International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:5, No:1, 2011

ReveaLing Casein Micelle Dispersion under Various Ranges of Nacl: Evolution of Particles Size and Structure

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***Abstract*—**Dispersions of casein micelles (CM) were studied at a constant protein concentration of 5 wt % in high NaCl environment ranging from 0% to 12% by Dynamic light scattering (DLS) and Fourier Transform Infrared (FTIR). The rehydration profiles obtained were interpreted in term of wetting, swelling and dispersion stages by using a turbidity method. Two behaviours were observed depending on the salt concentration. The first behaviour (low salt concentration) presents a typical rehydration profile with a significant change between 3 and 6% NaCl indicating quick wetting, swelling and long dispersion stage. On the opposite, the dispersion stage of the second behaviour (high salt concentration) was significantly shortened indicating a strong modification of the protein backbone. A salt increase result to a destabilization of the micelle and the formation of mini-micelles more or less aggregated indicating an average micelles size ranging from 100 to 200 nm. For the first time, the estimations of secondary structural elements (irregular, ß-sheet, α-helix and turn) by the Amide III assignments were correlated with results from Amide I.

***Keywords*—**Casein, DLS, FTIR, Ionic environment.

I. Introduction

MILK proteins are the most valuable leading component of milk and are often commercialised in a dehydrated form to extend their shelf-life, facilitate their use and reduce transportation costs. They play a range of valuable roles regarding nutrition, physical functionality. Furthermore, their use under controlled conditions to produce nutritional, functional or flavourful dairy products and occasionally biological activities was often demonstrated [1].

Caseins are the most important class of milk proteins and are widely used as food ingredients mainly due to their water binding, emulsifying, foaming, gel forming and thickening capacities [2]. These are the network formers in dairy products such as yoghurt, cheese… In milk, casein exists as micelles, comprising α_{s1}-, α_{s2}-, β- and κ-casein as well as minerals, and exists in proportions of approximately 4:1:4:1 by weight respectively [3].

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Addition of salts was found to have a major influence on the rehydration process of NMC powder by modifying its structure and the mineral composition [4] [5]. Moreover, few studies were done on the estimation and quantification of spectral structures in whole casein with reference of Amide I [6] [7]. FTIR is becoming an increasingly important method to study protein secondary structure, mostly by the analysis of the amide I region. With the use of FTIR, various elements of the secondary structures of individual casein for Amide I (α-helix, β turns, random coils and β-sheets) have already been determined [8]. Although Amide I spectral region (1700-1600 cm^{-1}) has a strong signal but presents also important limitations including a significant interference from water vibrational bands (around 1640-1650 cm^{-1}), a relatively unstructured spectral outline, and a bands overlap being able to correspond to various secondary structures. In contrast, Amide III bands (1350-1200 cm^{-1}) are easily resolved, better defined therefore are quite suitable for quantitative analysis of protein secondary structure as they do not have the precedent limitations [9]. In earlier investigations, various but limited techniques have been employed to study the effect of NaCl on the structural properties of casein micelles or milk systems [10], [11] including DLS [12], [13].

Here we report the influence of the ionic environment (distilled water, NaCl solution) on CM powder rehydration and secondary structure. In this work, micelle casein dispersions under high NaCl environments were characterized at various structural levels; including a study of molecular changes occurring upon salt increase by means of Fourier transform infrared spectroscopy, and dynamic light scattering. From an industrial point of view, our project earns vital significance due to a lack of basic data on the dynamics of hydration in connection with environment and poorly controlled rehydration step in food industry.

II. Materials And Methods

A. Samples

Micellar casein powder was obtained from International Dairy Ingredient (IDI, Arras, France). This industrial powder (Promilk 872B) is prepared by microfiltration from milk and presents a high percentage of native micellar casein. The composition of the powder (wet basis) was 86.9 wt % proteins (Kjeldahl, N x 6.38), 0.3 wt % fat, 0.4 wt % lactose, 7.6 wt % ashes and 4.8 wt % moisture. NaCl salt was furnished by VWR (Prolabo, Belgium). Sodium azide (Merck, KGaA,

Germany) was added as antimicrobial agent in each rehydration media (0.2 $g.L^{-1}$). Milli-Q water was used in all sample preparation.

B. Rehydration Setup

The experimental setup used to follow the rehydration kinetics was already described in detail [14]. The rehydration was carried out in a 2-L vessel equipped with a 4-blade 45° impeller (R 100 impeller: 8 cm diameter) rotating at 450 rpm (Lightnin Lab Master Mixer, Axflow, France). The turbidity sensor was positioned through the vessel wall to avoid disturbances during stirring. A turbidity meter (Analite NEP 160, McVan Instruments, Australia) was used to monitor turbidity changes accompanying powder rehydration. A measurement system for continuous monitoring (Almemo 8990-8V5, Ahlborn, and Holzkirchen, Germany) was connected to the turbidity meter. Turbidity data were collected automatically every 5 second for 30,000 s at least in triplicate. For all experiments, the powder concentration was fixed at 5% (wt/vol). The powder was poured in less than 5 seconds, 60 seconds after starting the monitoring in order to obtain a correct stabilization of turbidity. All analyses were performed on totally rehydrated micellar casein powder at 5 wt % in NaCl media at 8 different concentrations ranging between 0 and 12% salt.

