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Whey proteins modify the phase transition of milk fat globule phospholipids

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Abstract — The phospholipids extracted from milk fat globule membranes were composed of three major classes, phosphatidylethanolamines (37.2 %), phosphatidylcholines (30.0 %), sphingomyelin (28.2 %), and two minor classes, phosphatidylinositols (2.0 %) and phosphatidylserines (2.6 %). The thermogram showed that the extracted phospholipids presented a single wide-phase transition centered at 18.3 °C. The effects of temperature on the phase behaviour of milk phospholipids in the presence of β-lactoglobulin and α-lactalbumin have been studied at pH 4.0 and 7.0 (with or without calcium) using Fourier transform infrared spectroscopy. Principal component analysis carried out on the spectra in the 3 000–2 800 cm^{-1} region showed that the binding of the whey proteins to the phospholipids modified the lipid phase transition in different ways, depending on the in-solution protein. © Inra/Elsevier, Paris

milk / phospholipid / protein / interaction / phase behaviour / infrared

Résumé — L'arrangement des phospholipides extraits des globules gras du lait est modifié par la présence de la β-lactoglobuline et de l'α-lactalbumine. Les phospholipides extraits de la membrane du globule gras comprennent des phosphatidylethanolamines (37,2 %), des phosphatidylcholines (30,0 %), des sphingomyélines (28,2 %), des phosphatidylsérines (2,6 %) et des phosphatidylinositols (2,0 %). Le thermogramme des phospholipides du lait présente un pic large avec une seule transition de phase à 18,3 °C. L'effet de la température sur l'arrangement des phospholipides extraits des globules gras du lait en présence de la β-lactoglobuline et de l'α-lactalbumine a été étudié par spectroscopie moyen infrarouge à pH 4,0 et 7,0 (en présence ou en absence de calcium). L'analyse en composantes principales réalisée sur les spectres correspondant à la région 3000–2800 cm^{-1} montre qu'aux deux pH étudiés, l'arrangement des phospholipides est modifié suite à leurs interactions avec les protéines du lactosérum. © Inra/Elsevier, Paris

lait / phospholipide / protéine / interaction / transition de phase / infrarouge

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1. INTRODUCTION

The surface of milk fat globules is partly stabilized by a protective layer of phospholipids, the fat globule membrane [40]. It has been estimated that the milk fat globule membrane comprises some 27 % (w/w) of phospholipids and 41 % of proteins. Milk phospholipids consist mainly of phosphatidylethanolamine, phosphatidylcholine and sphingomyelin [10]. These phospholipids are commonly found in dairy products. Phospholipids, particularly soya phosphatidylcholine, and milk proteins are also added to certain processed foods to act as emulsifiers.

Protein/phospholipid interactions are significant with regard to the behaviour of food systems. The interfacial properties of proteins have substantial consequences within a wide range of food and medical systems. For example, the milk homogenization, modifying the composition of fat globule/water interface [12, 32], has dramatic effects on milk coagulation and cheese texture.

Among the bovine milk proteins, β-lactoglobulin and α-lactalbumin, the two major whey proteins, have been extensively studied and detailed structural information is available. β-Lactoglobulin is a whey protein found in the milks of many mammals (e.g. ruminants, sow, mare, dog). Structural studies of β-lactoglobulin [14, 28, 30] show that this protein (52 % β-sheet and 10 % α-helix) has a three-dimensional structure known as a β-barrel. The binding of Ca^{2+} by β-lactoglobulin has been reported [4, 13, 20]. Native β-lactoglobulin is also able to interact with phospholipids, but only in well-defined conditions. The protein interacts with mixed phosphatidylcholine/ phosphatidylglycerol monolayers at pH 4.4 and at low ionic strength [11], and with phosphatidylcholine or phosphatidylglycerol liposomes at pH 6.2 and 160 mmol·L^{-1} NaCl [8].

α-Lactalbumin, the second most significant protein of the bovine whey, is the modifier protein of the lactose synthase complex in the mammary cell. The secondary structure of this protein yields 45 % of α-helix and 5 % of β-sheet [1]. Ptitsyn [31] showed that α-lactalbumin has a 'molten globule state' conformation at low pH. This conformational state may facilitate the insertion of proteins in membranes [18, 19, 24].

