JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Changes and Roles of Secondary Structures of Whey Protein for the Formation of Protein Membrane at Soy Oil/Water Interface under High-Pressure Homogenization

SANG-HO LEE,*,† THIERY LEFÈVRE,§ MURIEL SUBIRADE,§ AND PAUL PAQUIN§

Busan regional Korea Food & Drug Administration, 123-7 Yongdang-dong, Busan, Korea and Centre de Recherches en Sciences et Technologie du Lait (STELA), Faculté des sciences de l'agriculture et l'alimentation, Pavillon Paul-Comtois, Université Laval, Sainte-Foy, Québec, Canada

The conformational changes of whey proteins upon adsorption at the soy oil/water interface were investigated using Fourier transform infrared (FT-IR) spectroscopy. Significant changes were observed in the bands assigned to β-sheets and α-helix structures following the adsorption of proteins at the oil/water interface. The remaining interfacial proteins after Tween 20 desorption revealed small changes in β-sheet and α-helical structures, whereas in the desorbed whey proteins the unordered structures largely increased, and β-sheet structures almost disappeared. These FT-IR results provide important knowledge about the conformational modifications in whey proteins occurring upon adsorption at the oil/water interface. Finally, specific conformational changes are necessary to stabilize emulsions: adsorption-induced unfolding, increase in α-helical structures to establish interactions with the oil phase, and aggregation between adsorbed whey proteins to form protein membranes. Moreover, the structural changes in whey protein adsorbed at the oil/water interface under high-pressure homogenization are irreversible.

KEYWORDS: High-pressure homogenizer; emulsion; whey protein; interfacial structure; protein adsorption

INTRODUCTION

In the food and dairy industry, emulsions and their byproduct are stabilized by surface-active constituents (i.e., low molecular weight surfactants and proteins). In many studies, changes in the stability of protein-stabilized emulsions are explained by the droplet size and the protein concentration on droplets (protein load) (*1, 2*). It has recently been emphasized that the structure and properties of proteins adsorbed at interfaces are also an important factor for determining emulsion stability (*3–5*). Given their amphiphilic nature, resulting from polar and nonpolar functional groups, proteins can adsorb at oil/water and air/water interfaces. The adsorbed proteins can form a protective membrane against destabilizing phenomena such as flocculation and coalescence, because proteins cause steric and electrostatic stabilization that can prevent the aggregation of droplets. It is generally admitted that there are differences between the conformation of adsorbed proteins at the interface and that of native proteins in the bulk solution. Some studies (*6–8*) have provided particular structural properties of proteins adsorbed at the interface. Moreover, there may be the relation between protein conformation and emulsion stability. The investigation on the conformational changes of proteins after adsorption at the oil/water interface could be an important key to understanding the stabilizing process of emulsions by proteins.

To obtain information on the structure of proteins adsorbed at interfaces, the system similar to an emulsion interface, such as the phospholipids monolayer, has been used. However, only a few experimental techniques can be used to determine protein structure in real emulsion systems because of their two-phase heterogeneity (e.g., lipid and serum phases). To overcome this problem, previous studies have tried to mimic interfacial conditions encountered in emulsions, through the use of hydrophobic solvents, nonplanar liquid–liquid interfaces, polymer solid surfaces, or air/water interfaces. Jackson and Mantsch (*9*) showed the structural protein and polypeptide changes in halogenated alcohols that could mimic the membrane environment. Kondo et al. (*3*) used Ultrafine silica particles, and Maste et al. (*10*) used hydrophobic particles of a tetrafluorethylene and perfluorovinylether copolymer to determine the structural changes in several proteins during adsorption. Attenuated total reflection (ATR) infrared spectroscopy has also been used to study the adsorption-induced structural changes in proteins. Green et al. (*8*) investigated the possible interfacial effects of protein unfolding and aggregation by adsorbing hen egg lysozymes and bovine serum albumin onto an ATR crystal. In

* Corresponding author. Telephone: +82-51-610-6182, fax: +82-51-610-6199, e-mail address: salutsh@hanmail.net.
† Busan Regional Korea Food & Drug Administration.
§ Centre de Recherches en Sciences et Technologie du Lait (STELA).

10.1021/jf0726076 CCC: $37.00 © 2007 American Chemical Society
Published on Web 11/29/2007

spite of such results, information on conformational changes in proteins at real oil/water interfaces of emulsion systems remains limited.

In the present study, conformational changes in whey proteins adsorbed at the oil/water interface of real emulsions were investigated by FT-IR spectroscopy. Progress in IR spectroscopic instrumentation generated high expectations for the analysis of the secondary structures of proteins. Among the many benefits of FT-IR is its capacity to study protein structure in a large number of environments, including optically turbid media. In addition, the potential usefulness of amide I spectra for the quantitative and qualitative determination of the secondary structure of proteins under various conditions allows for the accurate measurement of conformational changes in proteins (*9*). Then, FT-IR spectroscopy is one of the rare techniques that can be used to determine the protein structure at interfacial conditions. Some authors recently studied the spectral and conformational changes in proteins after adsorption in emulsion systems using FT-IR technique (*11–13*). In brief, through FT-IR spectroscopy, it may be possible to detect adsorption-induced changes in the secondary structure of whey proteins at the soy oil/water interface of emulsions. In addition, the competitive adsorption phenomenon between proteins and small molecule emulsifier (Tween 20) will be used to obtain more information on the interfacial structure of whey proteins. Considerable progress has been made in recent years in the area of competitive adsorption of milk proteins and small molecule emulsifiers. Although many studies (*14–16*) report that Tween 20 can replace most adsorbed proteins, some proteins remain at the oil/water interface. In this state, oil droplets are stabilized by fewer proteins and Tween 20, but the remaining proteins may have more effective interactions or/and structures than displaced ones. The determination of this structure may provide insight into more effective methods of stabilizing emulsions. Also, proteins desorbed by Tween 20 could provide some information for conformation that are modified by adsorption at the oil/water interface. There are no studies on the structural modifications in adsorbed proteins after desorption by Tween 20. The analysis of such structural changes could provide a better understanding of the essential adsorption-induced structure modifications at interfaces.

Given that there is little information on the conformational changes of proteins after adsorption at the oil/water interface of real emulsions and that it has an important relationship with emulsion stability, we investigated how proteins are adsorbed and how their structures are changed at the oil/water interface of emulsions. We have focused on the structure of the proteins that are desorbed, as well as those that remained at the interface after competitive adsorption with Tween 20. The secondary structure of whey proteins adsorbed at the soy oil/water interface was investigated using FT-IR spectroscopy. The purpose of this paper is to explore the adsorption-induced conformational transition of whey proteins at the soy oil/water interface and to establish a relationship between structural properties of adsorbed proteins and emulsion stability.

