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Protein Conformational Modifications and Kinetics of Water−Protein Interactions in Milk Protein Concentrate Powder upon Aging: Effect on Solubility

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Protein conformational modifications and water−protein interactions are two major factors believed to induce instability of protein and eventually affect the solubility of milk protein concentrate (MPC) powder. To test these hypotheses, MPC was stored at different water activities (a_w 0.0−0.85) and temperatures (25 and 45 °C) for up to 12 weeks. Samples were examined periodically to determine solubility, change in protein conformation by Fourier transform infrared (FTIR) spectroscopy and principal component analysis (PCA), and water status (interaction of water with the protein molecule/surface) by measuring the transverse relaxation time (T_2) with proton nuclear magnetic resonance (^{1}H NMR). The solubility of MPC decreased significantly with aging, and this process was enhanced by increasing water activity (a_w) and storage temperature. Minor changes in protein secondary structure were observed with FTIR, which indicated some degree of unfolding of protein molecules. PCA of the FTIR data was able to discriminate samples according to moisture content and storage period. Partial least-squares (PLS) analysis showed some correlation between FTIR spectral feature and solubility. The NMR T_2 results indicated the presence of three distinct populations of water molecules, and the proton signal intensity and T_2 values of proton fractions varied with storage conditions (humidity, temperature) and aging. Results suggest that protein/protein interactions may be initiated by unfolding of protein molecules that eventually affects solubility.

KEYWORDS: Principal component analysis; proton NMR; milk proteins; water activity; solubility

1. INTRODUCTION

Milk protein concentrate (MPC) powder is produced from skim milk by ultrafiltration/diafiltration followed by spray drying (*1*, *2*). It contains varying amounts of protein up to 85%. The use of MPC as a food ingredient is increasing worldwide due to its favorable functional properties and high nutritional value. However MPC gradually loses solubility upon storage (*2*−*4*). Generally it is believed that the loss of solubility is linked with conformational modification of protein molecules during processing and storage (*5*). Proteins are stable in their low-energy, native (folded) state; unfolding or denaturation exposes hydrophobic regions of proteins with greater adoption of β-sheet structures, which promote aggregation via protein−protein interactions, leading to destabilization (*6*). It is possible to determine unfolding (denaturation) of protein by monitoring changes in the β-sheet structure. Fourier transform infrared (FTIR) spectroscopy is a well-established method of probing the secondary structure of proteins (*7*, *8*). The amide I (1600−1700 cm^{-1}) band of polypeptides and proteins is sensitive to changes in secondary structure, and analysis of this band is used to investigate these changes (*8*, *9*). In this study, we used FTIR techniques together with principal component analysis (PCA) and partial least-squares (PLS) regression, a multivariate statistical technique for simplifying complex data sets, to extract information from FTIR data on changes in spectral features (*5*, *10*) to probe protein conformational changes upon storage of MPC.

Water plays an important role in the physical or textural characteristics as well as the chemical stability of food systems (*11*). Nuclear magnetic resonance (NMR) offers a variety of methods for characterization of protein systems that can be used to investigate the nature of water−protein interactions and physical states of water over a wide range of time scales (*12*, *13*). In the dairy protein field, the technique of NMR relaxometry has been applied to the study of rehydration of casein, caseinates and milk powders (*14*, *15*), monitoring hydration and solubility of β-lactoglobulin (*16*), hydration of rennet casein and emulsification of fat during cheese manufacture (*17*), and immobilization of water in differently treated whey protein concentrates (*18*). The relaxation times of water protons are sensitive indicators of the local chemical and physical environment in heterogeneous

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materials. This is a result of the dependence of both the longitudinal (T_1) and particularly the transverse (T_2) relaxation times on the strength of residual dipole−dipole couplings with near-neighbor protons. These couplings are moderated by molecular motion and exchange with protons on protein molecules. Thus it is often possible to identify water molecules in a range of distinct environments from the distribution of (T_2) relaxation times of the water protons. The aim of this study was to investigate the changes in water−protein interactions and to relate this to protein conformational modifications and changes in solubility upon aging of MPC.

2. MATERIALS AND METHODS

2.1. MPC Powder. Milk protein concentrate powder was supplied by Murray Goulburn Co-operative Co. Ltd. (Brunswick, Victoria, Australia). Analysis of the powder was carried out by the manufacturer using the method described in Australian standard 2300 (standards Australia 1995). It showed the composition on a dry weight basis was proteins (82.4% w/w), lactose (4% w/w), fat (1.6% w/w), and ash (7.3% w/w). The moisture content of MPC was measured as 5.5% w/w in our laboratory. The proportion of casein to whey proteins was as in the milk used for powder manufacture (ca. 80:20). MPC samples were kept at −4 °C (as powder solubility remained unchanged for long time at or below this temperature) prior to use for experimentation.

2.2. Experimental setup. MPC was stored under two conditions: (i) 24 ± 1 °C at different relative humidity and (ii) at 45 ± 1 °C and high humidity conditions to enhance protein instability. In the first case MPC samples were equilibrated to relative humidity in the range 0−85% by storing them in vacuum-sealed desiccators containing saturated salt solutions (P_2O_5 powder was used for zero water activity) at 24 ± 1 °C for up to 11 weeks. Samples were examined after 4 and 11 weeks of storage for change in solubility. Protein conformational modifications were examined by FTIR after 3 and 10 weeks and change in water status by measuring the proton transverse relaxation time using ^{1}H NMR techniques after 3 and 11 weeks of storage. In the second case, samples were equilibrated to water activity (a_w) = 0 for one week at 24 °C prior to storing them in vacuum-sealed desiccators at equilibrium relative humidity (ERH) 0% and 74% in an incubator at 45 ± 1 °C for up to 7 weeks. Solubility was examined after 3, 4, and 5 weeks of storage. FTIR and ^{1}H NMR experiments were carried out periodically from 12 to 49 days of storage. The same samples (stored under the same conditions) were used for both FTIR and NMR analysis, and all analyses were done in duplicate.

