsions were prepared

with 2% w/v thymol using 2% w/v CH with 0 or 1% w/v SS and heating at 90 °C for 20 min. Emulsions were acidified at 75 °C to pH 3.0-6.8 with 1.0 M and 0.5 M HCl. After stirring for 2 min at 75 °C, samples were cooled in an ice water bath. The final pH was verified and readjusted after reaching ambient temperature. The samples were then diluted 40 times with 0.01 M PBS adjusted to sample pH before ζ -potential measurement. Because of the low solubility of SS in water at ambient temperature, a control emulsion was prepared with SS only using the anti-solvent precipitation method. Briefly, SS and thymol were dissolved in PG at 10% and 20% w/v, respectively, by heating at 75 °C, and the PG solution was then mixed at 2.5% v/v into room temperature water on a stirring plate. The prepared emulsions were adjusted to pH 3.0-6.8 with 0.1 M HCl at 75 °C, stirred for 2 min at 75 °C, and cooled to room temperature in ice water, followed by dilution for 10 times with 0.01 M PBS adjusted to sample pH before ζ -potential measurement.

2.9. Statistical analysis

Three independent emulsion replicates were studied throughout. Statistical analyses were performed using the SAS software (version 9.4, SAS Institute, Cary, NC). One-way analysis of variance was carried out. Differences between pairs of means were compared using a Tukey's test. The significance level was set at 0.05.

3. Results and discussions

3.1. Effects of hydrolysis duration on emulsification ability of hydrolysates

Figure 1 shows the Abs₆₀₀ of emulsions prepared using 2% w/v thymol, 0.5% SS w/v and 2% w/v NaCas or CH before and after heating at 90 °C for 20 min. Before heating, only the samples prepared with CH hydrolyzed for 10 min (10mCH) resulted in a clear emulsion, which

was stable for more than 1 week. The Abs₆₀₀ of the emulsion prepared with NaCas was significantly higher than the 10mCH treatment but was significantly lower than those with CH hydrolyzed for 1-20 h. Emulsions prepared with NaCas or 1-20 h CH all precipitated after one-day storage. After heating at 90 °C for 20 min, Abs₆₀₀ of the emulsion prepared with NaCas significantly reduced, while no influence on other emulsions ($p > 0.05$) was observed. The heated emulsions prepared with NaCas and 10mCH were optically stable for more than 60 days, while other heated emulsions showed precipitation after one-day storage.

When examined using SDS-PAGE (Figure 2), a partial reduction of NaCas molecular weight was observed after 10-min hydrolysis, while other CHs had a substantial reduction of molecular weight, with the 20-h treatment showing no visible bands. Results in Figure 1 and Figure 2 show that a limited extent of hydrolysis improved the emulsifying activity of NaCas but a sufficient molecular weight of CHs is needed to maintain emulsifying properties. The overall trend agrees with a previous study reporting the reduced emulsifying capacity of CHs produced by tryptic hydrolysis at pH 5.5-8.0 for a longer duration (Chobert, Bertrand-Harb, & Nicolas, 1988). It was also suggested that a minimum length of 20 amino acid residues and distinct zones of hydrophobic and hydrophilic amino acid residues are required for emulsifying and interfacial properties of peptides (Turgeon, Gauthier, Molle, & Leonil, 1992). Based on the results in Figure 1, the 10mCH was selected for further studies.

3.2. Influence of sucrose stearate concentration on nanoemulsion formation

To further study the nanoemulsion formation, the first group of emulsions was prepared with 2% w/v 10mCH, 0-3% w/v thymol, and 0, 0.25, or 1% w/v SS, with and without heat treatment. For emulsions without SS, there was no big difference of Abs₆₀₀ between samples with and without heat treatments when thymol was used at 1% w/v or higher (Figure 3A).