MATERIALS AND METHODS

Materials. Whey protein isolate (WPI, 95% protein content) was obtained from Davisco Food International Inc. (Le Sueur, Minnesota, USA) and was used without further purification. Soy oil was purchased from a local store. Tween 20 was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Emulsion Preparations. WPI was dissolved in deionized water at a 0.5% (w/w) concentration and was stirred for 1 h at room temperature. The pH of the protein solution was adjusted with 0.1 M HCl and 0.1 M NaOH to pH 6.0, and this protein solution was used as the aqueous phase. Premixed emulsion was prepared by mixing 90% (w/w) aqueous phase with 10% (w/w) soy oil using Ultraturrax (Hansen Co., Germany). Emulsions were produced with a high-pressure homogenizer, Emulsi-Flex-C50 (Avestin Inc., Ottawa, Canada). A first pass was operated at 500 bar and then a second one at 30 bar. To prevent an increase in the temperature of the final product, cold water was circulated at the outlet of the homogenizing valve.

Droplet Size Measurement. The emulsions were diluted 500× with a dissociating buffer (pH 7.0) containing 8 M urea, 50 mM EDTA, and 10 mM β-mercaptoethanol to disperse the aggregated/flocculated oil droplets formed during emulsification (*17*). This diluted solution was then stirred for 5 min. Emulsion globule size was estimated by photon correlation spectroscopy (PCS: Pacific Scientific, Hiac/Royce Instruments Division, Model 370, CA, USA).

Protein Load. To measure the concentration of whey proteins adsorbed on oil droplets, the method of Zahar and Smith (*18*) was used with modification. Fresh emulsion was centrifuged at 40 000*g* for 60 min to separate the serum and cream phrase. The separated cream layer was removed with a spatula and was stored at 4 °C until protein and oil content were determined. Protein and oil content in the cream phases were measured by the Kjeldahl and Mojonnier methods, respectively. Protein load was calculated as protein quantity (mg) adsorbed per m^2 of interface.

Separation of Cream Phase. Fresh emulsions were centrifuged at 40 000 g for 60 min to separate the serum and cream phrases. The separated cream layer was removed using a spatula and was stored at 4 °C until it was analyzed for the structure of whey proteins adsorbed at the interface. The whey proteins fraction adsorbed at the oil/water interfaces could be obtained by this process. The cream phase was used for FT-IR spectroscopic measurement. A part of the collected cream phase was washed twice with deionized water to remove residual unadsorbed whey proteins from the aqueous phase. The washed cream phase was dispersed in deionized H_2O at a 10% (w/w) concentration with a magnetic stirrer to ensure that the emulsion's aqueous phase would be free of unadsorbed proteins.

Displacement of Adsorbed Proteins. To desorb the adsorbed whey proteins from the interface, 8 mM Tween 20 was added to both the fresh and the washed emulsions. The emulsion containing Tween 20 was stirred for 1 h for competitive adsorption with whey proteins to take place. After the desorption process, the emulsion sample was centrifuged at 40 000*g* for 60 min to separate the oil droplets from the aqueous serum phase. The aqueous phase was carefully removed using a syringe, and this sample was then used to analyze the structure of desorbed whey proteins using a FT-IR spectrometer.

FT-IR. Infrared spectra were measured with a Magna 560 Nicolet Fourier transform spectrometer (Madison, WI) equipped with a liquid nitrogen cooled MCT (mercury cadmium telluride) detector. Continuous purging of dry air within the spectrometer was maintained during measurements to remove water vapor from the chamber. Samples for IR analysis were prepared between CaF_2 windows separated by polyethylene terephthalate film spacers having a 6 μm path length for the protein aqueous solutions and 23 μm for the cream phases, respectively. For each spectrum, a 128 scan interferogram was collected with a 2 cm^{-1} resolution, from 4000 to 1000 cm^{-1}. The solvent subtraction and Fourier self-deconvolution, which were used to study the amide I region of whey proteins, were performed with the software provided with the spectrometer (Omnic software). Spectra of media without proteins were recorded as reference spectra, and the subtraction of reference spectrum from samples spectrum was carried out. To obtain the spectrum of adsorbed whey proteins in the cream phase, the spectrum of soy oil was subtracted from the sample spectrum. The spectrum of emulsion made with Tween 20 as surfactant was used for the reference spectrum in emulsions after Tween 20 desorption. The overlapped components in the broad amide I band contour were identified through Fourier self-deconvolution.

RESULTS AND DISCUSSION

Structural Changes in Whey Proteins Adsorbed at Interface. Before determining the behavior of whey proteins at the

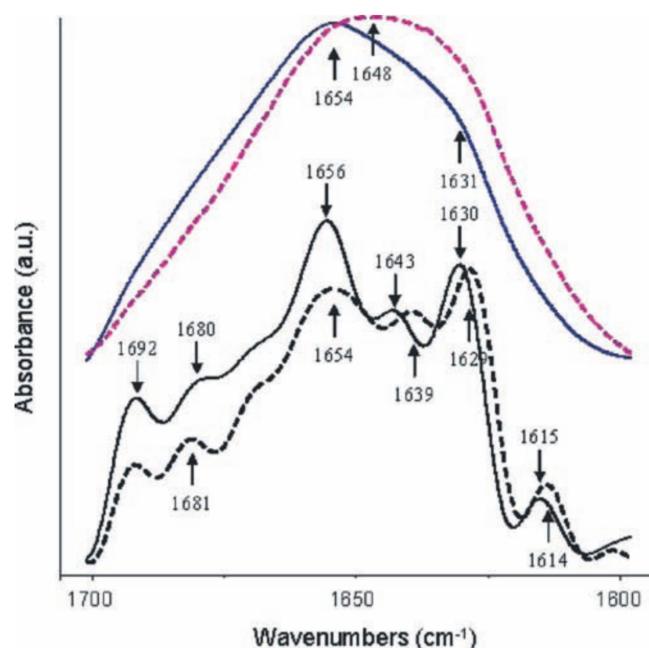

Figure 1. The original (upper spectra) and deconvoluted (lower spectra) amide I region of the IR spectra for whey proteins, in their native (dashed line) and adsorbed (solid line) states. The concentration of whey protein for the native state is 2%. Cream phase separated from emulsion is used to measure protein structure in the adsorbed state.

interface, we should consider that whey proteins are composed of several proteins such as β-lactoglobulin, α-lactalbumin, bovine serum albumin, etc., and that they have different properties to the state where the protein is individually used. However, the fact that the protein mixture is assumed to be homogeneous in behavior and major proteins of whey proteins, β-lactoglobulin and α-lactalbumin have globular structure, can help us to describe the interfacial conformation of whey proteins, even when we have a protein mixture.

