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Multilevel Characterization of Milk Proteins: Influence of Ionic Environment & Temperature

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UNIVERSITE DE LORRAINE

École Nationale Supérieure d'Agronomie et des Industries Alimentaires

Ecole Doctorale Sciences et Ingénierie Ressources Procédés Produits Environment (RP2E)

Laboratoire d'Ingénierie des Biomolécules – LIBio

THESE

Par

Raza HUSSAIN

Pour obtenir le grade de Docteur de Université de Lorraine (UL)

Spécialité : Procédés Biotechnologiques et Alimentaires

Caractérisation multi-niveau de protéines laitières: influence de l'environnement ionique et de la température

Multilevel Characterization of Milk Proteins: Influence of Ionic Environment & Temperature

Directeur de thése : Joël SCHER

Co-directeur de thése : Claire GAIANI

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Avant-propos

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Nomenclature

ATR : réflexion totale atténuée

ANS: 1-anilinonaphthalene-8-sulfonic acid

α-La: alpha-lactalbumin

BSA: bovine serum albumin

β-Lg: beta-lactoglobulin

$CaCl_2$: chlorure de calcium

CIE : commission interactionnelle de l'éclairage

NMC : caséines micellaires natives

D_T : coefficients de diffusion translationnelle

DLS: dynamic light scattering

DSC: differntial scanning calorimetry

DTGS : deuterated tri glycine sulfate

FTIR : fourier transfer infrarouge

FIL : féderation international laitière

G' : module élastique

G'' : module visqueux

L : litre

MAT : matière azotée totale

MCT : mercury cadmium telluride

TEM: microscopie électronique à transmission

M : moles

m M: milli moles

NaCl : chlorure de sodium

nm : nano mètres

WPI : protéines de lactosérum natives

PSH : l'indice d'hydrophobicité des protéines de surface

NPN : azote non protéique

rpm : rotation par minutes

SLS: static light scattering

T_D : températures de dénaturation

µg : microgramme

µm : micromètre

ZnSe : séléniure de zinc

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Chapitre 1. Introduction générale et objectifs de l’étude

Introduction générale et objectives de l'étude

Une meilleure compréhension des propriétés physicochimiques, morphologiques et de conformation des protéines laitière sous l'addition de sel est importante non seulement pour la recherche fondamentale mais également pour le développement de nouveaux produits laitiers. Dans le contexte actuel ces protéines trouvent des débouchés majeurs en tant qu'ingrédients, dans le but d'améliorer la saveur et de développer des produits naturels ayant de bonnes propriétés nutritionnelles et fonctionnelles (gélifiantes, et moussantes…).

Les protéines sont les principaux composants du lait, elles sont souvent commercialisées sous une forme déshydratée pour prolonger leur durée de conservation, faciliter leur utilisation et réduire les coûts de transport.

Le lait entier en poudre contient principalement du lactose (environ 38%), des protéines sériques (environ 4%), des caséines (environ 20%) et de la matière grasse laitière (environ 26%). Les caséines constituent la majeure partie des protéines laitières, elles sont principalement utilisées comme ingrédients de fait de leurs liaisons avec l'eau et de leurs propriétés émulsifiantes, moussantes et épaississantes. Elles sont à la base de la fabrication de nombreux produits laitiers tels que les yaourts et les fromages. Dans le lait, les caséines sont sous formes de micelles contenant des fragments α_{s1}-, α_{s2}-, β et κ, à des proportions en poids respectivement de 4:1:4:1. Les caséines sont la partie essentielle du lait de bovin et sont généralement les plus étudiées. Les micelles de caséines sont hétérogènes de par leurs compositions, leurs tailles et leurs grandes porosités **(Swaisgood, 2003).**

La structure détaillée des micelles de caséine a fait l'objet de nombreux modèles. Trois modèles de structure de caséine sont généralement acceptés : un modèle enveloppe – noyau, un modèle de structure interne et un modèle sub-micellaire **(Horne *et al.*, 1989 ; Rollema., 1992 ; Schmidt,1982, Walstra *et al.*, 1999).**

Les protéines de lactosérum restent solubles dans le sérum après précipitation des caséines à pH 4,6 à 20°C. Grâce à leurs intérêts nutritionnel et fonctionnel, les protéines de lactosérum sont parmi les plus utilisées comme ingrédients dans les industries agroalimentaires.

Sur le marché, les protéines de lactosérum sont sous forme d'isolats ou de concentrés. Les isolats de protéines sériques contiennent de plus de 90% de protéines, les concentrés ne contiennent que 50 à 75% de protéine. Ces protéines sont souvent très couteuses parce qu'elles exigent des traitements d'obtention et de purification importants. Avec le développement des méthodes de séparation membranaire et de filtration, le

fractionnement des protéines sériques natives est devenu possible. Par conséquent, les fractions (α-lactalbumine, β-lactoglobuline, glycolmacropeptide, lactoferrine…) et des hydrolysats de protéine sont maintenant facilement disponibles.

Les protéines de lactosérum sont composées de deux fractions majeures la β-lactoglobuline (β-Lg) et l'α-lactalbumine (α-La) qui représentent respectivement 54% and 21% de la masse totale des protéines du lactosérum. La β-Lg renferme 162 résidus d'acides aminés pour une masse moléculaire de 18400 $g.mol^{-1}$. A pHi de 5.2 la β-Lg se trouve sous forme de dimère et la structure secondaire est composée principalement de feuillets β**.** L**'**α-La est une protéine globulaire aussi, sa structure secondaire est principalement en hélice. Elle renferme 123 résidus d'acides aminés pour une masse moléculaire de 14 200 $g.mol^{-1}$ et un pHi compris entre 4.2 et 4.5. **(Kinsella, 1984).**

Il est maintenant possible d'obtenir des caséines micellaires natives (NMC) ainsi que des protéines de lactosérum natives (WPI) grâce aux méthodes de microfiltration sur membrane de lait écrémé. Ces produits sont facilement séchés par atomisation ou par procédés par rouleaux et les poudres de protéines résultantes contiennent une forte teneur en protéines (90% d'extrait sec). Les poudres de NMC constituent des ingrédients très intéressant pour les industries agroalimentaires en raison de leur teneur en protéines et aussi par le fait qu'elles peuvent se substituer aux micelles de caséines de lait. Ces poudres présentent aussi une excellente aptitude à la coagulation enzymatique (par la présure) et sont utilisées pour augmenter les rendements fromagers.

Néanmoins ces poudres des caséines micellaires natives se dispersent lentement ce qui rend le procédé de réhydrations très long. Les poudres de NMC présentent des applications spécifiques dans l'alimentation grâce aux différentes propriétés fonctionnelles comme la solubilité, la viscosité, les liaisons avec l'eau, les propriétés moussantes ou émulsifiantes, etc.

Les WPI présentent des propriétés de réhydratation différente de celles des NMC. En effet, son étape de mouillage s'avère extrêmement lente contrairement à son étape de dispersion. Finalement, le processus de rehydratation est plus rapide que celui des NMC **(Gaiani *et al.*, 2007).**

Le Chlorure de sodium (NaCl) améliore l'aptitude à la réhydratation des poudres de caséines micellaires, il provoque des changements dans la structure des micelles des caséines et la composition minérale micellaire. L'addition de NaCl aux protéines de lactosérum accentue généralement l'agrégation, ceci se traduit par une par augmentation de la turbidité, de la taille moléculaire, et une diminution de la solubilité**.** Le chlorure de sodium affecte le

processus de réhydratation par plusieurs mécanismes : (1) interactions électrostatiques, (2) interactions ioniques hydrophobes spécifiques (3) diminution du taux de dissolution. Cependant, il ne présente aucun effet notable sur la structure secondaire des protéines du lactosérum **(Boye *et al*., 1996 ; Schuck *et al*., 2002)** .

Pour une meilleure compréhension des relations protéines-sel, différentes techniques permettant d'étudier leurs structures moléculaires ainsi que les interactions avec les ions Na^+ et Cl^- dans différents environnement ionique aqueux ont été mise en œuvre.

Dans les premières études, différentes méthodes sont utilisées pour étudier l'effet de NaCl sur les propriétés structurelles des caséines et des protéines de lactosérum ainsi que l'effet sur la morphologie et sur la taille des particules. (DLS, SLS, TEM) en prenant en considération divers paramètres (pH, concentration en protéines, force ionique…).

Divers méthodes sont également utilisées pour la mise en œuvre de la structure des protéines. La cristallographie de rayons X est ainsi une méthode qui permet de fournir plus de détails sur la conformation et la structure des protéines et plus particulièrement sur la formation de monocristaux. Néanmoins cet objectif est difficile atteindre dans le cas des caséines de lait de vache car ces dernières appartiennent à la classe des protéines non cristallisable. Seulement une petite partie de micelles peut être étudiée par cette technique en raison de la limite technique concernant la longueur d'onde de rayonnement.

La spectroscopie de résonance magnétique nucléaire est une autre technique permettant l'analyse de la structure des protéines en solution. Cette méthode est inapplicable sur les micelles de caséine à cause de leurs tailles importantes d'une part et d'autre part dans le cas des protéines de lactosérum cette méthode présente des limites pour déterminer les structures secondaires de façon précise des feuillets β en comparaison avec la méthode de cristallographie aux rayons X.

La spectrométrie de dichroïsme circulaire des protéines peut donner des informations importantes sur la structure secondaire des protéines mais cette technique présente quelques limites. En effet, cette méthode si elle est la plus fiable pour la quantification des hélices est limitée par le fait que les mesures sont effectuées sur des échantillons fortement dilués. En outre, des structures non périodiques comme les feuillets β ne peuvent pas être distingués par cette technique.

La méthode de Spectroscopie Infrarouge (FTIR) est devenue une des méthodes parmi les plus importantes pour étudier la structure secondaire des protéines et plus particulièrement pour l'analyse de la région de la bande amide I. Avec l'utilisation de la FTIR, divers éléments

des structures secondaires de la caséine pour la bande amide I ont été déjà déterminés (hélice α, feuillets β, …) **(Curley *et al*., 1998).**

La région spectral de la bande amide I (1700-1600 cm^{-1}) a un fort signal mais présente des interférences significatives avec les bandes de vibration de l'eau (environ 1640-1650 cm^{-1}), le contour spectral n'est pas très structuré et le chevauchement des bandes peut correspondre à diverses structures secondaires. Les bandes amide III (1350-1200 cm^{-1}) sont elles facilement identifiables et bien définies, elles sont associées à l'analyse quantitative de la structure secondaire des protéines. L'estimation et l'attribution de la structure secondaire des éléments pour la région spectrales de l'amide III sont très étudiées et sont corrélées à la structure secondaire obtenue à partir des données de la méthode de cristallographie aux rayons X. **(Cai and Singh, 1999 ; Cai and Singh, 2004).**

L'objectif de ce travail de thèse est triple. Il consiste dans un premier temps à étudier l'influence de l'addition de sels sur le processus de réhydratation de différentes poudres laitières, dans un second temps nous avons étudié l'influence de la concentration en sels sur la structure des protéines laitières et enfin certaines propriétés fonctionnelles des poudres sont étudiées en fonction des l'ajout des sels et de la température.

Le document présenté se compose de cinq chapitres qui s'attachent à considérer un point précis de l'étude globale. Ce document présente ainsi de manière chronologique les différents articles scientifiques, publiés, ou soumis pour publication, qui seront le fil conducteur de ce travail.

Le document est structuré de la façon suivante :

- **Chapitre I**: Introduction (en Français)
- **Chapitre II** : État de l'art
- **Chapitre III** : Matériels et Méthodes (en Français)
- **Chapitre IV** : Résultats et Discussion
  - **IV. I** : Réhydratation de poudres de protéines laitières dans des milieux complexes
  - **IV. II** : Caractérisation multi-niveau des solutions protéiques réhydratées
  - **IV. III :** Propriétés fonctionnelles des solutions protéiques réhydratées.
  - **Chapitre V** : La rédaction se termine par une **conclusion générale** permettant de faire un bilan sur les résultats obtenus et d'ouvrir sur les nombreuses **perspectives** que soulèvent cette thèse.
  - **Chapitre VI** : **Références**

Les résultats obtenus dans le cadre de cette thèse ont fait l'objet des publications et communications suivantes :

Publications dans des journaux internationaux:

1. **R. Hussain**, C. Gaiani, L. Aberkane, & J. Scher, (2011). Characterization of high-milk-protein powders upon rehydration under various salt concentrations. *Journal of Dairy Science*, 94 14-23.
2. **R. Hussain,** C. Gaiani, & J. Scher, (2011). Relation entre les propriétés morphologiques et la rehydratation des poudres de lait. Séminaire 2011 de l'Ecole Doctorale RP2E : *Ingénierie de Ressources, Procédés, Produits et Environnement*, ISBN 2-9518564-9-0.
3. **R. Hussain**, C. Gaiani, L. Aberkane, J. Ghanbaja, & J. Scher, (2011). Multiscale Characterization of Casein Micelles under NaCl Range Conditions, *Food Biophysics*, 6:503–511
4. **R. Hussain**, C. Gaiani, J. Ghanbaja, & J. Scher, (2012). Morphological characterization of salt induced whey protein aggregated species, *Milchwissenschaft*, (In Press).
5. **R. Hussain**, C. Gaiani, R. Shukat, M.C. Karam, & J. Scher, (2012). Multiscale Characterization of Whey Protein Isolates under High NaCl Environments, *Milchwissenschaft*, (In Press).
6. **R. Hussain**, C. Gaiani, C. Jeandel, J. Ghanbaja, & J. Scher, (2012). Effect of Combined Heat Treatment and Ionic Strength on the Functionality of Whey Protein Isolates, *Journal of Dairy Science*, JDS-12-5416 (Accepted).
7. **R. Hussain**, C. Gaiani, & J. Scher, (2012). From high milk protein powders to the rehydrated protein in variable ionic environment: A Review, *Journal of food engineering*, JFOODENG-D-12-00489R1 (In Press).
8. **R. Hussain**, C. Gaiani, C. Jeandel, & J. Scher (2012). Use of FTIR combined with 2D correlation spectroscopy to study sequential events occurring during salt and temperature induced unfolding and aggregation process of whey proteins, *Food Hydrocolloids*, (under redaction).
9. C. Gaiani, P. Boyanova, **R. Hussain**, I.M. Pazos, M.C. Karam, J. Burgain, J. Scher, (2011). Morphological descriptors and colour as a tool to better understand rehydration properties of dairy powders. *International Dairy Journal*, 21-462-469.

10. L. Aberkane, J. Jasniewski, C. Gaiani, **R. Hussain**, J. Scher, C. Sanchez, (2012). Structuration mechanism of β-lactoglobulin- Acacia gum assemblies in presence of quercetin, *Food Hydrocolloids,* 29- 9-20.

Conférences avec comité de lecture:

Communication Orale

11. **R. Hussain**, C. Gaiani, & J. Scher, Revealing Casein Micelle Dispersion under Various Ranges of NaCl: Evolution of Particles Size and Structure, *ICABBBE,* 25th-27th January 2011, Dubai, United Arab Emirates.

12. **R. Hussain**, C. Gaiani, & J. Scher, Influence of ionic strength on the size, structure and morphology of casein micelles during rehydration, *International Conference on Emerging Issues in Food Safety*, 14th -16th November, 2011, Faisalabad, Pakistan.

13. **R. Hussain**, C. Gaiani, & J. Scher, Elucidation/formation and physicochemical characterization of whey protein isolates aggregates, *11th International Hydrocolloids Conference, Biofunctionality and Technofunctionality of Hydrocolloids,* 14th-18th May, 2012, Purdue University, West Lafayette, Indiana, USA.

14. C. Gaiani, I. Murrieta Pazos, **R. Hussain**, P. Boyanova & J. Scher, Relationship between milk powders morphology and its rehydratation properties, 2011, 5th *International Symposium on Granulation*, 15th-18th June 2011, *Lausanne, Switzerland.*

15. M.C. Karam, C. Gaiani, **R. Hussain** & J. Scher, Influence of powder rehydration state on gel formation and stability during yogurt, 2012, *5th International Symposium on Spray dried Dairy Products*, 19th -21th June, 2012, St. Malo, **France.**

Communication par Poster

16. **R. Hussain**, C. Gaiani, P. Boyanova, I. Murrieta Pazos, P. Schuck & J. Scher, Relationships between morphology and rehydration properties of milk powders, November 2010, *International Dairy Summit*, Christchurch, New Zealand.

17. **R. Hussain**, C. Gaiani, & J. Scher, Morphological descriptors of milk powders, January 2011, *Seminar of Graduate School (RP2E),* Nancy, France

18. **R. Hussain**, C. Gaiani, J. Burgain, & J. Scher, Influence of the Ionic Strength on the Size, Structure and Morphology of Casein Micelles during Rehydration, *International Dairy Summit,* October 2011, Parma, Italy.

19. **R. Hussain**, C. Gaiani, & J. Scher, Formation and Multiscale characterization of salt Induced Whey Protein Isolates Aggregates, January 2012, *Seminar of Graduate School (RP2E),* Nancy, France.

20. **R. Hussain,** C. Gaiani, M.C. Karam, & J. Scher, Combined effect of heat and ionic environments on rheological and physicochemical properties of whey proteins, *5th International Symposium on Spray dried Dairy Products*, 19th -21th June, 2012, St. Malo, France.

Chapitré II. Étude bibliographique

Des poudres de protéines laitières à leur dispersion dans différents environnements ioniques : une revue.

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Résumé

Cette revue présente l'état actuel de la recherche liée aux poudres de protéines laitières ainsi que l'effet des sels sur leur profil de réhydratation, leurs propriétés structurales, morphologiques et fonctionnelles. La première partie de cet article est consacrée à la description des protéines majeures des poudres de lait en fonction de leur composition, leur production et leur utilisation dans l'industrie alimentaire.

Dans la seconde partie, nous abordons le rôle et l'utilisation de sels dans l'industrie laitière qui sont d'une grande importance non seulement sur le plan de la qualité des produits laitiers (goût, saveur, texture, durée de conservation…), mais aussi sur leur consommation qui est directement liée au mode de vie, facteurs culturels, sociaux, sensoriels, économiques et psychologiques.

L'effet des sels à des concentrations différentes et les profils de réhydratation et d'hydratation liés aux propriétés fonctionnelles sont illustrés dans la troisième partie. La quatrième partie de cette revue couvre les dernières découvertes englobant l'impact des sels sur les dispersions protéiques (structure secondaire, taille des protéines). Dans la dernière partie, une évaluation détaillée des différentes propriétés fonctionnelles des dispersions protéiques sous l'influence de sels et d'autres facteurs complémentaires (température, la concentration en protéines, pH, …) sont résumées.

From high milk protein powders to the rehydrated dispersions in variable ionic environments: A Review

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ABSTRACT

This review presents the current status of research related to the milk protein powders and impact of salts on their rehydration properties, structure, morphlogy and functional properties. The first part of this paper deals with the description of major milk proteins powders according to their composition, production and use in food industry. In the second part the role and utilization of salts in dairy industry is covered, demonstrating their importance not only for quality parameters of dairy products (taste, flavor, texture, shelf life extension) but also their consumption is directly related to life style, cultural, social, sensory, economical, psychological and technological factors of food. Effect of different salts at various concentrations on powder rehydration properties and hydration related to functional properties are illustrated in third part. Fourth part of this manuscript covers latest findings narrating salts impact on the secondary structure, protein sizes, and morphological transformations of milk proteins in aqueous ionic environments coupled with different biophysical techniques. In the last part a detailed evaluation of different functional properties of milk protein powder under influence of salts and other complimentary factors (temperature, pH, protein concentration etc…) are discussed.

Keywords: casein, whey proteins, salts, rehydration profiles, multiscale characterization, functional properties.

Abbreviations: AFM: Atomic Force Microscopy, α-La: Alpha-Lactalbumin, β-Lg: Beta-Lactoglobulin BSA: Bovine Serum Albumin, CD: Circular Dichroism, COC: Coagulated Casein, CSLM: Confocal Scanning Laser Microscopy, CM: Casein Micelle, CMP: Caseinmacropeptide, Cryo TEM: Cryogenic Transmission Electron Microscopy, DLS: Dynamic Light Scattering, FTIR: Fourier Transfer Infrared Microscopy, HMPP: High Milk Protein Powders, HP-GPC: High-performance Gel Permeation Chromatography, Lf: Lactoferron, MALS: Multi Angle Light Scattering, MF: Microfiltration, MPC: Milk Protein Concentrates, NMC: Native Micellar Casein, NMR: Nuclear Magnetic Resonance, SANS: Small Angle Neutron Scattering, SAXS: Small Angle X-ray Scattering, SEC-MALLS: Size Exclusion Chromatography Multi-Angle Laser Light Scattering, SEM: Scanning Electron Microscopy, SLS: Static Light Scattering, SWP: Sweet Whey Powders, TEM: Transmission Electron Microscopy, Tr: Rehydration time, Ts: Swelling time, Tw: Wetting time, UF: Ultrafiltration, WPC: Whey Protein Concentrate, WPI: Whey Protein Isolate, WHC: Water Holding Capacity,

1. Introduction

A good understanding of physicochemical, structuctural and functional properties of milk proteins under addition of salts is not only momentous for fundamental research but also for the improvement of dairy products in which this fraction appears to be complex, dynamic and in strong interaction with minerals. In this context, formulation of these ingredients can provide dairy industry with a unique opportunity to develop tasty, healthy, convenient, and natural food products with good functional and nutrional properties. Milk proteins are the most valuable leading component of milk and are often commercialized in a dehydrated form to extend their shelf-life, facilitate their use and reduce transportation costs. There play a range of valuable roles regarding nutrition and physical functionality. Furthermore, their use under controlled conditions to produce nutritional, functional or flavorful dairy products and occasionally biological activities was often demonstrated **(Sawyer et al., 2002).** Whole milk powders mainly consists of lactose, whey proteins, caseins, and milk fat (respectively 38, 4, 20 and 26%) **(Walstra et al., 1999).**

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 **(Walstra, 1999).** These are the network formers in dairy products such as yogurt, 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 **(Swaisgood, 1996).** Caseins constitute an extensive part of bovine milk and due to their nutritional acuteness and commercial significance; these are one of the food proteins the most extensively studied. Casein micelles are heterogeneous, both in composition and size, with a great polydispersity, a loose packing and a higher porosity than many other protein assemblies **(Liu and Guo, 2008)**.