208 Without thymol, the heated 10mCH solution had significantly higher Abs₆₀₀ than the unheated
209 sample, which may be caused by the aggregation of hydrophobic hydrolysates during heating.
210 The Abs₆₀₀ of both heated and unheated emulsions was higher at a higher content of thymol, and
211 there was visible oil on the top when thymol concentration was higher than 1% w/v, which
212 indicated the emulsifying capacity of 2% w/v 10mCH was about 1% w/v thymol at the studied
213 conditions. The stability of heated and unheated emulsions with 1% w/v thymol was quite
214 different. The unheated samples turned turbid and precipitated after one month, while the heated
215 sample with $d_{3,2}$ of around 40 nm was stable for two months (Table 1, Figure 4). This set of
216 results showed that emulsifying thymol as colloidal particles stabilized 10mCH from aggregation,
217 and the stability improved after heat treatment. This improved stability should be attributed to
218 complex formation between 10mCH and thymol due to hydrophobic interactions, as previously
219 studied for NaCas and thymol (Pan et al., 2014). Heating can cause restructuring of the
220 complexes to improve the stability of the heated emulsion (Pan et al., 2014).

221 When 0.25% w/v SS was added into the 10mCH-thymol mixture to prepare emulsions,
222 samples had the improved clarity after heat treatment, except for the one with 3% w/v thymol
223 showing no significant difference (Figure 3B). Because the water solubility of SS is poor at
224 ambient temperature, the reduced Abs₆₀₀ after heating can be attributed to both the reduced
225 amount and size of insoluble SS particles and the reduced oil droplet size. Heating improves
226 water-solubility of SS and therefore interfacial activity to restructure oil droplets. SS solubilized
227 after heating also can aggregate as smaller particles after cooling to ambient conditions. These
228 two aspects can reduce emulsion turbidity after heating. The improved interfacial activity of SS
229 after heating was evident for the ability to emulsify a higher content of thymol as stable
230 nanoemulsions (Table 1). For the heated emulsions, Abs₆₀₀ increased with the increase of thymol

concentration. Because there is no significant difference in particle size of the samples with thymol (Table 1), the higher Abs₆₀₀ at a higher thymol concentration resulted from the increased number of oil droplets. At a thymol concentration of 3% w/v, the contribution to Abs₆₀₀ may be similar for oil droplets and SS particles, resulting in no difference in sample turbidity before and after heating. Although there is no difference in the Abs₆₀₀ of emulsions with 3% thymol, the heated sample with $d_{3,2}$ of 59.0 nm was stable for two months (Table 1; Figure 4), while the unheated sample precipitated after only one day. The results indicate SS particles are not stable, while the co-adsorption of 10mCH and SS on oil droplets stabilized the emulsions.

When more SS (1%) was studied, the Abs₆₀₀ of emulsions was overall lowered after heating, except at 1% w/v thymol concentration (Figure 3C) that can be attributed to similar effects of SS and heating at this thymol level. The lowest Abs₆₀₀ was observed 1.5% and 2% w/v thymol after heating. The data further supports the improved emulsifying ability of SS after heating. For samples with 1.5-3% w/v thymol, unheated samples precipitated after one day, while heated samples with $d_{3,2}$ of around 45 nm (Table 1), were stable for at least one month.

To further investigate how SS concentration influenced the emulsion clarity, another set of emulsions was prepared with 2% w/v 10mCH, 2% w/v thymol and 0-2% w/v SS with and without heating at 90 °C for 20 min. Without heating, the Abs₆₀₀ of emulsions increased with an increase of SS concentration (Figure 5A), which likely is due to the increased concentration of insoluble SS particles. In contrast, the heated emulsions had the lowest Abs₆₀₀ at 1% SS (Figure 5A) due to the enhanced interfacial activity of SS at an elevated temperature, and the highest Abs₆₀₀ of the 2% w/v SS treatment can be contributed by an excess amount of SS. For unheated samples, the storage stability, measured by the appearance of visible precipitation, decreased from less than 30 days to 1 day at 0-2% w/v SS. For heated samples, emulsions prepared with 0-

2% w/v SS were all stable for at least one month, and the stability also decreased with an increase of SS concentration.