Figure 1 shows the original and deconvoluted spectra of whey protein in the aqueous solution and at the oil/water interface. The amide I band consists of overlapping bands at characteristic frequencies corresponding to different types of secondary structures. In the original spectra, the amide I band of the native whey proteins exhibits maximum absorbance at 1648 cm^{-1}. After adsorption, the maximum absorbance is displaced to 1654 cm^{-1}, and the band becomes slightly narrow. This indicates that the structure of whey protein is changed after adsorption and is more ordered than in the solution.

To further resolve the overlapping bands within the amide I band, Fourier self-deconvolution is carried out. On the basis of previous IR studies of β-lactoglobulin, the major protein of whey, in H_2O, the bands observed near 1629, 1639, and 1692 cm^{-1} are due to β-sheet structures (*19*). The band at 1654 cm^{-1} is assigned to unordered and α-helical structures, and those located at 1663, 1671, and 1681 cm^{-1} are attributed to turns. It is found that the FT-IR spectrum of whey proteins in H_2O before adsorption is in good agreement with that of native β-lactoglobulin reported in previous studies. Significant spectral changes after the adsorption are also revealed between both deconvoluted spectra. In the adsorbed state, the deconvoluted spectrum in the amide I region exhibits bands that are ascribed to β-sheet (1630, 1643, and 1692 cm^{-1}), α-helix and unordered (1656 cm^{-1}), and turn structure (1669 and 1679 cm^{-1}). By comparison between the deconvoluted spectra before and after adsorption, we can observe what kind of conformational changes have to form at the interfacial layer under high-pressure homogenization. Interfacial adsorption causes two major changes in the amide I spectrum: (1) α-helix and unordered structures (1656 cm^{-1}), and (2) β-sheet structures (1630 and 1692 cm^{-1}). These adsorption-dependent changes are similar and highly reproducible in emulsions prepared at other pH values and homogenization pressures (data not shown).

There are several adsorption-induced changes in the 1654 cm^{-1} band of the native protein spectrum. It is generally shown that an unordered structure may exhibit amide I absorbance near 1652 cm^{-1}, and a band near 1658 cm^{-1} can generally be assigned to an α-helical structure (*19*). Thus, the band forming these structures is partially overlapped. Although this band is closer to the unordered structure (1654 cm^{-1}) before adsorption, adsorption causes a shift in its frequency toward that of the α-helical structure (1656 cm^{-1}). Moreover, its intensity is significantly increased, and it becomes sharper in the adsorbed state. These modifications may be indicative of an increased content of α-helical structure as whey proteins adsorb at the oil/water interface. It is reported that changes in a predominantly α-helical configuration were found in various interface circumstances, such as organic solvents (*9, 20, 21*) and phospholipids monolayer (*22*). These indicate that the α-helical structure, which is exposed to the hydrophobic region, plays an important role in the adsorption and interaction with oil phase of emulsions.

However, from previously cited studies, it could be proposed that interfacial changes in α-helix structure may be dependent on the conditions encountered at the interface. Maste et al. (*10*) also found that the α-helix content in protein was unaltered at full surface coverage, whereas it was increased upon adsorption at low surface coverage. FT-IR spectrum results reveal behaviors and conformations that varied according to pH and protein concentration when β-lactoglobulin was adsorbed at the oil/water interface (*11*). Our study shows that the intensity of bands assigned to the α-helix and to unordered structure of the adsorbed proteins decreases with increasing homogenization pressure (data not shown).

The second important change in adsorbed whey proteins concerns the band assigned to the β-sheet structures (i.e., bands near 1630 and 1692 cm^{-1}). In fact, an adsorption-induced increase in intensity at the 1692 cm^{-1} band is observed. This band is specific to antiparallel β-sheets. The major β-sheet structure related to the band at 1629 cm^{-1} is slightly shifted to 1630 cm^{-1}. To characterize these changes in β-sheet structures in adsorbed whey proteins, we carried out two experiments. First, the thermal denaturation of whey protein in solution (H_2O at pH 6.0) was performed (**Figure 2**) to enable direct comparisons with adsorbed whey proteins. As the temperature increases, the intensity of the 1629 and 1691 cm^{-1} bands increases, whereas the intensity of the 1614 and 1642 cm^{-1} bands decreases and finally disappears at 80 °C. Heating to temperatures exceeding 65 °C causes denaturation and aggregation of whey proteins (*23*). The spectrum at 80 °C highlights the structural disruption responsible for aggregation and gelation. The spectrum at 70 °C shows changes in the β-sheet structure region that are analogous to those observed in adsorbed whey proteins, with the exception of the 1656 cm^{-1} band. This comparison also reveals that the adsorbed whey proteins undergo structural changes similar to the initial stages of thermal denaturation, and the structure of whey protein is disrupted by adsorption to form new interactions. Our results are in agreement with Fang and Dalgleish (*11*), who also compared the changes in secondary structure in β-lactoglobulin induced by adsorption and thermal treatment using FT-IR spectroscopy. They explained

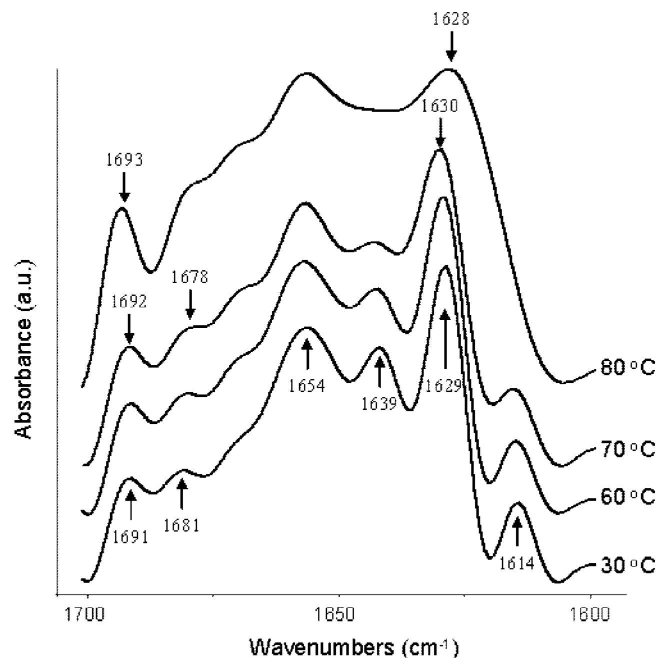

Figure 2. Deconvoluted amide I spectra of whey proteins in H_2O (10%, w/w) at pH 6.0 as a function of temperature.