Whey proteins are defined as the group of milk proteins that remain soluble in milk serum or whey after precipitation of casein at pH 4.6 and 20°C **(Farrell et al., 2004).** Whey proteins are among the most employed and versatile source of functional and nutritional food proteins. These represent a high nutrional profile with specific physiological properties and are widely used in food industry **(Madureira et al., 2010).** Most probable applications are in bakery products, processed meats, pasta, ice cream, confectionary, infant foods, spreads, dips and beverages **(Mulvihill and Ennis, 2003).** Whey proteins are composed of two major fractions, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) representing respectively 54% and 21% of the mass of whey proteins. β-Lg and α-La are globular proteins consisting respectively in 162 and 123 amino-acid residues **(Kinsella, 1984)**.

With the advent of industrial filtration and separation methods, recovery and fractionation of whey proteins in their native form had become possible. Consequently, a wide variety of whey protein isolates, fractions and hydrolysates are currently available **(Abd El-Salam et al., 2009)**. Milk proteins powders are frequently dry in order to stabilize and transport.

This is the reason why rehydration is an essential quality attribute of the powder. Consequently, rehydration is often realized by the industry before use and for a long time (**King, 1966**). It is well-known that microenvironments such as pH and/or salt can significantly alter the behavior and overall performance of proteins. Whey proteins also have an ample capacity for cations binding **(Hussain et al., 2011b)**. Globular proteins present compact and ordered conformations because of the presence of disulphide bridges which give them well defined secondary and tertiary structure.

Elucidation of the structure of biomolecules is the primary factor that determines their biological functions. Among biomolecules, some biopolymers, especially proteins are technologically important due to their texturing, hydrating and interfacial stabilizing capacities. In recent decade, literature contains a large number of references on the effect of salts on physicochemical, morphological, and functional properties of milk proteins using various analytical techniques and different parameters (pH, temperature, protein concentration, ionic strength). **(Hussain et al., 2011a; Hussain et al., 2011b ; Schmitt et al., 2011)**. Some reviews have been published recently on physiological **(Madureira et al., 2010; Totosaus et al., 2002),** nutritional **(Albarracin et al., 2011; Cruz et al., 2011)** and functional properties of milk **(Augustin and Udabage, 2007)** and milk proteins products **(Abd El-Salam et al., 2009; Nicolai et al., 2011).**

But, information and details related to the role and effect of salts on these properties are often lacking. Following recent data, in this review, various milk protein powders are defined and also role of salts regarding dairy industry is mentioned. The influence of salts on milk protein rehydration process is described and special attention is paid on multiscale characterization of milk protein under various salt ranges. Finally, functional properties of milk proteins are elaborated with reference to salt addition.

2. Milk Protein Powders

Dairy industrials have expended a great amount of efforts during the last 20 years to build up commercial-scale processes for manufacturing dairy powders with good functional, nutrional and flavor properties suitable for use as food and animal feed ingredients. These products can be easily spray dried and the resulting proteins powders present high total protein content (90% of total solids). The main characteristic of these

powders is that proteins can now be obtained in their native form. A schematic diagram describes the steps involved in fabrication of different dairy milk powders at an industrial level **(Figure 1).** A brief description of some of frequently used High Milk Protein Powders **(HMPP)** in dairy industry and fundamental research is given below.

2.1. Milk Protein Concentrate powders

Milk protein concentrates (MPC) are manufactured by means of membrane separation from skim milk. A well known manufactured MPC powder contains a ratio of casein to whey proteins of about 82% casein and 18% whey proteins. Caseins are present in their natural, native structure known as a micelle while whey proteins are in undamaged/undenatured form **(Schuck et al., 1998).** Developments in current years have resulted in MPCs of diverse compositions being produced and incorporated into a broad range of dairy products. For example, MPCs powders are often used in recombined cheese industry, and to standardise the protein content in normal milk without loss of product properties. MPC forms a white opaque dispersion of low viscosity when dispersed in water, looking very similar to milk. It is sold as a fine white powder with no odor, bland flavor and a smooth creamy mouth feel. The drying of these high proteins concentrates led to loss of functionality.

This is generally illustrated by a loss in solubility, a poor reconstitutability and hydration properties **(Mulvihill and Ennis, 2003)** Infact, solubility is a prerequisite for many other functional properties of proteins (viscosity, gel, foaming, emulsions…) because it enables the functionality of the protein ingredients to be fully realized **(Schuck et al., 1999)**. The solubility of MPC powders deteriorates during storage and it could be improved by addition of monovalent salts to the ultrafiltered retentate or by removal of calcium ions earlier to drying **(Augustin and Udabage, 2007).** Recently, the effects of storage time and temperature on the solubility of MPC were investigated indicating that, the solubility of MPC decreased exponentially with time **(Anema et al., 2006)**.

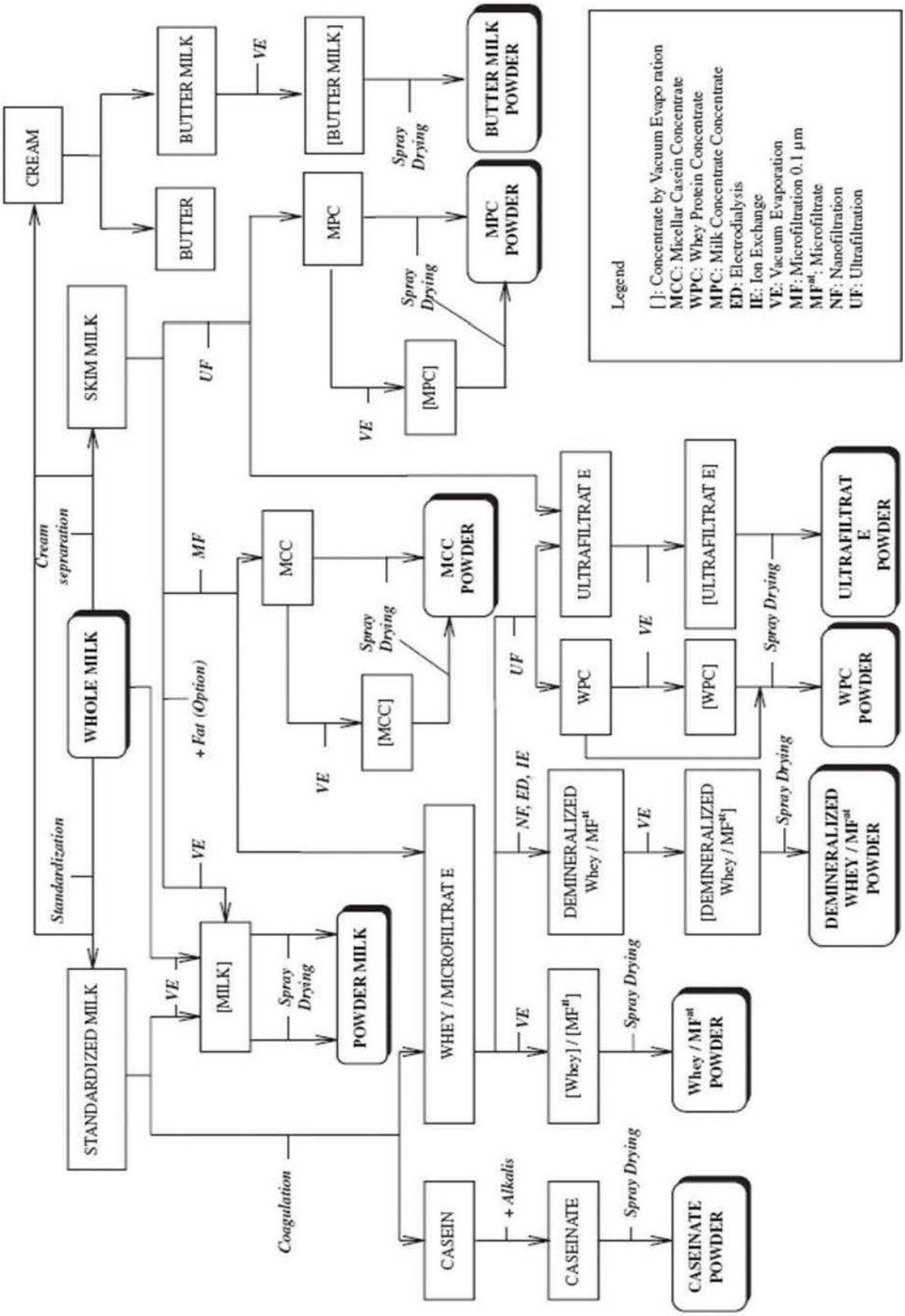


Figure 1. Schematic descriptions of different steps involved in high milk protein powders (HMPP) preparations **(Adopted from Schuck, 2002).**

2.2. Native Micellar Casein powders

Native Micellar Casein (NMC) is basically a whey-protein-free milk protein concentrate powder which may be obtained by tangential membrane microfiltration of milk followed by spray drying diafiltrated retentate **(Schuck et al., 1994)**. Due to its high protein contents this powder can be used as a relevant model of milk micelles **(Famelart et al., 1999a)**. NMCs reveal high water holding and cheese making capacities but mediocre reconstitution properties. Moreover, the reconstitutability of these powders can be significantly improved by lowering both inlet and outlet air drying temperatures. Another solution is to enrich the concentrate with suitable soluble proteins or carbohydrates before spray drying **(Schuck et al., 1994; Schuck et al., 1998).**

In recent years, several studies are made to improve the rehydration properties of NMC powders with or without salt addition **(Gaiani et al., 2005; Hussain et al., 2011b; Schuck et al., 2002).** Indeed, by improving its rehydration properties, this powder would be able to serve as an attractive material for dairy and food industry.

2.2. Sweet Whey Powders

Sweet Whey Powders (**SWP**) are significant byproducts of the cheese industry. Due to their abundant functional properties, such as browning, bulking, foaming, solubility and water binding; SWPs are utilized as ingredients in different food industries. For several reasons, the food industry has yet to fully identify the potential of SWP as a food ingredient, such as whey protein concentrate (**WPC**) or whey protein isolate (**WPI**). The leading structural component of a SWP particle is lactose (amorphous or crystalline) whereas fat exists in either globular or non globular (free fat) forms. **(Mulvihill and Grufferty, 1997).**

Nevertheless, on the basis of processing conditions and the resulting physical state of lactose, sweet whey powder can be either hygroscopic or non not. Being a food ingredient, the qualities of final manufactured food items are numerously affected by distinctive physicochemical properties of SWP, particularly during storage and distribution **(Saltmarch and Labuza, 1980).** Indeed, a substantial quantity of SWP is used as a constituent of animal feeds **(Banavara et al., 2003).**

2.3. Whey Protein Concentrates

The manufacture of Whey Proteins Concentrates (WPC), with a protein content ranging from 35-80%, involves different phases of whey treatment: ultrafiltration/ diafiltration, concentration by evaporation under reduced pressure, and spray drying. Novel modifications and advancements have been made in recent years to obtain a better feed of WPC with less lactose and more proteins **(Barba et al., 2001; Yee et al., 2007).** WPC powders that contain between 35 and 55% of proteins are mainly used in animal feed manufacture **(Onwulata et al., 2003; Yun et al., 2005).** Commercial WPC develops off flavor during storage and this problem is one of the major factor limiting its applications in delicate formula **(Javidipour and Qian, 2008; Liaw et al., 2010).** Two mechanisms (lipids oxidation and Maillard browning) are supposed to be the main causes of off flavor **(Whitfield, 1992).**

2.4. Whey Protein Isolate powders

Whey Proteins Isolate (WPI) powders are manufactured by a stirred-bed ion exchange chromatography or microfiltration process. The ion exchange method is based on several steps: (1) the pH of whey is adjusted to have proper charge on protein molecules; (2) these molecules are adsorbed by a ion exchanger when passed through it and deproteinzed whey is eluted from the reactor; (3) the pH is readjusted to desorb the proteins and after their subsequent elution from ion exchanger, desorbed proteins are concentrated by ultrafiltration and spray-dried. Hence, resulting product contain more than 90% of proteins. In the microfiltration method (MF), lipids, protein aggregates and microbial debris are removed from whey by a microfiltration process using a suitable membrane (pores diameters 1μm).

The MF permeate is ultrafiltrated/diafiltered, concentrated and spray dried to give final WPI powder form. WPI prepared by former method has less casein glycomacropeptide content than prepared by the latter one **(Abd El-Salam et al., 2009; Morr and Ha, 1993).** For the purpose of this review, only NMC and WPI powders are briefly discussed, no peculiarity will be made between WPI and WPC and both will be considered as WPI which differs only with WPC that former has higher protein and proportionality lower concentration of lactose and minerals.

3. Role of salts in dairy industry

The evolution of new food products that may add to attenuate issues related to public health in a positive way is a big challenge for the dairy industry. Salt played a significant role throughout human olden times. History reveals that Egyptian called it ’natron’ and the Latin term ‘salarium’ originates from salt and refers to the amount of salt that was given to the worker as his salary. In the present circumstances, salt content in food is the core focus of consideration of the world food industry especially in the dairy one. Salt is one of the most commonly and extensively used additives in dairy industry for the reason of its low cost and varied properties. It brings out particular functions for example improves taste, texture, and enhances shelf life of dairy products **(Albarracin et al., 2011).** Moreover, its consumption is directly related to life style, cultural, social, sensory, economical, psychological and technological factors **(Purdy and Armstrong, 2007).**

Sodium Chloride (NaCl) is an alimentary salt widely used in dairy industry as it is a major determinant of water activity. In addition, it acts as preservative, contributes directly to the flavor as a consequence of its effect on different biochemical mechanisms and is a source of dietary sodium **(Guinee, 2004).** The most important dairy product that involves the major utilization of salt is cheese. Cheeses differ in their composition of their nutrients such as proteins, lipids, carbohydrates, minerals, calcium, phosphorus, and vitamins A and B. It is one of the most nutrious food products with large variety of shapes, packaging and tastes containing 48% fat and 23-25% proteins that play an important role in the nutrition of people of all ages **(Perry, 2004; Walther et al., 2008).** During production process of cheese, salt is added at the end of process, after shaping and/or molding and pressing except in the case of Domiati cheese **(Cruz et al., 2011).**

Cheese salting is carried out by following three methods: (1) the product is engrossed deeply in a solution of NaCl of known concentration, this method is called immersion in brine and is applied to the types of cheese such as Edam, Gouda, Provolone and Swiss; (2) essentially the same mechanism of salt absorption occurs as in brine salted cheeses, but salt is in the form of crystals and directly distributed over the surface of curd granules or milled curd chips prior to its molding and/or pressing, this method also called dry salting is applied to Cheddar and Stilton; and finally (3) some cheeses (Blue cheeses…) are prepared by rubbing dry salt on the surface of molded cheese over a period of days, this is called dry salting at surface **(Guinee and O’Kennedy, 2007)**. Various factors such as, brine concentration or type, salting time, pH, temperature of curd, cheese geometry, initial salt and salt-in-moisture level of curd refer to the quantity of salt absorbed during brine-salting or surface dry salting

(Sutherland 2002; Guinee and Fox 2004). Salt level in cheese ranges from 0.7% (w/w) in Swiss-type to 6% (w/w) in Domiati.

Table 1 shows different types of cheeses according to salt ranges. Generally, salt in cheese is added to prevent the undesirable microbial growth by controlling the growth of lactic acid bacteria **(McMahon, 2010)** as well as provides an additional flavor to wise bland-tasting cheese **(Rowney et al., 2004)** by improving cooking performance **(Guo et al., 1997)** and rheological properties **(Guinee and O'Kennedy, 2007).**

In addition to these functions, concentration and distribution of salt in cheese influences enzymatic activities and biochemical changes such as glycolysis, proteolysis, lipolysis **(Floury et al., 2009; Guinee and O'Kennedy, 2007; Katsiari et al., 2000)** and para-casein hydration **(Guinee, 2004)** that take place during ripening. Moreover, salt level has major effect on cheese composition **(Farkye, 2004),** texture **(Kaya, 2002)** and microbial growth **(Guinee, 2004)** modifying the water binding capacity of casein within the cheese matrix **(Pastorino et al., 2003)** and, hence, its overall quality **(Fox et al., 2000).**

Table 1. NaCl and Na approximate values in different Cheeses (**Adopted from Cruz et al., 2011**)

Cheese	NaCl (%)	Sodium (%)	Quantity of cheese (g) equivalent to 100% of the recommended daily intake of sodium[a]
Quark	0.15	0.06	4000
Emmental	0.70	0.28	857
Brie	1.60	0.63	381
Cottage	1.04	0.41	585
Gruyere	0.86	0.34	706
Provolone	2.24	0.88	273
Mozzarella	1.40	0.55	436
Cheddar	1.70	0.67	358
Gouda	2.40	0.94	255
Blue	3.30	1.30	185
Parmesan	4.07	1.60	150
Roquefort	4.10	1.61	149
Romano	4.10	1.61	149
Feta	4.50	1.77	136

% = weight Na or NaCl/Weight Cheese
[a] Based on the value of recommendation of NaCl (2.4g) or only Na (6g).

Nevertheless, despite the comprehensive and integral use of salt in dairy industries especially in cheese production, it faces some criticism. Infact daily consumption recommended for adult person is approximately 2.4 g Na or 6 g of NaCl/ day that is naturally available in food **(Kaplan, 2000),** but in developed countries the average total daily intake per

individual is 4-5 g of Na per day **(Johnson et al., 2009).** Due to its negative effects on health such as hypertension and consequently an increased risk of cardiovascular diseases, high intake of salt derived from dairy based products is also a matter of significant concern for modern society, producers and researchers. According to **Famelart et al. (1999b),** NaCl replacement has an effect on the casein micelles and the sodium/phosphorus equilibrium.

On the contrary, another study carried out by **Floury et al. (2009)** elaborates that diminution in NaCl concentration influence the fat droplet size and the rheological properties of cheeses with the presence of air bubbles. It may be explained by a distortion between casein and water molecules. Recently, efforts are made to develop various types of cheeses with reduced sodium contents by reducing NaCl or its partial/whole substitution with KCl, $MgCl_2$, and $CaCl_2$. Some encouraging results with acceptable varities of cheese are on board but at times sour residual tastes are produced by this substitution **(Albarracin et al., 2011; Cruz et al., 2011)**.

4. Milk protein powders rehydration in salt media

Milk protein powders are normally dried in order to stabilize and transport. That's why rehydration is an essential quality attribute of a dairy powders as a food ingredient **(Gaiani et al., 2007a; Martin et al., 2007). Freudig et al. (1999)** confined powder rehydration to four stages. The wettability stage entails the capability of the powder to absorb water. Afterward, the sinkability stage is the ability to sink into the water. The third stage is the dispersability, which is the capacity to disperse in single particles throughout the water. And the last stage, also called dissolution, relates to the separation between molecules during the rehydration process. These four phases occur in the sequence indicated above with occasional overlap of steps as wetting/dispersion or dispersion/dissolution... The term "instant properties" is used for the physical properties of powder linked with these four stages of rehydration **(Schubert, 1993).**

Table 2 presents the rehydration behaviors of two major milk protein powders in aqueous ionic medium. In addition, it is quite familiar that microenvironments such as pH, nature of mineral salts, size and structure of the powders and the rehydration conditions (agitation speed, temperature…) can notably amend the behavior, water transfer properties and overall performance of proteins **(Hussain et al., 2011a; Hussain et al., 2011b; Schuck et al., 2007).** Due to lack of basic data on the dynamics of hydration in connection with salt environment and inadequately controlled rehydration step in food industry; there is a strong need to understand the process of interaction between salts and milk protein constituents and to know its effect on overall rehydration mechanism.