2.3. Water Activity, Moisture Content, and Solubility Test. Water activities (a_w) of the samples were measured using an AquaLab 3TE water activity meter (Decagon Devices. Inc., Pullman, WA). Moisture content (dry basis) of the samples was determined by the AOAC Official Method 927.05 (AOAC International, 2000). The solubility of MPC was determined by a modification of the method described by Anema et al. (*4*). Duplicate 5% MPC solutions were prepared from stored samples using Milli-Q water. The solutions were continuously stirred using a mechanical stirrer (rotor speed 400 rpm) at room temperature for 30 min. Sample solutions (40 mL) were transferred into 50 mL centrifugation tubes and were centrifuged at 1000*g* for 10 min at 20 °C. The supernatant was then filtered under vacuum (GF/A microfiber filter paper, 1.6 μm pore size, Whatman), and duplicate 5 g aliquots of the filtrate were weighed into preweighed aluminum dishes containing ∼20 g of acid washed sand and dried at 105 °C for 24 h. The sample dishes were cooled to room temperature in a desiccator containing dry silica gel prior to weighing. The solubility of the MPC sample was calculated using the following equation:

$$\%\ \text{solubility} = \frac{\text{solids in the supernatant}}{\text{solids in the solution}} \times 100$$

2.4. FTIR Analysis. The protein conformational study of MPC as a function of storage at different a_w and temperature was carried out using a Perkin-Elmer Fourier transform infrared (FTIR) spectrometer (Perkin-Elmer, Spectrum 100, FT-IR spectrometer) with an attenuated total reflection (ATR) cell (Universal ATR). About 2−3 mg of MPC powder (stored at different RH and temperature conditions) was placed on the ATR crystal, and the absorbance intensity was measured at 4 cm^{-1} resolution in the wavenumber range from 4000 to 400 cm^{-1}.

2.4.1. Data Processing. Spectra were baseline corrected and normalized, and second derivatives of the amide I and II (1700−1500 cm^{-1}) bands were determined to indicate the position of individual component peaks within the amide envelope. The amide I region (1700−1600 cm^{-1}) of the spectra was deconvolved using PeakFit 4.11, SeaSolve Software Inc., USA. The concept of Fourier self-deconvolution is based on the assumption that a spectrum of single bands (each narrow band is characteristic of a secondary structure) is broadened in the liquid or solid state. Therefore, the bands overlap and cannot be distinguished in the amide envelope. A curve-fitting procedure was applied to estimate quantitatively the area of each component representing types of secondary structure (*7*). In this study, the deconvoluted spectrum was fitted with Gaussian band shapes by an iterative curve fitting procedure (second-derivative deconvolution, baseline linearization, automatic smoothing). The resultant peaks were assigned to different protein secondary structures (α-helical, β-sheet and turn). Peak assignment of deconvolved amide I bands (1700−1600 cm^{-1}) was done using the results of refs *19* and *20* as a primary guide. Peaks between 1640 and 1620 cm^{-1} are assigned as β-sheet, 1650 ± 2 cm^{-1} as α-helix and between 1700 and 1660 cm^{-1} as β-turns. The peak at ∼1648 cm^{-1} was considered to represent a loop or helix and the peak at ∼1658 cm^{-1} a large loop and assigned as an α-helix in the fitting routine.

Statistical significance of the change in secondary structural components between different sample groups (aged and nonaged, stored at high and low a_w etc.) was determined by calculating one-way ANOVA (analysis of variance) using the software Minitab 15, Minitab Inc., USA.

2.4.2. Multivariate Analysis of FTIR Data. The total number of data sets used for PCA analysis was 23. These included data for MPC samples stored at various water activity conditions at room temperature as well as samples stored at high (45 °C) temperature. All FTIR spectra were baseline-corrected and normalized. Data transformation was done by multiscattering correction (MSC) prior to PCA calculations. PCA was performed on both the whole FTIR spectra and the amide I (1700−1600 cm^{-1}) region of the spectra using the chemometric software LatentiX 2.0, Latent5, Copenhagen, Denmark.

The relationship between FTIR measurements and solubility was obtained by applying partial least squares (PLS) regression analysis using the Simca-P+ software (Umetrics AB, New Jersey). The PLS regressions were carried out on centered multiscattering-corrected (MSC) FTIR data, and the goodness of fit was analyzed by assessing the validated correlation coefficients (R^2) and root-mean-square error of cross validation (RMSECV).

2.5. NMR Analysis. A Bruker MSL-300 spectrometer with resonance frequency 300.13 MHz was used for proton transverse relaxation time T_2 measurement. T_2 relaxation curves were obtained using the single point Carr−Purcell−Meilboom−Gill (CPMG) pulse sequence. T_2 relaxation times were measured using CPMG echo trains with pulse spacing T_E ranging from 10 to 100 ms and with a maximum of 512 pulses. The T_2 distributions were obtained from the decay of the signal intensity plotted against time using the open source software CONTIN, which is based on Laplace transform algorithm (*21*). The experiments were carried out at 290 K (17 °C).

3. RESULTS AND DISCUSSION

3.1. Solubility. The initial solubility of fresh MPC powder was 70 ± 2.7%, and moisture content (mc) on a dry weight basis was 5.4%. Solubility declined (**Table 1**) even during storage at room temperature for only 4 weeks (65% for samples stored at ERH 23%, mc 5.5% and 30% for sample stored at ERH 85%, mc 17%). After 11 weeks of storage, solubility was negligible for samples stored at high ERH (> 50%). It should be noted that the test used measures solubility after a fixed time of 30 min; increased solubility occurs after longer times of stirring in water (*3*).