The $d_{3,2}$ and polydispersity index (PDI) of heated emulsions prepared with 2% w/v 10mCH, 2% w/v thymol and 0-2% w/v SS are shown in Figure 5B. The $d_{3,2}$ was around 50 nm and not significantly different among samples prepared with up to 1.5% w/v SS. The $d_{3,2}$ of the emulsion with 2% SS (>150 nm) was significantly larger than other emulsions with less SS, which is due to an excess amount of SS on the interface or SS aggregates in the aqueous phase. Free oil was observed on the top of emulsions for those prepared with 2% w/v 10mCH and 0-0.125% w/v SS, which indicates the limited emulsifying capacity of systems with a low SS concentration. When more than 0.25% w/v SS was used, PDI increased with an increase of SS concentration. This result indicated that various forms of SS existed at a high SS concentration, likely due to the heterogeneity of SS aggregates.

3.3. Effects of thymol concentration on nanoemulsion formation

The $d_{3,2}$ of emulsions prepared with 2% w/v 10mCH, 0, 0.25 or 1% w/v SS and 0-3% w/v thymol are shown in Table 1. For freshly made samples, $d_{3,2}$ decreased with the presence of thymol in the system, and was not significantly different at different thymol concentrations. An exception was observed for the treatment with 1% thymol and 1% SS, which likely had an excess amount of free SS. The reduction of droplet size after addition of thymol may have resulted from the enhanced packing of both 10mCH and SS molecules due to hydrophobic attraction by thymol, which otherwise would result in bigger particles with a higher thymol concentration (Pan et al., 2014). This was further evidenced for the precipitation of treatments with low concentrations of thymol (0 and 1% for 0.25% SS; 0-2% for 1% SS) after 60-day storage (Figure 4A).

3.4. Emulsion structures studied by AFM

Emulsions containing 2% w/v thymol prepared with 2% w/v 10mCH and 0, 0.25, and 1% w/v SS were imaged using AFM (Figure 6). Particles were discrete and mostly spherical. The average particle dimension estimated in AFM was similar for samples prepared with 0 and 1% w/v SS ($p > 0.05$), while that prepared with 0.25% w/v SS was significantly smaller ($p < 0.05$). Differences in particle dimensions measured in AFM (Figure 6) and dynamic light scattering (Table 1) may be due to their differences in the principle and sample preparation conditions.

3.5. Interactions between casein hydrolysate and sucrose stearate

Binding between proteins and small molecules in aqueous solutions can be conveniently investigated using intrinsic fluorescence properties of proteins. For example, fluorescence data showed that thymol formed complexes with caseins by binding with tyrosine (Tyr) (Pan et al., 2014). Due to composition similarities between amino acid residues of caseins and 10mCH, similar interactions are expected between thymol and 10mCH. In the present study, fluorescence spectroscopy was used to further investigate interactions between 0-4 mg/mL SS and 0.5 mg/mL 10mCH before and after thermal treatment. As shown in Figure 7, fluorescence intensity at an excitation wavelength of both 295 and 280 nm significantly increased with an increase of SS concentration, especially after heating, and the increases were greater at 280 nm. At an excitation wavelength of 280 nm, both tryptophan (Trp) and Tyr residues have fluorescence emission, while at 295 nm, it only shows fluorescence emission of Trp residue (Sahu, Kasoju, & Bora, 2008). The excitation of both Trp and Tyr residues (Figure 7) indicates a global change of protein structure (Mir et al., 2010). It has been shown that hydrophobic tails of SS molecules prefer to interact with hydrophobic regions of caseins to change the conformation of caseins and therefore the exposure of Trp and Tyr residues (Chakraborty & Basak, 2008), which in turn result in the

increase of fluorescence intensity with increasing concentrations of SS. The more significant increase in fluorescence intensity at both excitation wavelengths after heating indicates the strengthened interactions between SS and 10mCH after heating, partially due to the enhanced solubility and therefore mobility and flexibility of SS. In thymol emulsions, the increased exposure of hydrophobic residues of 10mCH and the increased flexibility of SS during thermal treatments can provide more binding sites for thymol (Wu, Hettiarachchy, & Qi, 1998), which enhances the emulsification properties of 10mCH with and without SS, as presented previously.