Table 2. Rehydration times obtained for casein and whey proteins powders in different media in comparison with the same powders rehydrated in water (**Adopted from Hussain et al., 2011b)**

powder	salt	Concentration (%)	wetting time (s)	swelling time (s)	total rehydration time (s)
native micellar casein	NaCl	0	10	120	28 000
		0.75	33*	880***	nr
		1.5	37*	1233***	nr
		2.25	40*	1405***	nr
		3	45*	3295***	nr
		6	55**	no	14 291**
		9	56**	no	13 100**
		12	62**	no	12 999**
	$CaCl_2$	0	10	120	28 000
		0.75	45*	3410***	nr
		1.5	48*	no	15 690**
		2.25	50*	no	11 212***
		3	50*	no	11 320***
		6	51*	no	11 561***
		9	49*	no	12 888***
		12	50*	no	11 901***
native whey isolate	NaCl	0	23	no	1 525
		0.75	25^{ns}	no	1 616^{ns}
		1.5	24^{ns}	no	1 623^{ns}
		2.25	31^{ns}	no	1 981*
		3	35^{ns}	no	1 613^{ns}
		6	38^{ns}	no	1 599^{ns}
		9	41*	no	1 678*
		12	45*	no	1 821*
	$CaCl_2$	0	23	no	1 525
		0.75	35^{ns}	no	1 497^{ns}
		1.5	34^{ns}	no	1 560^{ns}
		2.25	36^{ns}	no	nr
		3	35^{ns}	no	nr
		6	41*	no	nr
		9	44*	no	nr
		12	44*	no	nr

$^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{NS}P > 0.05$

4.1. Effect of salt on casein powders rehydration properties

Native micellar casein powders present very slow dispersion properties making the total rehydration process time-consuming. Addition of salts usually enhances hydration, micelle voluminosity, screening of charges of caseins and calcium ion activity in the milk serum whereas it drops off the pH and the amount of supernatant casein **(Karlsson et al., 2005; Le Ray et al., 1998).** In addition, it improves rehydration of casein micellar powder, **(Hussain et al., 2011b)** and imparts changes in casein micellar structure and micellar mineral composition **(Famelart et al., 1999a; Hussain et al., 2011a; Le Dean et al., 2004).** In water, NMC powders demonstrates a quick wetting/swelling and very long dispersion stage making the total rehydration a time consuming process **(Gaiani et al., 2005; Hussain et al., 2011b).**

These authors found dispersion stage as a rate controlling step while **Baldwin and Sanderson, (1973)** showed for whole milk powders that, the most significant improvement in rehydration properties was attained by improving wetting properties. Usually, at room temperature a significant portion of powder material remains undissolved after a reasonable reconstitution time **(Havea, 2006). Schuck et al., (2002)** showed that addition of NaCl (at 420 mM) during rehydration process improved water transfer (reconstitution time) in the casein suspension due to the more hygroscopic nature of NaCl rather than by extensive modification of the micellar structure.

By using NMR, **Schuck et al., (2007)** reported changes in the micellar structures resulting in the modification of protein-protein interactions during rehydration related to the mineral environment. Moreover, it is well known that NaCl brings about an increase in micellar hydration by a decrease in the activity coefficients of the diffusible ions and as a result increases the dissociation of ions pairs, leading to solubilization of calcium and phosphate **(Famelart et al., 1999a).** Rehydration (solubility, dispersability) was found to be limiting factor for micellar casein **(Schuck et al., 2007).** With salt addition, an increase in protein solubility during rehydration process was reported as a result of large protein-protein electrostatic repulsion (salting-in) **(Machado et al., 2007)** or because of the 'salting-out' effect of hydrophobic interactions at higher salt concentrations >1M **(Albarracin et al., 2011).**

Nayak et al., (2006) observed the solubilities of phosphorylated milk proteins in water and ionic (0.1M NaCl or 10–70mM $CaCl_2$) systems over various pH ranges (3-9). At 0.1M NaCl concentration these solubilities were pH dependent while there was a decrease in solubilities of these phosphorylated milk proteins with increase in calcium ion concentration. In the presence of NaCl (0.05–0.2 M), a solubility maximum was observed at pH 5.5 **(Roman**

and Sgarbieri, 2006). The water holding capacity (WHC) is one of the most important functional properties of milk protein powder **(Stanley and Yada, 1992).**

It is defined as the capacity of the powder to avert water release from the three-dimensional structure **(Chantrapornchai and McClements, 2002).** This property is strongly influenced by protein ionic strength, type and concentrations of salts, pH, temperature and balance between protein and water **(Choi et al., 2000). Roman and Sgarbieri, (2006)** illustrated various functional properties (solubility, WHC, foaming and emulsifying) of CM, enzymes (chymosin) and coagulated casein (COC). They reported a higher WHC for CM than COC as a function of pH (3.5-7.5) in the presence of NaCl and $CaCl_2$ (0-0.2M)**.**

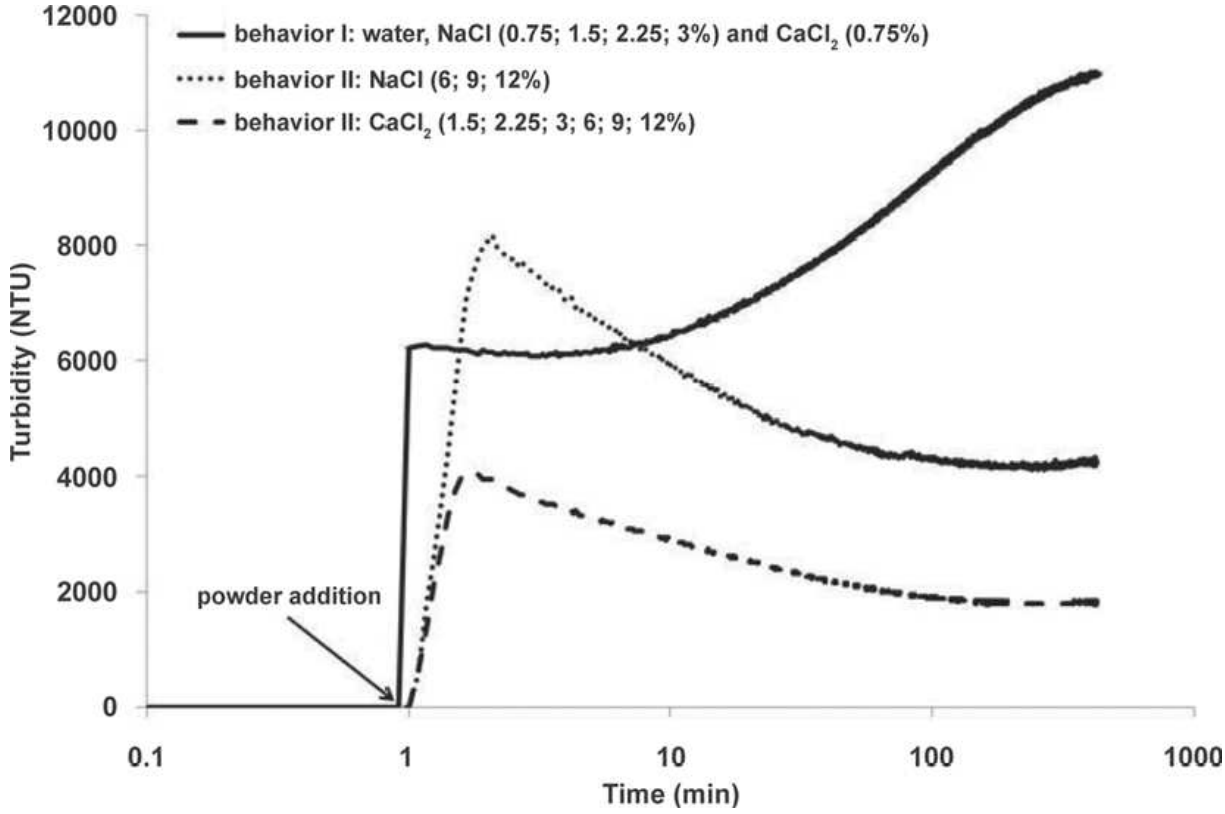


Figure 2. Turbidity profiles (NTU, nephelometric turbidity units) obtained during rehydration of 5% native micellar casein powder at 24°Cfor 1,000 min in different ionic environments (mean of 3 independent analyses). Behavior I: wetting stage, swelling stage, and long total rehydrationstage. Behavior II: wetting stage, no swelling stage, and short total rehydration stage **(Adopted from Hussain et al., 2011b)**

Recently, **Hussain et al., (2011b)** observed two rehydration behaviors depending upon the salts concentrations. Solutions of NaCl and $CaCl_2$ (0-2M) were used as rehydration media **(Figure 2).** The first behavior (with low salt concentration) represents typical rehydration

profile while the dispersion of the second behavior (with high salt concentration) was shortened for NMC powders indicating a strong modification of protein backbone.

4.2. Effect of salt on whey powders rehydration properties

In comparison with NMC, WPI powders present opposite rehydration properties. The wettability was found to be very poor and is a limiting factor **(Schuck et al., 2007)** whereas the dispersion stage was rather quicker resulting in faster rehydration times **(Gaiani et al., 2007a; Hussain et al., 2011b).** Addition of NaCl to whey proteins normally enhances the formation of denser aggregates by an increase in turbidity, molecular size, and decrease in protein surface hydrophobicity and solubility (**Hussain et al., 2012b; Vardhanabhuti and Foegeding, 2008).** NaCl also influences the hydration process of proteins by: (1) electro-static shielding; (2) ion-specific hydrophobic interactions **(Boye et al., 1996b; Hussain et al., 2011b).** It's weird that in spite of overwhelming the use of salt in the dairy industry and fundamental research; very short literature is available on salt-WPI rehydration phenomenon.

For example, **Gaiani et al., (2005)** developed a turbidity sensor method to study the rehydration properties of dairy powders (NMC and WPI) under standardized conditions. These powders were characterized and differentiated by wetting time (Tw), swelling time, (Ts) and rehydration time (Tr). Furthermore, these authors **Gaiani et al., (2007a)** studied protein powder rehydration with reference of protein state, incorporation mood and agglomeration. Among salts divalent cations ($CaCl_2$) are more effective at screening electrostatic interactions than monovalent (NaCl) but at comparatively lower concentration **(Mercade-Prieto et al., 2007; Mercade-Prieto et al., 2009)** due to higher valency of counterions and ability of $CaCl_2$ to form ion bridges between negatively charged protein molecules **(Bryant and McClements, 2000).** With addition of salts, rehydration of whey protein is less influenced showing a typical profile of wetting with lump formation, no swelling and a quick dispersion. **(Hussain et al., 2011b)** due to screening of electric charges by electrolyte and association between protein molecules **(Kinsella, 1982).**

(Figure 3) shows effect of salts on rehydration behavior of WPI powders. Solubility of whey proteins is an important determinant for the functional properties of whey protein products. Solubility of proteins is governed by the balance between attractive and repulsive intermolecular forces between them, which are determined by the protein and water structures, as well as salting-in or salting-out of proteins. Adjusting the ionic strength to 0.1 M NaCl, **deWit and van Kessel, (1996)** reported minimum solubility for WPI near the isoelectric point of β-lactoglobulin (pH 5.2) in the pH range 4.6-5.0, for WPC. Whey proteins

exhibited good water holding capacity under influence of monovalent (**Boye et al., 1997b; Choi et al., 2000)** and divalent salts **(Barbut, 1995; Kuhn et al., 2011; Vardhanabhuti et al., 2001).** Recently, **Ryan et al., (2012)** studied the stability and the mechanism of whey protein soluble aggregates (7% w/w, pH 6.8) thermally treated (90°C, 5 min) with different NaCl concentrations (0-108 mM). At 108 mM NaCl, high solubility, low viscosity, low turbidity and smaller final aggregate size illustrated that soluble aggregates of whey proteins were more thermally stable than the native proteins.

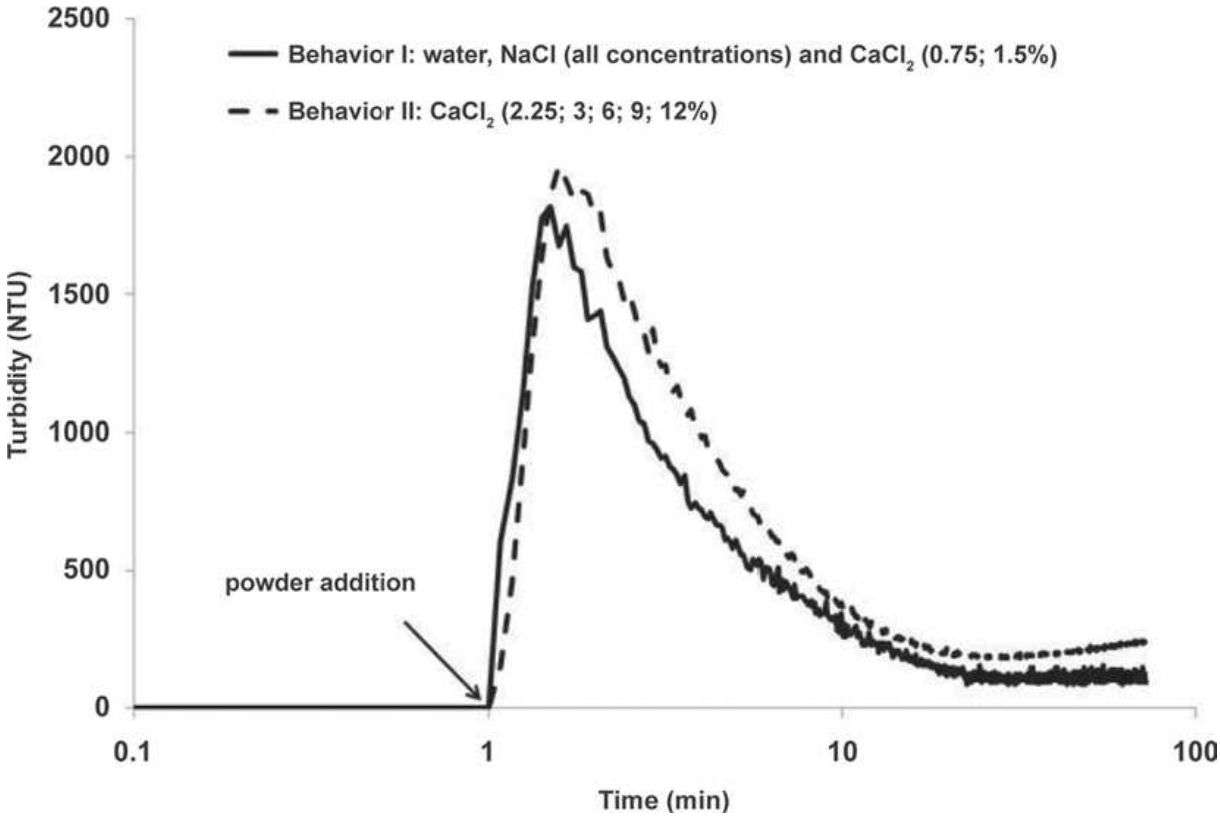


Figure 3. Turbidity profiles (NTU, nephelometric turbidity units) obtained during rehydration of 5% native whey protein isolate powder at 24°C for 1,000 min in different ionic environments (mean of 3 independent analyses). Behavior I: wetting stage, no swelling stage, and quick total rehydration stage. Behavior II: wetting stage, no swelling stage, and rehydration stage not observed **(Adopted from Hussain et al., 2011b).**

5. Multiscale characterization of milk proteins under salt conditions

The subject matter of this section has been already treated by using many instrumental, physicochemical, and theoretical approaches to characterize milk proteins (casein and whey proteins) regarding the influence of salts on their secondary structure elements correlated with their size, and morphology. The coverage here is highly selective, reviewing mainly most specific and recent findings intended to draw out comparisons and

conflicts between them and to provide an overview of the 'state of art'. An effort is made to characterize these results with respect of techniques used along with different microenvironments (pH, type and concentration of salts, size and structure of the proteins…) to give a better idea and foot print even for non specialist readers.

5.1. Effect of salt on casein micelles secondary structure

One of the great dogmas of molecular biology is that protein structure reflects function and that protein function dictates its three dimensional structure **(Horne, 2002).** Among milk proteins, the caseins (αs_1-, αs_2-, β- and κ) are characterized as unusual type of proteins because they are clearly neither globular nor fibrous in nature but contain an "open structure" **(Alaimo et al., 1999).** They are suggested to be comprised of predominantly random structure (**Swaisgood, 2003**) with only small segments of secondary structure present as a consequence of their high proline content **(Rose et al., 1985).** CMs are colloidal protein-calcium-transport complexes whose structure has not been unequivocally elucidated **(De Kruif and Holt, 2003).**

Three categories of models micelles are broadly accepted that combine theoretical and experimental data: coat-core model, internal structure model and sub-unit model. It is generally considered that κ-casein is present at the surface of micelles while αs_1, αs_2 and β caseins constitute the inner hydrophobic core **(Fox and Brodkorb, 2008; Glantz et al., 2010; McMahon and McManus, 1998)**. Assignments of secondary structure elements to whole casein and various individual caseins have been compared in **Table 3.**

Several instrumental, physicochemical, and theoretical approaches have been used to characterize caseins, casein micelles, and casein peptides including nuclear magnetic resonance (NMR) **(Bansal et al., 2006; Le Feunteun et al., 2012),** Circular Dichroism (CD) **(Creamer et al., 1981; Farrell et al., 2001; Wong et al., 2012),** Raman spectroscopy **(Ashton et al., 2010; Ashton et al., 2011),** small ray-angle scattering (SAXS**) (Mata et al., 2011; Zhai et al., 2012)** and infrared spectroscopy (FTIR) **(Gaiani et al., 2011; Hussain et al., 2011a; Sikand et al., 2011).**

Table 3. Comparison bewteen predictions of secondary elements percentages of caseins (α-helix, β-sheet, turns and irregular structures) measured by FTIR with literature.

	α-helix	**β-sheet**	**turns**	**irregular**	**References**
αs_1-casein	30	25	39	11	**(Farrell et al., 1996)**
αs_2-casein Amide I	32	27	31	9	**(Farrell et al., 2001)**
αs_2-casein Amide III	29	37	34	34	**(Farrell et al., 2001)**
β-casein	29	34	32	4	**(Kumosinski and Unruh, 1996)**
κ-casein	17	35	25	23	**(Farrell et al., 2001)**
casein micelle Amide I	25	30	36	9	**(Kumosinski and Farrell, 1993)**
casein micelle Amide III	14	49	23	14	**(Hussain et al., 2011a)**

Irrespective of a vast and important role of salts and casein in dairy industry (**see section 3**), we find a dearth of literature corresponding to its effect on the secondary structure of this milk protein. Addition of $CaCl_2$ to α_{s1}- or β- casein in neutral solutions (3mM) resulted in no change in the CD spectrum below 250 nm **(Creamer et al., 1981)** while **Byler and co-workers** used Laser Raman Spectroscopy and FTIR to investigate the secondary structures of lyophilized α_{s1}-, β- and whole-caseins disclosing the interaction of Ca^{+2} ions with carboxylate side chain. These observation suggested that both α_{s1}- and β-caseins have around 10% helical structure, around 20% β-structure, and from 20 to 35% turns with a subsequent role of carboxylate groups for Ca^{+2} ion binding in caseins (**Byler et al., 1988; Byler et al., 1989**).

Likewise, FTIR studies of **Curley et al., (1998)** showed a concomitant increase in the percentages of turns and irregular structures at the expense of α-helical and irregular constituents of bovine casein submicelles by addition of Ca^{2+} at 15 and 37°C. Addition of NaCl to native micellar casein (**NMC**) dispersions revealed an increase in irregular structures and a concurrent decrease in ß-sheet structures **(Figure 4) (Hussain et al., 2011a).**

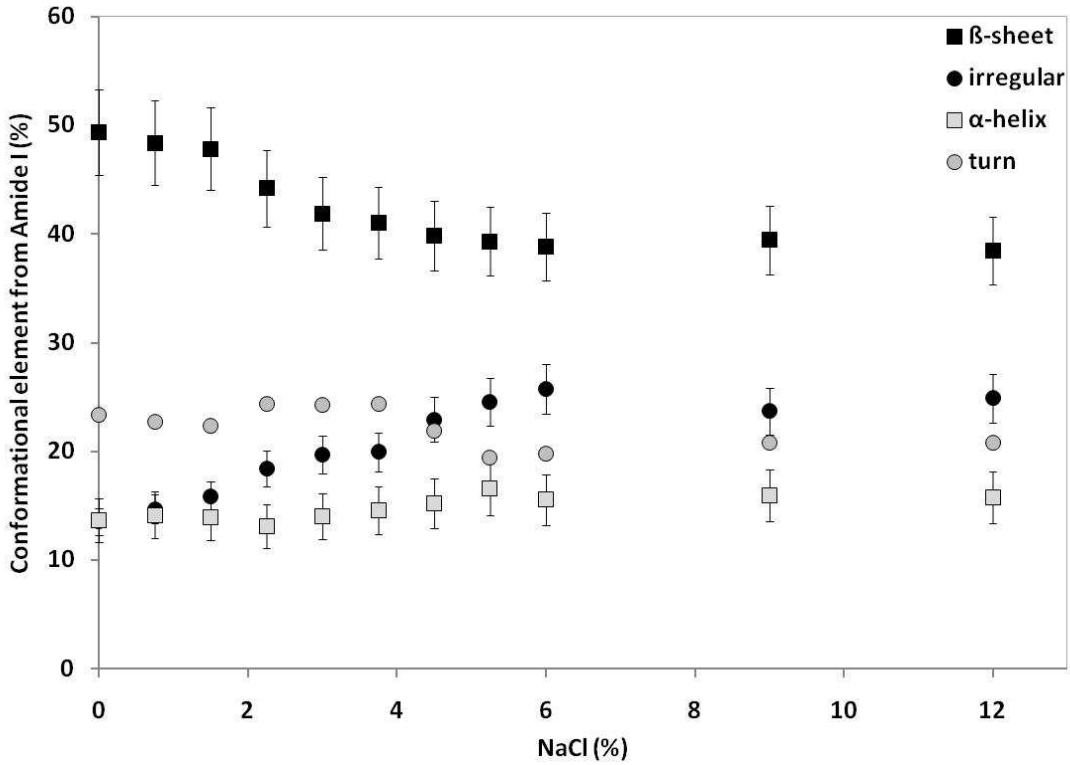


Figure 4. Structural components (ß-sheet, Irregular, Turn and α-helix) percentage peaks areas obtained for casein micelle dispersions in different NaCl environment measured from Amide I **(Adopted from Hussain et al., 2011a).**

In this report, for the first time, the estimations of secondary structural elements (irregular, ß-sheet, α-helix and turn) from Amide III assignments were correlated with results from Amide I showing almost similar structural elements percentages from both bands but an easy deconvolution and refined spectral contour for Amide III compared to Amide I.