In the case of storage at high temperature (45 °C) and high humidity conditions (ERH = 74%), the solubility decreased rapidly from around 70% initially to 24.2 ± 0.3% and 14.3 ± 2.9% for samples stored for 3 weeks at ERH 0% and 74%, respectively. After 4 weeks of storage, the solubility was negligible (**Table 1**). This observation is consistent with previous work on the effect of temperature and aging time on the solubility of high

Table 1. Solubility of MPC 85 Powder after 3 and 9 Weeks of Storage at Room Temperature at Different a_w and Storage at 45 °C and at a_w 0.0 and 0.75 for 3−4 Weeks

		solubility %		
a_w	storage temp (°C)	3 weeks	4 weeks	11 weeks
0.23	24 ± 1		65 ± 0.8	35 ± 14
0.45	24 ± 1		48 ± 2.2	16.6 ± 0.2
0.69	24 ± 1		42 ± 0.04	10.8 ± 2.7
0.85	24 ± 1		29.6 ± 0.01	7.5 ± 10.6
0.00	45 ± 1	24.2 ± 0.33	14.1 ± 7.3	
0.74	45 ± 1	14.3 ± 2.9	6.82 ± 0.2	

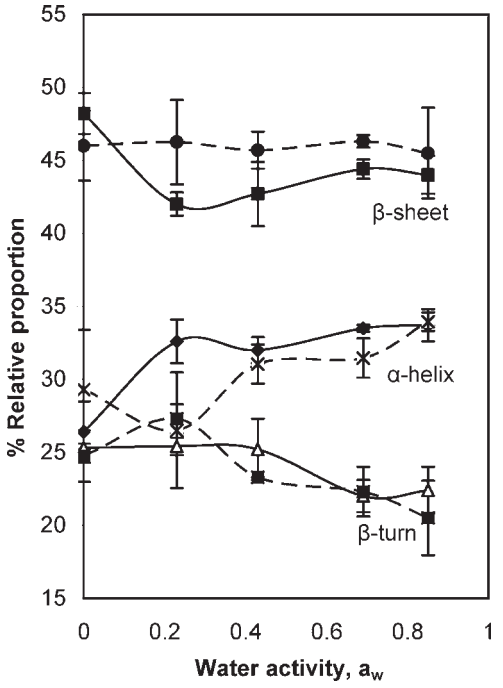

Figure 1. Relative fractions of different structural components after room temperature (24 ± 1 °C) storage of MPC 85 for 3 and 10 weeks at different a_w. Solid and dashed lines represent corresponding structural components after 3 and 10 weeks of storage, respectively.

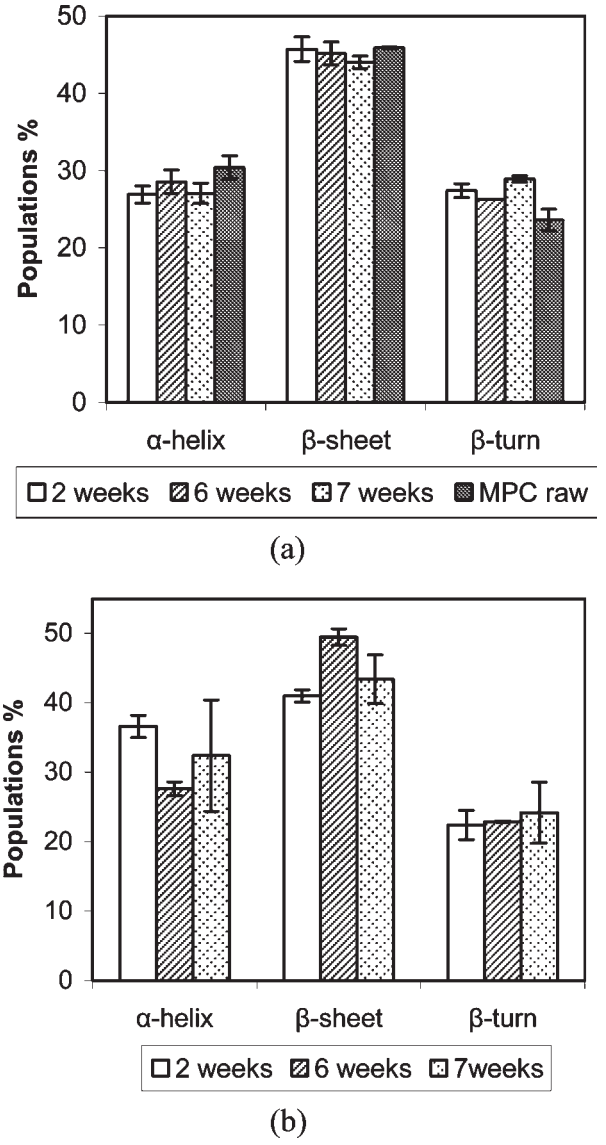

Figure 2. Relative fractions of different structural components after high-temperature (45 °C) storage at (**a**) a_w 0 and (**b**) a_w 0.74.

protein powder (*4*). The study by Anema et al. indicated that MPC solubility decreased with increasing aging time and temperature and suggested that casein was the insoluble fraction of MPC powder and cross-linking of the protein at the surface could be the cause of insolubility indicated by a gel electrophoresis study (*4*). Havea et al. showed that insoluble material obtained during MPC rehydration consisted of large particles (~100 μm) where the casein micelles were fused together by some form of protein−protein interactions predominantly hydrophobic in nature (*2*). In a recent study, Mimouni et al. showed that the low solubility of MPC85 powder (the same one as used in the present study) is a consequence of slow dissolution kinetics rather than presence of a large amount of insoluble matter, at least for the samples of less than 6 months old. They also showed that the rehydration process consists of two overlapping steps, namely, disruption of agglomerated particles and release of material from the powder particles to the surrounding phase. Both occur simultaneously, and the latter process is suggested to be the rate-limiting step (*3*). However, this study did not show the importance of irreversible aggregation and whether the protein molecules after solubilization attained altered structure with respect to storage at different conditions (a_w, temperature, time). This is another important aspect which needs to be explored. These studies, along with our current results, suggest that aging induces physicochemical changes in MPC powder particles/molecules which lead to a rapid decline in solubility. High temperature and a_w greatly enhance this process.