Studies of lipid–protein interaction in reconstituted model systems have been an active topic for a numbers of years [9, 17, 25, 27, 36, 39]. These studies provide basic information about the molecular organization of biological membranes. Biophysical studies on model membranes such as liposomes have also been quite successful in characterizing the organization and dynamics of a lipid matrix. The development of Fourier transform infrared (FTIR) spectroscopy in recent years affords the possibility of obtaining unique information about protein structure [2, 15, 34, 35, 37], lipid organisation [9, 12] and lipid–protein interaction [27, 36] without introducing perturbing probe molecules. The infrared bands appearing in the 3 000–2 800 cm^{-1} region are particularly useful because they are sensitive to the conformation and the packing of the phospholipid acyl chains [9, 27, 39]. For example, the phase transition of phospholipids (sol to gel state transition) can be followed by mid infrared spectroscopy: increasing temperature results in a shift of the bands associated with C-H (~ 2 850, 2 880, 2 935 and 2 960 cm^{-1}) and carbonyl stretching mode of the phospholipids. The plot of the maximum frequency of the C-H band at about 2 850 cm^{-1} versus the temperature allows the determination of the melting temperature of 1,2-dipalmitoyl-glycero-3-phosphocholine in fully hydrated multibilayers [9]. These methods work very well for model systems containing only one or two phospholipid species. For more complex systems such as biological membranes, the lipid bands broaden and it becomes increasingly difficult to extract valuable information from the spectra. Univariate analysis techniques are not always appropriate for the study of

complex mixtures. Multivariate analysis techniques such as principal component analysis (PCA) may make it possible to extract information related to the conformation of lipid acyl chains from the mid-infrared spectra [12].

Even though it is clear that whey proteins interact with charged lipids in model membranes, the published data have been obtained using mainly one kind of phospholipid molecule such as 1,2-palmitoyl-*sn*-glycero-3-phosphocholine. We will report in this paper the interactions between phospholipids from milk fat globule membrane and two milk whey proteins, β-lactoglobulin and α-lactalbumin. The experiments were conducted at pH 4 (close to the pH of acid whey) and pH 7 (close to milk pH) in the absence or in the presence (30 mmol·L^{-1}) of calcium. The interactions of two whey proteins with milk phospholipid multibilayers and the modification of milk phospholipid acyl chain conformations induced by protein insertion into phospholipid multibilayers are reported and discussed.

2. MATERIALS AND METHODS

2.1. Purification of β-lactoglobulin and α-lactalbumin

β-Lactoglobulin variant A (BLG) was obtained from homozygote cow milk following the method of Mailliart and Ribadeau Dumas [26]. α-Lactalbumin (ALA) was purified as described by Mailliart and Ribadeau Dumas [26].

2.2. Purification and identification of milk phospholipids

Dialysed and lyophilized industrial buttermilk was hydrated to obtain a 20 % (w/v) solution [38]. Lipids were extracted from buttermilk with a chloroform/methanol (2/1) mixture [16].

The extract was then fractionated into neutral lipids, glycolipids and phospholipids by chromatography on silicic column [22]. Phospholipids were quantified by phosphorus analysis [3] and analyzed by high performance liquid chromatography coupled with a light-scattering detector [33].

The phase transition temperature of a 25 % milk phospholipid suspension in water (w/v) was determined by differential scanning calorimetry (DSC) (DSC121 Setaram, France). The thermograms (duplicate) were recorded between 2 and 50 °C.

2.3. Infrared spectroscopy studies of lipid-protein complexes

Samples containing about 7 % (w/w) of lipids dispersed in phosphate solution (100 mmol·L^{-1}, pH 4.0), or in 100 mmol·L^{-1} Tris buffer, pH 7.0, containing 0 or 30 mmol·L^{-1} calcium, were heated to approximately 65 °C for 10 min, stirred on a vortex mixer and cooled down to the gel-to-liquid crystalline phase transition temperature. This cycle was repeated three times to obtain milk phospholipid multibilayers (PL). Lipid-protein complexes (PL + ALA or PL + BLG) were prepared by adding appropriate amounts of a stock protein solution (ALA or BLG) to phospholipid dispersions to obtain the required lipid-to-protein molar ratio (Ri = 10).

Infrared spectra were recorded with a Nicolet Magna 560 Fourier transform spectrophotometer equipped with a mercury-cadmium-telluride detector and a thermostated cell. Samples of lipids and lipid-protein complexes were inserted between two BaF_2 windows separated by a 6 μm Mylar spacer and spectra were recorded between 6 and 70 °C. For each spectrum, a total of 250 scans were collected at 2 cm^{-1} resolution. The experiments were done in duplicate.