5.2. Effect of salt on casein micelles size and morphology

Casein micelles are naturally occurring colloidal protein aggregates present in dispersed state in milk. The casein micelles harbor the main part of casein with an average hydrodynamic diameter between 100-200 nm. Quantifiable changes in particle size of casein furnish valuable indications of aggregation or dissociation phenomena, which might be helpful to predict stability and a range of microscopic properties of colloidal systems **(Gaiani et al., 2007b; Hussain et al., 2011a; Hussain et al., 2011b).** Size measurements based on electron-microscopic studies are subject to inaccuracies caused by possible artifacts which result from fixation, staining and dehydration process.

Morphological studies of proteins carried out only with microscopic techniques do not provide complete information about its structure and complete structural interpretations

cannot be ruled out **(Clark et al., 1981)** unless it is complimented by other physical techniques i.e. light scattering techniques (**Schmidt, 1982**). In the case of casein micelles, different sample preparation techniques for electron microscopy have been shown to induce diverse changes **(McMahon and McManus, 1998)** and no optimal technique for preparation of exists up to now. Three different preparation methods were proposed by **Karlsson et al., (2007)** during microscopic studies of casein micelles in ultrafiltrated (UF) skim milk concentrate at pH 6.5 and 5.8.

Casein micelles have been characterized for their size and morphology in aqueous solution, using different light scattering techniques (DLS, SLS, MALS, SANS, SAXS) alone **(Beliciu and Moraru, 2009; de Kruif et al., 2012; Glantz et al., 2010; HadjSadok et al., 2008; Panouille et al., 2004)** and/or with combination of various electron microscopic (TEM, SEM, AFM) **(Anal et al., 2008; Auty et al., 2005; Dalgleish et al., 2004; Hussain et al., 2011a; Karlsson et al., 2005; Lencki, 2007; Marchin et al., 2007; Mimouni et al., 2010; Mueller-Buschbaum et al., 2007),** fluorescence **(Jean et al., 2006; Liu and Guo, 2008)** and nanoparticle tracking analyses **(Le et al., 2008)** with and without addition of salts and reflect sometimes conflicting results.

Mueller-Buschbaum et al., (2007) extensively studied the effect of calcium concentration on structure of casein micelles in thin films and used small angle X-ray scattering and atomic force microscopy. Addition of $CaCl_2$ resulted in the formation of increased sized aggregates (> 500 nm) along with mini micelles (20 nm) while the size of micelles slightly decreased. In their view, this fact was explained in the framework of particle-stabilizing properties of the hairy layer of κ-casein surrounding the CMs. However, binding of subsequent Ca^{2+} to ß-casein is weaker than the binding to αs_1-casein **(Parker and Dalgleish, 1981)**.

Invariably, an evidence of fibril-like structure cross-linked by dense aggregates junction zones in bovine casein micelles (7 to 10 nm) upon addition of 25 mM $CaCl_2$ is discussed in literature **(Lencki, 2007)**. Similarly, **Horne and Davidson, (1986**) showed that NaCl addition to casein micelles in skim milk stabilized them against aggregation by an increase of the hairy layer (κ-casein) but with no modification of the casein hydrodynamic radius. More recently, **Hussain et al., (2011a)** illustrated that an addition of NaCl to casein micelles dispersions may cause a destabilization of the micelle and the formation of mini-micelles (around 20 nm) more or less aggregated. Without salt, casein micelles seem to be compact and spherical with a size around 100-200 nm corresponding to the micelle size measured by DLS. When salt is added, the micelles changed in lumpy shaped, disintegrated, constellated aggregates of sub-micelles (around 20 nm) **(Figure 5).**

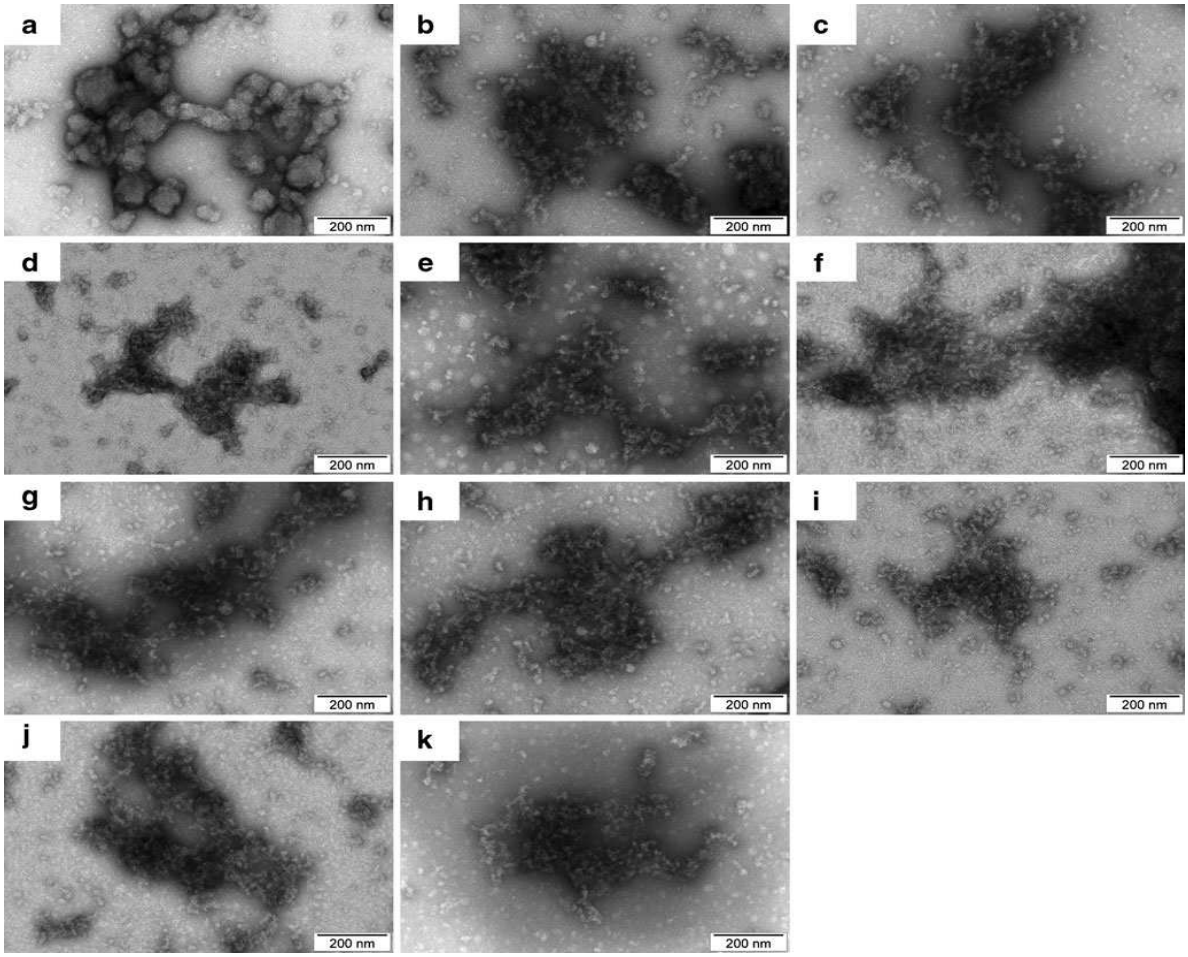


Figure 5. Transmission electron micrographs of casein micelles in different NaCl environment. A: 0%; B: 0.75; C: 1.5; D: 2.25; E: 3.0; F: 3.75; G: 4.5; H: 5.25; I: 6.0; J: 9.0 and K: 12.0 % NaCl. Imaging was at 50,000x magnification. Each image is representative of at least 6 images. Scale bar = 200nm **(Adopted from Hussain et al., 2011a)**

Many other studies also dictate presences of these mini micelles with addition of monovalent and divalent cations in αs1, β, κ and whole caseins **(HadjSadok et al., 2008; Morris, 2002; Panouille et al., 2004)**. These authors concluded that when higher amounts of salt are considered, more chloride ions could bind to the protein surface so that the preferential hydration by water is overcome. Furthermore, the amount of calcium bound to casein micelles decreases **(Huppertz and Fox, 2006)** leading to the observed salting-out effect. These effects could lead to less connected networks **(Walstra et al., 1999**). As a consequence of these changes, the hydration of casein micelles is increased, even if their sizes stay constant **(Gaucheron, 2005). (Table 4)**

Table 4. Estimation of the hydrodynamic diameters of casein micelles dispersions in water and added NaCl concentrations.

NaCl concentration (%)	1st peak Hydrodynamic diameter (nm)	2nd peak Hydrodynamic diameter (nm)
0.00	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
2.25	180 ns ± 14.5	37 ns ± 9.2
3.00	175 ns ± 22.0	33 ns ± 14.1
3.75	165 ns ± 22.7	37 ns ± 12.1
4.50	162 ns ± 18.8	30 ns ± 10.2
5.25	175 ns ± 13.3	35 ns ± 19.7
6.00	161 ns ± 4.6	31 ns ± 2

0.001, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{ns}P > 0.05$

5.3. Effect of salt on whey proteins secondary structure

As mentioned earlier, addition of salts not only allows making a distinction between the role of electrostatic and hydrophobic interactions **(Gaucheron et al., 2000; Kinsella, 1984)** but also it plays an important role in the conformation stability of whey protein secondary structure illustrated by FTIR technique **(Boye et al., 1996b; Hussain et al., 2012a)** Secondary structure elements are affected more or less, depending on the amount of NaCl added. The effects of pH (2-10), protein concentration (20-300 mg WPC/g H_2O), NaCl (0-2M), heating temperature (60-90°C) and heating time (15-60 min) on the gelation of WPC and the associated changes in the molecular conformation of the individual whey proteins (β-Lg and α-Lac) were studied using different spectroscopic techniques **(Boye et al., 1995).**

These authors observed that protein unfolds to a lesser extent in the 2M solutions than it does in the 0.5 M due to salting out effect leading to an aggregation followed by a precipitation. In some other studies, addition of NaCl (0-2M) seems to bring no changes in the secondary structure of whey proteins at room temperature **(Boye et al., 1996b; Hussain et al., 2012a) (Table 5).**Upon heating (30-95°C), a phenomenon of heat denaturation of β-Lg was proposed that involves unfolding of β-type structures in the interior of proteins, which results

	Structural Component (%)	NaCl concentration (%)								
		0	0.75	1.5	2.25	3	3.75	4.5	5.25	6
from Amide I	ß-sheet	46.2±1.2	46.1±1.0	45.9±0.9	45.7±1.5	45.9±2.1	46.0±1.7	45.7±1.5	45.4±2.1	46.1±1.4
	unordered	19.4±0.8	19.2±1.3	19.2±1.3	18.3±1.2	19.0±1.7	19.2±1.5	18.7±1.2	19.2±2.1	19.2±2.2
	α-helix	13.1±0.9	13.1±1.5	13.1±0.9	13.2±1.1	13.1±1.8	13.2±1.4	13.2±1.3	12.7±1.8	12.8±2.1
	ß-turn	21.3±1.5	21.5±1.5	21.8±1.0	22.7±0.6	22.0±1.6	21.7±1.0	22.4±1.4	22.7±1.8	21.9±1.9
from Amide III	ß-sheet	48.3±2.2	49.2±1.1	50.8±1.1	50.4±1.0	50.4±1.1	51.8±0.8	49.3±1.5	55.7±1.3	54.2±1.4
	unordered	18.1±1.2	17.0±1.0	15.8±1.4	15.0±1.1	15.1±0.9	15.1±1.0	16.6±1.1	12.3±1.2	13.3±0.4
	α-helix	17.2±1.1	18.1±0.8	16.4±0.8	17.8±0.8	18.2±0.9	16.7±0.9	16.0±1.3	17.0±0.5	17.2±0.7
	ß-turn	16.4±07	15.6±0.7	17.0±0.7	16.8±0.7	16.3±0.7	16.4±1.2	18.1±0.3	16.0±0.4	15.3±0.5

Table 5. Structural component (ß-sheet, unordered, α-helix and ß-turn) percentage areas obtained for native whey proteins dispersions in different NaCl environments measured from Amide I and Amide III (mean of three independent analyses). **(Adopted from Hussain et al., 2012a)**

in β-sheet structures formations and more extensive random coils, followed by an unfolding of α-helical structures resulting in aggregate formation **(Boye et al., 1996b).**

Moreover**,** following β-Lg denaturation upon heating with NaCl (30-60 mM), FTIR results accompanied by CD measurements have been related to the formation of a molten globule structure above 70°C **(Qi et al., 1997).** This conformational state is generally believed to hold ordered native-like secondary elements with a loss of tertiary structure. The results of both techniques showed a progressive loss of β-sheet structure with increasing temperature, beginning below the denaturation temperature (65°C). Undoubtedly, β-Lg provides an excellent model protein system for β-to-α conformational changes but its behavior varies when the change is induced by lipid vesicles. CD and FTIR were used to probe secondary and tertiary structural changes in β-Lg upon interaction with lipids **(Zhang and Keiderling, 2006)**.

The addition of NaCl (up to 0.5 M) largely dissociates β-Lg from the negatively charged lipid at neutral pH. Near UV-CD spectra revealed that tertiary structure of lipid-bound β-Lg is highly expanded but not completely disrupted while FTIR spectra showed the orientation of the α-helix and β-sheet axes in membrane-bound β-Lg to be parallel and perpendicular, respectively, to the membrane film normal. By using Raman spectroscopy technique, **(Ikeda and Li-Chan, 2004)** reported that the secondary structure of the proteins was better preserved in particulate gels (pH 5.4 with 0.3 M NaCl) than in finely stranded gels (pH 2.0 or 7.0 by heating at 80°C for 60 min gels).

A decrease in α-helix was more pronounced in fine stranded gels, while considerable fractions of ß-sheet structures remained in both types of gels. Previously, the structure of heated systems (25-95°C) obtained by two important globular proteins (α-Lac and BSA) have been described at various pH levels (from 3.0 to 9.0) and different salt concentrations (from 0.5 to 2 M) **(Boye et al., 1996a, 1997a).** The major effects of salt and heat treatment of α-Lac were: loss in the bands attributed to α-helix, 3_{10}-helix and β-sheet and increase in β-turns. This process of heat denaturation is reversible in the presence of cationic salts. Conversely, denaturation of BSA resulted in the loss of the 1654 cm^{-1} band attributed to α -helical structure and the rise of two bands at 1616 and 1684 cm^{-1} attributed to the formation of ordered non-native β-sheet structure associated with aggregation.

These results were similar to that observed for β-Lg **(Boye et al., 1996b)** but not for α-Lac **(Boye et al., 1997a)**, which demonstrated the 1616 and 1684 cm^{-1} band only in the presence of NaCl concentrations above 1 M. In a most recent study, 5% w/v whey protein isolates **(WPI)** dispersion (pH 6.5), with or without NaCl (0-500 $mmol.kg^{-1}$) was submitted to dynamic heat treatment (30-90°C) **(Hussain et al., 2012c submitted)**.

Upon heating, without salt increase, the infrared spectroscopy data reveal an increase in intermolecular β-sheets primarily at the cost of β turns and random coils with no change in α-helical structures for Amide 1. Similarly, intermolecular β-sheets contents increased significantly for Amide III. However, NaCl addition along with dynamic heat treatment of WPI dispersion showed a stabilizing effect on the secondary structural elements of both Amide I and Amide III bands.

5.4. Effect of salt and other microenvironments on whey proteins size and morphology

Among whey proteins, β-Lg the most extensively studied protein since its isolation and purification from bovine milk by acidic precipitation **(Palmer, 1934).** Ionic strength influences the structure of proteins to get desired functionalities of proteins **(Taheri-Kafrania et al., 2010).** Effect of ionic strength on size, structure, denaturation, aggregation or gelation mechanisms have been extensively studied by different research groups by thermal, light scattering, X-ray diffraction or by microscopy. Differential scanning calorimetry **(Haug et al., 2009)**, light scattering (SLS or DLS) **(Ako et al., 2010)**, multi-angle laser light scattering (SEC MALLS) **(Vardhanabhuti and Allen Foegeding, 2008)**, small angle X- ray scattering (SAXS) **(Pouzot et al., 2005),** confocal laser scanning microscopy (CSLM) **(Balakrishnan et al., 2011),** Atomic force microscopy **(Arnaudov and de Vries, 2006)** and transmission electron microscopy (TEM) **(Loveday et al., 2010)** are the widely common techniques used in this era to study salt's effect n proteins.

During denaturation, aggregation and subsequent gelation process of β-Lg, upon varying ionic concentrations (from lower to higher), salt type, pH and rate of heating, β-Lg depicts enormous changes in particle size and morphology. Even a very small salt addition to whey proteins may result in significant aggregates formation with other complimentary environmental factors. **Kavanagh et al., (2000)** used TEM to investigate heat-induced (80°C) gels and aggregates with 0.05M NaCl at pH 2.0 and 7.0. This work generated images apparently well defined linear aggregates at neutral pH unlike **Aymard et al., (1999)** who reported randomly branched structures (clusters).

For ionic strength less than 0.05M and pH > 6.0 the size of aggregates increase weakly with increasing NaCl and found to depend slightly on the protein concentration **(Baussay et al., 2004; Pouzot et al., 2004).** Cryo-TEM images demonstrated that addition of a small concentration of NaCl (0.001M-0.1M) resulted into large aggregates formed by cross-linking short curved strands in head to tail manner, with a diameter of about 10 nm and

a length of 50 nm **(Durand et al., 2002; Pouzot et al., 2005).** Aggregation of native β-Lg is favored by adding low amount of NaCl (60 mM) **(Unterhaslberger et al., 2006)** observed in DSC experiments appearance of the exothermic peaks is linked to protein aggregation, in particular to a strong increase in aggregate size (372 nm). In addition, recently by using TEM, worm-like fibrils were seen at ≥ 60 mM NaCl or 33 ≥ mM $CaCl_2$ addition for β-Lg protein having a persistence length **(Loveday et al., 2011; Loveday et al., 2010)** much shorter than the long semi-flexible fibrils formed without salts. Contrarily, a particle size of 23.46 µm was reported for WPC gels produced at pH 7.0 and 55 °C with a minimum concentration of $CaCl_2$ (2.5 mM) **(Gu et al., 2011).**

The formation of β-Lg fibrils at low NaCl (0.01-0.2 M) and at pH 2.0 was initially described as a nucleation and growth mechanism of partially unfolded monomers. Further at different ionic strengths studied (0.01-0.1M), the fibrils had similar thicknesses of about 3.5 nm and a periodic structure with a period of about 25 nm with a shorter and curvier microstructure **(Arnaudov and de Vries, 2006; Aymard et al., 1999).**

O'Kennedy and Mounsey, (2009) observed the denaturation/aggregation behavior of heated (78 °C, 10 min) β-lactoglobulin (1%, w/w) in the pH range 5.0–7.0 and reported that more proteins were denatured after 10 min at 78°C in 5 mM $CaCl_2$ than in 100 mM NaCl. In the same way, **Balakrishnan et al., (2011)** very recently investigated the mobility of tracer particles in globular protein gels s using CLSM combined with multi particle tracking. Globular protein gels were prepared with a variety of structures by heating (80°C) β-Lg solutions at different concentrations (20- 120g/L) and ionic strengths (0.1-0.25M NaCl).