3.2. Protein Conformational Modifications. Based on infrared analysis of the amide I band, small changes in secondary structural components with time of storage and/or water activity conditions were observed (**Figure 1** and **Figure 2**) for both moderate (24 ± 1 °C) and high (45 ± 1 °C) temperature storage. In the case of storage at 24 ± 1 °C, the relative fraction of α-helix was slightly higher in samples stored for 3 weeks than that of samples stored for 10 weeks for all levels of water activity (**Figure 1**). Consequently, the relative fractions of β-sheet components were a little higher after 10 weeks than after 3 weeks for all levels of water activity (**Figure 1**). The content of β-turn showed a slight increase after 10 weeks compare to that after 3 weeks for lower a_w samples but decreased for higher a_w (> 0.23) samples. Thus there was a decrease of helical components and a corresponding increase of β-components (specifically β-sheet) with aging of MPC. These transformations of helical and loop components (folded structure) to sheet and turn components (unfolding) indicate changes in the secondary structural conformation of the

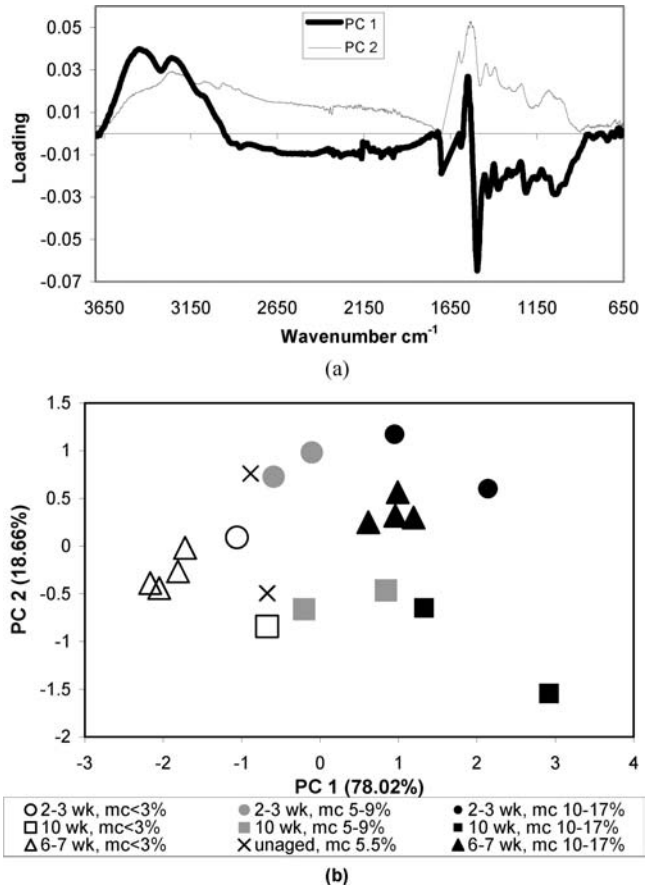

Figure 3. PCA loading (**a**) and score (**b**) plots of first two principal components calculated using whole FTIR spectra excluding the amide I region. Legend name prefix number followed by wk indicates number of storage weeks, and prefix mc followed by number indicates percent moisture content.

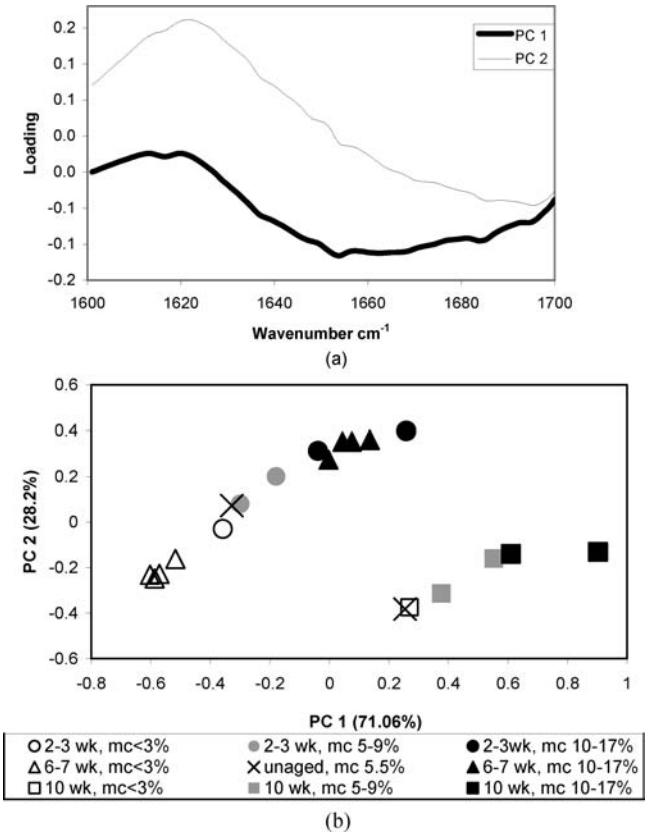

Figure 4. PCA loading (**a**) and score (**b**) plots of first two principal components calculated from amide I region of the FTIR spectra. Legend name prefix number followed by wk indicates number of storage weeks, and prefix mc followed by number indicates percent moisture content.

proteins during storage. However, these changes of structural components at different water activities were minor (±1−9%).