2.4. Mathematical processing and principal component analysis of the spectra in the 3 000–2 800 cm^{-1} region

Principal component analysis (PCA) was applied to the normalized data [6]. PCA is a multidimensional statistical method which optimizes the description of the data with a minimum loss of information [21]. From a data set, PCA assesses principal components and their corresponding eigenvectors. The principal components are used to draw maps that describe the physical and chemical variations observed between the samples and make it possible to study them without any calibration step [7]. Moreover, the eigen-

vectors are homologous to spectra and are called 'spectral patterns'. Both positive and negative peaks of the spectral pattern can be interpreted as characteristic wavelengths of chemical constituents. The PCA software was written by D. Bertrand (Phytec, Inra, Nantes, France) and is described elsewhere [7].

3. RESULTS AND DISCUSSION

3.1. Characterization of milk phospholipids

The characterization of the lipids extracted from the buttermilk showed that phospholipids accounted for 18.6 % of the total dry weight. The analyses indicated that the extracted phospholipids were made up of three major classes, phosphatidylethanolamines (37.2 %), phosphatidylcholines (30.0 %), sphingomyelin (28.2 %), and two minor classes, phosphatidylinositols (2.0 %) and phosphatidylserines (2.6 %). As deduced from the thermogram (data not shown), the extracted phospholipids presented a single wide-phase transition centered at 18.3 °C. The wide transition, beginning at 10 °C and ending at 30 °C, was the consequence of the great number of phospholipid molecular species constituting the milk fat globule membrane.

3.2. Effect of temperature on the packing of phospholipid multibilayers in the presence of whey protein solutions, pH 4.0

The protein/phospholipid interactions and the effects of whey proteins on the conformation of the PL have been studied using the spectral features associated with the C-H stretching mode region (2 800–3 000 cm^{-1}). The infrared bands appearing in this region are particularly useful because they provide valuable structural and conformational information about the changes that occur in the phospholipid acyl chains [9, 27, 39]. *Figure 1* shows the spectra in the acyl chain C-H stretching mode region recorded at two different temperatures and at acidic pH for lipids both alone and in the presence of ALA or BLG. This spectral region was dominated by two strong bands at 2 920 and 2 850 cm^{-1}, assigned to the methylene antisymmetric and symmetric stretching mode, respectively. Weaker bands resulting from the asymmetric and symmetric stretching modes of the terminal methyl groups were also present at 2 956 and 2 872 cm^{-1}. The C-H stretching mode region is known to be sensitive to the conformation and the dynamics of the phospholipid acyl chains. As can be seen in *figure 1*, the two methylene bands broaden and

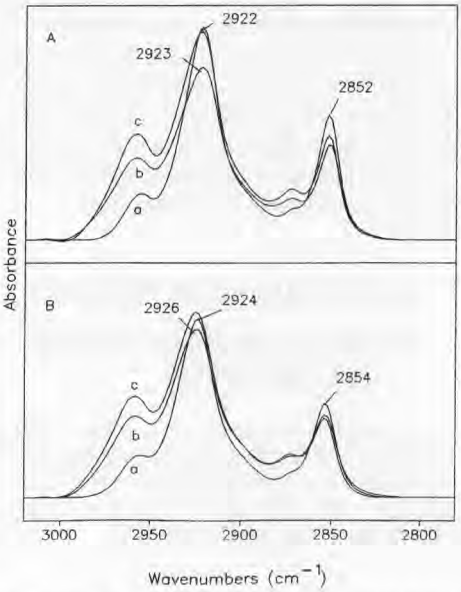

Figure 1. Infrared spectra in the C-H stretching mode region, at acidic pH in (A) the gel state (10 °C) and in (B) the liquid-crystalline state (70 °C) for (a) milk phospholipids, (b) milk phospholipids–ALA complexes and (c) milk phospholipids–BLG complexes.

Figure 1. Spectres infrarouge dans la région des modes d'élongation C-H, à pH acide, (a) des phospholipides du lait, (b) des complexes phospholipides-ALA et (c) des complexes phospholipides-BLG à (A) l'état gel (10 °C) et à (B) l'état liquide (70 °C).