that the interfacial denaturation was similar to the initial stages of heat denaturation but that the heated protein was much less structured than the adsorbed protein. Second, we have measured the interfacial changes in proteins using model emulsions with β-lactoglobulin, n-hexadecane, and D_2O. **Figure 3** presents the deconvoluted amide I spectra of β-lactoglobulin adsorbed on the surface of n-hexatetradecane, when H_2O and D_2O are used as the aqueous phase. In H_2O emulsions, spectral changes in β-lactoglobulin upon adsorption are found to be similar to those observed for whey proteins, especially the increase in intensity of the 1631 and 1687 cm^{-1} bands and the slight shift in frequency of the 1631 cm^{-1} band. In D_2O emulsions, significant adsorption-induced changes in β-sheets are also noted. However, it is pointed out that an IR spectrum of interfacial β-lactoglobulin shows intermolecular β-sheet structure with two bands at 1622 and 1680 cm^{-1} (*24*). Such intermolecular β-sheet structures are commonly found in aggregated proteins during protein gel formation, because of strong interactions between proteins. Thus, it may be that intermolecular β-sheet formation after adsorption reflects the formation of strong interactions and aggregations between neighboring adsorbed proteins to form gel-like interfacial layers. Because the two emulsions made with H_2O and D_2O are produced under identical conditions, the corresponding spectral changes should reflect the same interfacial β-lactoglobulin structure. Therefore, with emulsions made with H_2O, the changes in β-sheet structures upon adsorption are caused by the enhancement of interactions between adsorbed proteins due to formation of intermolecular β-sheet structures. At this point, we must estimate the possibility that protein structure may be altered by replacement of H_2O by D_2O. The H_2O solution is considered to be a media providing a more native environment than D_2O, but D_2O is widely used as solvent to overcome the problem related to the overlap of the H_2O spectrum with that of amide I of proteins. In D_2O solution, the hydrogen–deuterium exchange lead to a shift in frequencies because of strongly bonded hydrogen, whereas Goormaghtigh et al. (*25*) explained that it does not significantly modify the assignment of resolved components. Although the effects of this exchange on protein structural properties are not fully understood, D_2O solution could

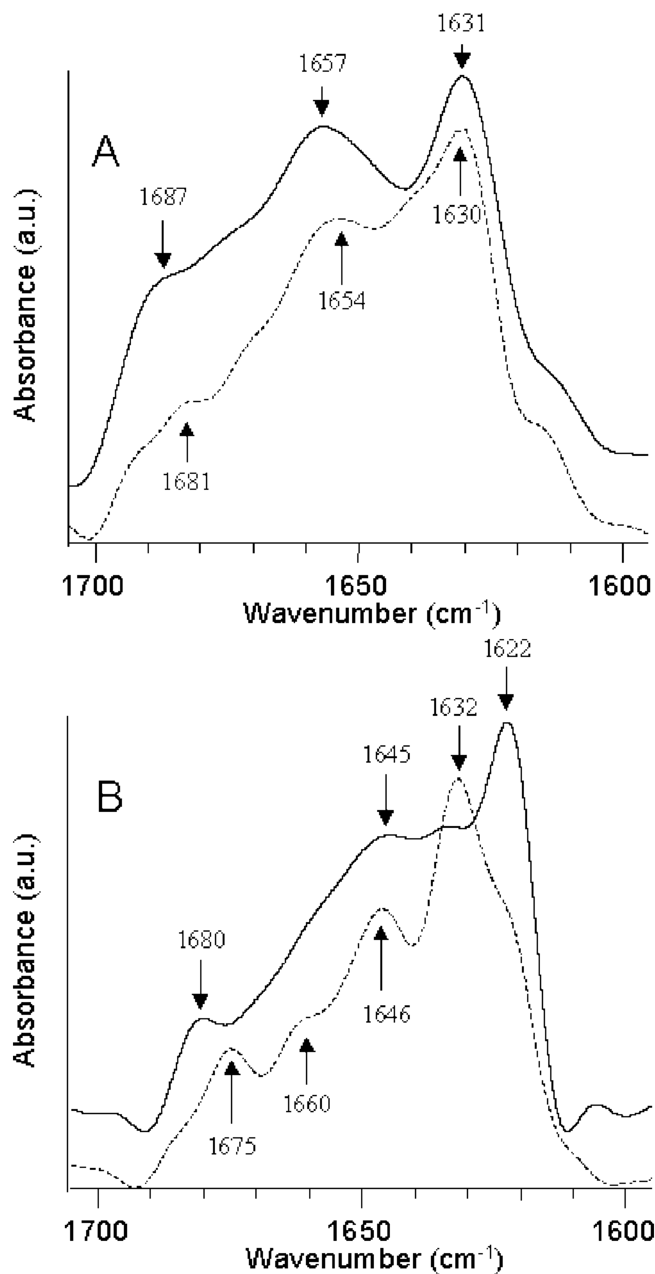

Figure 3. Deconvolved spectra in the amide I region of β-lactoglobulin in H_2O (**A**) and D_2O (**B**) adsorbed at the oil/water interface (solid lines) and in 1% aqueous solutions (dashed lines). In these emulsions, n-hexadecane was used as the lipid phase.

be a useful alternative method for exactly determining the change in protein structure.

From these results, it is reasonable to suggest that changes in β-sheet structures in whey protein (i.e., the increase in the band at 1691 cm^{-1} accompanied with that at 1630 cm^{-1} (**Figure 1**)) might show the extent of interactions between interfacial proteins. Moreover, it may be inferred from previous results that the adsorbed whey proteins could make an interfacial layer with interactions between proteins that are similar to the structure of aggregates observed during the initial stages of thermal denaturation. Other FT-IR studies revealed modifications in β-sheet structures after interfacial protein adsorption. Fang and Dalgleish (*11, 12*) reported an increase in the number of β-sheet structures in β-lactoglobulin and α-lactalbumin after adsorption, although such a change was dependent on pH and protein concentration. Corredig and Dalgleish (*26*) measured

Table 1. Effect of the Addition of Tween 20 on the Characteristics of a Soy Oil (10% wt) Emulsion[a] Stabilized with WPI (0.5 wt%)

Tween 20 concentration (mM)	protein load (mg/m^2)	droplets diameter (nm)
0	1.51	467
0.5	1.42	470
5.0	0.66	447
8.0	0.57	442

[a] Emulsion prepared at homogenization pressure of 500 bar and pH 6.0.