A similar result was obtained for high-temperature storage (**Figure 2**). For samples stored at 0% ERH (mc 2.8%), different structural components remain almost unchanged with storage time, whereas at ERH 74% (mc 10.8%) helical components tended to decrease and sheet and turn components correspondingly increased with increasing period of storage especially at high ERH (**Figure 2**). Overall, the effect of storage temperature and a_w on the relative fractions of different secondary structural components covered a restricted range (α-helix 26−36%, β-sheet 42−49% and β-turn 20−29%) (**Figure 1** and **Figure 2**) consistent with relatively minor average changes in protein conformation. For both low and high temperature storage statistical analysis did not show any significant difference between different sample groups. In all cases p values greater than 0.05 were obtained which indicate the limited change in protein conformation was statistically not significant.

The solubility of MPC powders declined rapidly with time of storage and increasing water activity. High temperature enhanced this process (**Table 1**). It is generally believed that unfolding of the secondary structure of a protein leads to alteration of its functional properties such as solubility. It was expected that such a change would be detected in the FTIR analysis, but this was not apparent in these results. The reason for this could be that the protein conformational change is partial (or not extensive), but that a minor average change could lead to a major change in solubility by influencing the protein−protein interactions which seems to be responsible for the slow dissolution kinetics of MPC (*22*). This is further discussed below.

3.3. Multivariate Analysis of FTIR Data. Principal component analysis on whole spectra excluding the amide I region showed that 96.6% of the data variations were explained by the first two principal components (PCs) and 100% was explained by the first four PCs, thus higher PCs were not relevant. Around 78% of the variations was explained in PC 1 and was related to the water content (moisture content and water activity which are correlated) of the samples. Score plots of the first two PCs showed that samples were grouped according to low or high moisture content (**Figure 3b**) along PC 1. A similar grouping was observed for water activity as it was correlated with moisture content. The storage period was approximately explained along PC 2, which explained 18.6% of the data variations (**Figure 3b**). From **Figure 3b** it is clear that low MC samples (white symbols in **Figure 3b**) are grouped together with one exception (for 10 week storage). Intermediate MC samples (MC 5−9%, gray symbols in **Figure 3b**) along with unaged samples (MC ∼5%, cross symbol in **Figure 3b**) were separated into two subgroups i.e. shorter aging period (2−3 weeks) and longer aging period (10 weeks) groups. Samples that were aged for 10 weeks remained separated from other groups (rectangle in **Figure 3b**). The PCA score plot with respect to solubility did not show well-defined groupings of samples (figure not included). The results indicate that FTIR spectral features do not directly correlate with solubility.

PCA analysis of the amide I band data showed a similar result to that of whole spectra except amide I region with 71% and 28% data variation being explained in PC 1 and PC 2 respectively (**Figure 4a** and **4b**). The major difference between the amide I

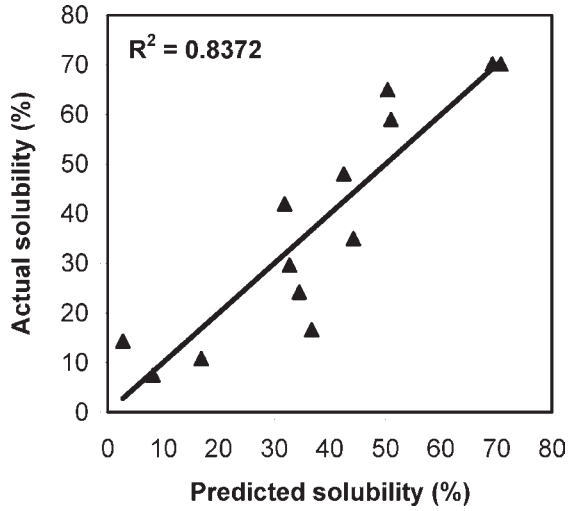

Figure 5. PLS regression of predicted vs actual solubility percentage of MPC samples. PLS regression calculated on 1734−650 cm^{-1} region.

band data and those for the other spectral region was the samples aged for 10 weeks which remained isolated from the rest of the samples. The score plot of the first two PCs showed a similar grouping of samples according to moisture content and storage period though not as precise as that for the whole spectra excluding the amide region (**Figure 4b**). In this case also no well-defined grouping of samples was observed for the solubility data. It should be noted that the main differences in FTIR spectra of MPC samples was in the region around ~3260 cm^{-1} (assigned to OH bond stretching) due to the different moisture contents. The spectral intensity in this region increased with increasing moisture content of the samples although other parts of the spectra remained almost unchanged. This spectral feature (change in band intensity at 3260 cm^{-1} region as a function of varying moisture content of the samples) could be represented in the score plot of the PCs. From the score plot of principal components, a correlation was also observed between spectral features and time of storage.

This observation was analogous to the result obtained by Kher et al. (2007) on spray-dried MPC powder by FTIR analysis of second-derivative spectra followed by PCA. They found a change in band intensity/position in the 1631 cm^{-1} region (associated with β-sheet) among samples that lost nitrogen solubility significantly upon storage, whereas the band in the 1660−1650 cm^{-1} region (associated with α-helix) did not change significantly. PCA discriminated among samples of a particular MPC where nitrogen solubility changed significantly upon storage, but did not discriminate where sample solubility change was negligible (*5*). In our case, samples were stored at different ERH and temperature which affected the solubility greatly and PCA was able to discriminate between samples according to mainly moisture content and aging period.

In order to determine if infrared data could predict solubility, a partial least-squares analysis was performed. Using data from 650−1734 cm^{-1}, predictions of solubility were found to account for approximately 60% of the observed variation (RMSECV 10.9). For data from 1741−4000 cm^{-1}, prediction was not as reliable, explaining ca. 55% of the observed variation (RMSECV 12.8). **Figure 5** showed the PLS regression of predicted vs actual solubility percentage calculated for 1734−650 cm^{-1} region.