C. Infrared Measurements

FTIR scans were obtained with a Tensor 27 mid-FTIR Bruker spectrometer (Bruker, Karlsruhe, Germany) equipped with an ATR cell (Total Attenuated Reflection mode) and a MCT (Mercury-Cadmium-Telluride) detector cooled with liquid N_2. Scanning rate was 20 kHz and 256 scans were used for reference and samples between 4000 and 850 cm^{-1}. The nominal instrument resolution was 2 cm^{-1}. References were recorded on water or NaCl salt solutions according to their concentrations. Then, 2 mL of the micellar casein solution was put on the ZnSe crystal of the optical cell and left 5 minutes allowing for proteins adsorption onto the crystal. This considerably ameliorated the signal-to-noise ratio of FTIR spectra. The ATR equipment was purged with dry air for the duration of the measurements. Three to five separate experiments were done for each salt concentration.

All treatments were carried out using OPUS software (Bruker, Karlsruhe, Germany). Raw absorbance spectra were smoothed using a nine-points Savitsky-Golay smoothing functions. Spectra were cut between 1720 and 1580 cm^{-1} or between 1200 and 1350 cm^{-1} for analyzing amide I and amide III bands respectively. Elastic baseline correction using 200 points was then applied to spectra. After that, spectra were centered and normalized using OPUS software. Second derivative spectra were calculated on centered and normalized data with an additional nine-points Satvitsky-Golay smoothing function. These second derivative spectra were used only for identifying individual peak positions as already described by many researchers in detail. [7] [8] [15]. Finally, the treated spectra were deconvoluted by a non linear regression curve fitting program of Gaussian peaks to the original spectra (Opus Software).

D. Dynamic Light Scattering

A Nanosizer ZS (HPPS 5001, Malvern Instrument, England) was used to follow the evolution of the hydrodynamic diameter (also called "z-averaged" diameter) of the caseins dispersions. The apparatus is equipped with a 4 mW He/Ne laser emitting 633 nm, a measurement cell, a photomultiplier and a correlator. The samples were placed in vertical cylindrical cells (10 mm diameter) for size measurements. Scattering intensity was measured at a scattering angle of 173° relative to the source using an avalanche of photodiode detector. This setup allows considerable reduction of the signal due to multiple scattering events and enables working in slightly turbid media. The D_T parameter is related to the hydrodynamic radius (R_h) of particles through the Stokes-Einstein relationship (eq. 1):

$$D_T = k_B T / 6\pi\mu R_h \quad (1)$$

Where μ is the solvent viscosity ($N.s.m^{-2}$), k_B (1.38×10^{-23} $N.m.K^{-1}$) is the Boltzmann constant, T is the temperature (K) and R_h is the equivalent hydrodynamic radius of sphere having the same diffusion coefficient than the particles (nm). Before measurements, samples were prepared by suspending 12.5 µL of different rehydrated CM powder solutions in about 50 ml of their corresponding brine solutions. Each sample was filtered on 0.20 µm pore size filter (Minisart RC 15 Sartorius) at 25°C. All measurements were carried out in triplicate.

E. Statistical Analyses

The average and standard deviations were calculated for each parameter. Descriptive statistical analysis was carried out by using the software KyPlot version 2.0. For comparisons between casein micelle sizes with and without NaCl, a parametric multiple test was performed (Dunnett test with respectively casein micelle size in water as control). The significance level was: ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ and $^{NS}P > 0.05$.

III. Results And Discussion

The rehydration of 5% native micellar casein in water is followed with a turbidity sensor. The profiles obtained in water are presented as Figure 1**.** The turbidity profile is interpreted in term of wetting, swelling, dispersion and total rehydration stages as already described in detail [16].

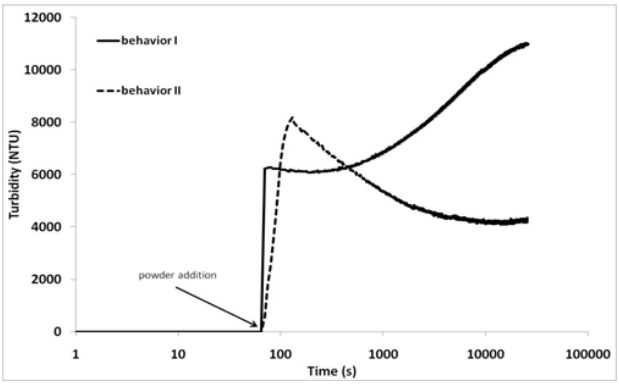

Fig. 1 Turbidity profiles obtained during rehydration of 5% micellar casein (MC) powder at 24°C for 30,000 seconds in NaCl solutions [23].

