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IMPROVED HEAT STABILITY BY WHEY PROTEIN – SURFACTANT INTERACTION

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Abstract

One of the main changes that occur during heat treatment of milk is whey protein denaturation, which in its turn may lead to protein aggregation and gelation. In this contribution, the effect of lysophospholipids, the main components of lysolecithins, as well as alternative surfactants, on heat-induced whey protein aggregation has been studied. Hereby, attention was paid to the relation between polar lipid molecular structure (e.g. effect of alkyl chain length, effect of polar head group) and heat stabilising properties. Residual protein determination in the supernatant obtained after centrifugation of heated whey protein solutions learned that whey protein aggregation was at least partly prevented in the presence of surfactants. As the short alkyl chain lysophospholipids were particularly effective heat stabilisers, hydrophilic surfactants seemed to be most effective, which may be ascribed to their higher critical aggregation concentration. Upon more severe heat treatment, protein aggregation was probed either in-situ by oscillatory rheology, or ex-situ by yield rheometry. As some surfactants significantly reduced the gel strength, or even prevented heat-induced gel formation, these experiments corroborated the heat-stabilising effect of hydrophilic surfactants. Nuclear Magnetic Resonance (NMR) enabled a more direct evaluation of the protein-surfactant interaction. A strong hydrophobic interaction between small molecular weight surfactants and whey proteins became obvious from the chemical shift of the surfactant hydrophobic groups in the NMR spectrum and could be quantified by pulsed field gradient NMR (pfg-NMR) diffusiometry. The results indicated that protein-surfactant interaction did not occur upon thermal denaturation, but already took place at room temperature. However, the effect of this interaction became mainly obvious during thermal treatment.

Overall, this work indicated that bound surfactants largely minimise heat-induced protein intermolecular interactions and hence prevent heat-induced protein aggregation. As the surfactant molecular structure plays a decisive role, it follows that the heat stability of whey protein containing products may be optimised by appropriate selection of ingredients such as (lyso)phospholipids.

Key words: Whey protein isolate (WPI), heat coagulation, lysophospholipids, surfactants, NMR, rheology

1. Introduction

Heat treatment of milk leads to numerous changes in its chemical and physical properties. An extreme change is heat coagulation, a phenomenon in which heat-induced serum protein-casein interactions play an important role (Fox, 1981; Fox, 2003; Jeurnink and de Kruif, 1993; Van der Meeren, El-Bakry, Neirynck & Noppe, 2005). Although the exact mechanism of this undesired effect is not yet fully understood, still it is known that heat-induced whey protein interactions play an important role (Brown, 1988; Havea, 1998; Havea, Carr & Creamer, 2004). Upon heating β-lactoglobulin, the major whey protein, at neutral pH conditions to 70°C, dimers first dissociate into monomers, and a thiol group as well as hydrophobic residues become solvent accessible. Subsequently, aggregates are formed via intermolecular thiol-disulphide exchange, thiol-thiol oxidation and noncovalent interaction (Hoffmann and van Mil, 1997; McSwiney, Singh, Campanella & Creamer, 1994; Mulvihill and Kinsella, 1987). This may lead to heat-induced gelation of whey protein.

A number of studies have shown that lecithins may overcome undesirable heat coagulation effects upon severe heating (Hardy, Sweetsur, West & Muir, 1985; McCrae, 1999; Tran Le, El-Bakry, Neirynck, Bogus, Hoa & Van der Meeren, 2007; Van der Meeren *et al.*, 2005; van Nieuwenhuyzen and Szuhaj, 1998). In addition, the effect of hydrolyzed lecithin on the characteristics of thermally induced protein gels and protein stabilized emulsion gels has been reported. Thus, Jost, Dannenberg and Rosset (1989) showed that introducing hydrolysed lecithin before or during emulsification reduced substantially the strength of a heat-set whey protein emulsion gel. Dickinson and Yamamoto (1996a), on the other hand, indicated that addition of pure egg-yolk L-α-phosphatidylcholine after emulsification caused an increase in strength of a heat-set β-lactoglobulin emulsion gel. Therefore, the investigation of whey protein-lecithin interaction is important to better understand the heat-stabilising properties of lecithin.

Interestingly, lecithin is only one type of low molecular weight surfactant. The interaction between whey proteins and low molecular weight surfactants has been extensively investigated by

Goddard and Ananthapadmanabhan (1993), Chen and Dickinson (1995), Dickinson and Hong (1995), Chen and Dickinson (1998), Chen, Dickinson, Langton & Hermansson (2000) as well as Roth, Murray & Dickinson (2000). A general model proposed to explain how anionic surfactant interacts with globular whey proteins has been suggested. According to Jones (1992) and Oakes (1974), three successive mechanisms may occur upon increasing the surfactant concentration, i.e. specific binding at low surfactant concentration, non-cooperative binding at higher surfactant concentration and cooperative binding at still larger concentration. Giroux and Britten (2004) indicated that the formation of protein-anionic surfactant complexes depends on the surfactant concentration, pH, ionic strength and temperature.

The aim of this study was to investigate the effect of hydrolysed lecithin on whey proteins during heat treatment. Whereas some effects may occur on the molecular level (leading to changes in protein conformation and/or its temperature dependence), our study focused on surfactant effects on (the prevention of) whey protein aggregation. Hereby, it is important to mention that whey protein gelation strongly depends on the electrolyte composition (Bryant & McClements, 2000). In order to ensure pH and ionic strength conditions that are representative for milk, a calcium-containing imidazole buffer was selected in all our experiments. For comparative purposes, the effect of hydrolysed soybean lecithin was compared to that of pure lysophospholipids, anionic surfactants, nonionic sucrose esters and POE-based nonionic surfactants. Oscillatory rheology was preferred for the in-situ determination of the effect of heating and subsequent cooling on whey protein solutions, whereas vane spindle rheometry enabled the ex-situ characterisation of the gels formed. Chemical analysis of the supernatant obtained by centrifugation of moderately heated whey protein solutions enabled the quantification of the residual amount of soluble whey proteins, as well as surfactants. Finally, Nuclear Magnetic Resonance (NMR) was used to study the whey protein-surfactant interactions into more detail.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI) was obtained from Davisco Foods International, Inc. (BiPRO®, Le Sueur, MN, USA). Kjeldahl analysis revealed that this minimally heat-treated WPI contained 92.6% of protein, whereas polyacrylamide gel electrophoresis (PAGE) indicated that approximately 85% of the total protein consisted of β-lactoglobulin. It contains 1.6% ash (by incineration at 525 ºC) and 5.0% moisture (from weight loss at 102 ºC) and 0.8% fat.

Hydrolyzed soybean lecithin (Emultop HL50 IP) was obtained from Cargill Texturing Solution (Germany). According to the manufacturer, this hydrolyzed lecithin contains 95% acetone insolubles, 14% phosphatidylcholine, 8.5% lysophosphatidylcholine, 2% moisture and 3% oil.

Three different lysophosphatidylcholine (LPC) products were purchased from Anatrace (USA): 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (C14-LPC; MW = 467.58 g/mol; CMC = 0.036 mM), 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (C16-LPC; MW = 495.64 g/mol; CMC = 0.0032 mM) and 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (C18-LPC; MW = 532.69 g/mol), in which the purity was over 99% (by HPLC analysis).

Sodium dodecylsulfate (SDS; MW=288.38 g/mol; CMC=8.3 mM), sodium laurate (Na-laurate, MW=222.3 g/mol; CMC=27.8 mM), Tween 20 and Brij78 originated from Sigma-Aldrich Logistik GmbH (Germany), whereas Tween 80 was obtained from ICI surfactants (Belgium). Three sugar ester products of Ryoto sugar ester (Mitsubishi-Kagaku food corporation, Japan) were used: sucrose palmitate (P-1570), sucrose oleate (OWA-1570), as well as sucrose laurate (LWA-1570) in which 70% pure palmitic acid, oleic acid and lauric acid are present, respectively.

Ca-imidazole buffer containing 20 mM imidazole, 5 mM $CaCl_2.H_2O$, 30 mM NaCl and 1.5 mM NaN_3 was prepared according to Anema (1997). Its pH was adjusted to 6.55 by 1N HCl. This buffer was selected to have an electrolyte composition that resembled dairy products.