A strong coarsening of the gel structure was observed upon increasing the NaCl concentration showing spherical particles with diameters between 0.2 and 2 µm in solution and gels. Nevertheless, The effect of adding NaCl (0-1M) at different pH values have been studied in detail on the association behavior and kinetics of heat induced aggregation of β-Lg and its variants (β-Lg A, B). At low ionic strength (0.1M) and lower pH (4.5) larger oligomeric structures were formed whereas β lactoglobulin A associates more strongly than β–lactoglobulin B **(Verheul et al., 1999; Verheul et al., 1998).** Similarly higher ionic strength (1M) showed a stabilizing and salting out effect with formation of larger aggregates. Stable oilgomers that were formed at low NaCl concentration tend to aggregate rapidly when salt was added. Fewer oilgomers were observed in the presence of NaCl **(Bauer et al., 1998; Croguennec et al., 2004).**

Denaturation properties of protein in concentrated systems found to be effected by different salts and ionic strengths. **Haug et al., (2009)** elaborated the thermal treatment (22-95°C) of β-lactoglobulin (14% w/v) and its denaturation as a function of pH (2–10) and ionic

strength (0-1M) followed by DSC thermograms. Their findings revealed that the quarternary structure of β-Lg at pH-values close to the isoelectric point was influenced by the presence of salt and the nature of the salt (NaCl, KI and LiI). At higher salt concentrations, **Ako et al., (2009)** using CLSM proposed a critical salt concentration (Cs) (0.2 M NaCl) for the preparation of heat induced (80°C) β-Lg gels that show homogeneous images but at higher NaCl (0.2-2M) concentrations micro-phase separation was observed leading to the formation of dense protein domains with a diameter of a few microns. In the same vein of study, with similar experimental conditions, these authors prepared globular protein β-Lg gels with two different salts (NaCl, $CaCl_2$) and the dependence of the kinetics and the final gel structure on the protein concentration, the temperature, the salt concentration, and the aggregate size was quantified **(Ako et al., 2010).**

In some sense, At low ionic strength (<0.05 M NaCl), homogeneous transparent gels were formed, while at higher ionic strength (0.2 M), the gels became turbid but had the same self-similar structure as reported for pure β–Lg **(Mahmoudi et al., 2007).** The effect of adding salt on denaturation/aggregation and foaming properties at various pH values has not been studied in much detail. **Schmitt et al., (2007)** found that particle size and morphology of WPI dispersions (10g L^{-1}) after 15 min heating at 85°C changed significantly with increasing pH from 6.0 to 7.0 by addition of NaCl (5-120mM) respectively. With addition of salt, particle average size of WPI aggregates increased from 180 to 327 nm associated with a morphological transformation (compact/spherical to fibrillar aggregates).

In a series of articles, **(Nicorescu et al., 2008a, b; Nicorescu et al., 2009)** reported the effect of dynamic heat treatment (100°C) using a tubular heat exchanger of a WPI solution (20g L^{-1}) at pH 7 to investigate denaturation/aggregation and foaming properties, with and without NaCl addition (100 mM). It was shown that not only denaturation (10%) and size (less than 10 µm) of aggregates was reduced by the presence of NaCl, but also the aggregates (diameter about 200 nm) obtained in the presence of 0.1 M NaCl played a key role on the stabilization of gas-liquid interfaces. However, destabilization of the foam was observed caused by the breakdown of thin films if too many if too many (>10%) insoluble aggregates (>100 µm) were present.

Mahmoudi et al., (2010) investigated the structure of heat-induced assemblies of WPI at 80°C and pH 7.0 in presence or absence of 0.1 M NaCl and showed that aggregates of various sizes generated by heating were able to form viscoelastic films by using small angle neutron scattering (SANS), static and dynamic light scattering (SLS and DLS), and cryogenic transmission electron microscopy (Cryo-TEM). NaCl induced-gels showed much higher hardness and elasticity than $CaCl_2$ induced gels. By adding these two cationic salts (150 mM),

Kuhn et al., (2011) evaluated cold set WPI-Flaxseed Gum (FG) gels with respect to their mechanical properties, water-holding capacity (WHC) and SEM microscopy. Use of soluble aggregates improved thermal stability in the presence of salt (0-108 mM) at neutral pH due to their altered aggregate shape and higher charge, and resulted in final aggregates that were smaller and less dense compared to native proteins.

Furthermore, Atomic force microscopy (AFM) was applied by **Ikeda and Morris, (2002)** to visualize fine-stranded and particulate whey protein aggregates induced by NaCl (0-0.3M) heated at 80°C and pH 2 and 7 in order to gain insights into the heat-induced gelation of globular proteins. Nevertheless, the structure of large aggregates and gels formed at steady state in WPI at pH 7 was found to be almost identical to those formed in pure β-Lg solutions.

Size exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALLS) and dynamic light scattering were used to determine aggregate size and TEM was used to evaluate aggregate shape **(Ryan et al., 2012).**

The structure of stable aggregates at steady state depends on the salt concentration. This was very recently illustrated by **Hussain at al., 2012b** who studied WPI aggregates at a constant protein concentration (5%) but in varying NaCl concentrations (0-1M). TEM images revealed that protein aggregates were spherical in shape with two populations around 6 and 70 nm. The size of these edifices was further confirmed by (**DLS**) and **(SLS)**. Salt addition resulted in an aggregation of the proteins and the formation of clumpy denser aggregates as observed by both techniques **(Figure 6).**

6. Use of salt in milk proteins functional properties

Protein molecules must undergo a series of physicochemical reactions that include hydration, dispersion, dissociation, molecular unfolding and rearrangement, diffusion, adsorption, interaction and ligand binding in a specific sequence of events in order to provide functionality. Milk proteins are frequently used as food ingredients in a number of food products because of their nutritional, technological and functional properties.

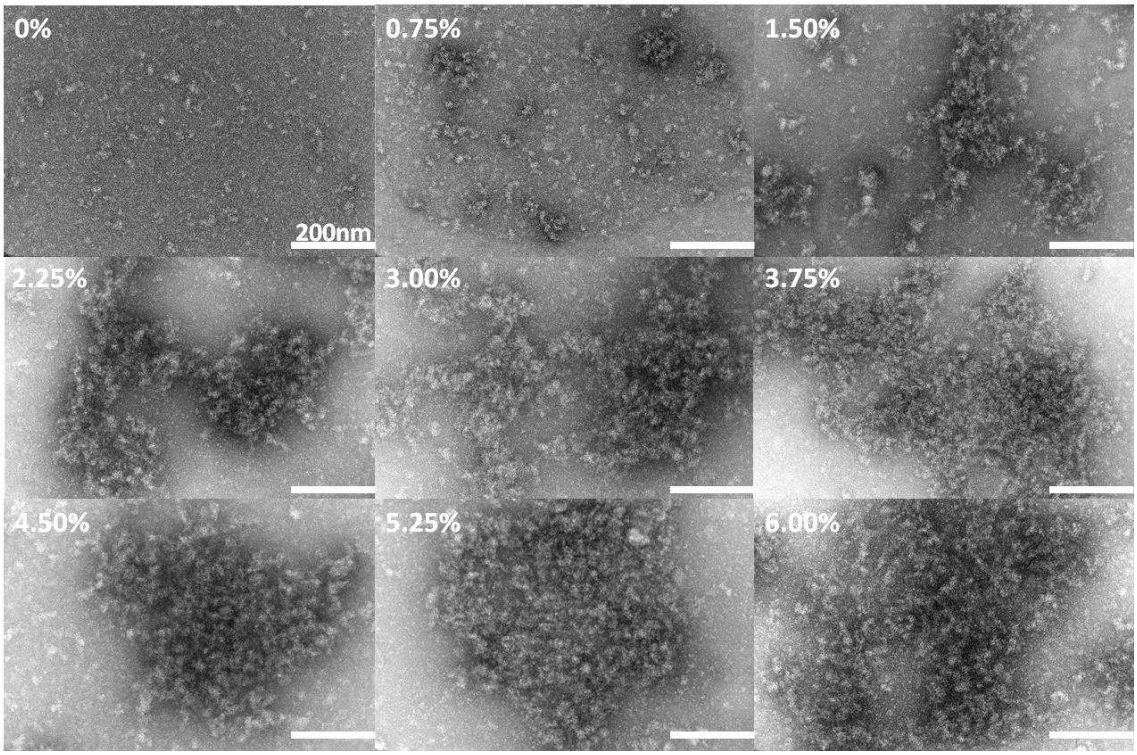


Figure 6. Transmission electron micrographs of whey proteins dispersions in variable NaCl environments ranging from 0 to 6%. Imaging was performed at 50,000x magnification (scale bar = 200 nm). Each image is representative of at least 6 images observed **(Adopted From Hussain et al., 2012b).**

The functional properties of food proteins may be classified into three major groups **(Nicorescu et al., 2008a):**

(a) Hydration related functional properties that include wettability, swelling, dispersability, solubility, viscosity, water absorption and water holding capacity;

(b) Interfacial properties including emulsification and foaming characteristics;

(c) Aggregation and gelation properties which are related to protein–protein interactions **(Kresic et al., 2006).**

A schematic diagram illustrates several functionalities of milk proteins **(Figure 7).**

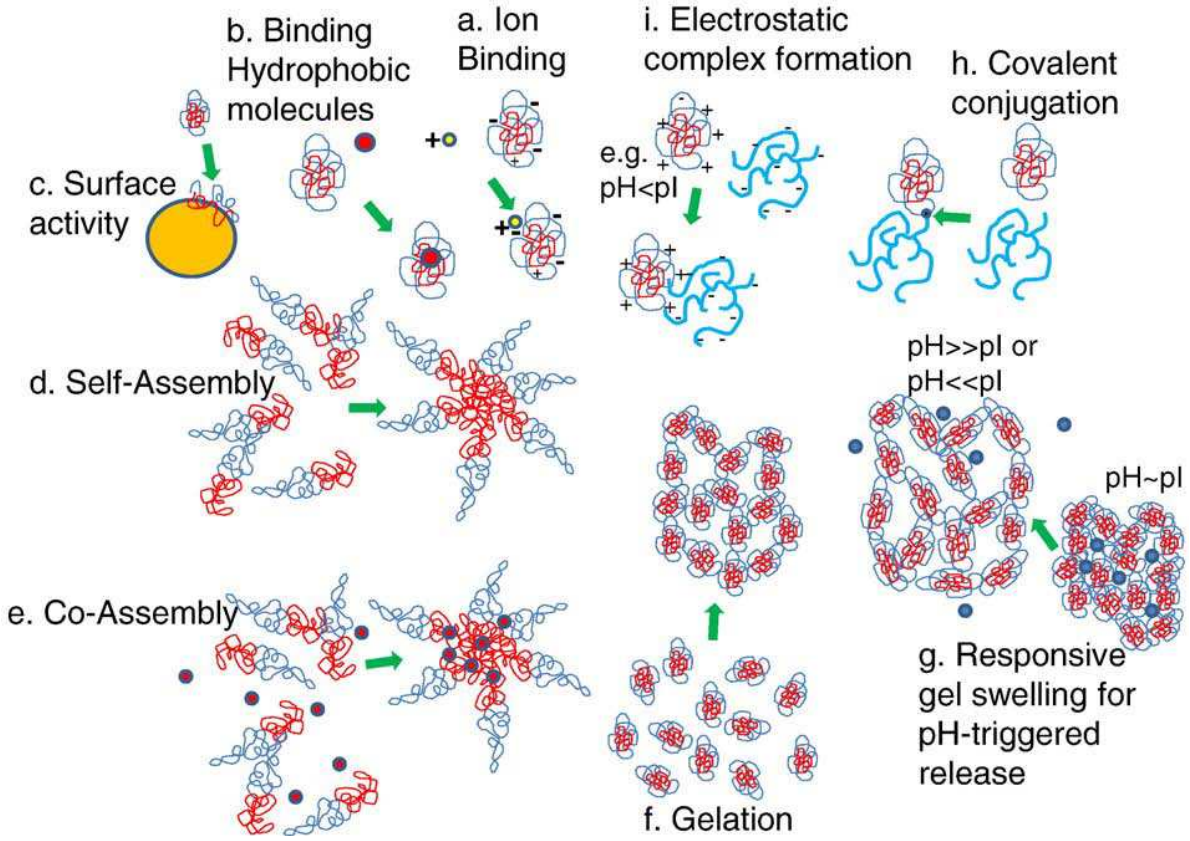


Figure 7. Schematic illustrations of several functionalities of milk proteins **(Adopted from Livney et al., 2010)**

6.1. Effect of salts on hydration related functional properties of milk proteins

The subject matter of this section has already been reviewed in one of the previous sections (See section **4**). A brief description of most recent findings relevant to effect of salt on viscosity of milk proteins are only given here. Viscosity is one of the essential properties of food additives that holds significant effect on food manufacturing processes and is considered as an index of protein quality. Viscosity not only provides information on physicochemical interactions between proteins but also used to indicate the degree of protein denaturation and aggregation.

Minerals are found to affect casein micelles functional properties generally **(Schuck et al., 1999). Schkoda et al., (1999)** investigated the effect of minerals and pH on rheological properties and syneresis of milk-based acid gels and they reported that if the concentration of NaCl is increased before acidification (pH 4.6), the viscosity decreases. Addition of NaCl led to an increased apparent viscosity and a shear thinning non-Newtonian fluid behavior of casein micelles mainly due to collapse of κ-casein layer, changed ionic strength, and altered distance between casein micelles **(Karlsson et al., 2005)**.

Caseinmacropeptide (CMP) isolated from cow and goat cheeses were studied for their viscoelasticity properties. Analyses at different pH (2-10), NaCl (0-0.7 mol L^{-1}) and protein concentration (50–200 $g.kg^{-1}$) at temperatures from 10 to 90°C showed no appreciable difference in viscosity **(Martin-Diana et al., 2006).** Moreover, a linear relation was observed between NaCl concentration and the decrease in viscosity for solution of WPC and its fractions (β-Lg and α-Lac). **El-Shibiny et al., (2007)** reported an increased viscosity for β-Lg and α-Lac solution with addition of NaCl (50 mg/100ml) and $CaCl_2$ (50 mg/100ml) but further increase of salt decreased their viscosity.

A very recent study conducted on whey proteins by **(Ryan et al., 2012)** showed that soluble aggregates thermally treated (90°C, 5 min) in the presence of 108 mM NaCl had lower viscosity and yield stress than native proteins at the same experimental conditions. Higher thermal stability and the formation of linear aggregates of native proteins were attributed to their increased viscosity when heated in the presence of NaCl.

6.2. Effect of salts on interfacial related functional properties of milk proteins.

Among interfacial or surface related properties of milk proteins are emulsifying and foaming properties. Indeed, proteins must disperse, diffuse to an air/water or oil/water interface immediately after they have been formed during processing, then unfold and reorient at the interface to lower interfacial tension. The adsorbed protein molecules must then interact to form a protective film (membrane) around the oil droplet (for emulsion) or air cell (for foam) with sufficient strength and elasticity to stabilize the final structure. The amphipathic nature of proteins, arising from the mixture of polar and non polar amino acid residues is strongly influenced by the pH and ionic strength and therefore these factors could affect the emulsifying and foaming properties of milk protein solutions **(Mohanty et al., 1988**).

6.2.1. Emulsifying Properties

Interface related functional properties of milk proteins include foaming and emulsifying properties. In foams, air droplets or fat droplets are covered by protein coatings. Emulsions are of 2 types, oil in water and water in oil emulsions. Proteins are at interface of oil and water and act as emulsifiers to reduce interfacial tension and hence to reduce mechanical energy required to form an emulsion. Milk proteins and particularly whey proteins are commonly used as emulsifying agent in diverse food products thanks to their unique interfacial properties **(Nicorescu et al., 2008a)**.

In emulsions, five types of destabilization mechanisms exist such as flocculation, creaming, sedimentation, coalescence and ostwald ripening **(McClements and Rao, 2011)**. During shelf storage, emulsion droplet size increase depending upon proteins adsorbed at emulsion droplet surface **(Shukat and Relkin, 2011**) and fat composition **(Shukat et al., 2012)** which may cause a protein destabilization. In protein-coated oil droplets, destabilization mechanisms are exhibited mainly by flocculation and creaming **(Walstra et al., 1999).** Both mono and divalent salts affect emulsification properties differently depending upon not only the concentration of salt but also the type of milk protein used to formulate such emulsions. In case of casein stabilized emulsions, cationic salts such as NaCl and $CaCl_2$ have positive effect on the emulsifying properties like emulsifying capacity, emulsifying activity index and emulsion stability **(Roman and Sgarbieri., 2006; Vieira et al., 2006).**

NaCl addition is reported to show stabilizing effect **(Dickson, 2010)** and enhance creaming stability due to increase in protein surface coverage because of increased NaCl contents in emulsions **(Srinivasan et al., 2000).** Apart from creaming stability, addition of NaCl up to 2M concentration (whether added before or after the emulsification) enhanced the emulsion stability against flocculation **(Surh and McClements, 2008)**. Polysaccharides (carrageenan) are normally added in emulsions to enhance the viscosity of systems to make it more stable. It was observed that ionic strength even at low concentrations (0.2 M NaCl) play their role in protein-polysaccharide complexation **(Mena-Casanova and Totosaus, 2011)** due to electrostatic interaction **(Ye, 2008).**

In case of whey protein stabilized emulsions, ionic strength significantly affects emulsions properties. NaCl addition could favor aggregation of the oil droplets and stimulate creaming destabilization mechanisms due to reduction in electrostatic repulsion and height of energy barrier between the droplets. **Sünder et al., (2001)** observed that the size of fat droplets increased by addition of 0.5-1.5% NaCl in emulsions (containing sunflower oil and phospholipids). Moreover, if WPI stabilized emulsions were heated in presence of 200 mM NaCl, total surface protein concentration across fat droplets increases due to heat treatment (90°C for 5 min). But analysis of aqueous phase showed aggregates formation (in presence of more than 50 mM NaCl) of β-Lg and α-La, which could ultimately reduce emulsion stability **(Ye, 2010).**

The role of salt concentration on bovine serum albumin (BSA) stabilized emulsions was carried out by **Saitoa al., (2006).** They found that emulsions are stable until NaCl concentration is less than 150 mM. **Hunt and Dalgleish, (1996)** investigated the role of another monovalent salt on adsorption of whey protein stabilized emulsions. They observed similar results with KCl as other authors observed with NaCl (monovalent salt). As

concentration of KCl increases, emulsion droplet size progressively increases. KCl concentrations above 50 mM increased the droplet size from 0.64 to 3.35µm. These authors reported that added salt produced an increase of the emulsion viscosity, indicating their aggregation as consequence of the electrostatic interactions.

Divalent salts at lower concentrations have stabilizing emulsification trends, while at higher concentrations; they cause coalescence of droplets due to aggregation of whey proteins in aqueous solution. **Chanamai and McClements, (2002)** found that a minimum concentration of 0-25 mM $CaCl_2$ enhance the emulsion stability mainly due to electrostatic repulsions. While higher concentrations (0.50 or 3.0 %) of $CaCl_2$ in whey protein solution prior to emulsion (30% soya oil) resulted in increase of average particle size and a decrease in the creaming stability of emulsions. Furthermore, aggregation of whey protein in solution occurs due to binding of $CaCl_2$, leading to the coalescence of small droplets, resulting in decreased emulsifying capacity **(Ye and Singh, 2000).**

From the above discussion, it is evident that ionic strength in casein stabilized emulsion enhance stabilization mechanism while in whey protein stabilized emulsions, salt addition induce protein aggregation which reduce emulsifying capacity.

6.2.2. Foaming Properties

The dynamics that influence the emulsifying properties of proteins are similar to those that affect their foaming properties (**Damodaran, 1994).** The basic requirements for a protein to be a good foaming agent are the ability to adsorb rapidly at the air–water interface during bubbling; and the ability to undergo rapid conformational changes and re-arrangement at the interface **(Fidantsi and Doxastakis, 2001).** NaCl addition showed also a major role on foam stability, as it acts on electrostatic repulsion forces **(Davis et al., 2004)** and increases the amount of adsorbed proteins **(Nicorescu et al., 2008b). Zhang et al., (2004)** produced aqueous foams by mixing skim milk powder (SMP) and whey protein isolate (WPI) at 0.5% w/w proportions at different pH values (0-8) and NaCl concentrations (0-8M).

An increase in concentration of NaCl demonstrated a continuous improvement in foamability of SMP due to the gradually increasing dissociation of β-casein. According to the authors, a higher concentration of NaCl (e.g. > 0.6 M) caused less whey protein to be adsorbed to the air/water interfaces and resulted in decreased foamability of WPI by altering their extent of solubility and polymerization. Foaming properties of whey proteins are normally determined as foam stability or maximum overrun or foam expansion (**Nicorescu et al., 2010**). In a more recent study, whey protein soluble aggregates were generated by heating

at 85°C for 15 min at pH ranging from 6.0 to 7.0 in the presence of NaCl (0-120 mM) **(Schmitt et al., 2007).**

They noted a clear increase of foam stability upon increase of pH/NaCl content. NaCl addition after a given coarsening time led to significantly smaller air bubble diameters for foam stabilized with WPI soluble aggregates if compared to aggregates generated without salts. Different foaming properties (overrun, foam stability and texture) of WPI (2% w/v) were studied by heating at 100 °C with and without NaCl addition (0-100 mM) (**Nicorescu et al., 2008b; Nicorescu et al., 2009).** The aggregates (about 200 nm diameter) obtained in the presence of salt and heat treatment led to smaller and more homogeneous air bubble size distributions in foams, with improved texture and increased stability against drainage.