The prolonged rehydration stage may be related to the interaction of water protons with casein, the mobility of which is greatly reduced by their structuration in micelles. As a consequence, micellar casein powders are generally considered to be poorly soluble powders for which rehydration of micelles is a time consuming process [17] [18]. Two rehydration behaviours are obtained for micellar casein rehydration in NaCl as summarized in Table 1.

TABLE I
REHYDRATION TIMES OBTAINED FOR CASEIN MICELLAR POWDER IN WATER AND VARIOUS RANGES OF NaCl SALT [23]

NaCl (%)	Wt(s)	St(s)	TRt(s)	Rehydration Behavior
0	10	120	28 000	I
0.75	33*	880***	nr	I
1.5	37*	1233***	nr	I
2.25	40*	1405***	nr	I
3	45*	3295***	nr	I
6	55**	no	14 291**	II
9	56**	no	13 100**	II
12	62**	no	12 999**	II

Wt: wetting time; St: swelling time; TRt: total rehydration time

The first behaviour is found for casein rehydration in water up to 3% salt (0, 0.75, 1.5, 2.25 and 3%). For these five profiles, it is possible to distinguish the following stages: wetting, swelling and dispersion (behaviour I in Fig. 1). The second behaviour (Behaviour II in Fig. 1) is obtained for higher salt concentrations (6, 9 and 12%).

The wetting stage is still longer than in water (55, 56 and 62 seconds) but the total rehydration is shortened (around 13,000 seconds for each concentration). For this behaviour, the swelling stage is not observed. In agreement with [18], the addition of NaCl improves the reconstitution period whatever the incorporation mode.

Particle size of MC rehydrated at 5 wt % in NaCl media was followed by DLS. The changes in micelle size with and without addition of different concentration of NaCl are presented in Table 2. Two populations are systematically observed. A first hydrodynamic diameter (D_h) corresponding to the 1st peak was found around 153 nm for casein dispersions in water. A 2nd peak was observed around 46 nm for the same casein dispersion. This peak represents the mini-micelles called submicelles upon addition of NaCl (ranging from 0.75% to 12%), no significant differences was observed for each D_h; the particle size ranging between 149 and 182 nm for the first peak and between 31 and 46 nm for the second peak. The first peak decreases and the second peak increases with the increment of percentages of s alt (Table2). Upon addition of NaCl, no significant changes in micellar size were reported. According to [12], there is a loss of Ca^{2+} from the micelles upon addition of NaCl, furthermore the amount of calcium bound to α_{s1}-casein or β-casein decreases with increasing ionic strength.

TABLE I
VARIATION OF THE HYDRODYNAMIC DIAMETERS WITH ADDED NACL CONCENTRATION.

NaCl (%)	1st peak Hydrodynamic diameter (nm)	2nd peak Hydrodynamic diameter (nm)
0	153 ± 2.9	46 ± 3.5
0.75	177 ns ± 5.3	36 ns ± 7.3
1.50	182 ns ± 8.2	38 ns ± 9.0
2.25	180 ns ± 14.5	37 ns ± 9.2
3	175 ns ± 22.0	33 ns ± 14.1
6	161 ns ± 4.6	31 ns ± 2.0
9	157 ns ± 29.8	34 ns ± 1.0
12	149 ns ± 14.3	34 ns ± 4.0

Moreover, this release of Ca^{2+} is not associated with loss of phosphorous, indicating that it originates from Ca^{2+} bound to casein molecules and not from the calcium phosphate nanoclusters. Accordingly, this Ca^{2+}/Na^{+} exchange may dissociates some of the calcium bridges that holds the casein molecules together and also increase the ionic pressure inside the micelle. These effects could lead to a less connected network. As a consequence of these changes, the hydration of casein micelles is increased, even if their sizes stay constant [19], [20].

Another study [21] showed that NaCl addition to casein micelles in skim milk stabilized them against aggregation by an increase of the hairy layer but with no modification of the casein hydrodynamic radius. In conclusion, a variety of

molecular forces are involved and the structure and stability of casein micelles depends on a balance between electrostatic repulsion and hydrophobic interactions.