2.2. Oscillation rheological measurements

An AR2000 Rheometer (TA instruments, Belgium) equipped with a 28 mm conical concentric cylinder measuring system was used in all experiments. An amount of 20 ml of a mixture of equal volumes of whey protein (5.5%) and surfactant (2%) solution in Ca-imidazole buffer was poured gently into the cup and covered by aluminium foil to prevent water evaporation during the experiment. Gels were formed by heating the samples from 20 to 80°C at a constant rate of 2°C/min, holding at 80°C for 15 minutes, and cooling to 20°C at a constant rate of 2°C/min. Measurements were taken at a frequency of 1 Hz and at a strain of 0.002. A preliminary strain sweep at a frequency of 1 Hz indicated that the linear visco-elastic region of a 2.75% whey protein isolate gel in the absence of surfactants extended up to about 10%.

Yield stress measurements were performed using a YR-1 Yield Rheometer (Brookfield, USA) with vane spindle 73. The EZ-Yield V 1.0 software was used to fix the parameters of the spindle and to transfer data from the rheometer to the computer. The parameters were set as follows: zero speed = 0.01 rpm, run speed = 0.03 rpm, and torque reduction = 110 %. An amount of 16 ml of a mixture containing equal volumes of WPI solution (5.5%) and hydrolysed lecithin solution (2%) in Ca-imidazole buffer was transferred into a glass tube with a diameter of 20 mm and a length of 80 mm, before heating at 80°C for 5, 10 or 15 minutes, and immediately cooled down in water at room temperature. The 4-bladed spindle was slowly inserted into the sample tube, to a point that the top of the gel is at the same height as the primary immersion mark.

2.3. Residual solubility

One ml of 5.5% (w/v) whey protein stock solution was mixed with either 0.0 or 1.0 ml of 2% (w/v) of either hydrolysed lecithin or alternative surfactant stock solutions, all in Ca-imidazole buffer. After adjusting the total volume to 2 ml with Ca-imidazole buffer, the mixture was heated for 0, 1 and 2 minutes in a water bath at 80°C and then cooled with tap water at room temperature. All heated samples were centrifuged for 15 minutes at 2900 g in a Labofuge GL (Heraeus) centrifuge.

The residual protein content present in the supernatant was determined based on the colorimetric

method of Schacterle and Pollack (1973) by spectrophotometry using a PerkinElmer Lamda 35 UV-spectrophotometer at a wavelength of 650 nm. The residual phospholipid (PL) content of the samples containing LPCs was determined by colorimetric phosphate analysis after acid digestion (Van der Meeren, Vanderdeelen & Baert, 1988) at a wavelength of 820nm. The total organic carbon (TOC) content in mixtures containing alternative surfactants was determined by an Anatoc series II SGE Total Organic Carbon Analyser (Singapore).

2.4. NMR measurement

Protein-free samples with the following concentrations were prepared: 5 mg/ml of surfactant and 5 mM of sodium acetate trihydrate (MW=136.08 g/mol; Normapur) in D_2O (Armar Chemicals 99.8 atom % D). In addition, whey protein containing samples (1 for each surfactant in the presence of protein) with the following concentrations were prepared: 5 mg/ml of surfactant, 10 mg/ml of WPI and 5 mM of sodium acetate trihydrate in D_2O. Sodium acetate was used as internal standard. All the NMR measurements were performed by a Bruker spectrometer operating at a ^1H frequency of 500.13 MHz. The samples were analyzed twice at a temperature of 25°C, i.e. before and after a heating/cooling process. In the latter case, the samples were heated at 80°C for 15 minutes in a water bath and subsequently cooled down at 25°C.

Diffusion coefficients were measured by pulsed field gradient NMR (abbreviated as pfg-NMR) with a convection compensated double-stimulated-echo experiment using monopolar smoothened square shaped gradient pulses and a phase cycle modified according to Connell *et al.* (2009). The echo-decay of the resonance intensity obtained with the double stimulated echo sequences obeys equation (1), from which the diffusion coefficient (*D)* is derived as a function of the parameter *k*. A detailed description of the pfg-NMR method and the sequences mentioned above is given in a review written by Johnson (1999):

$$I/I_0 = \exp\{-D\,(\gamma\,\delta\,G\,\Delta\,s)^2\,\Delta'\}$$

$$I/I_0 = \exp(-D\,k) \qquad [1]$$

I = echo intensity with gradient

I_0 = echo intensity at zero gradient

γ = gyromagnetic ratio

G = gradient amplitude

s = gradient shape factor (here 0.9)

δ = duration of the gradient pulse

Δ' = diffusion delay corrected for the finite gradient pulse duration ($\Delta' = \Delta - 0.602\ \delta$)

The determination of the diffusion coefficient was based on the fitting of a mono-exponential curve to the echo-decay of the peak integral as a function of k; the latter was varied by variation of the gradient amplitude within a range going from 2% to 95% of the maximum gradient strength (i.e. 56.1 G/m) while keeping all other parameters constant. The determination coefficient (r^2) was at least 0.90 for the (less intense) protein signals, and at least 0.999 for both SDS and sodium laurate.

3. Results and discussion

3.1. Effect of hydrolysed lecithin on WPI gelation

Oscillatory rheology is a powerful tool for monitoring protein gelation and has been utilized in studying the effect of surfactants on heat-induced gelation properties of β-lactoglobulin by Chen and Dickinson (1995), Dickinson and Hong (1995), Dickinson and Yamamoto (1996a; b), Chen and Dickinson (1998), Chen et al. (2000), as well as Roth et al. (2000). Figure 1 shows the change in the rheological properties of a 2.75% (w/v) WPI dispersion in Ca-imidazole buffer at pH 6.55 alone and in the presence of 1 % (w/v) of either hydrolysed lecithin or some alternative low molecular weight surfactants. The gelation curves are shown in Figure 1 by the behavior of the complex modulus, G*, as well as the phase angle, through the different temperature stages. The heat-induced gelation behavior of WPI in Ca-imidazole buffer in the absence of surfactants is shown by gelation curve (1) in Figure 1A. Previous studies of Bowland, Foegeding & Hamann (1995), Clark, Kavanagh & Ross-Murphy (2001), de La Fuente, Singh & Hemar (2002) and Singh & Havea (2003) explained that the formation of heat-induced whey protein gels is irreversible mainly due to disulfide bridges and hydrophobic interaction. Figure 1B revealed that the phase angle largely dropped at a temperature of 76 °C, which is a clear indication of the transition from a fluid to a gel state.

Gelation curve (2) in Figure 1A shows that hydrolysed lecithin addition to the WPI solution decreased the complex modulus G* significantly, both in the heating and cooling period. That means that the consistency of the WPI gel was reduced when hydrolysed lecithin was added. This heat-protecting effect is consistent with the results of DSC measurements performed by Van der Meeren *et al.* (2005) who found that hydrolysed lecithin addition shifted the denaturation temperature of the whey proteins (74.4°C) to a higher point, by about 7°C (to 81.1°C). This shift in denaturation temperature is also obvious from the phase angle data in Fig1B: whereas the phase angle became less than 45 degrees at 76 °C for the WPI without lecithin, this drop only occurred at 80 °C in the presence of lecithin. The question may arise whether the lower complex modulus in the presence of lecithin is (at least partly) due to the upward shift in denaturation temperature

which might result in limited protein denaturation at the gelation temperature (i.e. 80 °C) used. To answer this question, oscillatory measurements were repeated using a (similar) temperature program going up to 90 °C (to ensure that the gelation temperature was above the denaturation temperature) with 2.75% of WPI in the absence and presence of 1% of lecithin (results not shown). Also in this case, the complex modulus of the gel formed in the absence of lecithin was more than twice the value obtained in the presence of lecithin.

The lower complex modulus observed in the presence of lecithin (Fig.1A) is in contradiction with the results of Ikeda and Foegeding (1999a; b) who reported that adding lecithin had an increasing effect on the rheological properties of heat-induced WPI gels at low to moderate electrolyte concentrations. Only in the presence of 500 mM NaCl, a smaller value of the storage modulus was observed in the presence of lecithin in their experiments. According to our opinion, the difference between our results and those obtained by Ikeda and Foegeding (1999a) may be mainly ascribed to the concentrations used. In fact, Ikeda and Foegeding (1999a) used samples containing 10% of WPI to which either no or 10% of lecithin was added. This means that the water content was only 4 times larger than the combined protein and lecithin content, and hence water limitation is highly probable. Under these conditions, addition of more water-binding material will logically increase the rheological properties. On the other hand, our experiments were based on 2.75% of WPI in the absence or presence of 1% of lecithin, which means that the water to solute ratio is at least 96.25 over 3.75, which is equal to about 26. Under these conditions, much more water is available and the rheology will be much more affected by the number and strength of the interactions between the protein molecules within the gel. In fact, the salt effect observed by Ikeda and Foegeding (1999a) also provides an indication for the importance of the availability of water. As the water binding properties of both proteins and polar lipids are known to decrease with increasing salt content, it follows logically that salt addition will reduce the water limitation and hence may induce a transition from a gel reinforcing to a gel weakening effect of added lecithin.