In addition, concentration of added salts determines different mechanisms by which neutral salts affect the foaming properties of milk proteins **(Zhang et al., 2004).** The foam volume and stability of WPI was reported to decrease significantly at higher concentration of NaCl (>500 mM) **(Erçelebi and Ibanoglu, 2009).** These authors assessed the effect of ionic strength on the foaming properties of WPI and egg white (EW) proteins protein–polysaccharide mixtures in the presence of NaCl (0.2-2.0 M). It was concluded that at higher concentration, NaCl alters the extent of protein solubility (salting out effect), thereby reducing the foam stability.

6.3. Effect of salts protein–protein interactions properties of milk proteins

6.3.1. Aggregation Properties

In general, it is believed that the screening of electrostatic repulsion, which is a prerequisite to initiate aggregation process for all proteins, can be achieved by sufficient addition of salts. Additions of NaCl to the native casein micelles without thermal treatment have caused a slight “salting-in” effect but did not induce any considerable aggregation. **(Hussain et al., 2011a).**

Simons et al., (2002) demonstrated that calcium likewise sodium induces screening effect but because it binds specifically to the carboxylate, much lower concentrations are required than sodium to yield the same effect. $CaCl_2$ showed a significant effect on the aggregation of casein micelles in milk renneted at 15ºC at lower concentration (6 mM) as compared to NaCl (50 mM) **(Bansal et al., 2008).** A combined influence of ionic strengths and heat treatments on the aggregation properties of whey proteins has been exclusively reported in recent years **(Hussain et al., 2012a, Hussain et al., 2012c Submitted; Majhi et al., 2006; O'Kennedy and Mounsey, 2009; Ryan et al., 2012).** The rate of association of

aggregates increase strongly with increasing salt, protein concentration **(Ako et al., 2010; Bryant and McClements, 2000; Marangoni et al., 2000)** and temperature **(Baussay et al., 2004; Schokker et al., 2000)** as higher temperature promote hydrophobic interactions **(Nicolai et al., 2011)**.

In a serial of publications, **(Ikeda, 2003; Ikeda and Morris, 2002)** confirmed salt induced aggregation (0.1M NaCl) of heat denatured whey proteins as a two step process i.e. the formation of primary aggregates and their subsequent aggregation. They reported transitions from translucent to opaque and/or fine stranded to particulate gels **(Durand et al., 2002)** of heat denatured whey proteins with increasing ionic strength. Using in situ calorimetric, light scattering and microscopic techniques, it was confirmed that NaCl addition (0-0.2M) accelerated the appearance of exothermic peaks linked to whey protein aggregation, **(Unterhaslberger et al., 2006)** particularly a strong increase in aggregate size with the appearance of bimodal distributions **(Majhi et al., 2006)** and a self-similar structure with a fractal dimensions approximately ~ 2.5 **(Ako et al., 2010; Marangoni et al., 2000; Wu et al., 2005).**

According to **Hussain et al., (2012b)** adding NaCl gradually (0.1-1M) to WPI aqueous solution resulted in association of primary aggregates and producing large scale clumpy denser aggregates (0.1 µm-10 µm) caused by increasing degree of branching **(Baussay et al., 2004)** observed by TEM. Besides monovalent salts, $CaCl_2$ addition to whey proteins also affect aggregation properties of whey proteins in the same manner but comparatively at very low ionic strengths **(Mercade-Prieto et al., 2009; O'Kennedy and Mounsey, 2009).**

Nevertheless, similar phenomenons of salt/heat induced aggregation were reported for other milk proteins like BSA **(Donato et al., 2005)** and Lactoferron (Lf) **(Bengoechea et al., 2011).** Furthermore, the formation of whey protein aggregates (soluble aggregates) by adjusting various salt concentrations and heating temperatures have been investigated to improve their foaming properties **(Schmitt et al., 2007)** and thermal stability **(Hussain et al.,2012c Submitted)** with their possible applications in beverages **(Ryan et al., 2012).**

6.3.2. Gelling Properties

Among milk proteins, caseins have good emulsifying properties but they do not form gels whereas whey proteins possess excellent gelling properties therefore are widely used as ingredients in foods **(Kinsella and Whitehead, 1989). Section 5.4** of this review briefly covers the gelling properties of whey proteins powders and its dispersions induced by various ranges of salt and heat regarding modification in their particle size and morphological

characteristics. In opposition with heat induced gelation of whey proteins, cold gelation process may be defined as when salt is added to heat denatured whey proteins or when pH is adjusted close to the isoelectric point (pI), further association of aggregates led to gelation and it happens even at room temperature. Cold gelation carried out by salt induction is termed as salt-induced gelation **(Bryant and McClements, 1998).**

The phenomenon of cold gelation and influence of different salts on the cold-set gelation of heat denatured whey proteins has been extensively studied and have immense applications in wide variety of food products **(Ako et al., 2010; Barbut and Foegeding, 1993; Bryant and McClements, 1998, 2000; Hongsprabhas and Barbut, 1997; Vardhanabhuti et al., 2001).** However, a most recently published review **(Nicolai et al., 2011)** extensively cover this section related to salt induced impact on the gelling properties of whey proteins and their possible applied use in food industry can be found elsewhere.

7. Conclusion

There are abundant information concerning the behavior of milk proteins powders rehydration profiles, physicochemical properties, multiscale characterization and their functionality under various aqueous ionic environments. Reports to date are helpful in determining the relative behavior of major milk proteins in different experimental conditions; however data reviewed do not always predict the performance of such salts and proteins in actual food system. Such informations are essential to expedite appropriate methods of processing in order to improve rehydration step at industrial scale, to characterize milk protein powder size, structure, morphology, extent of denaturation, onset of aggregation and formation of gels under an influence of various salts, their types and ionic strength alone or/and in combination of heat treatments (heating time, heating method).

Careful adjustment of salt and heat treatments can result in the production of milk protein preparations, with reliable developed protocols and standardize functional properties which can establish the effectiveness of milk proteins powder as valuable functional ingredient. Nevertheless, remarkable advancement has been already made in this respect for the period of last two decades, however, more research is still needed to unlock new horizons and prospective in this regard.

Synthèse et positionnement de l'étude

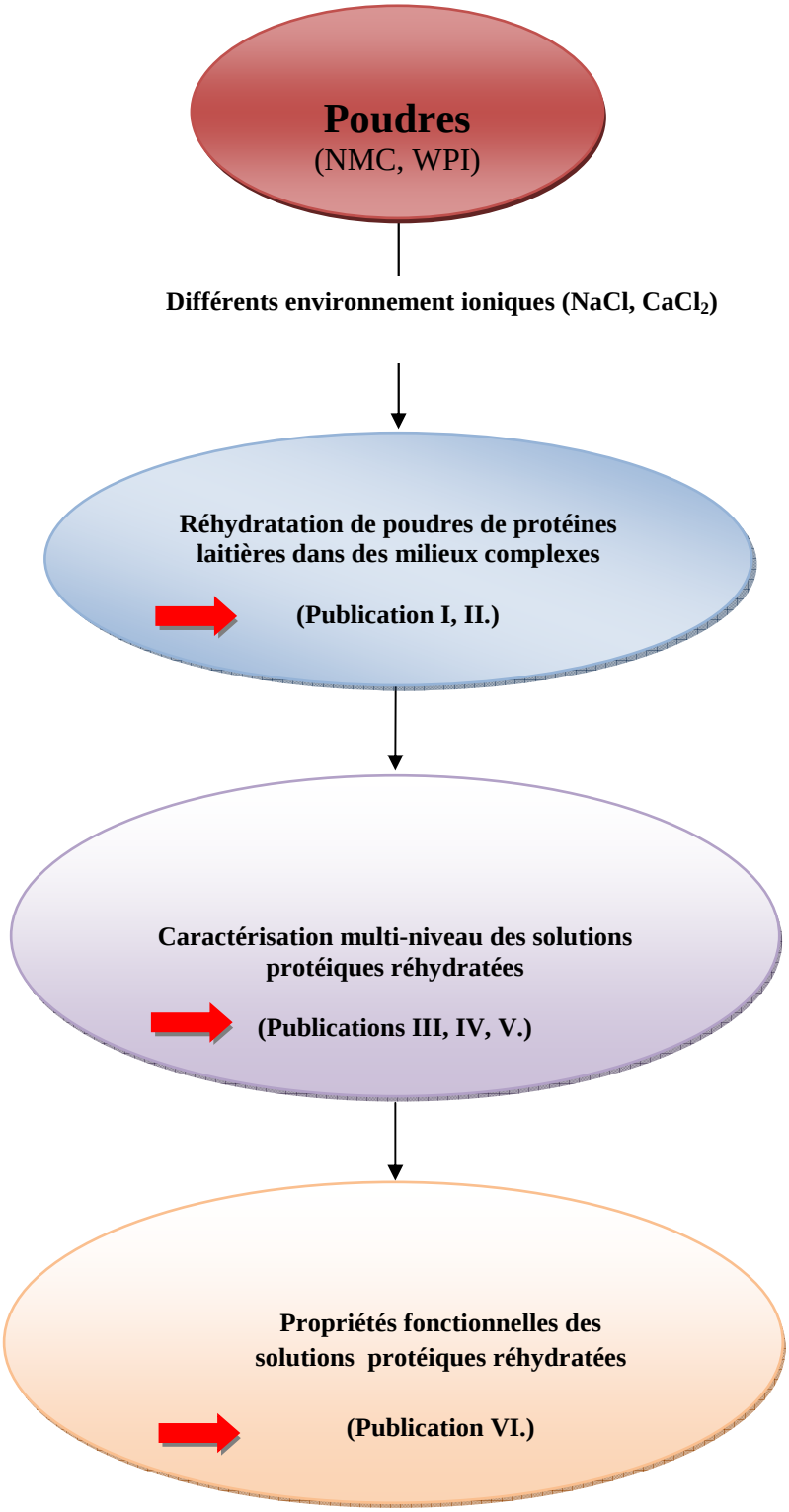
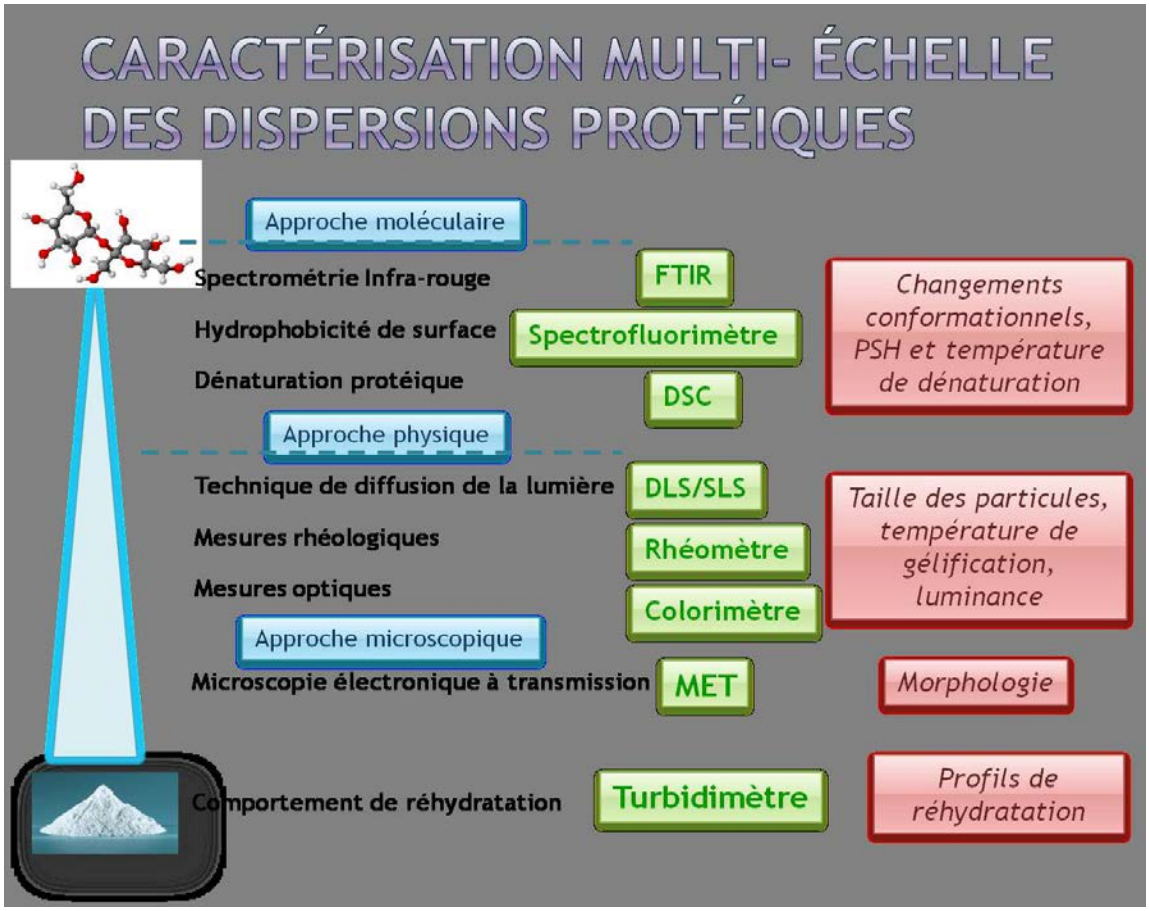


Schéma synthétique des différentes parties de thèse

Chapitré III : Matériel et Méthodes



Caractérisation des poudres

Les poudres de caséine micellaire (872B) et de protéines solubles (852FB) proviennent de la société International Dairy Ingrédient (IDI, Arras, France, lot 232-555-1).

1.1. Composition chimique

1.1.1. Dosage de l'eau

La teneur en eau a été déterminée par pesée et étuvage à 103°C suivant la norme AFNOR (1978). Deux grammes de poudre sont étuvés pendant trois heures, puis refroidis pendant une heure au dessiccateur (P_2O_5) avant d'être pesés.

1.1.2. Dosage des cendres

Le taux de cendres a été déterminé par minéralisation de 2 g de poudre dans des creusets de porcelaine dans un four à moufles à 550 degrés selon la norme décrite par l'ADPI (2002).

1.1.3. Dosage des protéines

Les teneurs en protéines ont été dosées par la méthode de Kjeldahl (AOAC, 1984). Les teneurs en azote total (NT), en azote non caséinique (NCN) et en azote non protéique (NPN) sont déterminés. Les pourcentages en protéines (g de protéines pour 100 g de poudre), en caséines (g de caséines pour 100 g de poudre) et en protéines sériques (g de protéines sériques pour 100 g de poudre) sont respectivement calculés d'après les relations suivantes : (NT-NPN) x 6,38 ; (NT-NCN) x 6,38 et (NCN-NPN) x 6,38.

Le pourcentage de dénaturation des protéines solubles a été déterminé par la mesure de la quantité d'azote soluble après centrifugation d'une solution protéique à 5% pendant 30 minutes à 3100g (FIL, 1995). Les protéines sont précipitées à pH 4,6 et le filtrat est dosé par Kjeldahl. À ce pH, les protéines dénaturées sont insolubles. Par centrifugation, on sépare les protéines dénaturées (culot) des protéines natives (surnageant).

1.1.4. Dosage du lactose

Le dosage du lactose est réalisé par voie enzymatique, avec un kit de dosage: ENZYTEC Lactose/D-galactose. Les échantillons sont préalablement clarifiés avec les réactifs de Carrez. Le lactose est hydrolysé en glucose et D-galactose en présence d'eau dans une réaction catalysée par la β-galactosidase. Le D-galactose est ensuite oxydé en acide galactonique avec libération de NADH. La formation de NADH mesurée par l'augmentation de l'absorbance à 340 nm est proportionnelle à la quantité de lactose présente dans le milieu.

1.1.5. Dosage des lipides

La teneur en matière grasse a été déterminée suivant la norme FIL (1987) de Röse Gottlieb. Son principe repose sur une extraction des lipides au moyen d'éther diéthylique et d'éther de pétrole. Enfin, le mélange de solvants est évaporé et les lipides extraits sont pesés.

1.2. Caractérisation physique

La distribution de taille des particules est déterminée par diffusion statique de la lumière grâce à un granulomètre Malvern Mastersizer S (Malvern Instruments Ltd, UK). L'appareil est équipé d'un laser de type He/Ne d'une puissance de 5 mW et opérant à une longueur d'onde de 632,8 nm. L'optique est composée d'une cellule de mesure de 0,5 mm sur laquelle est montée une lentille de type 300RF possédant une distance focale de 0,45 mm. L'intensité lumineuse diffusée par l'échantillon est récupérée par 42 photodiodes localisées à différents angles.

La lumière étant diffusée par une particule avec un angle inversement proportionnel à sa taille, les grosses particules dévient une quantité importante de lumière avec des angles faibles, alors qu'une petite particule va dévier des quantités faibles de lumières avec des angles plus importants. L'acquisition est réalisée à l'aide du logiciel de traitement Malvern (Sizer Sv2.17) qui permet la récupération et l'analyse des données brutes.

La distribution de taille des particules dans l'air est mesurée à l'aide d'un module spécifique aux poudres : dry powder feeder. Le code choisi est le modèle optique classique pour les particules dispersées dans l'air. La taille des particules détectées par ce système varie de 4,88 à 3473 µm.

2. Obtention des cinétiques de réhydratation avec le réacteur instrumenté

2.1. Montage expérimental

L'ensemble du montage expérimental est représenté sur la figure 1. Le réacteur instrumenté comprend une cuve à double paroi et un module d'agitation (figure 2). Le module d'agitation (Lightin Labmaster Mixer, Axflow, France) est composé de 6 pales (R 100 impeller ; 8 cm diamètre). L'acquisition des données est réalisée à l'aide d'un data logger (Almemo 8990-8V_5, Ahlborn, Holzkirchen, Allemagne). L'ordinateur relié au data logger utilise le logiciel AMRWinControl pour centrale Almemo. Ce réacteur est instrumenté et comprend un certain nombre de sondes : turbidité, conductivité et pH.

2.2. Mode opératoire

La réhydratation est suivie dans une cuve contenant 2 L d'eau distillée. La quantité de poudre versée est calculée en pourcentage de MAT ; les analyses sont effectuées à 5% de MAT. Le système est thermostaté à 24°C et la vitesse d'agitation est fixée à 400 tours par minutes et reste constante tout au long des mesures. Pendant les 60 premières secondes, les mesures se font sur de l'eau distillée, ceci permet une stabilisation des trois capteurs sur la solution de réhydratation. Au bout d'une minute, la poudre est versée d'un seul coup dans la cuve. Les données sont collectées automatiquement toutes les secondes pendant les 1000

premières secondes puis toutes les 5 secondes. Tous les résultats présentés correspondent au moins à trois expérimentations.

Figure1 : Vu d'ensemble du montage expérimental permettant le suivi de la réhydratation

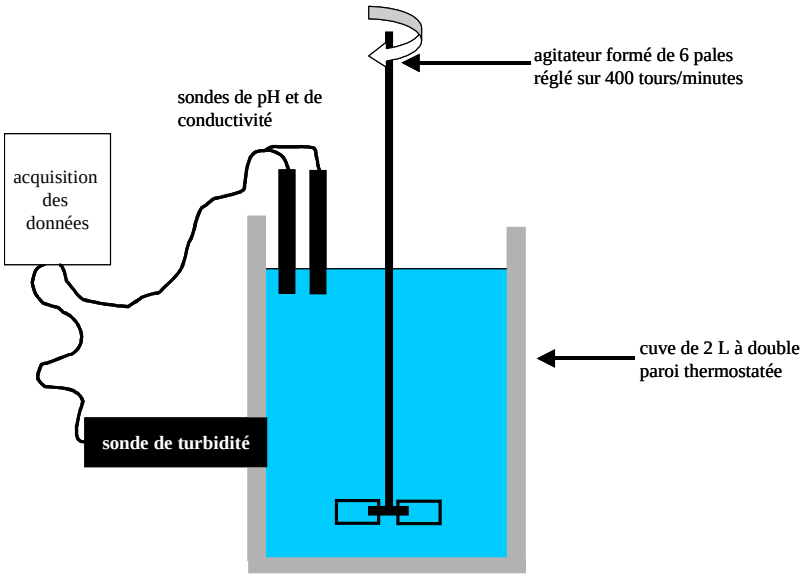


Figure 2 : Schéma du réacteur comprenant les sondes de turbidité et de pH

(d'après Gaiani *et al.*, 2005)

2.3. Les sondes

2.3.1. Sonde de turbidité

La sonde de turbidité (Analyte NEP 160 Mc Van Instruments, Mulgrave, Australie) est placée perpendiculairement à la cuve à travers la double paroi et affleure la suspension. Son diamètre étant important, une introduction horizontale à travers la paroi plutôt que verticale en haut de cuve permet d'éviter la création de turbulences en cours de réhydratation.

La mesure est basée sur la réflexion d'un rayonnement monochromatique dans le proche IR. Le rayon incident (860 nm) transmis dans la dispersion par l'intermédiaire de fibres optiques est réfléchi par les particules en suspension. Le rayonnement réfléchi à 180° est capté par un second réseau de fibres optiques jusqu'à un récepteur électronique amplifiant le signal. Le signal reçu est enfin transformé en unité néphélométrique de turbidité (NTU).

2.3.2. Sonde de pH

Les cinétiques de pH sont réalisées avec un pH-mètre 210 (Radiometer Analytical SAS, France) muni d'une électrode de pH adaptée aux produits laitiers (pHC3101). La sonde est placée verticalement en haut de cuve (Figure 2).