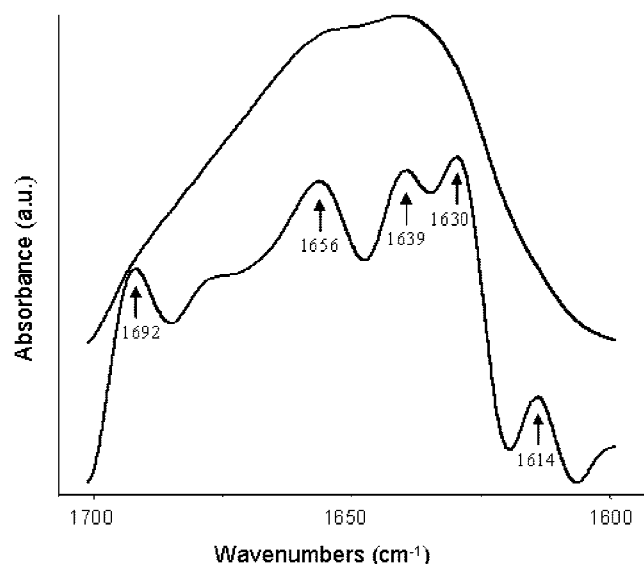

Figure 4. Original and deconvoluted amide I spectra of the whey proteins remaining in the cream phase after protein desorption by the addition of Tween 20. The emulsion was formed at pH 6.0 and at a homogenization pressure of 500 bar.

the DSC thermogram of whey proteins adsorbed in emulsion, showing that the protein is denatured by the adsorption process. Recent research with an air/water interface showed the images for the formation of elastic (gel-like) protein networks at the interface using atomic force microscopy (*5*).

Structure of Desorbed Interfacial Whey Proteins. To determine which conformational changes in whey proteins resulted in more efficient interfacial adsorption, we used the competitive adsorption of Tween 20. When 8 mM Tween 20 is added to fresh emulsion stabilized by whey proteins, approximately 60% of the interfacially adsorbed whey proteins could be extracted without changes in the droplet size (**Table 1**). **Figure 4** shows the original and deconvoluted amide I spectra of whey proteins remaining in the cream phase after Tween 20 desorption. It is found that there are additional spectral changes in the amide I region of the protein remaining at the interface. Two β-sheet components are always observed at 1630 and 1692 cm^{-1}, without any change in frequency, and the 1643 cm^{-1} band is shifted to 1639 cm^{-1}. Although the increase in β-sheet bands is also observed at the oil/water interface after adsorption, as well as after Tween 20 treatment, there is a substantial difference between the two states. In the adsorbed form, the 1630 cm^{-1} band was the single major band, whereas in the remaining proteins after Tween 20, there is no major band for the β-sheet. The 1643 cm^{-1} band in the adsorption state is shifted back to 1639 cm^{-1}, like the native form of whey protein, but the relative intensity and frequency of the 1656 cm^{-1} band in the spectrum of whey proteins remaining after Tween 20 treatment has been shown to be identical to those of adsorbed whey proteins attributing to interaction with the lipid phase.

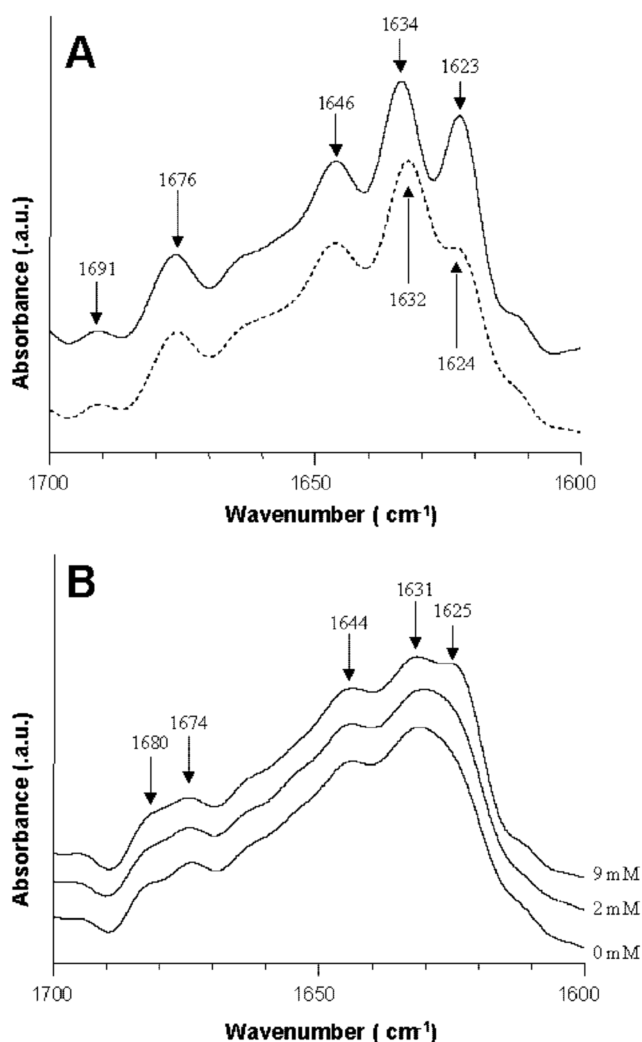

Figure 5. Deconvoluted spectra in the amide I region of β-lactoglobulin interacted with Tween 20. (**A**) Native β-lactoglobulin (1% w/w); the solid line is protein only, and the dashed line is protein with Tween 20 (9 mM). (**B**) The change in the spectrum of denaturated protein (80 °C, 10 min) as a function of Tween 20 concentration.