An alternative and simpler method to characterise the rheological properties of WPI gels is to determine the yield stress, which may be defined as the minimum stress required to initiate flow

after gel formation. In this experiment, the yield stress of a 2.75% (w/v) WPI gel in Ca-imidazole buffer (pH 6.55), containing 0% and 1% (w/v) hydrolysed lecithin, was determined. To that end, the stress was recorded as a function of the angular displacement of the vane spindle (Figure 2). As long as the material behaved as an elastic solid, the stress increased steadily with the rotational angle. The slope of this part is proportional to the elastic modulus of the gel. As the stress increases, a point is reached at which the gel network cannot withstand the exerted stress anymore and the gel starts to flow. From this point onward, the stress decreases due to an increasing gel breakdown. Hence, the yield stress corresponds to the maximum in the stress versus strain curves, as shown in Figure 2. It can be seen that both the elastic modulus and the yield stress of the WPI gels increased when increasing the heating time from 5 minutes to 15 minutes. The presence of 1% hydrolysed lecithin significantly reduced the elastic modulus and the yield stress of WPI gels for each heating time. Hence, these ex-situ measurements are completely in line with the results of the in-situ measurements by oscillatory rheology.

In conclusion, the rheological results in Figure 1 and 2 show that the presence of lecithin reduced the strength of the whey protein isolate gel. Hence, both in-situ and ex-situ characterisation of WPI gels clearly indicated the heat stabilising effect of lysolecithin. According to Van der Meeren *et al.* (2005), this may be explained from the fact that hydrolysed lecithin largely reduced attractive protein-protein interaction during heating.

3.2. Effect of alternative surfactants on WPI gelation

In order to check whether the observed effect is specific for phospholipids, some alternative surfactants were tested. They were divided into two main groups, i.e. anionic surfactants and nonionics. Hereby, sucrose esters of lauric, palmitic, and oleic acid, as well as polyoxyethylene (POE)-based nonionic surfactants (Tween 20, Tween 80 and Brij 78) were selected. The effect of the surfactants on the heat-induced whey protein isolate gels is quantified by the change in the complex modulus (G*) during heating and cooling. The gelation curves (3-7) of samples containing WPI and surfactants were compared to the gelation curves in the absence (1) and

presence of lysolecithin (2) in Figure 1. The results from Figure 1 show that a significant difference in the gelation curves occurred by adding surfactants. The presence of both ionic surfactants SDS and sodium laurate strongly affected the effect of heating on whey protein isolate solutions: in fact, no gelation occurred when adding these anionic surfactants (Figure 1, curve 3 and 4). The effect of sodium laurate is in line with the results of the heat stability experiments on β-lactoglobulin by Puyol, Perez, Peiro & Calvo (1994): using differential scanning calorimetry (DSC), they concluded that the binding of fatty acids to β-lactoglobulin may be an important factor in the thermal stabilization of this protein. Considering ovalbumin, Mine, Chiba & Tada (1993) also found an enhanced heat stability by coupling with either fatty acids or lysophosphatidylcholine (but not with egg phosphatidylcholine). For the sake of completeness, it can be mentioned that Ikeda, Foegeding & Hardin (2000) observed enhanced β-lactoglobulin gel formation upon fatty acid addition, with oleate and palmitate even inducing gelation during protein hydration at room temperature. However, these experiments were done using 12% of protein and 12% of fatty acid so that water limitation was very probable in the latter experiments.

Visual observation learned that gelation occurred in a sample containing 2 ml of 2.75% (w/v) whey protein isolate solution after heating at 80ºC for 2 minutes. However, a low viscosity transparent liquid in resulted upon heating a mixture a 2.75% WPI and 1% SDS, from which it may be deduced that there was no aggregation in the presence of SDS. Similarly, only small aggregates were formed in samples containing 1% sodium laurate resulting in a slightly turbid, but still very liquid-like aspect. The lower turbidity of the SDS-containing WPI solution indicated that the heat-stabilising effect of SDS on the whey protein isolate solution was stronger than that of sodium laurate.

Gelation curves (5), (6) and (7) in Figure 1 show that POE-based non-ionic surfactants, in comparison to lecithin, had an increasing effect on the complex modulus of the WPI gels. Addition of POE-based non-ionic surfactants had only a minor (for Tween 20) to no discernible effect (for Tween 80, as well as Brij 78) on the WPI gels, compared to the corresponding sample containing whey protein isolate without lecithin. The behaviour of these surfactants is known to be highly temperature dependent. In fact, POE-based non-ionic surfactants are known to become less

hydrophilic upon increasing the temperature. The resultant surfactant clouding is expected to badly affect the protein thermal stability. In order to overcome this complicating effect, fatty acid sucrose esters were investigated as alternative non-ionic surfactants (data not shown). However, these non-ionics also yielded only a small beneficial effect (in the case of lauric ester) or even a small deteriorating effect (in the case oleic ester). Hence, the effect of the addition of 1% surfactant on the complex modulus of heat-induced WPI gels has the following order: anionic surfactants > lysolecithin > POE-based nonionic surfactants ≈ sucrose esters.

In conclusion, the addition of lecithin has a more pronounced weakening effect on heat-induced whey protein isolate gels as compared to other surfactants, except from anionic surfactants that even prevented the formation of heat-induced whey protein isolate gels. Hence, protein heat-stabilising properties are not limited to phospholipids but also occur with other surfactants. More precisely, the sodium salts of fatty acids are relevant since commercially available hydrolysed lecithins not only contain lysophospholipids, but mostly also contain the fatty acid salts released upon phospholipase treatment.

3.3. Effect of surfactants on whey protein aggregation

In an attempt to elucidate the interactions of whey proteins with the different above-mentioned surfactants, two series of experiments were performed using mixtures of whey protein isolate with surfactants in imidazole buffer that were heated for either 1 or 2 minutes at 80ºC and subsequently cooled down by tap water. After centrifugation for 15 minutes at 2900 g, the residual protein in the serum was determined. In order to estimate the residual surfactant concentration, the total organic carbon content of the serum was determined as well. Prior to measurement of the protein and total organic carbon content, the samples had to be diluted to a suitable concentration within the measurable range: the samples were diluted 250 times in Ca-imidazole buffer (pH 6.55) for protein determination and 400 times with distilled water for TOC analysis, respectively. Figure 3 shows that upon heating, the whey protein isolate as well as the total organic carbon recovery in the supernatant was significantly reduced. The residual protein

solubility and amount of total organic carbon of samples containing 1% lysolecithin were roughly twice the values found for the sample without lecithin. In the presence of the ionic surfactants (SDS and sodium laurate), the residual whey protein as well as the total organic carbon content was hardly affected even after 2 minutes of heating. The prevention of whey protein aggregation explains why the complex modulus G* of the samples containing 1% ionic surfactant remained negligibly small in Figure 1.

The higher residual protein content in the supernatant upon heating in the presence of Tween 80 is in line with the heat-protecting effect on bovine serum albumin of this surfactant described by Arakawa and Kita (2000). According to these authors, this stabilising effect was due to the fact that Tween 80 addition before heating reduced aggregation and increased the monomer content. Considering the non-ionic surfactants, the total organic carbon content in the aqueous phase is less reduced as compared to the protein content. This effect is especially pronounced for the POE-based non-ionics and indicates that the surfactant recovery after heating is higher than the protein recovery. This may either indicate that only part of the surfactant is protein bound and/or that especially complexes with a higher protein to surfactant ratio are precipitated.

3.4. Effect of lysophospholipid molecular structure on whey protein aggregation

The heat stabilisation properties of three different molecular species of lysophosphatidylcholine (LPC) were compared. Visual observation revealed that gelation was prevented in tubes containing 2 ml of WPI (2.75% w/v) solution in the presence of 1% LPC after heating for 2 minutes at 80°C. In fact, a highly turbid, but still flowable coagulum was obtained in the presence of 1% LPC, as well as in the presence of 1% soybean lysolecithin. The turbidity is an indicator of severe aggregation in all samples. Whereas large visually observable aggregates were mostly formed, only small aggregates were formed in the mixture containing C14-LPC.