shift towards higher frequencies when the phospholipids undergo the gel-to-liquid crystalline phase transition. Earlier studies have assigned this frequency shift of the methylene bands to the introduction of gauche conformers along the phospholipid acyl chains in the high temperature phase [9, 27, 39]. The effect of whey proteins on the thermotropic behavior of phospholipids were investigated using only the frequency at about 2 850 cm^{-1} band since this band is not affected by the spectral contribution of proteins. From our data, the centre of gravity of the band associated with the ν_s CH_2 was determined for each spectrum using Omnic software. *Figure 2* shows the effect of temperature on the frequency of the methylene symmetric stretching mode. This plot makes it possible to determine the melting temperature of phospholipids [9]. The curves of the wave number at about 2 850 cm^{-1} versus temperature varied in the same way with temperature for the three systems and showed three main regions. Below 10 °C, the wavenumber remained almost constant. The increase of the temperature from 10 to 25 °C induced an increase of the wave number. It was followed by a line segment exhibiting a smaller slope above 25 °C. For milk phospholipids alone, it can be observed that the phase transition (10–25 °C range of temperature) determined from the infrared data is in agreement with the DSC result (18.3 °C). The binding of the whey proteins induced sizeable modifications of the C-H stretching mode region. ALA and BLG caused a comparable shift of the band assigned to the methylene symmetric stretching mode toward higher frequency by approximately 2 cm^{-1} in the liquid-crystalline phase and about 1 cm^{-1} in the gel phase. In addition, the presence of whey proteins decreased the temperature of the phase transition of phospholipids and it is shown that ALA had a more pronounced effect on the

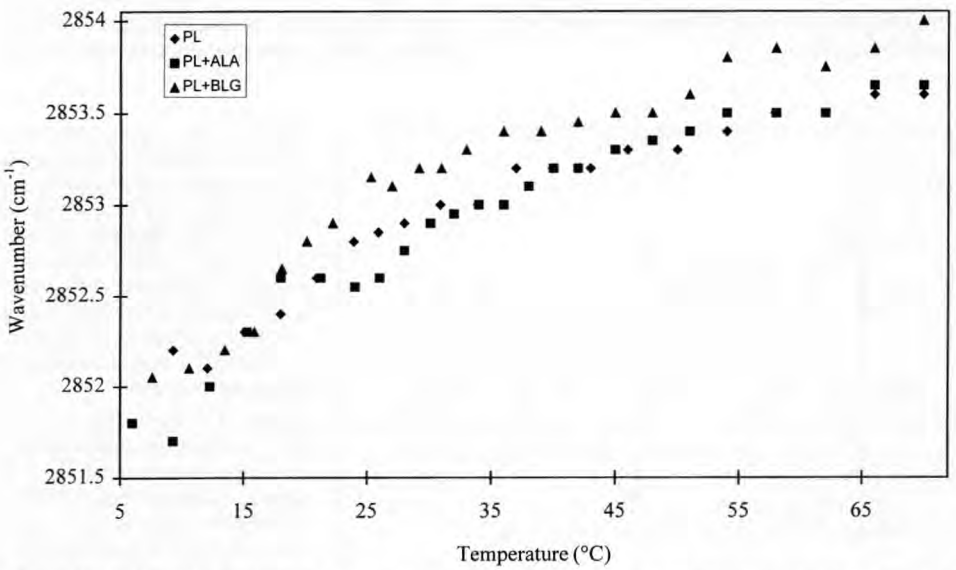

Figure 2. Shift of the maximum of the band assigned to the methylene symmetric stretching mode as a function of temperature PL (◆), PL+ALA (■), PL+BLG (▲).

Figure 2. Évolution du maximum d'absorbance de $\nu_s CH_2$ en fonction de la température pour les systèmes PL (◆), PL+ALA (■), PL+BLG (▲).

organization of phospholipid multibilayers than BLG. However, it was difficult from the data reported in *figure 2* to derive the temperatures of the phase transitions with a good accuracy. Even if the experiments done in triplicate lead to reproducible curves, the changes in the wavenumbers as a function of temperature are less pronounced for the three systems investigated than for a pure phospholipid [9, 27, 36]. This certainly results from the nature of the sample, a mixture of phospholipids, and from the fact that gel-to-fluid phase transition is much less cooperative for a mixture of phospholipids than for a pure phospholipid.

As can be seen in *figure 1*, the two methylene bands became broader in the presence of the two proteins, the broadening being greater in the presence of ALA than in the presence of BLG. Earlier studies showed that these spectral changes are associated with the introduction of gauche conformers along the phospholipid acyl chains and the increase in the width of the distribution of conformations [39]. This indicates hydrophobic interactions between proteins and lipid multibilayers.