There are several reasons that account for the observed structural changes in proteins remaining after desorption. First, the major changes in β-sheet structure may be induced by the fact that neighboring whey proteins are removed from the interface after desorption, probably indicating that interactions between proteins are broken and that whey proteins could return to their native structures. Because it is possible for remaining whey proteins to interact with Tween 20 as well as the lipid phase, changes in interactions after desorption may induce part of the conformational changes. To verify this possibility about Tween 20, we have measured the change in secondary structure of β-lactoglobulin under the Tween 20 coexistence condition (**Figure 5**). In native β-lactoglobulin (**Figure 5A**), the addition of Tween 20 shows only an increase in the intensity of 1624 cm^{-1} band, indicating that protein forms more dimers in this state (*27*). However, it is found that there are no other significant changes in secondary structure of β-lactoglobulin. In the case of partially denaturated protein (thermal treatment at 80 °C for 10 min, **Figure 5B**), 2 mM Tween 20 has no effect on protein structure, but 9 mM Tween 20 increase the intensity of 1625 and 1680 cm^{-1}. This change shows that high concentrations of Tween 20 could form aggregation from denaturated proteins. Therefore, the modification of protein structure induced by the

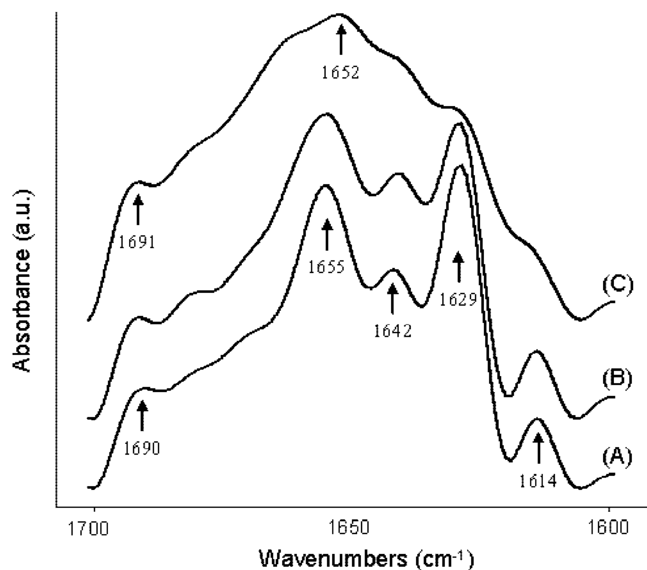

Figure 6. Deconvoluted spectra of whey proteins in aqueous phases separated (**A**) from fresh emulsion, (**B**) from fresh emulsion after Tween 20 desorption, and (**C**) from washed emulsion after Tween 20 desorption.

interaction with Tween 20 may be dependent on Tween 20 concentration and denaturation of protein. Although 8 mM Tween 20 was used in this study, which could lead the aggregation of denaturated proteins, half of the Tween 20 remained in the aqueous phase after desorption, because Tween 20 above 5 mM results in little additional removal of the adsorbed proteins (**Table 1**). From these results, it could be determined that the interaction of Tween 20 with proteins in aqueous phase and at the interface has a little effect on modification of the protein structure.

These findings provide direct evidence for the additional conformation changes when whey proteins are desorbed from the oil/water interface. Mackie et al. (*5*) proposed the mechanism of removal of protein by Tween 20 affecting the interfacial protein structure. Their results showed that the remaining protein is not in its normal adsorbed conformation, even though this adsorption occurred at air/water interface. The spectrum in **Figure 4** results from the formation of surface aggregates.

Figure 6 shows the amide I spectra of whey proteins presented in aqueous phase, which were separated from emulsion (**A**), after desorption (**B**), and after washing as well as desorption (**C**). When the aqueous phase is separated from the fresh emulsion (**Figure 6 A**), the spectrum shows several changes in comparison with the native state (**Figure 1**). The bands at 1629, 1642, and 1655 cm $^{-1}$ become sharper than the spectrum of the native protein. These changes indicate that the nonadsorbed proteins have a more structured form than the native ones. For adsorbed proteins, adsorptions and high pressure have an influence on the change in structure, whereas for nonadsorbed proteins, only the effects from high pressure have an impact on the spectrum. This result shows a little difference with the study of Fang and Dalgleish (*11*), which shows that the protein structure in the serum phase of the emulsion stabilized by β-lactoglobulin is identical to the native one. Because they used a high-speed blender to form an emulsion, not a high-pressure homogenizer, the comformational changes found in this study were not produced. This finding shows that high pressure homogenization affects not only the fat globules but also the protein structure in the aqueous phase.

Whey proteins desorbed from fresh emulsions and nonadsorbed whey proteins share similar amide I spectra (**Figure 6 B**). However, the 1655 cm^{-1} band becomes slightly larger, and its intensity is increased after desorption. These small variations in the sample may result from the mixing of nonadsorbed and desorbed whey proteins in the aqueous phase and because the fraction of protein desorbed is very small as compare to the protein in solution. If only desorbed whey proteins could be separated from this mixture, more information on the structural changes at the interface could probably be obtained. Therefore, washing the cream phase prior to Tween 20 desorption not only removes unadsorbed whey proteins from the emulsion, but also specifically measures the conformation of desorbed whey proteins.

Significant changes in both shape and intensity distribution in the amide I band are observed after desorption of whey proteins from the washed emulsion (**Figure 6C**). The amide I band is broad with a maximum peak at 1652 cm^{-1}, a strong shoulder at 1629 cm^{-1}, and a weak one at 1642 cm^{-1}. The significant changes after desorption are the marked decrease in the intensity of the 1629 and 1691 cm^{-1} bands and the increase in the intensity of the 1652 cm^{-1} band. The desorbed whey proteins do not recover their native secondary structure, even though neighbor proteins and interfacial conditions are removed after desorption. It means that proteins are denatured during adsorption. However, the weak-structured amide I band reveals that some of the protein structure modified by adsorption (i.e., α-helix and β-sheet structure) still remains after desorption. The structures, which disappeared during the desorption state, could play an important part in the formation of the protein layer during the adsorption state. The increased intensity in the 1652 cm^{-1} band and the shift to a lower frequency suggest that the protein structure consists of predominantly unordered components and that the α-helical structure is considerably decreased. A significant decrease in the number of β-sheet structures is also found in desorbed whey proteins. In particular, a weak amide I band component at 1629 and 1691 cm^{-1} is linked to a residual β-sheet structure, which appears to be indicative of the formation of an unordered conformation at the expense of the α-helical and β-type conformation. These spectra changes are, again, consistent with the importance of α-helical structure and intermolecular β-sheet structure that are related with the formation of interactions with the soy oil interface and those between adsorbed proteins, respectively.