The recovery of proteins and phospholipids in the supernatant after centrifugation of unheated and heated WPI (2.75% w/v) solutions in the absence and presence of 1% (w/v) hydrolysed lecithin or three different kinds of LPC is shown in Figure 4 (A). The results from Figure 4 show

that the soluble protein content was reduced in the supernatant of all samples by increasing the heating time. This reduction is due to aggregation and subsequent precipitation in the centrifugal field. However, the protein recovery was improved in the presence of 1% hydrolysed soybean lecithin (indicated as PL) and even more in the case of 1% LPC addition. Hereby, Figure 4(A) clearly reveals that the residual protein solubility was dependent on the length of the hydrophobic tail: comparing the residual protein solubility of samples containing different kinds of LPC, it follows that the shorter chain myristoyl LPC and palmitoyl LPC had a larger protective effect than the longer chain stearoyl LPC. This effect may be explained from the higher molar concentration of the shorter chain molecular species at a fixed mass concentration of 1%. In addition, the shorter chain LPC has a higher CMC and hence more monomers are available for binding to the proteins. The residual phospholipid content in Figure 4 indicates that the phospholipid recovery was also reduced by increasing the heating time. This is a clear indication of the binding of phospholipids to protein, resulting in their co-precipitation.

3.5. Quantification of whey protein-surfactant interaction by NMR measurement

In order to investigate the whey protein-surfactant interaction into more detail, NMR spectroscopy coupled with diffusion analysis was used. Hereby, the surfactants were selected that gave rise to the most pronounced heat-stabilising effects, i.e. SDS, sodium laurate and C14-LPC. Figure 5 (A) represents the ^{1}H-NMR spectrum of WPI at 25°C. In this spectrum, only two sharp peaks are present, which are due to residual H_2O (at about 4.8 ppm) and to the sodium acetate (at about 1.9 ppm) that was used as an internal standard. Besides these two sharp contributions, a broad range of contributions can be observed within the 0 to 8 ppm range that will form a noisy background for additional sharp peaks that may occur upon surfactant addition.

The chemical structure of sodium dodecylsulfate and the ^{1}H-NMR spectra of free SDS and SDS in the presence of WPI are shown in Figure 5 (B). The surfactant concentration was 5 mg/ml, which corresponds to 17.4 mM, i.e. about twice the reported CMC of this surfactant. The triplet around 4.1 ppm and the quintet arround 1.7 ppm are due to the methylene groups in α and β

positions with respect to the sulfate group, respectively. The intense signal around 1.3 ppm belongs to the long alkyl chain of the surfactant, while the triplet at 0.93 ppm comes from the terminal methyl group.

Comparing the SDS spectra in the absence and presence of WPI, a downward shift of the peak positions is observed in the presence of protein (Table 1). Hashimoto and Sakata (1995) observed that the proton NMR chemical shifts of macrocyclic compounds were dependent on the solvent used. Hereby, smaller chemical shift values were generally observed in solvents of smaller dielectric constant, i.e. in a less polar environment. Hence, the chemical shift depends on the molecular environment of the proton that is giving rise to a specific peak, from which it may be deduced that a shift in peak position upon WPI addition indicates a shift in environment which must be due to adsorption. In fact, this changes the environment from bulk water to a more hydrophobic environment on the protein surface.

A closer inspection of the NMR spectra shows that the peaks are not only displaced, but also slightly broadened as may be observed from the fact that less fine-structure can be seen e.g. in the peaks corresponding to the α and β methylene groups of SDS upon whey protein addition. The increased peak broadening is also an indication of a lower mobility, which must be due to binding to the proteins. In addition, Table 2 clearly reveals that the relative peak areas (as compared to the sodium acetate internal standard) are largely reduced in the presence of the proteins. This is a further indication for binding as it is generally known that the T2-relaxation time, i.e. the time constant for the exponential decay of the NMR signal intensity, becomes lower upon sorption; in fact, the latter phenomenon forms the basis of the determination of free and bound water by NMR. Considering the different groups within each surfactant molecule, the reduction in relative peak area is most pronounced for the long $(CH_2)_n$ alkyl chain, which also has the largest peak shift (Table 2). Both these observations indicate that the surfactant mostly interacts with the whey proteins by hydrophobic interactions.

Pfg-NMR diffusion measurements were used to quantify the surfactant interaction with WPI. Hereby, the observed diffusion coefficient of the surfactant in the presence of proteins (D_{obs}) is

the weighted average of non-bound surfactant molecules (with diffusion coefficient D_{free}) and protein bound molecules (with the same diffusion coefficient as the protein D_{pro}). If the bound fraction is represented by *p*, the weighted average may be calculated according to equation (2):

$$D_{obs} = p D_{pro} + (1 - p) D_{free} \quad [2]$$

from which:

$$p = \frac{D_{free} - D_{obs}}{D_{free} - D_{pro}} \quad [3]$$

Hence, the bound protein fraction follows from experimental values of the diffusion coefficient of surfactants in the absence (D_{free}) and presence (D_{obs}) of protein, as well as from the diffusion coefficient of the protein (D_{pro}).

Considering SDS, the diffusion coefficients (with 95% confidence limits mentioned within brackets) of surfactant (D_{free}), surfactant in protein (D_{obs}) and protein (D_{pro}) are $D_{free}=(1.50\pm0.00)\cdot10^{-10}$ m^2/s, $D_{obs}=(0.91\pm0.01)\cdot10^{-10}$ m^2/s, and $D_{pro}=(0.54\pm0.02)\cdot10^{-10}$ m^2/s, from which the bound fraction is calculated to be (61±2)% before heating. Based on the molar mass of SDS and β-lactoglobulin, this bound fraction corresponds to about 20 SDS molecules per β-lactoglobulin monomer (assuming that all WPI would be β-lactoglobulin). After a heating and cooling cycle, the diffusion coefficients are $D_{obs}=(0.93\pm0.00)\cdot10^{-10}$ m^2/s, and $D_{pro}=(0.49\pm0.02)\cdot10^{-10}$ m^2/s, giving rise to a bound fraction of (56±2)%. Hence, these data clearly indicate that the protein-surfactant interaction as such is not significantly affected by the temperature. However, the beneficial effect of the bound surfactant becomes only obvious upon heating.

For the sake of completeness, it should be mentioned that the diffusion coefficient of surfactant molecules is concentration dependent if the concentration is only slightly larger than the CMC (Söderman, Stilbs & Price, 2004). In fact, the observed value in that case is a weighted average of individual monomers (with a concentration equal to the CMC) and of micelles (with a concentration equal to the total surfactant concentration diminished by the CMC). As the latter only occur at surfactant concentrations larger than the CMC, only then the average diffusion coefficient will start to decrease. As the surfactant concentration is further increased, the

contribution of micelles is continuously increasing whereas the contribution of individual surfactant molecules remains constant and hence the average diffusion coefficient will gradually approach the micellar diffusion coefficient. Therefore, the diffusion coefficient of the unbound surfactant D_{free} in the presence of proteins (which is determined in a protein-free solution) is underestimated as it is determined at a higher concentration (in the protein-free solution) compared to the real residual concentration in the aqueous phase of the protein samples (where part of the surfactant has been bound to the protein). According to equation (3), the underestimation of the diffusion coefficient of the unbound surfactant results in an underestimation of the bound fraction.

The chemical structure of sodium laurate and its NMR spectra are shown in Figure 5 (C). The surfactant concentration was 5 mg/ml, which corresponds to 22.5 mM, i.e. slightly below the reported CMC. The signals around 2.2 and 1.58 ppm are due to the methylene groups in the α and β positions of the carboxyl group, respectively. The intense signal around 1.3 ppm belongs to the long alkyl chain of the surfactant, while the pseudo-triplet at 0.93 ppm comes from the terminal methyl group. Table 1 and 2 summarize the chemical shift and integral values of the surfactant signals. Also in the case of sodium laurate, the most pronounced shift in peak position, as well as the largest reduction in relative peak area are obtained for the aliphatic methylene groups of the fatty acyl chain, which again points to the fact that mainly these groups are involved in the (hydrophobic) interaction with the proteins.

The diffusion coefficients of free sodium laurate (D_{free}), sodium laurate in the presence of protein (D_{obs}) and protein (D_{pro}) were $D_{free}=(4.60\pm0.06)\cdot10^{-10}$ m^2/s, $D_{obs}=(2.34\pm0.02)\cdot10^{-10}$ m^2/s, and $D_{pro}=(0.47\pm0.06)\cdot10^{-10}$ m^2/s. Based on these values, the bound fraction was calculated as (55±4)%. Based on the molar mass of sodium laurate, the latter value corresponds to 22 fatty acid chains bound per β-lactoglobulin molecule. After a heating/cooling cycle the diffusion coefficients were $D_{obs}=(2.46\pm0.01)\cdot10^{-10}$ m^2/s, and $D_{pro}=(0.44\pm0.04)\cdot10^{-10}$ m^2/s. Based on these values, the bound fraction was calculated as (51±3)%, which again shows that heat-induced denaturation does not introduce additional surfactant sorption.