La mesure de pH fait intervenir deux électrodes : l'électrode de verre et l'électrode de référence. Ici, ces deux électrodes sont regroupées dans un même corps et donnent naissance à une seule électrode appelée électrode combinée. Lorsqu'elle est plongée dans la solution, une pile galvanique va se créer. Le potentiel de l'électrode de référence reste constant alors que le potentiel de l'électrode de verre va varier en fonction des variations de pH de la solution à mesurer. La différence de potentiel mesurée est définie par la Loi de Nernst.

Le pH étant défini par la relation pH = - log a_{H+}, la valeur de pH à une température T, peut être exprimée comme suit :

$$pH_T = pH_T^{\,0} - (E / R' \times S \times T)$$

avec : R' = constante = 0,1984 mV/K.

S = pente (relative), un facteur de correction qui prend en compte la différence de réponse entre l'électrode utilisée et la valeur théorique (pente mesurée divisée par pente théorique).

pH° = le zéro pH qui correspond à la valeur de pH pour laquelle le potentiel mesuré est 0 mV.

2.4. Etalonnage et validation des sondes

2.4.1. Linéarité de la réponse du turbidimètre pour des suspensions peu concentrées (gamme 0-4000 NTU)

Des solutions de formazine sont utilisées pour étalonner le turbidimètre. Elles sont préparées à partir d'un mélange de sulfate d'hydrazine et d'hexa-méthylène-tetramine. Ce mélange correspond à une turbidité de 4000 NTU, il sera ensuite au besoin dilué dans de l'eau microfiltrée pour obtenir une gamme étalon (EPA guidance manual, turbidity standard method, 1999).

Des mesures sont menées pour observer la linéarité du turbidimètre sur ces solutions. Plusieurs suspensions de formazine sont préparées à des concentrations variables permettant de couvrir la gamme 0 – 4000 NTU de l'appareil (figure 3). Il apparaît une linéarité entre la turbidité attendue et lue (R^2 = 0,99). Les valeurs de turbidité lues sont systématiquement légèrement supérieures aux valeurs attendues mais les différences ne sont pas significatives (Paired T-test de Kyplot).

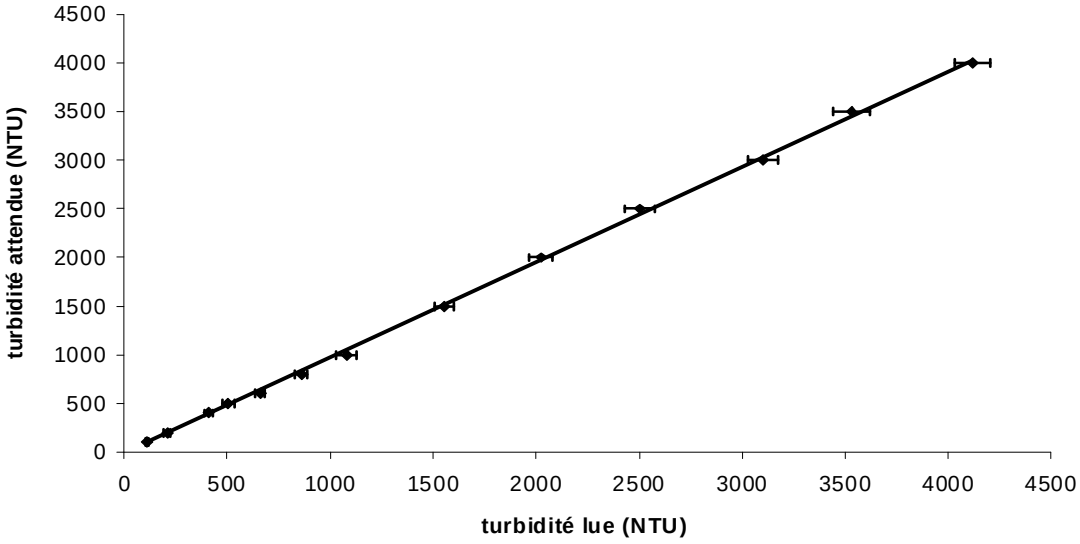


Figure 3: Comparaison entre la turbidité mesurée et attendue pour différentes suspensions de formazine (moyenne de 3 déterminations ± σ)

Linéarité de la réponse du turbidimètre pour des suspensions plus concentrées (> 4000 NTU)

La sonde de turbidité est utilisable sur la gamme 0 – 20 000 NTU. Or, au dessus de 4000 NTU, il n'existe plus de solution étalon disponible sur le marché. Si l'on suit la turbidité d'une solution de NMC contenant des pourcentages en protéine croissants, on constate qu'au dessus de 4000 NTU, l'évolution de la turbidité n'est plus linéaire. Sur la figure 5, la relation

entre la turbidité et le pourcentage en protéine n'est plus linéaire mais logarithmique et du type : y = 4920 x Ln (x) + 5970 avec un $R^2 > 0,99$. Au dessus de 16% de protéines la valeur de turbidité de la solution réhydratée atteint 20 000 NTU, valeur de saturation de la sonde. Au dessus de cette concentration, les valeurs ne sont donc plus dans la gamme de validité.

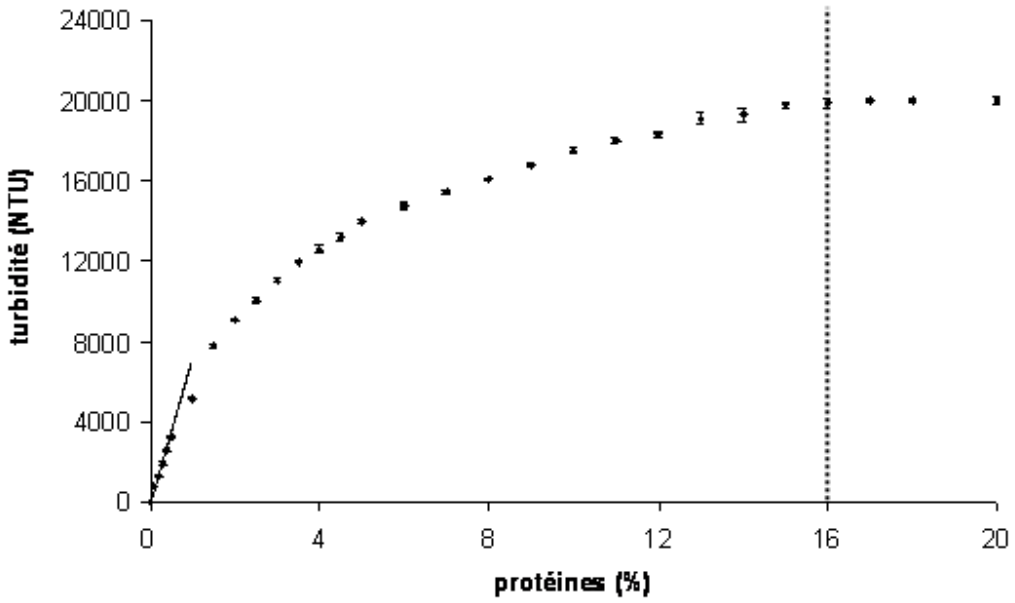


Figure 4: Relation entre la valeur de turbidité et le pourcentage en protéines dans des solutions contenant du NMC réhydraté à 24°C

Dérive au cours du temps de la réponse du turbidimètre

La formazine, de par ses contraintes d'utilisation (temps de préparation long et durée de conservation des solutions très courte), ne permet pas un étalonnage quotidien de la sonde. Pour cette raison, une évaluation de la dérive du signal de turbidité au cours du temps a été estimée pour évaluer le temps au bout duquel il est nécessaire de refaire l'étalonnage. Les valeurs de turbidité sur des solutions fraîches de formazine sans refaire l'étalonnage de l'appareil sont comparées au cours du temps sur la figure 6 Au bout de 10 jours, la turbidité commence à dévier des valeurs initiales. La dérive est ensuite de plus en plus importante au cours du temps. L'étalonnage de l'appareil a donc été fixé systématiquement tous les 6 jours.

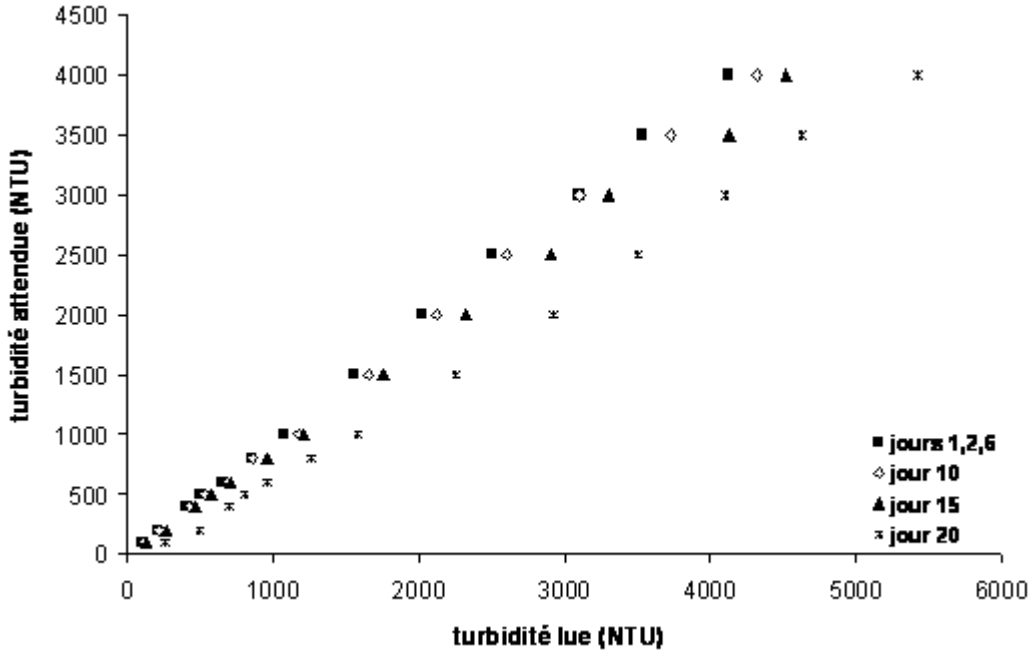


Figure 5 : Comparaisons des turbidités mesurées et attendues par différentes séries de suspension de formazine au cours du temps

2.4.4. Etalonnage et validation des sondes de pH et de conductivité

La sonde de pH est étalonnée tous les jours sur deux solutions étalons de pH 4,6 et 7. La constante de cellule de la sonde de conductivité est vérifiée une fois par semaine avec une solution étalon de NaCl à 0,05%. Toutefois elle est restée constante pendant toute la durée de la thèse.

3. Caractérisation des dispersions protéines

3.1. Préparation des dispersions protéiques

Pour la préparation des différentes dispersions les produits suivants sont utilisés: NaCl et $CaCl_2$ (VWR, Prolabo, Belgique) et de l'azide de sodium (Merck, KGaA, Allemagne).

Toutes les analyses ont été réalisées sur des poudres de caséine micellaire et de protéines solubles réhydratées à 5% (w/w total) dans des solutions de NaCl et $CaCl_2$ (variant de 0% à 12% maximum). Afin d'obtenir une réhydratation totale de ces poudres, elles ont été laissées sous agitation pendant une nuit entière. Une réhydratation correcte des poudres de caséine micellaire (connues pour présenter des temps de réhydratation très longs) a été confirmée par une analyse granulométrique. Une taille approximative de 0.2 - 0.3 µm correspondant à la taille de la micelle de caséine a confirmé la bonne réhydratation des caséines micellaires **(Gaiani *et al.*, 2006)**. Les dispersions obtenues après réhydratation totale

des poudres sont appelées dispersions protéines (de caséine micellaire ou de protéines solubles).

3.2. Observations macroscopique et microscopique des dispersions protéiques

3.2.1. Colorimétrie

Un colorimètre Minolta (CR-210 Minolta, Colombes, France) a été utilisé pour mesurer la couleur des dispersions protéiques. La couleur est un attribut perceptif, subjectif, élaboré dans notre système visuel à partir de la lumière renvoyé par les objets et leur environnement. La colorimétrie consiste en un ensemble de données et de méthodes permettant de quantifier la couleur objectivement. L'espace chromatique L*a*b a été défini par la commission interactionnelle de l'éclairage (CIE). Les trois coordonnées sont des correspondances de la luminance (L*), de la balance « rouge-vert » a* et de la balance « jaune-bleu » b* (Figure 6).

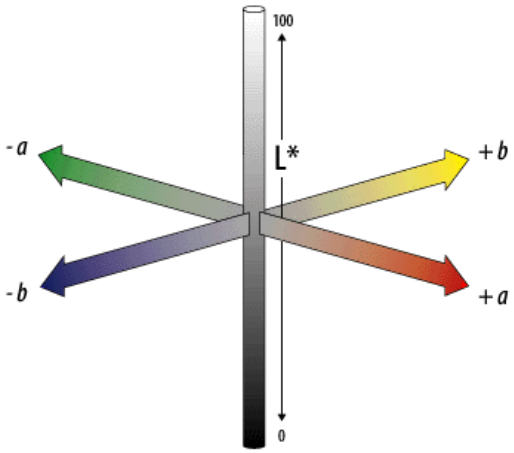


Figure 6 : Schéma de l'espace chromatique CIE L*a*b

Les différentes couleurs sont calculées en utilisant l'équation (1) ci-dessous

$$\Delta C = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$$

Les mesures sont effectuées sur les dispersions protéiques dans différents environnements ioniques. Ces dispersions sont placées dans des boites de pétri transparentes. Le volume de dispersion est systématiquement le même. Après une calibration de l'appareil avec les étalons noirs et blancs, la cellule de l'appareil est directement appliquée sur la boite contenant la dispersion pour effectuer la mesure. Les résultats présentés sont la moyenne de 3 mesures indépendantes.

3.2.2. Microscopie électronique à transmission

Principe général

La Microscopie Electronique à Transmission (TEM), dans ses différents modes (imagerie, diffraction, analyse chimique), constitue aujourd'hui l'une des techniques d'investigation la plus couramment utilisée dans tous les domaines de la science. Elle permet d'observer différents types de matériaux jusqu'à l'échelle atomique et d'en caractériser également la microstructure.

Les informations qui peuvent être obtenues par cet équipement sont très diverses : morphologiques, cristallographiques, chimiques, électroniques et même magnétiques. De plus, le TEM est la seule technique qui permet d'obtenir simultanément des informations structurales et chimiques pour une même position définie au nanomètre près.

Les principaux apports de la microscopie électronique à transmission sont d'une part son excellente résolution dans l'espace (pouvant atteindre l'échelle atomique) avec des grandissements variant de 10^3 à 10^6, et d'autre part la possibilité de combiner l'information obtenue en mode image avec l'information obtenue en mode diffraction.

Figure 7 : Détermination des changements morphologiques par TEM

Théorie

La caractéristique principale de tout microscope électronique est sa résolution ou pouvoir séparateur d. C'est la plus petite distance entre deux points de l'objet que le microscope permet de résoudre dans l'image. L'effet d'ouverture de taille finie (diffraction):

l'image d'un point est non pas un point mais une tache, dite tache d'Airy. Le rayon de la tache est donné par la formule ci-après :

$$d = \frac{0.61\ \lambda}{n \sin \alpha}$$

(avec α angle d'ouverture, λ: longueur d'onde du rayonnement et n : indice de réfraction):

Lorsqu'on diminue la valeur de résolution d, on dit qu'on améliore la résolution du microscope.

Pour y arriver, il y a 3 solutions:

- diminuer la longueur d'onde λ
- augmenter l'ouverture α
- augmenter l'indice de réfraction n

Pour le microscope électronique on ne sait pas construire des objectifs à grande ouverture et l'indice de réfraction du vide est égal à 1.

Le gain en résolution n'est donc lié qu'au gain considérable sur λ.

Pour les électrons, l'expression (6) se simplifie car sin α ~ α et n = 1

$$d = \frac{0.61\ \lambda}{\alpha}$$

Au début du XXème siècle, l'idée est venue de repousser cette limite imposée par la longueur d'onde relativement élevée de la lumière visible, de 400 à 700 nanomètres en mettant en jeu des électrons dont on savait, d'après la mécanique ondulatoire, qu'ils possédaient à la fois les propriétés des particules et celle des ondes. Cela suggérait que l'on pouvait traiter un faisceau d'électrons de la même manière qu'un faisceau d'ondes électromagnétiques afin d'obtenir une image de l'échantillon.

Dans un microscope électronique, les électrons sont générés au niveau d'une pointe (W ou LaB_6) puis accélérés par une anode.

Le faisceau d'électrons interagit avec l'échantillon avec un contraste spatial résultant de différences de densité ou de composition chimique, et mesuré par un détecteur permettant ainsi de former une image de l'échantillon.

Contrairement à la microscopie optique, le microscope électronique en transmission utilise, lui, comme rayonnement des électrons. Un système de lentilles magnétiques permet de focaliser le faisceau d'électrons sur un échantillon extrêmement mince. L'image (ou cliché de diffraction) obtenue est transformée en image photonique, par exemple par un écran phosphorescent. Cette image photonique est traitée de la même façon que dans un microscope photonique, avec la possibilité d'un enregistrement sur un film photographique ou, depuis la fin du XXème siècle un capteur CCD.

Différents contrastes en microscopie en TEM

En TEM, l'image est formée en sélectionnant une partie des électrons par le positionnement du diaphragme objectif au niveau du diagramme de diffraction. Si le diaphragme utilisé est suffisamment petit pour ne laisser passer qu'un faisceau (transmis ou diffracté) le contraste est dit de diffraction pour les objets cristallisés et de diffusion dans le cas particulier des matériaux amorphes.

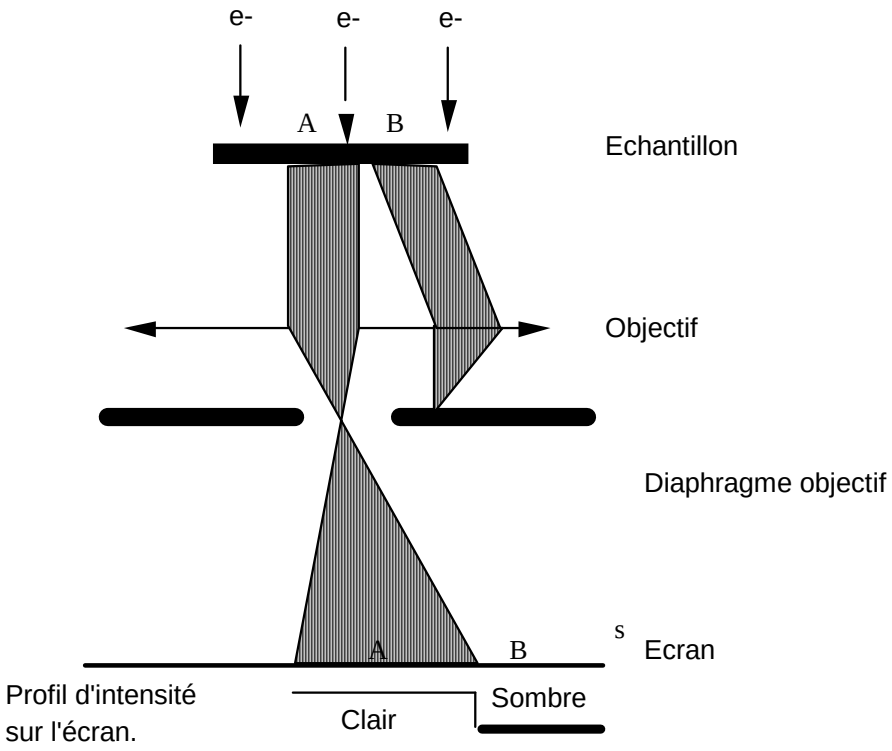
Dans le cas où le diaphragme est de taille suffisante pour sélectionner à la fois le faisceau transmis et un faisceau diffracté ou diffusé, le contraste est dit de phase car lié aux interférences entre des faisceaux présentant un déphasage.

A) Contraste des objets amorphes : contraste de diffusion.

La zone B de l'échantillon diffuse plus que la zone A ($Z_B > Z_A$). Il en résulte que l'image de la zone B est plus sombre car une grande proportion des électrons de la zone B ont été diffusés et arrêtés par le diaphragme objectif.

Le contraste augmente avec :

* le numéro atomique Z.
* l'épaisseur.
* le nombre d'atomes diffusants.
* la longueur d'onde des électrons.
* quand la taille du diaphragme objectif diminue.



B) Contraste de diffraction (matériaux cristallisés).

Il est obtenu en sélectionnant un seul faisceau par le diaphragme objectif.

- en champ clair:

Les parties qui diffusent peu, donc qui transmettent bien les électrons, donnent une image claire. C'est le cas des trous dans les membranes supports, des zones riches en éléments légers, des bords de cristaux, des zones cristallines loin de toute position de Bragg.

- en champ sombre:

Les parties qui diffusent beaucoup, ou les parties de cristal en position de Bragg pour le faisceau diffracté sélectionné par le diaphragme objectif donnent une image claire tandis que le reste est sombre.