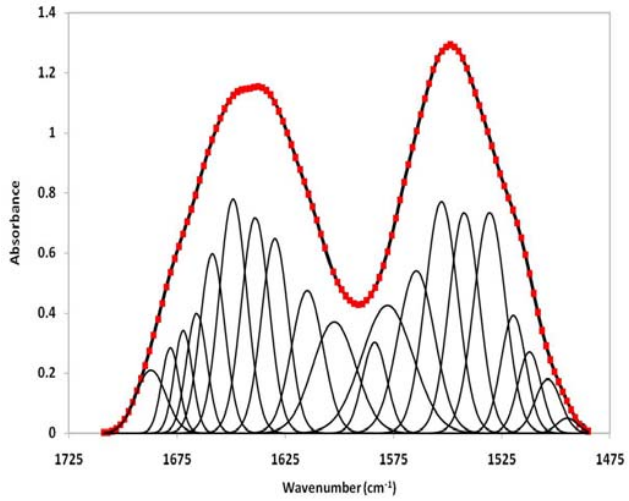

Fig. 2 FTIR spectra of casein micelle in water. Amide I and II regions were fitted at peak positions obtained from second-derivative spectrum followed by Gaussian curve fitting. Red dots represent experimental data; black curves represent individual Gaussian components and their sum.

Fig. 2 and Fig. 3 represent Structural changes that are introduced in solution by modifying the NaCl environment were followed by FTIR. The distributions of components within classes of structures are reproducible and the tendencies are exactly the same whatever the Amide band analysed. A strong modification of β-sheets (decreasing trend) and irregular structure (increasing trend) with salt increase was observed. Concurrently, α-helix and turns structures were mostly constant. These modifications occurred around 3% NaCl (Data not shown). Up to now, amide I spectral region is the most commonly used band because of its very strong signal but our results highlight some limitations in the use of this band. This band suffers principally from an unstructured spectral contour and an overlap of the random coil and α-helix bands. Amide III region was already found very sensitive to the protein secondary structure [9] [22]. The usefulness of this region was demonstrated in this work for casein micelle. Moreover, the shape of the band clearly facilitates the procedure of deconvolution; the band presenting a very structured spectral contour (Fig. 3) in comparison with the Amide I (Fig. 2).

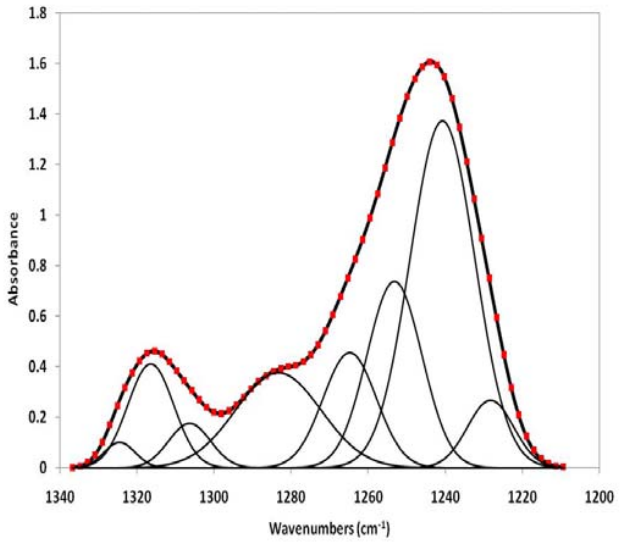

Fig. 3 FTIR spectra of casein micelle in water. Amide III region was fitted at peak positions obtained from second-derivative spectrum followed by Gaussian curve fitting. Red dots represent experimental data; black curves represent individual Gaussian components and their sum.

It was already observed that changes in temperature, pH, ionic strength, water activity etc., could lead to change in size distribution and in the proportion of free sub-micelles. [24] [25]. This is certainly the case in this work. By increasing the NaCl percentage, there may be a disintegration of casein micelles with the formation of sub-micelles (as viewed by DLS).

IV. CONCLUSION

The interpretation of the turbidity profiles was quite helpful to better understand the rehydration stages of native micellar casein powder in increasing salt environments. We observed two distinct behaviours depending by changing salt concentration. This study revealed structural details of casein micelle dispersions upon NaCl environment. These dispersions in water were composed of spherical casein micelles with an average size around 150 nm. Under NaCl increase, this open-hydrated micellar structure may be disintegrated in sub-micelles around 20-30 nm more or less aggregated. The preliminary, ATR-FTIR investigation highlights a significant redistribution of component areas. Nevertheless, the secondary structure prediction of casein micelle should be used with caution due to fairly interpretations already done in the literature. But, the simultaneous identification of secondary components (with the amide I and III band) greatly enhances our interpretation.

APPENDIX

Abbreviations used:
CM: Casein Micelles; DLS: Dynamic Light Scattering;
FTIR: Fourier Transfer Infrared; WT: Wetting Time;
SW: Swelling Time; TRT: Total Rehydration Time.

ACKNOWLEDGMENT

We thank C. Jeandel (LIBio technician) for assistance during this work. The authors are highly indebted to The Pakistan Ministry of Higher Education Commission (HEC) for financial support.

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