The ^{1}H-NMR spectra of free C14-LPC and C14-LPC surfactant in the presence of WPI can be seen in Figure 5 (D). The surfactant concentration of 5 mg/ml corresponds to 10.7 mM, i.e. much larger than the CMC. The triplet at 0.92 ppm belongs to the final methyl group of the alkyl chain. The intense peak at 1.4 ppm is representative of the aliphatic methylene groups. The small peaks at 1.65 ppm and 2.45 ppm belong to methylene groups in β and in α position to the carboxyl group, respectively, whereas the peak at 3.29 ppm is due to the three methyl groups bound to nitrogen. The peak around 3.74 ppm comes from the methylene group which is directly bound to a nitrogen atom. The quintet around 3.92 ppm is due to the proton which is present on the chiral carbon. The other 4 signals in the range 3.98-4.23 ppm are due to diastereotopic protons directly bound to the chiral centre. Finally, the peak at 4.36 ppm belongs to the methylene group directly bound to the phosphate group.

In Table 3 and 4, the chemical shift values for both free C14-LPC and C14-LPC in the presence of WPI are reported. Table 3 shows that the chemical shift difference in the presence versus absence of WPI is low for all protons belonging to the hydrophilic part of the lysophospholipid (first 6 columns), whereas a significant effect is observed for the protons of the hydrophobic part (i.e. last 4 columns). This is a clear indication of protein-lysophospholipid interaction by hydrophobic effects. The relative peak area values lead basically to the same conclusion, with the largest effect (i.e. more than halved) for the myristoyl methylene protons.

Considering C14-LPC without WPI, as well as WPI with C14-LPC, highly similar diffusion coefficients were observed. Hence, the dimensions of the diffusing units of proteins and surfactants must be of the same order of magnitude, which follows logically from the fact that the surfactant's CMC is largely surpassed. Indeed, micelles at low concentration are spherical aggregates whose radius is of the order of magnitude of some nm, which is quite similar to the dimensions of globular proteins. For this reason, it is not possible to evaluate the fraction of surfactant bound to the protein. On the other hand, the downfield shift of the resonances of the hydrophobic part of this surfactant, as well as the reduction in their relative peak area indicates that protein-surfactant interactions do occur also in this case.

4. Conclusions

Rheological measurements revealed that the addition of lysolecithin reduced the complex modulus of heat-induced WPI gels, which pointed towards an enhanced heat stability of the whey proteins against aggregation. The gelation curves (representing the complex modulus G* versus time) of the samples containing 2.75% WPI and 1% of surfactant showed that no gelation occurred upon addition of the anionic surfactants sodium dodecyl sulfate (SDS) or sodium laurate. On the other hand, the complex modulus of the WPI gels was increased in the presence of sucrose palmitate, sucrose oleate and sucrose laurate, whereas POE-based nonionic surfactants, such as Tween20, Tween80 and Brij78, had a smaller effect on WPI gelation as compared to lysolecithin.

As lysolecithin addition significantly improved the residual whey protein content in the supernatant after heating, it follows that whey protein aggregation intensity was less pronounced. The residual protein content in the presence of the above-mentioned anionic surfactants was roughly twice the residual protein content in the presence of lecithin. Hence, these hydrophilic surfactants were even more effective in minimizing the heat-induced whey protein aggregation intensity.

The interaction between whey protein and surfactants was studied into more detail by high resolution as well as diffusion NMR. The shift of the peak positions of the hydrophobic parts of surfactants indicated that these groups resided in a different environment upon whey protein addition. In addition, diffusion measurement revealed that a large portion of surfactant was protein-bound even if the protein was not thermally denatured.

Overall, the results indicated that hydrolyzed lecithin has a significant protective effect on whey proteins against heat-induced aggregation. However, the observed effect was not limited to lecithin, since similar or even larger effects could be observed for alternative surfactants, such as sodium laurate.

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6. References

Anema, S. G. (1997). The effect of chymosin on κ-casein-coated polystyrene latex particles and bovine casein micelles. *International Dairy Journal, 7*, 553-558.

Arakawa, T. and Kita, Y. (2000). Protection of bovine serum albumin from aggregation by Tween 80. *Journal of Pharmaceutical Sciences, 89*, 646-651.

Bowland, E. L., Foegeding, E. A. and Hamann, D. D. (1995). Rheological analysis of anion-induced matrix transformations in thermally-induced whey-protein isolate gels. *Food Hydrocolloids, 9*, 57-64.

Brown, R. J. (1988). Milk coagulation and protein denaturation. In N.P. Wong, R. Jenness, M. Keeney & E.H. Marth, *Fundamentals of Dairy Chemistry* (pp. 583-608), New York, USA: Van Nostrand, Reinhold & Co.

Bryant, C.M. and McClements, D.J. (2000). Influence of NaCl and CaCl2 on cold-set gelation of heat-denatured whey protein. *Journal of Food Science*, 65, 801-804.

Chen, J. S. and Dickinson, E. (1995). Protein/surfactant interfacial interactions. 2. Electrophoretic mobility of mixed protein plus surfactant systems. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 100*, 267-277.

Chen, J. S. and Dickinson, E. (1998). Viscoelastic properties of protein-stabilized emulsions: Effect of protein-surfactant interactions. *Journal of Agricultural and Food Chemistry, 46*, 91-97.

Chen, J. S., Dickinson, E., Langton, M. and Hermansson, A. M. (2000). Mechanical properties and microstructure of heat-set whey protein emulsion gels: Effect of emulsifiers. *Lebensmittel-Wissenschaft und Technologie - Food Science and Technology, 33*, 299-307.

Clark, A. H., Kavanagh, G. M. and Ross-Murphy, S. B. (2001). Globular protein gelation - theory and experiment. *Food Hydrocolloids, 15*, 383-400.

Connell, M. A., Bowyer, P. J., Adam Bone, P., Davis, A. L., Swanson, A. G., Nilsson, M. and Morris, G. A. (2009). Improving the accuracy of pulsed field gradient NMR diffusion experiments: Correction for gradient non-uniformity. *Journal of Magnetic Resonance, 198*, 121-131.

de la Fuente, M. A., Singh, H. and Hemar, Y. (2002). Recent advances in the characterisation of heat-induced aggregates and intermediates of whey proteins. *Trends in Food Science and Technology, 13*, 262-274.

Dickinson, E. and Hong, S. T. (1995). Influence of water-soluble nonionic emulsifier on the rheology of heat-set protein-stabilized emulsion gels. *Journal of Agricultural and Food Chemistry, 43*, 2560-2566.

Dickinson, E. and Yamamoto, Y. (1996a). Effect of lecithin on the viscoelastic properties of beta-lactoglobulin-stabilized emulsion gels. *Food Hydrocolloids, 10*, 301-307.

Dickinson, E. and Yamamoto, Y. (1996b). Viscoelastic properties of heat-set whey protein-stabilized emulsion gels with added lecithin. *Journal of Food Science, 61*, 811-816.

Fox, P. F. (1981). Heat-induced changes in milk preceding coagulation. *Journal of Dairy Science, 64*, 2117-2137.

Fox, P. F. (2003). Milk proteins: general and historical aspects. In P.F. Fox & P.L.H. McSweeney, *Advanced Dairy Chemistry*. Vol. 1- Proteins (pp.1-42), New York: Kluwer Academic/Plenum Publisher.

Giroux, H. J. and Britten, M. (2004). Heat treatment of whey proteins in the presence of anionic surfactants. *Food Hydrocolloids, 18*, 685-692.

Goddard, E. D. and Ananthapadmanabhan, K. P. (1993). *Interactions of surfactants with polymers and proteins*, CRC Press, Boca Raton, FL.

Hardy, E. E., Sweetsur, A. W. M., West, I. G. and Muir, D. D. (1985). Heat stability of concentrated milk: enhancement of initial heat stability by incorporation of food grade lecithin. *Journal of Food Technology, 20*, 97-105.

Hashimoto, M. and Sakata, K. (1995). Solvent effects on proton nmr chemical-shifts of macrocyclic and nonmacrocyclic compounds employed with n-h functional-group. *Analytical Sciences, 11*, 631-635.