As protein is the predominant component in these systems, infrared spectral intensities are likely to be determined primarily by protein features. The fact that PLS analysis reveals a reasonable level of prediction of solubility when taking account of a wide

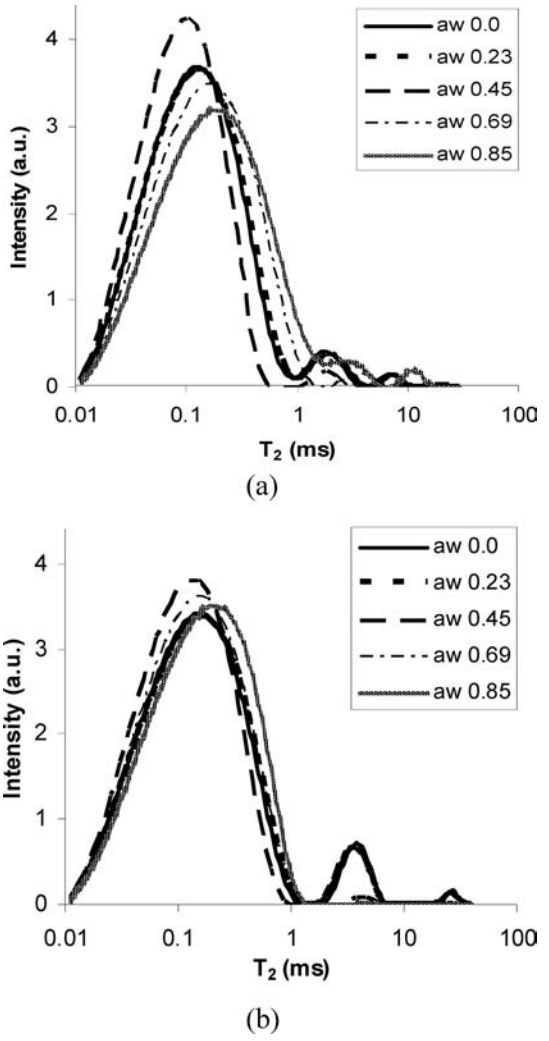

Figure 6. T_2 distribution profiles of MPC samples stored at room temperature (24 ± 1 °C) for (**a**) 3 weeks and (**b**) 11 weeks at different a_w. *X*-axis is in logarithmic scale.

spectral range, whereas ANOVA analysis of the amide I band did not show significant differences, suggests that a simple interpretation of solubility differences in terms of changes in α-helix and β-sheet contents may not be appropriate.

3.4. Water−Protein Interactions. In all cases, the T_2 (proton transverse relaxation time, millisecond time scale) distribution profile could be fitted by three populations of relaxation times (**Figure 6** and Figure **8**). In reality, this is likely to be an approximation of a broader distribution of exponential functions, but provides a minimum number of relaxation time ranges that can be interpreted in terms of water−protein interactions. The short T_2 component (~0.01−2 ms) is assigned to water protons in close proximity to the protein surface that exchange rapidly and may have low mobility. The longest T_2 component (~5−28 ms) is associated with the fraction of water that is furthest from a protein surface and which may have higher mobility. The intermediate T_2 component (~1.0−7.4 ms) relates to an intermediate form of water that is moderately mobile. Generally peak widths indicate uniformity of the samples with broader peaks corresponding to less uniform samples (*17*, *23*). The peak of the short T_2 component was narrow (from linear plot of T_2 distribution, figure not included), indicating that this protein-associated water fraction was relatively uniform. Peak width (distribution of relaxation time) became progressively wider for the other two water components, suggesting that there were greater variations in the

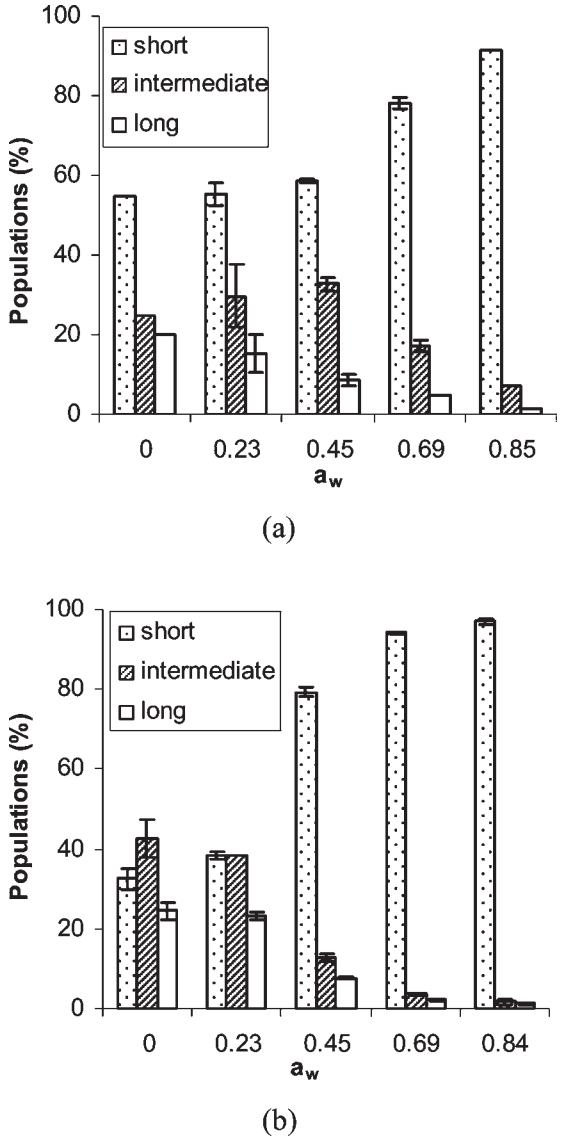

Figure 7. T_2 proton fractions of MPC stored at room temperature (24 ± 1 °C) for (**a**) 3 weeks and (**b**) 11 weeks at different a_w. Proton fractions were determined by calculating the corresponding peak area from linear T_2 distribution plot.