3.3. Multidimensional analysis of the spectra recorded at pH 4.0

PCA was applied to the set of spectra recorded at acidic pH and at various temperatures corresponding to PL, PL + BLG and PL + ALA systems, to obtain additional structural information. The results of PCA are presented and the map defined by principal components 1 and 2 is shown in *figure 3*. The first two principal components took into account 96 % of the total variation. A discrimination of the samples as a function of protein in the mixture was observed according to the principal component 1. The principal component 2 discriminated the samples as a function of temperature: at low temperature the scores were positive, while at high temperature the scores were negative. The eigenvectors corresponding to the principal components are homologous to spectra and are called 'spectral patterns'. They provide information about the characteristic absorption bands, which explain the similarities in the sample observed on the maps. The peaks characterized in the spectral patterns of the principal components 1 and 2 correspond to the wavenum-

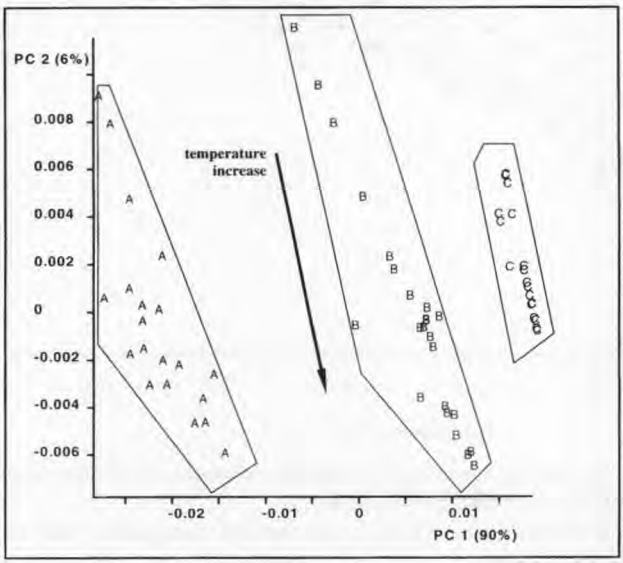

Figure 3. Principal component analysis similarity map defined by the principal components (PC) 1 and 2 for PL (A), PL + ALA (B), PL + BLG (C) spectral data of the 3 000–2 800 cm^{-1} region at acidic pH. The spectra were recorded between 6 and 70 °C. Each label corresponds to a spectrum. See Materials and Methods for details.

Figure 3. Carte factorielle 1–2 de l'analyse en composantes principales réalisée sur les spectres, dans la région 3 000–2 800 cm^{-1}, acquis à pH acide entre 6 et 70 °C pour les systèmes PL (A), PL + ALA (B), PL + BLG (C). Chaque étiquette correspond à un spectre. Se reporter au matériel et méthodes pour les détails.

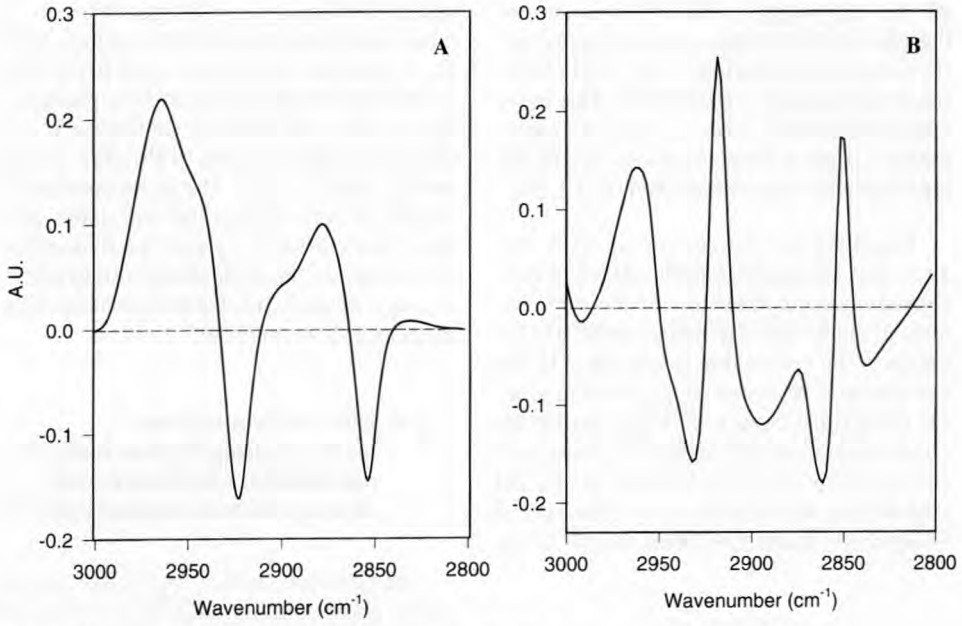

Figure 4. Spectral patterns corresponding to the principal components 1 (A) and 2 (B).
Figure 4. Vecteurs propres correspondant aux composantes principales 1 (A) et 2 (B).

bers that are the most discriminant. These bands could provide valuable structural information about the changes that occurred in the acyl chains during the phase transition.