Havea, P. (1998). *Studies on heat-induced interactions and gelation of whey protein. Massey University, Palmerston North, New Zealand. Ph.D. thesis.*

Havea, P., Carr, A. J. and Creamer, L. K. (2004). The roles of disulphide and non-covalent bonding in the functional properties of heat-induced whey protein gels. *Journal of Dairy Research, 71*, 330-339.

Hoffmann, M. A. M. and van Mil, P. J. J. M. (1997). Heat-induced aggregation of β-lactoglobulin: Role of the free thiol group and disulfide bonds. *Journal of Agricultural and Food Chemistry, 45*, 2942-2948.

Ikeda, S. and Foegeding, E. A. (1999a). Dynamic viscoelastic properties of thermally induced whey protein isolate gels with added lecithin. *Food Hydrocolloids, 13*, 245-254.

Ikeda, S. and Foegeding, E. A. (1999b). Effects of lecithin on thermally induced whey protein isolate gels. *Food Hydrocolloids, 13*, 239-244.

Ikeda, S., Foegeding, E. A. and Hardin, C. C. (2000). Phospholipid/fatty acid-induced secondary structural change in beta-lactoglobulin during heat-induced gelation. *Journal of Agricultural and Food Chemistry, 48*, 605-610.

1 Jeurnink, T. J. M. and de Kruif, K. G. (1993). Changes in milk on heating: viscosity
2 measurements. *Journal of Dairy Research, 60*, 139-150.

3 Johnson Jr, C. S. (1999). Diffusion ordered nuclear magnetic resonance spectroscopy: principles
4 and applications. *Progress in Nuclear Magnetic Resonance Spectroscopy, 34*, 203-256.

5 Jones, M. N. (1992). Surfactant interactions with biomembranes and proteins. *Chemical Society*
6 *Reviews, 21*, 127-137.

7 Jost, R., Dannenberg, F. and Rosset, J. (1989). Heat-set gels based on oil/water emulsions: an
8 application of whey protein functionality. *Food Microstructure, 8*, 23-28.

9 McCrae, C. H. (1999). Heat stability of milk emulsions: phospholipid-protein interactions.
10 *International Dairy Journal, 9*, 227-231.

11 McSwiney, M., Singh, H., Campanella, O. and Creamer, L. K. (1994). Thermal gelation and
12 denaturation of bovine beta-lactoglobulin-A and beta-lactoglobulin-B. *Journal of Dairy*
13 *Research, 61*, 221-232.

14 Mine, Y., Chiba, K. and Tada, M. (1993). Effect of phospholipids on conformational change and
15 heat stability of ovalbumin. Circular dichroism and nuclear magnetic resonance studies.
16 *Journal of Agricultural and Food Chemistry, 41*, 157-161.

17 Mulvihill, D. M. and Kinsella, J. E. (1987). Gelation characteristics of whey proteins and β-
18 lactolgobulin. *Food Technology, 41*, 102-111.

19 Oakes, J. (1974). Protein–surfactant interactions. *Journal of the Chemical Society. Faraday*
20 *Transaction 1, Journal of Physical Chemistry, 70*, 2200-2209.

21 Puyol, P., Perez, M. D., Peiro, J. M. and Calvo, M. (1994). Effect of binding of retinol and palmitic
22 acid to bovine β-lactoglobulin on its resistance to thermal denaturation. *Journal of Dairy*
23 *Science, 77*, 1494-1502.

Roth, S., Murray, B. S. and Dickinson, E. (2000). Interfacial shear rheology of aged and heat-treated β-lactoglobulin films: Displacement by nonionic surfactant. *Journal of Agricultural and Food Chemistry, 48*, 1491-1497.

Schacterle, G. R. and Pollack, R. L. (1973). A simplified method for the quantitative assay of small amounts of protein in biologic material. *Analytical Biochemistry, 51*, 654-655.

Singh, H. and Havea, P. (2003). Thermal denaturation, aggregation and gelation of whey. In: *Advanced Dairy Chemistry*. Vol. 1 - Proteins, Fox, P. F. and McSweeney, P. L. H. eds., Kluwer Academic/Plenum Publishers, New York, 1261-1282.

Söderman, O., Stilbs, P. and Price, W. S. (2004). NMR studies of surfactants. *Concepts in Magnetic Resonance Part A, 23A*, 121-135.

Tran Le, T., El-Bakry, M., Neirynck, N., Bogus, M., Hoa, H. D. and Van der Meeren, P. (2007). Hydrophilic lecithins protect milk proteins against heat-induced aggregation. *Colloids and Surfaces B: Biointerfaces, 60*, 167-173.

Van der Meeren, P., Vanderdeelen, J. and Baert, L. (1988). Quantification of phospholipid phosphorus in sub-micromolar concentrations. *Bulletin des Sociétés Chimiques Belges, 97*, 413-418.

Van der Meeren, P., El-Bakry, M., Neirynck, N. and Noppe, P. (2005). Influence of hydrolysed lecithin addition on protein adsorption and heat stability of a sterilised coffee cream simulant. *International Dairy Journal, 15*, 1235-1243.

van Nieuwenhuyzen, W. and Szuhaj, B. F. (1998). Effects of lecithins and proteins on the stability of emulsions. *Lipid - Fett, 100*, 282-291.

7. Tables

Table **1**. Chemical shift values (ppm) of selected SDS and Na-laurate protons in the absence and presence of WPI. In the latter case, measurements were performed both before and after heating/cooling cycle.

	α	β	$(CH_2)_n$	ω
Free SDS	4.08	1.72	1.35	0.93
SDS + WPI	4.04	1.66	1.25	0.86
SDS + WPI after heating	4.04	1.66	1.25	0.86
Free Na-laurate	2.20	1.58	1.32	0.89
Na-laurate + WPI	2.19	1.57	1.25	0.85
Na-laurate + WPI after heating	2.19	1.55	1.26	0.84

Table 2. Relative peak areas of selected SDS and Na-laurate protons in the absence and presence of WPI. In the latter case, measurements were performed both before and after heating/cooling cycle.

	α	β	$(CH_2)_n$	ω	NaAc
Theoretical	2	2	18	3	
Free SDS	4.30	4.43	40.78	6.31	1
SDS + WPI	2.64	3.23	21.17	4.17	1
SDS + WPI after heating	2.48	2.89	18.92	3.64	1
Theoretical	2	2	16	3	-
Free Na-Laurate	3.73	4.00	30.09	5.64	1
Na-Laurate+WPI	2.96	3.41	21.94	5.82	1
Na-laurate+WPI after heating	3.57	3.83	23.71	5.29	1

Table 3. Chemical shift values (ppm) of C14-LPC protons in the absence and presence of WPI

	α'	RCH_2OPO_3R	RCH_2OCOR	R_2HCOH	β'	$N(CH_3)_3$	α	β	$(CH_2)_n$	ω
Free C14-LPC	4.36	3.98-4.16	4.08-4.23	3.91	3.74	3.29	2.44	1.65	1.35	0.92
C14-LPC + WPI	4.36	3.99-4.15	4.08-4.22	3.92	3.72	3.26	2.40	1.61	1.29	0.88

Table 4. Relative peak areas of C14-LPC protons in the absence and presence of WPI

	α'	RCH_2OPO_3R	RCH_2OCOR	R_2HCOH	β'	$N(CH_3)_3$	α	β	$(CH_2)_n$	ω
Theoretical	*2*	*1-1*	*1-1*	*1*	*2*	*9*	*2*	*2*	*20*	*3*
Free C14-LPC	2.27	1.05-1.00	1.07-0.97	1.00	2.50	10.45	2.19	2.24	23.24	3.43
C14-LPC + WPI	1.39	0.66-0.59	0.73-0.66	0.65	1.11	4.60	1.42	1.85	10.27	3.02

8. Figure captions

Figure 1. Complex modulus, G* (A), and phase angle (B) of a 2.75% (w/v) WPI dispersion in Ca-imidazole buffer at pH 6.55 containing 0% and 1% (w/v) surfactant as a function of time during a heating and cooling cycle, observed by oscillation rheology. The solid line represents the temperature history.

Figure 2. Representative examples of stress-strain curves of 2.75% (w/v) WPI gels in the absence and presence of 1% phospholipids (PL; hydrolyzed lecithin) after 5 (A), 10 (B) and 15 (C) minutes heating at 80°C and subsequent cooling to room temperature using tap water.