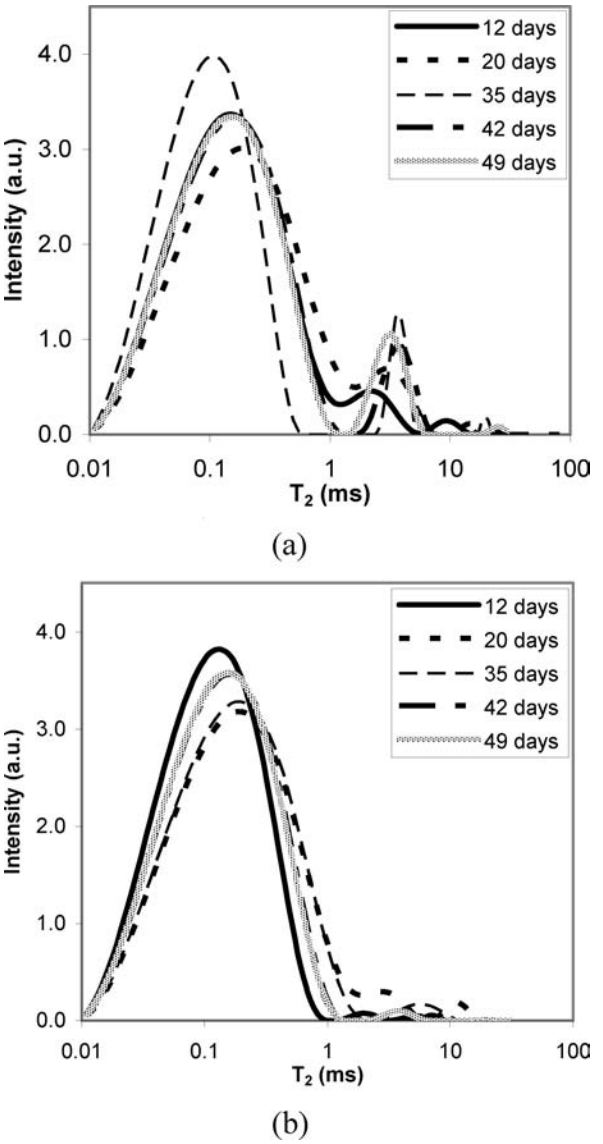

Figure 8. T_2 distribution profile of MPC samples stored at high temperature (45 ± 1 °C) for up to 49 days at a_w (**a**) 0.0 and (**b**) 0.74. *X*-axis is in logarithmic scale.

physical environments of the protons and that these fractions were less uniform. After 3 weeks of storage at room temperature (24 ± 1 °C) and at different a_w there was a decrease in T_2 with increasing water content up to a_w 0.45 (MC 8.2%), and then a further increase upon increase of a_w (MC) was observed (**Figure 6a**). The overall T_2 values increased with added water as expected for an increased average distance to protein surfaces. This indicates that the exchange (of water) became more important as water is added to the low-moisture-content samples. The proton fraction with short T_2 values increased progressively with increasing MC, and consequently proton fractions of intermediate- and long-T_2 pools decreased (**Figure 7a**). This indicates increased interaction of water with protein sites resulting in reduced mobilization of water.

After 11 weeks of storage at room temperature, the T_2 distribution pattern was similar to that after 3 weeks. That is a decrease in short T_2 with increase of a_w up to a_w 0.45, and subsequent increase with further increase of a_w was observed. The difference in short T_2 with change in a_w was less pronounced than after 3 weeks of storage (**Figure 6a** and **Figure 6b**). This change in short T_2 may suggest that proton spins close to the protein surface were experiencing a similar physical environment indicating a similar average arrangement of protein molecules at the different storage times. Another noticeable change was that the intensity of the signals from intermediate- and long-T_2 fractions decreased more for samples stored at high a_w (≥0.45, MC 8.2%) than for samples stored at lower a_w (**Figure 7b**). On the other hand, for samples kept at lower a_w (< 0.45), proton fractions with short T_2 values decreased and those of intermediate and long values increased after 11 weeks storage compare to that after 3 weeks of storage (**Figure 7a** and **Figure 7b**). This suggests that, at higher a_w (MC), water molecules from more mobile phases (intermediate and long pool) migrated to near the protein surface and became less mobile, with the distinction between short and intermediate T_2 fractions diminishing. However, at lower a_w, water molecules of the short-T_2 pool seem to move to outer (intermediate and long) fractions. This is a generic feature of proteins under variable (low) moisture conditions and will be reported on elsewhere (Strounina et al., in preparation).

In the case of storage at high temperature (45 ± 1 °C), for low-a_w samples (a_w 0.0, mc 2.86%) the short-T_2 pool decreased

slightly specifically after 35 days of storage though it did not change significantly afterward (**Figure 8a**). Also at high a_w storage (a_w 0.74, mc 10.8%), the short-T_2 pool did not change much with storage time (**Figure 8b**). In the case of MPC powder, the water−protein interaction pattern is most sensitive to moisture content of the samples, as was observed for low temperature storage (considering systematic change in T_2 distribution with respect to increasing moisture content). As for high temperature storage, the MC remained nearly unchanged (for both a_w 0.0 and a_w 0.74 samples) during the storage period, and little change was observed in the T_2 distribution profile. It was observed that, for the low-moisture-content samples (a_w 0.0, MC 2.8%), the proton fraction of the short-T_2 pool decreased (~15%) with increasing storage period up to 35 days and then remained unchanged (**Figure 9a**). In the case of high a_w samples (a_w 0.74, MC 10.8%), the proton fractions with different T_2 values remained mostly unchanged with aging (**Figure 9b**). This observation may indicate that changes in protein/water interactions occurred within the short period of storage under adverse conditions.