The examination of the spectral patterns confirmed that the environment of the methylene groups of phospholipid acyl chains was modified in the presence of proteins and as a function of temperature. The spectral pattern corresponding to the principal component 1 showed an opposition between positive peaks at 2 878 and 2 964 cm^{-1} and negative peaks at 2 854 and 2 923 cm^{-1} (*figure 4A*). This indicated that the ratio of $A_{\nu CH3}/A_{\nu CH2}$ increased in the presence of the whey proteins. *Figure 4B* shows that the spectral pattern corresponding to the principal component 2 was characterized by the opposition between bands at 2 932 and 2 918 cm^{-1} and bands at 2 861 and 2 852 cm^{-1}. This pattern indicated a shift of the methylene bands of phospholipids as a function of temperature. This agrees with the data reported in *figure 2*.

Spectral pattern 2 also presented two other discriminant peaks that were observed at 2 961 and 2 874 cm^{-1} and that corresponded to the regions of ν_{asCH3} and ν_{sCH3} bands, respectively. It indicated that the environment of methyl groups was also altered by proteins. The plot of the absorbance at 2 961 cm^{-1} versus temperature is shown in *figure 5*. The absorbance for PL and BLG + PL samples was roughly constant as a function of the temperature. The pattern for the ALA + PL sample was quite different since the curve showed two distinct regions. The absorbance increased from 0.09 to 0.1 for temperatures ranging between 9 and 30 °C. As shown by DSC, this temperature range corresponded to the melting region of milk phospholipids. Furthermore, the absorbance remained constant up to

80 °C. This suggests that ALA penetrates the phospholipid bilayer more deeply and it modifies the packing of the acyl chains more substantially than BLG. The more effective penetration of ALA into the multibilayers at pH 4.0 is also consistent with the data reported in the literature [18, 19, 24].

The different properties of ALA and BLG may originate from the different conformations and stabilities of the two proteins at acidic pH. The structure of BLG is known to be very stable at low pH [23]. On the contrary, ALA can adopt a molten globule form at pH close to 2.0. This particular conformation of the protein is, however, observed for a relatively more acidic pH than the one used in this study. However, it is generally understood that the pH at the surface of negatively charged bilayers is more acidic than the pH of the solution [29]. For a solution exhibiting a bulk pH of 4.0, ALA in the neighbourhood of the phospholipid polar heads will be at a pH close to 2.0 that corresponds to the formation of the molten globule state. It can be concluded that ALA in its molten globule state penetrates more effectively into the hydrophobic interior of the milk phospholipid bilayers, in a manner similar to that of integral membrane proteins [29].

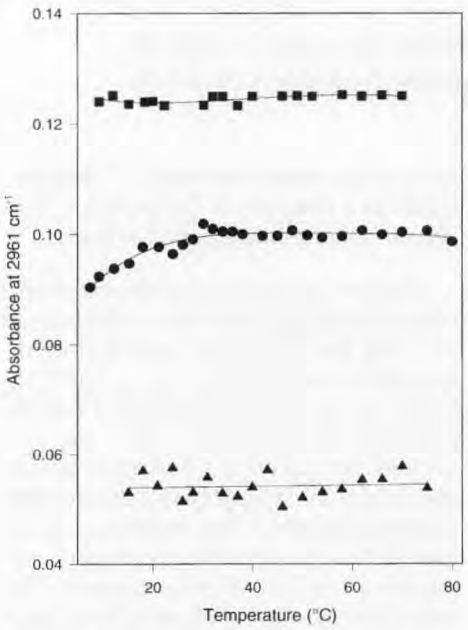

Figure 5. Plots of the absorbance at 2 961 cm^{-1} versus temperature for PL (▲), PL + ALA (●), PL + BLG (■).

Figure 5. Courbes de l'évolution de l'absorbance à 2 961 cm^{-1} en fonction de la température pour les systèmes for PL (▲), PL+ALA (●), PL+BLG (■).

3.4. Effect of temperature on the packing of phospholipid multibilayers in the presence of whey protein solutions, pH 7.0

Temperature studies were also carried out on phospholipid multibilayers, alone or complexed with the proteins, suspended in 100 $mmol \cdot L^{-1}$ Tris buffer pH 7 containing 0 or 30 $mmol \cdot L^{-1}$ calcium. *Figure 6* shows that the presence of the whey proteins induced modifications in the 2 800–3 000 cm^{-1} region, indicating that ALA and BLG bound to the milk phospholipids. In the temperature range investigated and for all the systems studied, the band assigned to the methylene symmetric stretching mode sh ifted by about 1 cm^{-1} when the conformation of the lipid membranes in the gel phase turned into the liquid-crystalline phase. For at least two systems, calcium did not seem to modify the lipid phase transition since the spectra in the C-H stretching mode region for PL and PL + ALA in the absence of calcium appeared to be similar to that obtained in the presence of calcium. On the contrary, the frequency of the methylene symmetric stretching mode of PL + BLG system in the absence of calcium was 0.3 cm^{-1} higher than that of the same system in the presence of 30 $mmol \cdot L^{-1}$ calcium for all the temperatures investigated. In addition, the values of ν_{sCH2} frequency for PL + BLG system in the presence of calcium were close to that of PL system for the investigated temperatures.