Figure 3. Residual protein solubility (A) and residual carbon solubility (B) of mixtures of 2.75% (w/v) WPI and 1% (w/v) phospholipids or 1% (w/v) of alternative surfactants upon heating at 80ºC for 1 and 2 minutes (relative to the corresponding unheated samples).

Figure 4. Protein recovery (A) and phospholipid recovery (B) in a mixture of 2.75% (w/v) WPI without and with 1% (w/v) lecithin as well as 1% (w/v) C14-LPC, C16-LPC and C18-LPC upon heating at 80ºC for 1 and 2 minutes (relative to the corresponding unheated samples).

Figure 5. ^{1}H-NMR spectra for WPI (A), free SDS and a mixture of SDS and WPI (B), free Na-laurate and a mixture of Na-laurate and WPI (C) and free C14-LPC and a mixture of WPI and C14-LPC (D), all measured before heating.

Figure 1

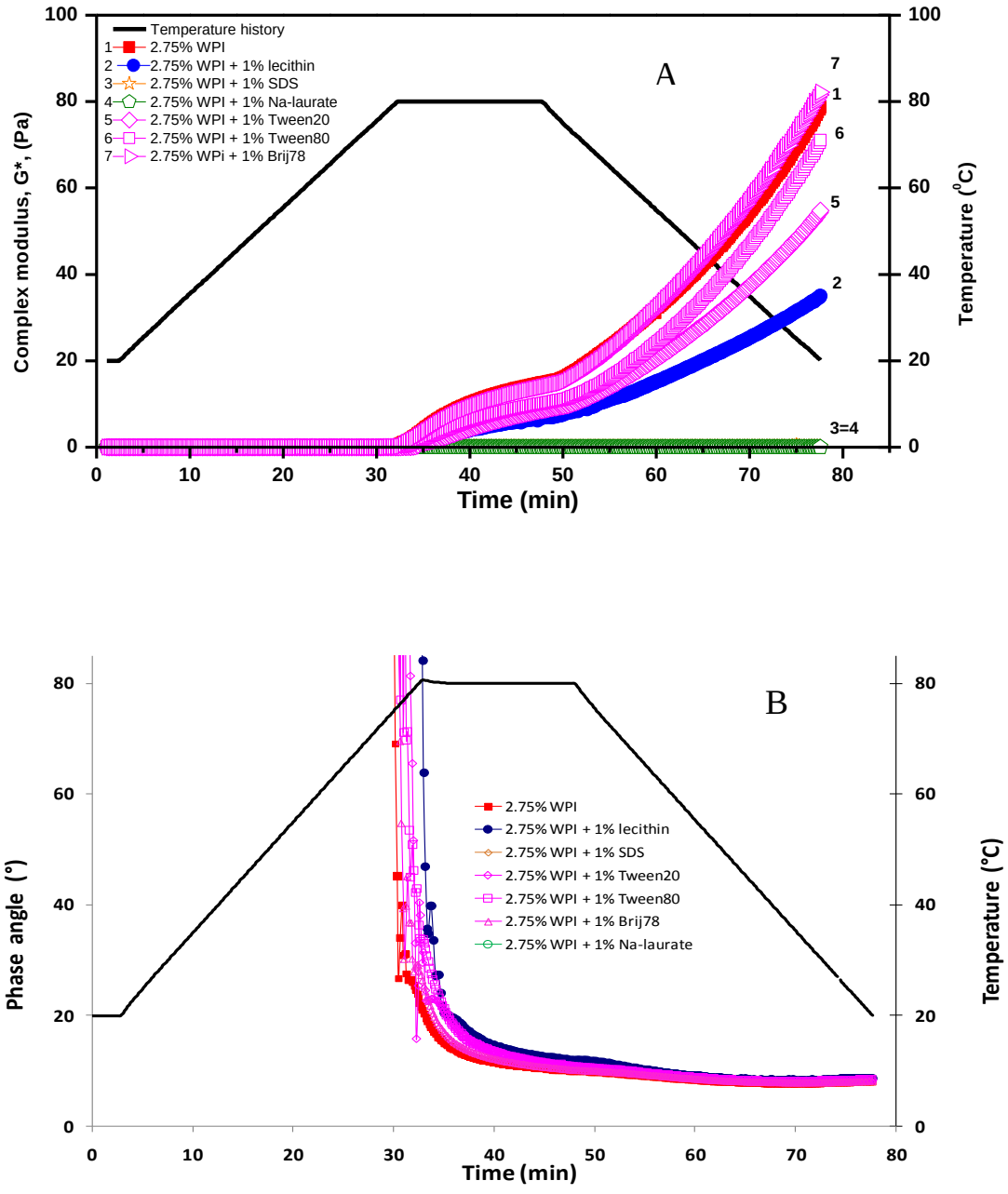

A
Temperature history
1 2.75% WPI
2 2.75% WPI + 1% lecithin
3 2.75% WPI + 1% SDS
4 2.75% WPI + 1% Na-laurate
5 2.75% WPI + 1% Tween20
6 2.75% WPI + 1% Tween80
7 2.75% WPi + 1% Brij78
Complex modulus, G*, (Pa)
Temperature (°C)
Time (min)
3=4
B
2.75% WPI
2.75% WPI + 1% lecithin
2.75% WPI + 1% SDS
2.75% WPI + 1% Tween20
2.75% WPI + 1% Tween80
2.75% WPI + 1% Brij78
2.75% WPI + 1% Na-laurate
Phase angle (°)
Temperature (°C)
Time (min)