3.6. Storage stability of emulsions

Destabilization of nanoemulsions may occur through mechanisms such as creaming/precipitation, flocculation, coalescence, and Ostwald ripening (McClements, 2004). As presented previously, 2% w/v 10mCH was able to stabilize only 1% w/v thymol, and an insufficient amount of thymol resulted in precipitation (of likely SS particles) at each combination of 10mCH and SS (Table 1). Particle size and PDI are two quantitative parameters comparing physical stability of emulsion systems, and a smaller growth of particle size and PDI after storage indicates a better physical stability (Anarjan & Tan, 2013a). The $d_{3,2}$ and PDI of thymol nanoemulsions before and after storage at room temperature for 60 days are listed in Table 1 and Table 2, respectively. For samples remaining dispersed after storage, no significant differences of $d_{3,2}$ and PDI were observed before and after storage (Table 1 and Table 2). These samples also showed similar appearance and particle size distributions before and after storage (Figure 4).

Compared to lemon oil nanoemulsions prepared with the combination of NaCas and Tween 20 that were only stable for 15 days during ambient storage (Su & Zhong, 2016), thymol nanoemulsions prepared with 10mCH and SS were much more stable (Tables 1 and 2), although

heating was used in both studies. Tween 20 reduces the oil/water interfacial tension effectively but interacts with NaCas very weakly, resulting a small fraction of NaCas on the interface (Jean-Luc Courthaudon, Dickinson, & Dalgleish, 1991; Su& Zhong, 2016). The improved stability of thymol nanoemulsions in the present study can be contributed by several factors. The longer hydrophobic tail of SS than Tween 20 – stearate vs. laurate - can be more effective in anchoring the interfacial layer and entrapping the oil within droplets to improve the storage stability of nanoemulsions. The stronger hydrophobic interactions between 10mCH and SS than those of Tween 20 and NaCas and differences in oil polarity are two other possible factors.

3.7. pH stability of emulsions

Nanoemulsions prepared with 2% w/v 10mCH and 2% w/v thymol with and without 1% w/v SS by heating at 90 °C for 20 min were studied for pH stability. Figure 8 shows the appearance of the 20-fold diluted emulsions acidified to pH 3.0-6.8 at 21 or 75 °C. No precipitation was observed for all samples at pH 6.0 and 6.8, while precipitation was observed for all samples at pH 4.0 and 4.6. The effect of temperature during pH adjustment was studied for nanoemulsions prepared with 10mCH and SS, showing no precipitation at pH 5.0 after acidification at 75 °C (Figure 8A vs. 8B). Precipitation was also observed when the nanoemulsion prepared with 10mCH only was acidified to pH 5.0 at 75 °C (Figure 8C). For nanoemulsions prepared with NaCas and SS, the sample acidified to pH 3.0 at 75 °C showed precipitation (Figure 8D), which was different from the treatment with 10mCH. Therefore, the stability of thymol nanoemulsions at pH 3.0-5.0 is affected by both emulsifier structure and acidification conditions.

To understand the role of electrostatic repulsion on the stability of emulsions, ζ -potential of the four groups of thymol emulsions in Figure 8 and the thymol emulsion prepared with SS

only and acidified at 75 °C was measured (Figure 9). For emulsions prepared with 10mCH only, the smaller ζ -potential magnitude at pH 4.0-5.0, nearby the isoelectric point of 10mCH, than other pHs corresponded to precipitation (Figure 8), indicating the significance of electrostatic repulsion on emulsion stability. For emulsions prepared with SS only, the ζ -potential was all negative at pH 3.0-6.8, likely due to carboxylate groups of free stearate molecules that are protonated at a lower pH, resulting in the reduction of ζ -potential magnitude. Because free SS was not removed in emulsions prepared also with 10mCH or NaCas, the role of electrostatic interactions on emulsion stability at pH 5.0 and 3.0 was not clear. For the emulsions prepared with 10mCH and SS and acidified to pH 5.0 at 21 or 75 °C, the magnitude of ζ -potential was similar, which suggested their stability difference at pH 5.0 (Figure 8) resulted from SS adsorbing differently on oil droplets at two temperatures. When comparing to emulsions prepared with SS and 10mCH or NaCas, the ζ -potential was similar for the two emulsions at pH 3.0, but the treatment with NaCas was not stable. It can result from differences in SS adsorption and/or the lower solubility of NaCas at pH 3.0 than CHs (Luo, Pan, et al., 2014). Therefore, although SS significantly improved the emulsifying capacity and stability of emulsions at neutral acidity, steric repulsion provided by the sucrose head group of SS is not evident at pH 4.0-5.0 at the studied conditions, which would require the fractionation of SS to further understand the mechanism.