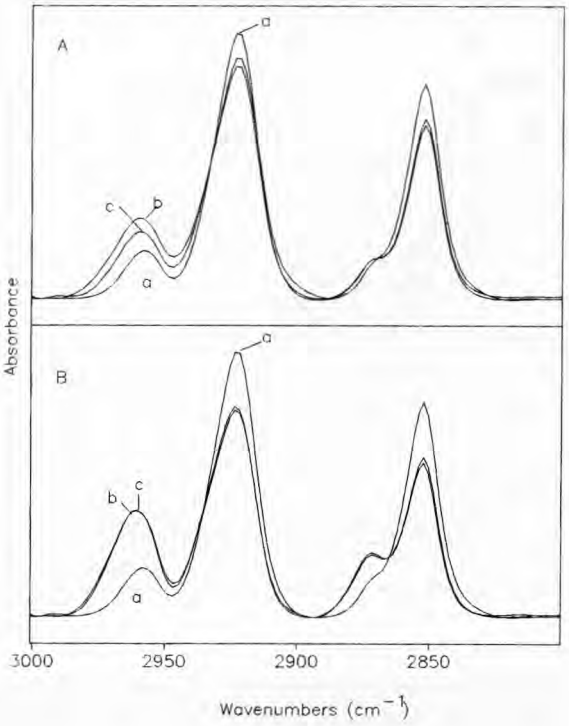

Figure 6. Infrared spectra in the C-H stretching mode region, at neutral pH and at 20 °C of A: milk phospholipids (a), milk phospholipids–ALA complexes (b) in the absence and (c) in the presence of calcium (30 mmol·L^{-1}). B: milk phospholipids (a), milk phospholipids–BLG complexes (b) in the absence and (c) in the presence of calcium (30 mmol·L^{-1}).

Figure 6. Spectres infrarouges dans la région des modes d'élongation C-H, à pH neutre et à 20 °C des A : des phospholipides du lait (a) et des complexes phospholipides-ALA en absence (b) et en présence (c) de calcium 30 mmol·L^{-1}. B : des phospholipides du lait (a) et des complexes phospholipides-BLG en absence (b) et en présence (c) de calcium 30 mmol·L^{-1}.

As the whey proteins induced smaller changes in the phospholipid packing at pH 7.0 than at pH 4.0, it was suggested that the binding of proteins by phospholipids was weaker at neutral pH, involving mainly electrostatic interactions.

3.5. Multidimensional analysis of the spectra recorded at pH 7.0

The weak interactions between proteins and phospholipids at pH 7.0 and the relatively broad methylene bands due to the large number of phospholipid molecular species make it difficult to analyze the data using only univariate techniques. Multivariate analysis techniques such as PCA may allow extraction of more structural information. PCA was applied to the set of spectra recorded at various temperature corresponding to PL alone, PL + BLG and PL + ALA in 100 mmol·L^{-1} Tris solution, pH 7, in the presence of 0 or 30 mmol·L^{-1} calcium. The map defined by principal components 1 and 2 is shown in *figure 7*. The first two principal components took into account 90 % of the total variation. A discrimination of the samples as a function of the protein in the mixture was observed according to the principal component 1 and the principal component 2 discriminated all the samples as a function of temperature: at low temperature the scores were positive, while at high temperature the scores were negative. Surprisingly, the spectra of PL and PL + ALA in the absence of calcium are superimposed on the spectra of the samples containing 30 mmol·L^{-1} calcium. This indicated that the transition phase of PL and PL + ALA systems was not modified by the presence of calcium. The binding of apoALA and Ca^{2+}-ALA to bilayer vesicles has been previously described at pH 7.4 [5]. These authors have shown that the fluores-