Our studies on protein conformational modification upon storage of MPC 85 powder at different water activities and storage temperatures indicate some limited unfolding of protein (transformation of helical components to β-components) upon aging. This protein denaturation was aggravated by adverse storage conditions such as high water activity and higher temperature and had a negative impact on solubility. A similar result was obtained in other studies (*5*). The water−protein interaction behavior observed by proton transverse relaxation time suggests that, as proteins unfold, water molecules can interact with a greater proportion of the surface of the protein. The traditional view is that charged groups on proteins alter the normal structure of the water in their vicinity, and water becomes oriented in layers around these groups. As a_w is increased (0.2−0.7), the nonionic groups and the accessible peptide bonds are thought to become progressively saturated. This interpretation of water binding to dry protein was described by Kinsella et al. (*24*). A similar feature of increased water binding to the protein surface (increase of short-T_2 fraction) of denatured whey protein compared to native whey protein was suggested for differently treated whey protein concentrates (*18*). However, the increase in the fraction of protons with short T_2 values with increasing moisture is inconsistent with this interpretation. As will be reported in detail elsewhere (Strounina et al., in preparation), it is more likely that low water contents result in limited interaction with protein surfaces, and that greater contact with protein surfaces occurs as water content increases up to ca. 20% w/v.

Recent studies on MPC solubility by scanning electron microscopy (SEM) suggest that during storage increased interaction between and within casein micelles results in compaction of micelles and formation of a closely packed monolayer skin of casein micelles that seem to be responsible for the slow dissolution kinetics of MPC (*22*). Our studies showed a gradual decline of solubility with aging at more adverse conditions such as high ERH (or a_w) and temperature. Also studies on protein conformational change and water−protein interactions showed comparatively more protein unfolding (although overall transformation was not extensive) and increased water−protein interaction with storage at more adverse conditions. PLS analysis also showed a correlation between solubility and FTIR spectral feature. Based on our observations, it could be suggested that unfolding of protein (specifically casein) molecules may be related to protein denaturation and may initiate casein−casein interaction within a micelle. Adverse storage conditions aggravate this transformation process. As solubility became negligible upon long time storage of MPC at adverse conditions, this result suggests that

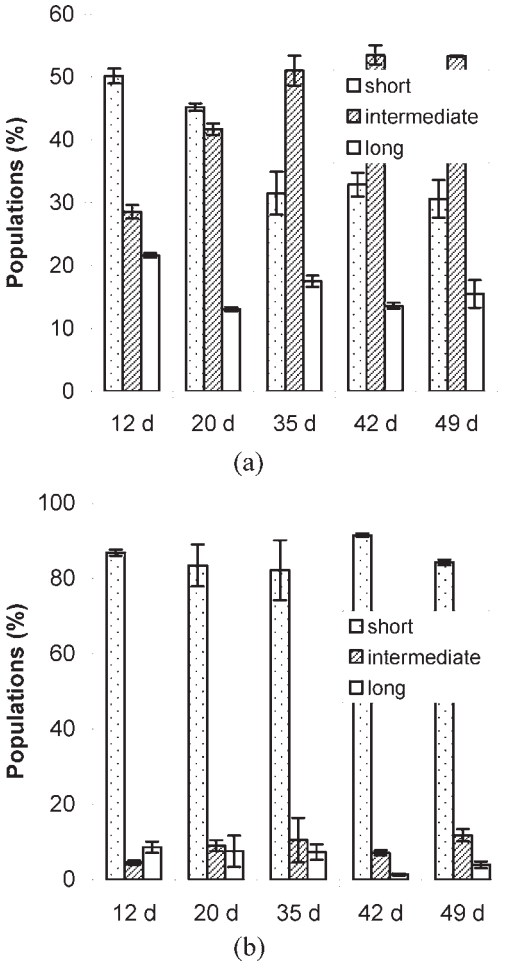

Figure 9. T_2 proton fractions of MPC samples stored at high temperature (45 ± 1 °C) for up to 49 days at a_w (**a**) 0.0 and (**b**) 0.74. Proton fractions were determined by calculating the corresponding peak area from linear T_2 distribution plot.

denaturation also affects whey protein solubilization. It is unlikely that protein unfolding is solely responsible for the whole transformation process, but it may be the initiation step. More studies are required to understand the molecular mechanism of subsequent steps. One suggestion is that the protein unfolding and subsequent association may be more extensive at the surface of particles, resulting in the "skin" observed by SEM (*22*). If this is the case, then the small average changes in protein secondary structure and protein/water interactions reported here could reflect a substantial change for surface components and little or no change for components in the interior of particles. This would be consistent with the observation of rapid solubilization of micelles from within a particle of MPC once the surface "skin" is breached (*22*).

In this study we investigated the interrelationship between loss of solubility of MPC, protein conformational modification and water−protein interactions. The results indicate unfolding of protein is occurring upon aging, and is enhanced with adverse (in terms of subsequent solubility) storage conditions. PCA showed some correlation of moisture content and aging period with FTIR spectral features. Also PLS analysis showed some correlation between solubility and spectral features. Study of proton transverse relaxation time showed that water−protein interactions increased as protein denaturation increased with aging. Protein unfolding seems to be the initiation step of possible casein−casein interaction within a micelle which eventually leads

to changes in casein micelle microstructure which affect solubility. It is possible that changes in protein secondary structure and water−protein interactions are greatest at the surface of MPC particles, which would explain why small average changes reported in this current study could lead to major differences in solubility.

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