4. Conclusions

In conclusion, several advantages have been shown in the present work for stable and transparent thymol nanoemulsions prepared using combinations of food grade emulsifiers 10mCH and SS. Interactions between SS and 10mCH enhanced the exposure of both Trp and

Tyr residues to favor the binding with thymol during heating and therefore improved the capability of emulsifying a greater amount of thymol as small droplets. The reorientation of SS molecules after heating and cooling improved the stability of nanoemulsions at neutral pH and also pH 5.0 that would otherwise be impossible with 10mCH alone. The transparent nanoemulsions had $d_{3,2}$ of around 50 nm and were stable during 60-day storage at room temperature. These stable transparent nanoemulsions prepared with food grade emulsifiers have great potential to incorporate lipophilic bioactive compounds like thymol in functional beverages.

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Figure captions

Figure 1. Absorbance at 600 nm (Abs_{600}) of emulsions prepared with 2% w/v NaCas (0 h) or casein hydrolysates produced by pancreatin hydrolysis for 10 min-20 h, 0.5% w/v sucrose stearate, and 2% w/v thymol with and without heating at 90 °C for 20 min. Error bars are standard deviations ($n = 3$). Different letters above bars indicate differences in the mean ($p < 0.05$).

Figure 2. SDS-PAGE showing protein markers (lane 0), sodium caseinate (lane 1), and its hydrolysates produced by pancreatin hydrolysis for 10 min (lane 2), 1 h (lane 3), 4 h (lane 4), and 20 h (lane 5).

Figure 3. Absorbance at 600 nm (Abs_{600}) of emulsions prepared with 0-3% w/v thymol, 2% w/v 10-min casein hydrolysate and (A) 0, (B) 0.25%, or (C) 1% w/v sucrose stearate with and without heating at 90 °C for 20 min. Error bars are standard deviations ($n = 3$). Different letters next to symbols indicate differences in the mean ($p < 0.05$).

Figure 4. (A) Appearance of samples prepared with 0-3% w/v thymol, 0, 0.25, or 1% w/v sucrose stearate (SS), and 2% w/v 10-min casein hydrolysate (10mCH) after 1 and 60 days of storage at 21 °C; (B) particle size distributions of samples prepared with 2% w/v 10mCH only and 1% w/v thymol, or with 2% w/v 10mCH, 0.25 or 1% w/v SS, and 3% w/v thymol, before and after 60-day storage.

Figure 5. (A) Absorbance at 600 nm (Abs_{600}) of emulsions prepared with 2% w/v 10-min casein hydrolysate, 2% w/v thymol, and 0-2% w/v sucrose stearate (SS) with and without heating at 90 °C for 20 min. Volume-area mean diameter ($d_{3,2}$) and polydispersity index of heated samples are shown in Figure (B). Error bars are standard deviations ($n = 3$). Different letters next to symbols indicate differences in the mean ($p < 0.05$).

Figure 6. AFM topography images of nanoemulsions prepared with 2% w/v 10-min casein hydrolysate, 2% w/v thymol, and (A) 0, (B) 0.25%, or (C) 1% w/v sucrose stearate. Image dimension is $2\ \mu\text{m} \times 2\ \mu\text{m}$.