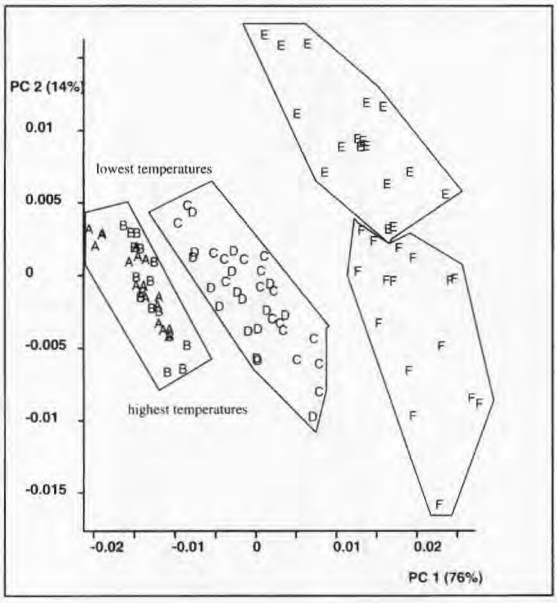

Figure 7. Principal component analysis similarity map defined by the principal components (PC) 1 and 2 for PL (A), PL + Ca^{2+} (B), PL + ALA (C), PL + ALA + Ca^{2+} (D), PL + BLG (E), PL + BLG + Ca^{2+} (F) spectral data of the 3 000–2 800 cm^{-1} region at neutral pH. The spectra were recorded between 6 and 60 °C. See Materials and Methods for details.

Figure 7. Carte factorielle 1-2 de l'analyse en composantes principales réalisée sur les spectres, dans la région 3 000–2 800 cm^{-1}, acquis à pH neutre entre 6 et 60 °C pour les systèmes PL (A), PL+Ca^{2+} (B), PL+ALA (C), PL+ALA+Ca^{2+} (D), PL+BLG (E), PL+BLG+Ca^{2+} (F). Chaque étiquette correspond à un spectre. Se reporter au matériel et méthodes pour les détails.

cence properties of the bound apoALA were altered only slightly. In addition, their fluorescence experiments led them to conclude that the calcium-binding site was not exposed to the surface, but was actually buried in the bilayer structure. From microcalorimetric and fluorescence studies, Hanssens et al. [18] concluded that ALA also interacts with the polar head groups of phosphatidylcholine at pH 7.0.

A different pattern is observed for PL + BLG samples. Principal component 2 allowed the discrimination of the BLG + PL sample without calcium from PL + BLG sample containing 30 $mmol \cdot L^{-1}$ calcium. These results suggested that BLG presented different conformations in the presence and in the absence of calcium modifying its interactions with the milk phospholipids. In order to investigate the effect of calcium on the PL + BLG system more precisely, PCA was applied to the set of spectra recorded at different temperatures and corresponding to PL + BLG system with or without calcium. A discrimination of the samples as a function of calcium in the mixture was observed according to the principal component 1 (data not shown). The spectral pattern corresponding to the principal component 1 showed an opposition between symmetric (positive) and asymmetric (negative) vibration bands of methyl and methylene groups. This result indicated that the ratio of $A_{vas(CH3 + CH2)}/A_{vs(CH3 + CH2)}$ is higher in the absence of calcium than with 30 $mmol \cdot L^{-1}$ calcium. As the calcium ions do not modify the shape of the phospholipid spectra in the 3 000–2 800 cm^{-1} region (*figures 6* and *7*), it can be assumed that the protein conformation is modified by the presence of the cations resulting in different lipid–protein interactions and lipid packings. In fact, it is known that one BLG molecule, at pH 6.6 and ionic strength of 0.1, binds about one calcium ion, suggesting that the protein possesses a specific calcium binding site [4]. The presence of calcium changes the outcome of BLG hydrolysis by thermolysin [13] indicating that the protein exhibits different conformations in the two experimental conditions. The region of the protein sensitive to calcium includes a cluster of five dicarboxylic

amino acids (E44, 45, 51, 55 and D53) that may be involved in the binding of the cation. The structural changes induced by the binding of calcium have also been shown by fluorescence spectroscopy [20].

4. CONCLUSION

The present results show that whey protein binding to milk fat globule phospholipids occur at the two pH investigated and in the presence of calcium. It can be expected from the data that hydrophobic and electrostatic interactions are both involved in lipid–protein binding, with the relative significance of the two varying from case to case. Hydrophobic interactions are predominant for ALA interacting with milk phospholipids at acid pH, whereas the interactions of ALA and BLG with membranes are mainly dependent on electrostatic interactions at neutral pH in the presence or absence of calcium. It also appears that the interfacial properties of the proteins depend on their conformations which can be modulated by the physicochemical characteristics of the medium. The present results show that spectroscopic methods and, in particular, mid-infrared spectroscopy, in combination with multivariate statistical analyses, have broad implications in the understanding at a molecular level of food structures and properties.

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