Our results reveal important facts concerning interfacial behavior when whey proteins are adsorbed at the oil/water interface. First, the conformational changes (α-helical and β-sheet structures) and denaturation of proteins happened after interfacial adsorption. Second, adsorption-induced structural changes at the oil/water interface are irreversible. Although structural changes in proteins at the oil/water interface are suggested in a number of studies, the irreversible nature of these changes has never been clearly demonstrated. Although Kondo et al. (*3*) insisted that adsorption-induced conformational changes in bovine serum albumin are highly reversible, the interface used in their study (i.e., the Ultrafine silica particles having 15 nm average diameter) was not a real oil/water interface of emulsion. The structural changes at such interfaces may not be identical to those taking place in a real emulsion interface. Thus, the interfacial aggregation and the interaction with the oil phase could cause the irreversible structural changes in whey proteins.

Whey Protein Adsorption Behaviors at the Soy Oil/Water Interface. Our results provide insight into the adsorption and stabilization process of whey proteins in emulsion. **Figure 7** provides a schematic drawing on the mechanisms involved in the adsorption of whey proteins at the oil/water interface and

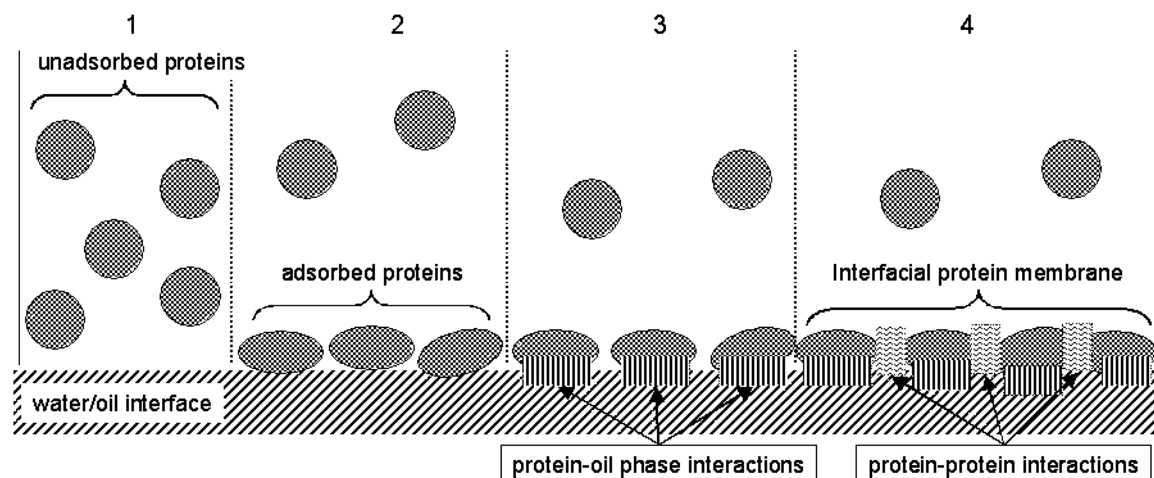

Figure 7. The structural changes in whey proteins during adsorption at the oil/water interface, and according to these changes, the mechanism and adsorption behavior for stabilizing emulsified oil droplets under high-pressure homogenization.

on the role they play in the stabilization of the emulsions. First, once the whey protein moves to the interface, the partial changes are needed for it to be adsorbed at the oil/water interface (panel 2 in **Figure 7**). Second, the α-helical structure is formed and/or exposed to ensure effective interactions with the oil interface (panel 3 in **Figure 7**). Changes in the intensity of the relative amide I bands, resulting from adsorption and desorption, seem to indicate that modifications in the α-helical structure are involved in contact with the interface after adsorption. Finally, whey protein interactions lead formation of aggregation at the interface (panel 4 in **Figure 7**). Besides interactions stated in this study (i.e. protein–protein interaction and protein–lipid interaction), other studies reported the formation of disulphide bonds between adsorbed proteins at the interface (*28, 29*). This bond enhances protein–protein interactions and contributes to formation of a more dense and rigid interfacial film around droplets. However, the extent of polymerization increased according to storage time (*14, 29*) and heating (*28*). The importance of polymerization via disulphide interactions was not been considered in the present study, because we measured the changes in protein structure at the interface without ageing and thermal treatment.

Consequently, the adsorption-dependent conformational changes involve both formations of interactions between adsorbed proteins to form a gel-like structure and increase of α-helical structure to generate lipid interactions. These changes could form an effective and stable interfacial protein layer. In addition, both structural changes in adsorbed whey proteins may occur simultaneously during the interfacial rearrangement process. However, the formation of gel-like structure on the interface is more important than the α-helical change, because the latter is easily diminished by variations in the emulsion condition and the desorption treatment (**Figure 4**). Actually, in previous studies, the change in α-helical structure was reported under specific conditions, such as phospholipid layer, whereas the increase of β-sheet structure was found after interfacial adsorption. This process could be altered by various conditions in the emulsion system, such as protein and oil concentration, salt addition, type of proteins and oils, pH, and temperature, so more studies are needed to confirm this.

In this study, we suggested a possible mechanism as well as provide explanations for adsorption behaviors of whey proteins in the formation of an interfacial protein layer. The deconvoluted amide I band of whey proteins gives direct evidence of the substantial differences in the secondary structures after adsorption. The results indicated that the adsorption at the soy oil/water interface causes significant conformational changes and some specific interactions, and the adsorption mechanism of whey proteins at the oil/water interfaces is complex. It was found that, after adsorption, the structural changes are mainly localized in the β-sheet structure region to form the aggregation with neighboring whey proteins. The changes in α-helical and unordered structures were consistent with the formation of interactions with the soy oil interface. However, the amide I IR spectra in detecting conformational transitions in proteins was admittedly limited, because it involved changes in the secondary structure, and the changes in tertiary structure could not be reflected in the amide I IR spectra. Therefore, in spite of revealing the structure of interfacial whey proteins, the continued efforts must be made to gain a better understanding of the relationship between the structure of proteins adsorbed at interface and the stability of emulsions.

ACKNOWLEDGMENT

We thank the industrial chair from NSERC and the partners (Novalait, Agropur, Parmalat).