Figure 7. Fluorescence emission spectra of 0.5 mg/mL 10-min casein hydrolysate aqueous solution at an excitation wavelength of 295 nm for (A) unheated and (B) heated samples, and at excitation wavelength of 280 nm for (C) unheated and (D) heated samples after interacting with 0-4 mg/mL sucrose stearate (SS) at pH 6.8 for 2 h. Unheated samples were incubated at ambient temperature, while heated samples were incubated at 90 °C for 2 h and cooled down to room temperature in an ice/water bath.

Figure 8. Appearance of 20-fold diluted nanoemulsions prepared with 10-min casein hydrolysate (10mCH) and sucrose stearate (SS) acidified to pH 3.0-6.8 at 75 °C (A) or 21 °C (B), or with 10mCH only (C) or NaCas and SS (D) acidified to pH 3.0-6.8 at 75 °C. Before acidification, nanoemulsions were prepared with 2% w/v thymol, 2% w/v 10mCH (or NaCas) and 0 or 1% w/v SS at pH 6.8 by heating at 90 °C for 20 min and cooling in an ice/water bath. The precipitated samples are highlighted with red arrows.

Figure 9. Zeta-potential of thymol emulsions prepared with 10-min casein hydrolysate (10mCH) alone, sucrose stearate (SS) alone, or both, in comparison to the emulsion prepared with NaCas and SS, acidified to pH 3.0-6.8 at 75 °C. The emulsion prepared with 10mCH and SS and acidified to pH 3.0-6.8 at 21 °C was also compared. All emulsions contained 2% w/v thymol, 2% 10mCH/ NaCas, and/or 1% w/v SS and were prepared by heating at 90 °C for 20 min and cooling in an ice/water bath before acidification. Error bars are standard deviations ($n = 3$).

Table 1. Volume-area mean diameter (nm) of samples prepared with 0-3% w/v thymol, 2% w/v 10-min casein hydrolysate, and 0, 0.25, or 1% w/v sucrose stearate (SS) before and after storage at 21 °C for 60 days. *

Thymol	0% SS		0.25% SS		1% SS	
(% w/v)	Day 0	Day 60	Day 0	Day 60	Day 0	Day 60
0	117.81±5.01 ^{bc}	Precipitated	75.13±23.19 ^{bcd}	Precipitated	132.63±65.17 ^b	Precipitated
1	40.93±5.72 ^d	40.14±0.93 ^d	42.28±4.97 ^d	Precipitated	193.38±29.83 ^a	Precipitated
2	51.67±6.91 ^d	N/A	58.08±3.50 ^d	38.67±0.93 ^d	41.09±.54 ^d	Precipitated
3	50.87±5.50 ^d	N/A	59.02±5.31 ^d	61.57±4.36 ^{cd}	45.94±1.37 ^d	43.33±1.56 ^d

* Numbers are mean±standard deviation (n = 3). Different superscript letters indicate differences in the mean ($p < 0.05$). “N/A” indicates samples showing free oil on the top, and “precipitated” indicates samples showing precipitation.

544 **Table 2.** Polydispersity index of samples prepared with 0-3% w/v thymol, 2% w/v 10-min casein hydrolysate, and 0, 0.25, or 1% w/v
 545 sucrose stearate (SS) before and after storage at 21 °C for 60 days. *

Thymol	0% SS		0.25% SS		1% SS	
(% w/v)	Day 0	Day 60	Day 0	Day 60	Day 0	Day 60
0	0.240±0.018 ^c	Precipitated	0.248±0.060 ^{bc}	Precipitated	0.290±0.026 ^{abc}	Precipitated
1	0.344±0.124 ^a	0.246±0.020 ^{bc}	0.333±0.014 ^a	Precipitated	0.329±0.024 ^{ab}	Precipitated
2	0.265±0.023 ^{abc}	N/A	0.242±0.011 ^c	0.288±0.050 ^{abc}	0.283±0.018 ^{abc}	Precipitated
3	0.291±0.025 ^{abc}	N/A	0.233±0.022 ^c	0.243±0.028 ^c	0.259±0.014 ^{abc}	0.268±0.034 ^{abc}

546 * Numbers are mean±standard deviation (n = 3). Different superscript letters indicate differences in the mean ($p < 0.05$). “N/A”

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(A)

(B)

(C)

(D)

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