Figure 2

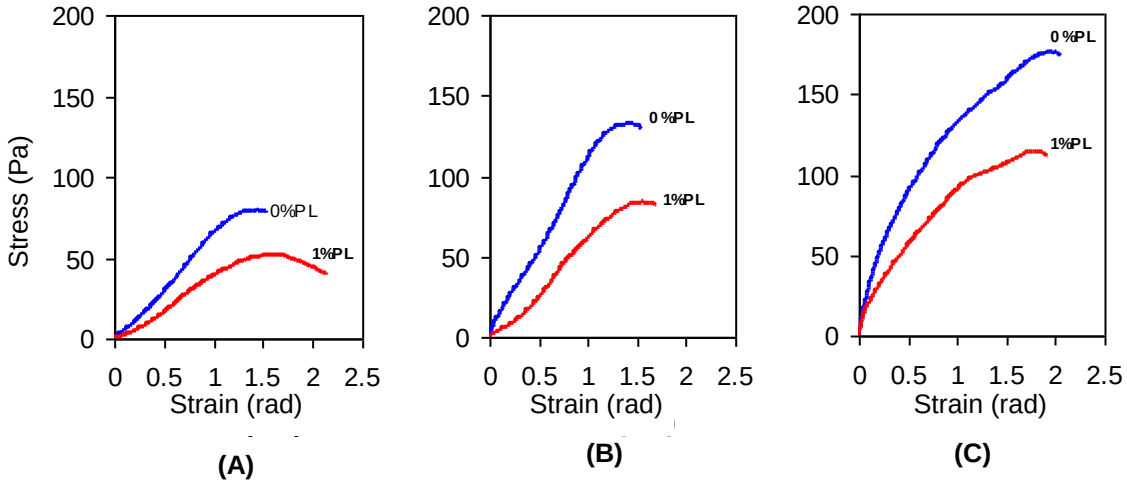


Figure 3

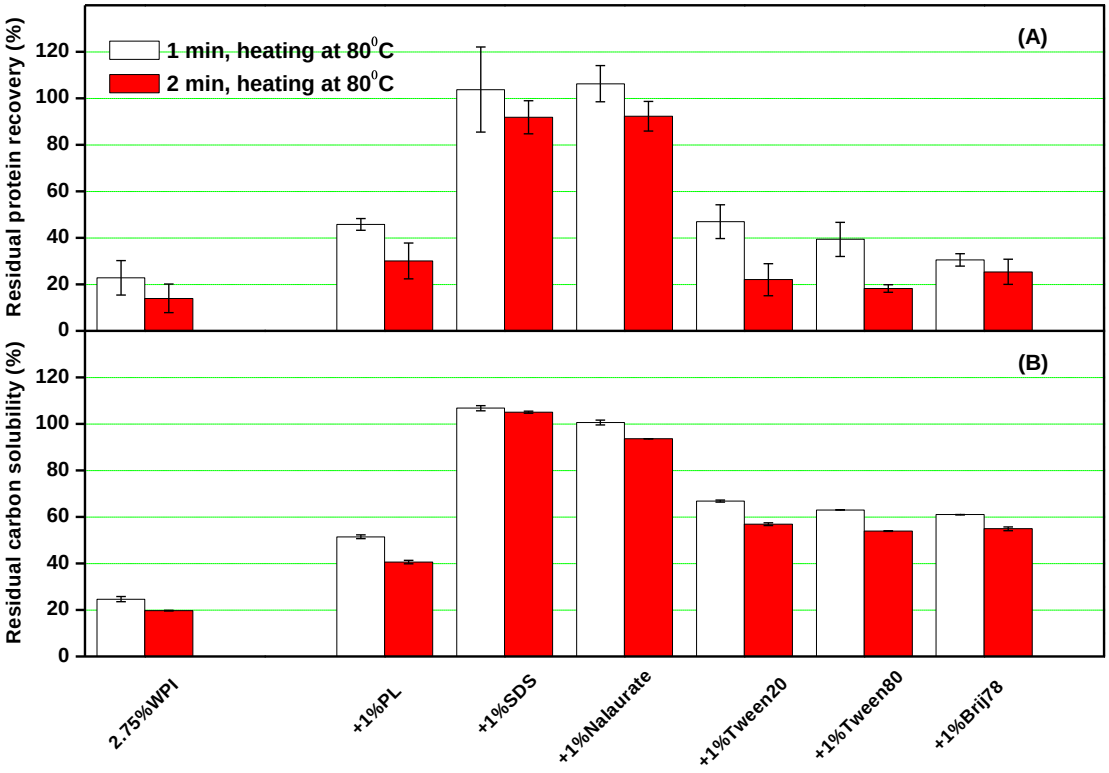

1 min, heating at 80°C
2 min, heating at 80°C
(A)
(B)
Residual protein recovery (%)
Residual carbon solubility (%)
0
20
40
60
80
100
120
2.75%WPI
+1%PL
+1%SDS
+1%Nalaurate
+1%Tween20
+1%Tween80
+1%Brij78

Figure 4

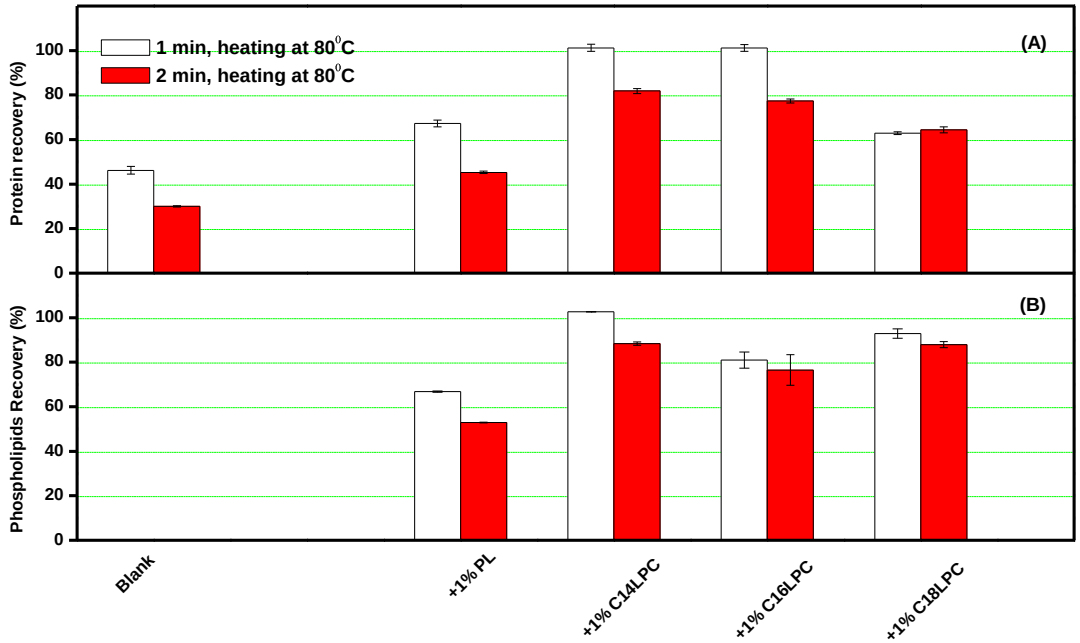
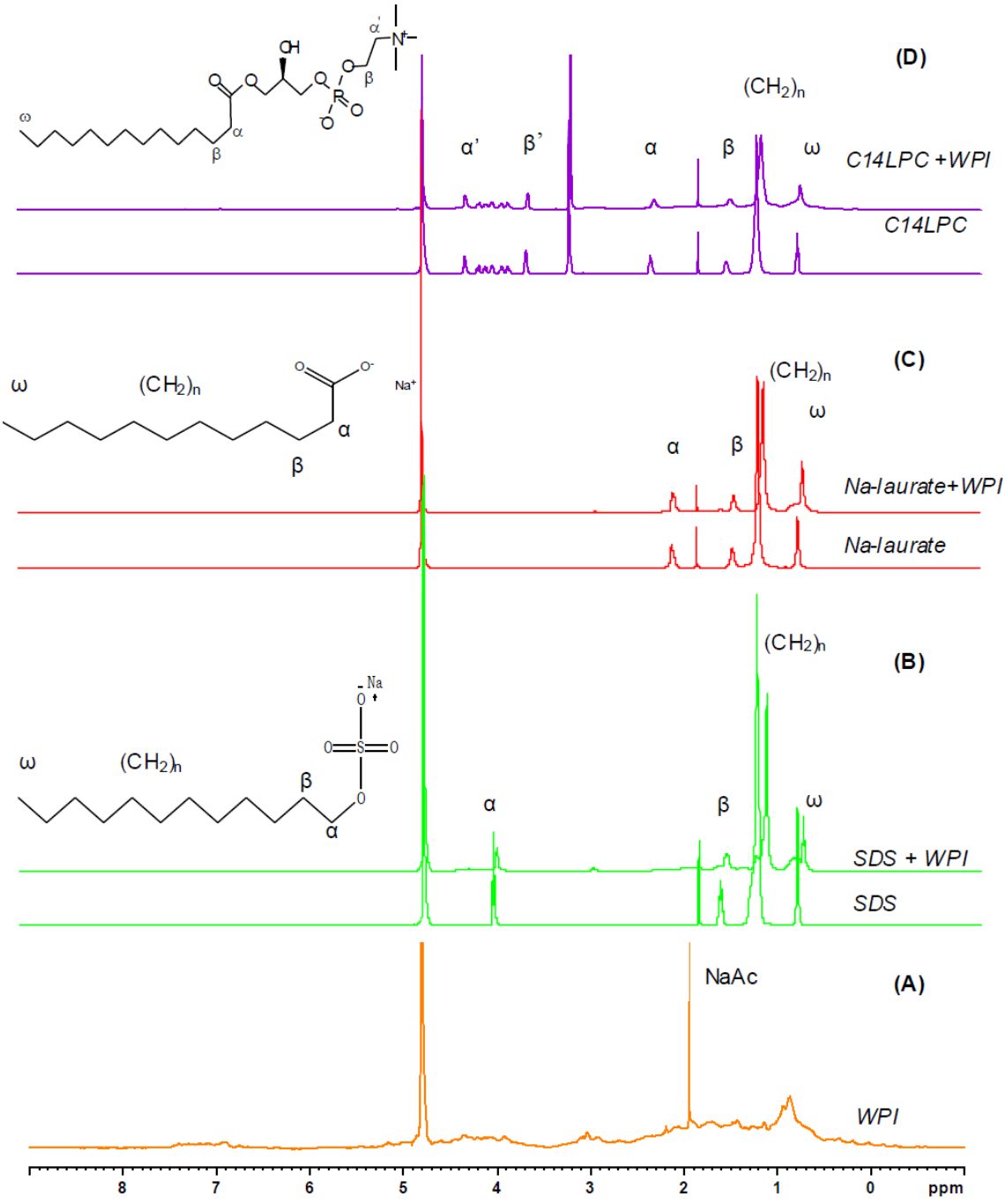

1 min, heating at 80°C
2 min, heating at 80°C
(A)
Protein recovery (%)
0
20
40
60
80
100
(B)
Phospholipids Recovery (%)
0
20
40
60
80
100
Blank
+1% PL
+1% C14LPC
+1% C16LPC
+1% C18LPC

Figure 5


(D)
(CH2)n
α'
β'
α
β
ω
C14LPC +WPI
C14LPC
(C)
ω
(CH2)n
Na+
α
β
Na-laurate+WPI
Na-laurate
(B)
ω
(CH2)n
α
β
ω
SDS + WPI
SDS
(A)
NaAc
WPI
8
7
6
5
4
3
2
1
0
ppm