LITERATURE CITED

(1) Hunt, J. A.; Dalgleish, D. G. Effect of pH on the stability and surface composition of emulsions made with whey protein isolate. *J. Agric. Food Chem.* **1994**, *42*, 2131–2135.
(2) Mohan, S.; Narsimhan, G. Coalescence of protein-stabilized emulsions in a high-pressure homogenizer. *J. Colloid Interface Sci.* **1997**, *192*, 1–15.
(3) Kondo, A.; Oku, S.; Higashitani, K. Structural changes in protein molecules adsorbed on Ultrafine silica particles. *J. Colloid interface Sci.* **1991**, *143*, 214–221.
(4) Dickinson, E. Proteins at interfaces and in emulsions. *J. Chem. Soc. Faraday Trans.* **1998**, *94*, 1657–1669.
(5) Mackie, A. R.; Gunning, A. P.; Wilde, P. J.; Morris, V. J. Orogenic displacement of protein from the air/water interface by competitive adsorption. *J. Colloid Interface Sci.* **1999**, *210*, 157–166.
(6) Leman, J.; Kinsella, J. E. Surface activity, film formation, and emulsifying properties of milk proteins. *Crit. Rev. Food Sci. Nutr.* **1989**, *28*, 115–138.
(7) Dalgleish, D. G. Adsorption of protein and the stability of emulsions. *Trends Food Sci. Tech.* **1997**, *8*, 1–6.
(8) Green, R. J.; Hopkinson, I.; Jones, R. A. Unfolding and intermolecular association in globular protein adsorbed at interfaces. *Langmuir* **1999**, *15*, 5102–5110.

(9) Jackson, M.; Mantsch, H. H. Halogenated alcohols as solvents for proteins: FTIR spectroscopic studies. *Biochim. Biophys. Acta* **1992**, *1118*, 139–143.

(10) Maste, M. C. L.; Pap, E. H. W.; Hoek, A. V.; Norde, W.; Visser, A. J. W. G. Spectroscopic investigation of the structure of a protein adsorbed on a hydrophobic latex. *J. Colloid Interface Sci.* **1996**, *180*, 632–633.

(11) Fang, Y.; Dalgleish, D. G. Conformation of β-lactoglobulin studied by FTIR: Effect of pH, temperature, and adsorption to the oil-water interface. *J. Colloid Interface Sci.* **1997**, *196*, 292–298.

(12) Fang, Y.; Dalgleish, D. G. The conformation of α-lactalbumin as a function of pH, heat treatment and adsorption at hydrophobic surfaces studied by FTIR. *Food Hydrocolloids* **1998**, *12*, 121–126.

(13) Husband, F. A.; Garrood, M. J.; Mackie, A. R.; Burnett, G. R.; Wilde, P. J. Adsorbed protein secondary and tertiary structures by circular dichroism and infrared spectroscopy with refractive index matched emulsions. *J. Agric. Food Chem.* **2001**, *49*, 859–866.

(14) Courthaudon, J. L.; Dickinson, E.; Matsumura, Y.; Williams, A. Influence of emulsifier on the competitive adsorption of whey proteins in emulsions. *Food Struct.* **1991**, *10*, 109–115.

(15) Dickinson, E. Competitive adsorption and protein-surfactant interactions in oil-in-water emulsion. In *Microemulsions and Emulsions in Foods*; Magda, E. N.; Cornell, D. , Eds.; American Chemical Society: Washington DC, 1991; pp 114–129.

(16) Chen, J.; Dickinson, E. Time-dependent competitive adsorption of milk proteins and surfactants in oil-in-water emulsions. *J. Sci. Food Agric.* **1993**, *62*, 283–289.

(17) Robin, O.; Paquin, P. Evaluation of the particle size of fat globules in a milk model emulsion by photon correlation spectroscopy. *J. Dairy Sci.* **1991**, *74*, 2440–2447.

(18) Zahar, M.; Smith, D. E. Adsorption of proteins at the lipid-serum interface in milk systems with various lipids. *Int. Dairy J.* **1996**, *6*, 697–708.

(19) Dong, A.; Matsuura, J.; Allison, S. D.; Chrisman, E.; Manning, M. C.; Carpenter, J. F. Infrared and circular dichroism spectroscopic characterization of structural differences between β-lactoglobulin A and B. *Biochem.* **1996**, *35*, 1450–1457.

(20) Jackson, M.; Mantsch, H. H. The use and misuse of FTIR spectroscopy in the determination of protein structure. *Crit. Rev. Biochem. Mol. Biol.* **1995**, *30*, 95–120.

(21) Dong, A.; Matsuura, J.; Manning, M. C.; Carpenter, J. F. Intermolecular β-sheet results from trifluoroethanol-induced nonnative α-helical structure in β-sheet predominant proteins: Infrared and circular dichroism spectroscopic study. *Arch. Biochem. Biophys.* **1998**, *355*, 275–281.

(22) Briggs, M. S.; Cornell, D. G.; Dluhy, R. A.; Gierasch, L. M. Conformations of signal peptides induced by lipids suggest initial steps in protein export. *Science* **1986**, *233*, 206–207.

(23) Morr, C. V.; Ha, E. Y. W. Whey protein concentrates and isolates: processing and functional properties. *Crit. Rev. Food Sci. Nutr.* **1993**, *33*, 431–476.

(24) Lefèvre, T.; Subirade, M. Molecular differences in the formation and structure of fine-stranded and particulate β-lactoglobulin gels. *Biopolymers* **2000**, *54*, 578–586.

(25) Goormaghtigh, E.; Cabiaux, V.; Ruysschaert, J. M. Determination of soluble and membrane protein structure by Fourier Transform infrared spectroscopy. In *Subcellular Biochemistry Volume 23: Physicochemical Methods in the Study of Biomembranes*; Hilderson, H. J.; Ralstion G. B., Eds; Plenum Press: New York, 1994; pp 363–403.

(26) Corredig, M.; Dalgleish, D. G. A differential microcalorimetric study of whey. proteins and their behavior in oil-in-water emulsions. *Coll. Surf. B* **1995**, *4*, 411–422.

(27) Lefèvre, T.; Subirade, M. Structural and interaction properties of β-lactoglobulin as studied by FTIR spectroscopy. *Int. J. Food Sci. Tech.* **1999**, *34*, 419–428.

(28) Monahan, F. J.; McClements, D. J.; German, J. B. Disulfide-mediated polymerization reactions and physical properties of heated WPI-stabilized emulsions. *J. Food Sci.* **1996**, *61*, 504–510.

(29) Damodaran, S.; Anand, K. Sulfhydryl—disulfide interchange-induced interparticle protein polymerization in whey protein-stabilized emulsions and its relation to emulsion stability. *J. Agric. Food Chem.* **1997**, *45*, 3813–3820.

Received for review September 3, 2007. Revised manuscript received October 10, 2007. Accepted October 22, 2007.

JF0726076