C) Contraste de phase:

Contraste de phase = image haute résolution

Un diaphragme objectif relativement grand est choisi pour laisser passer le faisceau transmis et un ou plusieurs faisceaux diffractés. Ce type de contraste est important à très fort grandissement et est utilisé pour former des images haute résolution.

Mode opératoire

Les dispersions de NMC et de WPI dans différents environnements ioniques (NaCl) ont été observées à l'aide d'un TEM Philips CM20 opérant à 200 kV. Les micrographies ont été réalisées à un grandissement de 25 000.

Les échantillons sont préparés en utilisant la méthode de coloration négative. Une goutte de la dispersion (0,05% en poids) a été déposée sur une grille de cuivre recouverte de carbone (3 mm de diamètre). L'excès de produit a été retiré au bout de 30 secondes en utilisant un papier filtre. Ensuite, une goutte d'acétate d'uranyle (2%) a été déposée sur la grille pendant 2 minutes. Après retrait de l'excès du colorant, la grille est séchée pendant 5 minutes à température ambiante.

3.2.3. Détermination de taille à l'échelle nanométrique: méthode de diffusion dynamique de lumière

Principe

La diffusion dynamique de lumière (aussi appelé PCS ou spectroscopie de corrélation photonique) mesure le mouvement brownien qui est rapporté à la taille des particules. Un faisceau laser éclaire les protéines en dispersion. L'intensité de la lumière diffusée est mesurée. En général, les particules en suspension dans un liquide ne sont jamais immobiles. Ces particules sont constamment en mouvement en raison du mouvement brownien (Figure 8). Il s'agit du mouvement des particules en raison de la collision aléatoire avec les molécules du liquide qui entoure la particule. Les petites particules se déplacent rapidement et les grosses particules se déplacent plus lentement. La relation entre la taille d'une particule et la vitesse liée au mouvement brownien sont définies dans l'équation d'Einstein par la formule ci-dessous (eq 3) :

$$D_H = \frac{kT}{f} = \frac{kT}{3\pi\eta D}$$

D_H : diamètre hydrodynamique (m)

k : constante de Boltzmann (1.38×10^{-23} $J.K^{-1}$)

f : coefficient de friction des particules

η : viscosité de solvant (Pa.s)

T : température (K)

D : coefficient de diffusion ($m^2. s^{-1}$)

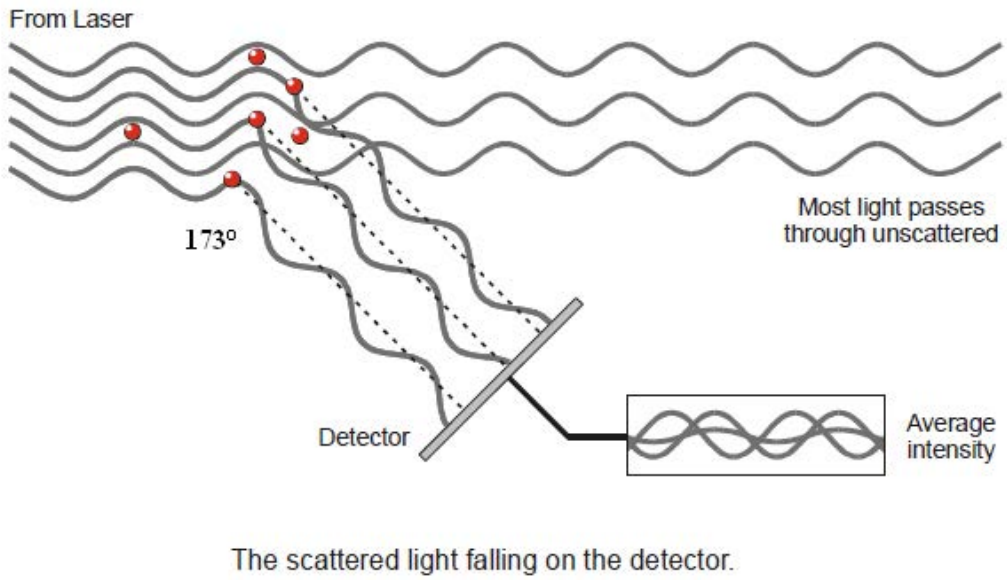


Figure 8: Light scattering in Zetasizer

Appareillage

Le Zetasizer comprend six composantes principales (Figure 9). Tout d'abord, un laser fournit une source de lumière permettant d'illuminer les particules de l'échantillon dans une cellule. La plupart du faisceau laser passe ensuite directement à travers l'échantillon, mais une partie est dispersée par les particules dans l'échantillon. Finalement, un détecteur est utilisé pour mesurer l'intensité de la lumière diffusée. Le signal d'intensité de diffusion est transmis à une carte de traitement de signal numérique appelé un corrélateur. Le corrélateur va comparer l'intensité de diffusion à des intervalles de temps successifs et le dériver. L'information est ensuite transmise à un ordinateur et les données sont analysées par un logiciel Zetasizer.

Le Zetasizer Nano ZS (HPPS 5001, Malvern Instruments, UK) a été utilisé pour suivre l'évolution de la taille des dispersions protéique dans la gamme de mesure allant de 0.3 nm à 10 µm . Il est équipé d'un faisceau laser de type He/Ne (d'une puissance 4 mW) de longueur d'onde 633 nm, d'un détecteur photodiode à avalanche, d'un photomultiplicateur et d'un corrélateur. Des cuvettes verticales de 10 mm de trajet optique ont été utilisées comme cellules de mesure. Les mesures ont été effectuées à un angle de diffusion de 173° et à température ambiante. Les fonctions d'auto corrélation ont été analysées par l'algorithme d'analyse CONTIN (modèle de calcul intégré dans le logiciel Malvern) permettant de déterminer la distribution des coefficients de diffusion translationnelle (DT).

Le coefficient de diffusion D_T ($m^2.s^{-1}$) est relié au rayon hydrodynamique (R_H) des particules qui diffusent selon la relation de Stokes-Einstein (eq 4):

$$D_T = k_B T / 6\pi\mu R_H$$

Avec : µ est la viscosité du solvant (Pa.s), k_B la constante de Boltzmann ($1,38 \times 10^{-23}$ $N.m.K^{-1}$) et T la température absolue (°K). Les résultats obtenus par l'algorithme CONTIN permettent de déterminer les distributions de Rh (m) et l'amplitude respective de chaque population de particules. Les résultats sont présentés en diamètre hydrodynamique (D_H).

Mode opératoire

Avant analyse, les différentes dispersions protéiques ont été diluées de la façon suivante : 12,5 µL des dispersions sont mélangées à 50 ml de leurs solutions de saumure correspondantes. Chaque échantillon a été filtré à l'aide des filtres de 0,45 µm (Minisart, Sartorius, Tullagreen, Ireland) à température ambiante.

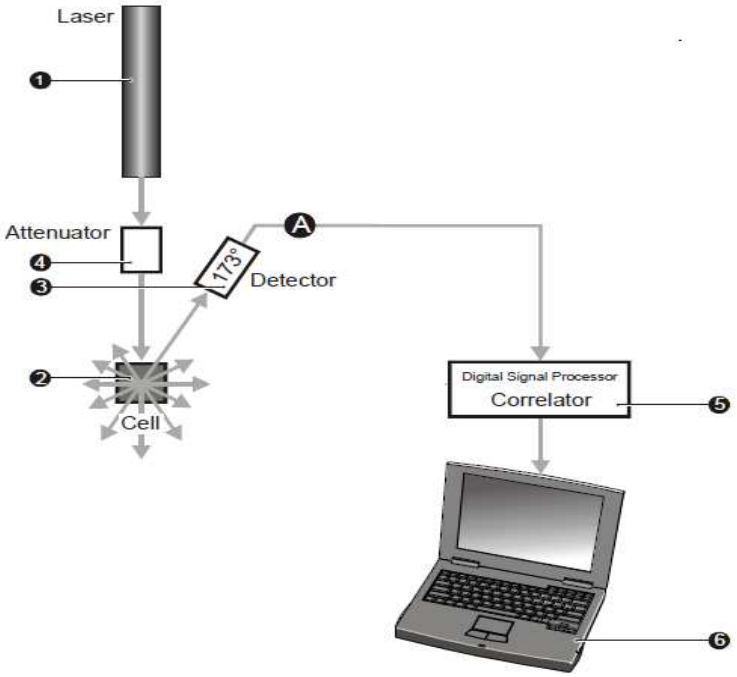


Figure 9: Représentation schématique du Zetasizer

3.2.4. Détermination de taille à l'échelle micrométrique: diffusion statique de la lumière

Principe

La distribution des tailles des particules à l'échelle micrométrique est déterminée par diffusion statique de la lumière grâce à un granulométrie Malvern Mastersizer S (Malvern instruments Ltd, UK). L'appareil est équipé d'un laser de type He/Ne d'une puissance de 5mVet opérant à une longueur d'onde de 623,8 nm. L'optique est composée d'une cellule de mesure de 0.5 mm d'épaisseur sur laquelle est montée une lentille de type 300RF possédant une distance focale de 0.45 mm. L'intensité lumineuse diffusée par l'échantillon est récupérée

par 42 photodiodes localisées à différents angles. La lumière étant diffusée par une particule avec un angle inversement proportionnel à sa taille, les grosses particules dévient une quantité importante de lumière avec des angles faibles, alors qu'une petite particule va dévier des quantités faible de lumières avec des angles plus importants. L'acquisition est réalisée à l'aide du logiciel de traitement Malvern (Sizer Sv2.17) qui permet la récupération et l'analyse des données brutes.

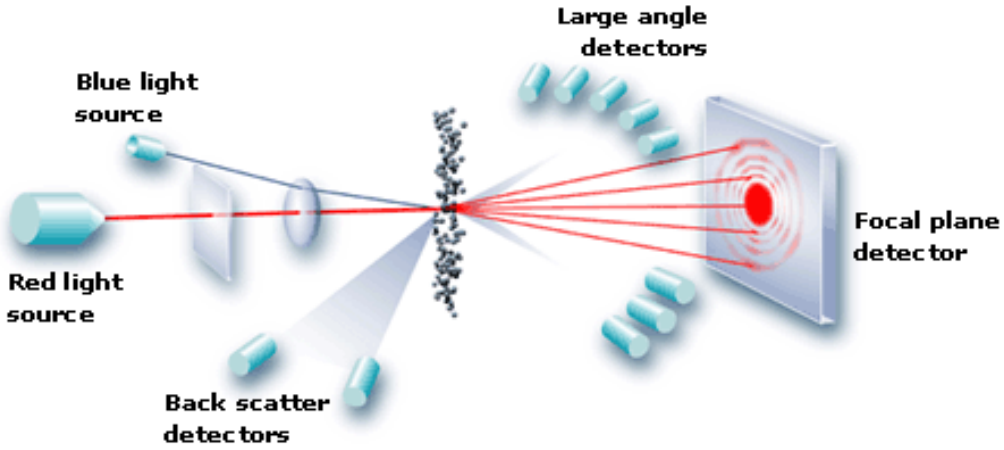


Figure 10 : Principe du Mastersizer de Malvern

Mode opératoire

Pour les tailles des particules les plus importantes (comprises entre 0.1 et 1000 µm), 50 ml de la dispersion protéique ont été ajouté à 200 ml d'eau. Cette dilution permet d'obtenir une obscuration correcte (autour de 15). La dispersion obtenue était stable tout au long de l'expérience et sous une agitation de l'ordre de 2000 rpm. Les résultats obtenus sont représentés par les diamètres moyens calculés selon la théorie de Mie. Le critère retenu était le d_{50}, représentant ainsi la moitié des particules ayant une taille inferieure à ce diamètre. Toutes les mesures ont été répétées trois fois.

3.3. Détermination de l'hydrophobicité de surface

3.3.1. Spectrofluorimètre

L'indice d'hydrophobicité des protéines de surface (PSH) a été déterminé par spectrofluorimétrie. De part l'énergie des photons, les électrons des molécules peuvent passer de l'état fondamental à l'état vibratoire excité. Une émission de florescence est observée au retour de l'électron excité à son état fondamental. Les mesures de fluorescence ont été réalisées à l'aide d'un spectrofluorimètre (AmicoBowman série II, Monaco, France) équipé d'une lampe au xénon 150W.

3.3.2. Protocole expérimental

Les dispersions protéiques diluées (0.05%) dans l'eau ou les différents sels (0-6 %) ont été introduites dans le spectrofluorimètre. L'échantillon est placé dans une cellule de quartz ayant une longueur de trajet optique de 1 cm. Les dispersions ont été placées dans le spectrofluorimètre et des quantités d'ANS (1-AnilinoNaphthalene-8-Sulfonic acid) ont été ajoutées successivement (de 0 à 50 µmol.L-1). Le titrage avec ANS a été contrôlé à λ_{ex} = 390 nm et λ_{em} = 480 nm. La lecture de l'intensité relative de fluorescence a été ajustée à 1 lorsque 50 µl de la solution ANS a été ajouté à tampon phosphate de sodium (à 0,10 mM et pH 7).

3.3.3. Traitement des données

Les protéines ont un pouvoir fluorescent grâce à leurs acides amines aromatiques (tyrosine, phénylalanine and tryptophane). Ces résidus d'acides aminés vont servir de sondes pour des études de conformation des protéines. Le protocole utilisé pour le traitement des données et la détermination du PSH a été celui de **(Schmitt *et al.*, 2009)** avec quelques modifications.

L'analyse des données de liaisons fut réalisée afin d'obtenir la fluorescence maximale accessible par la saturation des concentrations d'ANS. F_{max} dépend du nombre de sites hydrophobes accessibles au marqueur spectroscopique. La concentration en ANS nécessaire pour obtenir la moitié de la valeur de Fmax correspond à la constante de dissociation apparente du complexe protéique fluorescent ANS (Kd). Le rapport F_{max} /K_d ou le (PSH) représente un index cumulatif de l'hydrophobicité de la surface de la protéine tout en tenant compte à la fois du nombre et de l'affinité des sites hydrophobes.

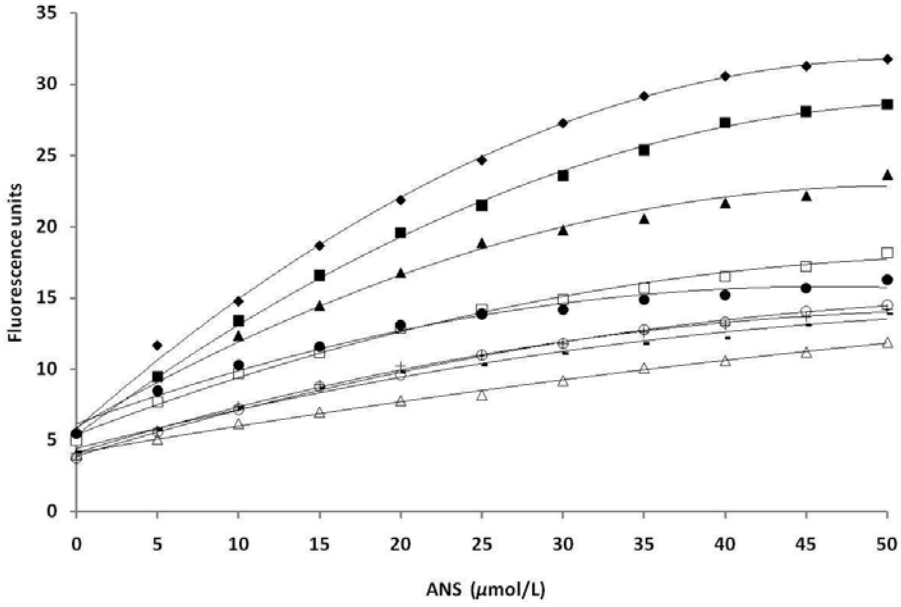


Figure 11 : Des courbes de titrage spectrofluorimétriques de dispersions de protéines de lactosérum dans des concentrations variable en sel

4. Caractérisation des dispersions lors des rampes de température

4.1. Spectroscopie FTIR

4.1.1. Principe de FTIR

La spectroscopie infrarouge à transformée de fourier (FTIR) est l'une des techniques les plus couramment utilisées en chimie organique et inorganique. Elle mesure l'absorption des fréquences infrarouges par un échantillon placé dans le trajet d'un faisceau infrarouge. L'objectif principal de l'analyse spectroscopique IR est de déterminer les groupements chimiques fonctionnels dans un échantillon. Chaque groupement fonctionnel a une fréquence d'absorption caractéristique de rayonnement infrarouge. Une large gamme d'échantillons tels que les gaz, liquides et solides peuvent être analysés par cette technique. La spectroscopie IR est un outil important et populaire pour l'élucidation de la structure et l'identification des composés.

4.1.2. Appareillage

Les spectromètres FTIR utilisent une méthode destinée à obtenir un spectre infrarouge en collectant d'abord l'interférogramme d'un signal échantillon à l'aide d'un interféromètre, puis en exécutant une transformation de fourier de cet interférogramme afin d'obtenir le spectre. Les spectromètres FTIR collectent et numérisent l'interférogramme, exécutent la transformation de fourier (une opération mathématique) et affichent le spectre. L'interféromètre le plus couramment utilisé est un interféromètre de Michelson. La représentation schématique ci-dessous (Figure 12), présente les différentes étapes pour analyser un échantillon.

Comme son nom l'indique, l'interféromètre produisant des signaux d'interférence, qui contiennent des informations spectrales infrarouge générées après passage à travers un échantillon.

Les deux détecteurs les plus utilisés en spectrométrie FTIR sont le DTGS (Deuterated TriGlycine Sulfate) et le MCT (Mercury Cadmium Telluride). Le détecteur DTGS est un détecteur pyroélectrique qui fournit des réponses rapides, car il mesure les variations de la température plutôt que la valeur de la température. Le détecteur MCT est un détecteur de photon qui dépend de la nature quantique du rayonnement, il présente également des réponses très rapides. Les détecteurs DTGS fonctionnent à température ambiante contrairement aux détecteurs MCT qui doivent être maintenus à température de l'azote liquide (77 ° K) pour être efficace. En général, le détecteur MCT est plus rapide et plus sensible que le détecteur DTGS. L'interférogramme détecté ne peut pas être directement interprété. Les analyses ont été

réalisées par réflexion totale atténuée (ATR) (Figure 13). Ces dernières années, la spectroscopie par ATR a révolutionné l'analyse des échantillons solides et liquides. Cette technique permet une préparation des échantillons plus aisée et une meilleure reproductibilité spectrale. Elle permet désormais

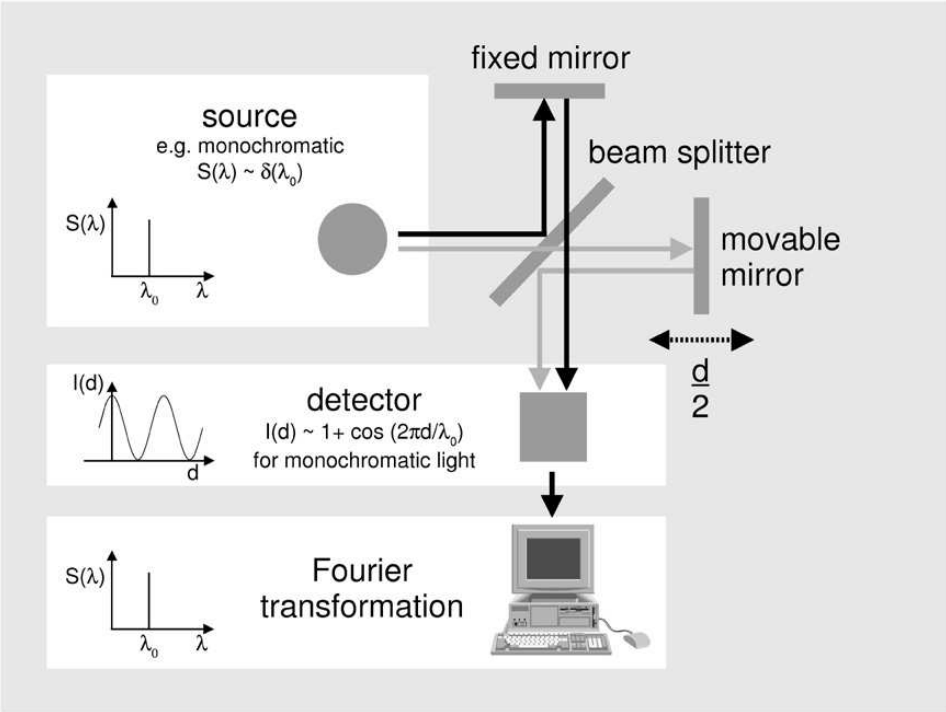


Figure 12: Schéma de fonctionnement d'un spectromètre FTIR

- un échantillonnage plus rapide
- une reproductibilité fiable
- Et une minimisation de la variabilité spectrale

Par ATR, les variations du faisceau de réflexion interne qui se produisent quand le faisceau infrarouge entre en contact avec l'échantillon sont mesurées. En fait, un faisceau infrarouge est dirigé sur un cristal optiquement dense avec un indice de réfraction élevé pour un angle déterminé. Ce facteur de réflexion interne crée une onde fugace qui s'étend au-delà de la surface du cristal sur l'échantillon maintenu en contact avec le cristal. Il s'avère plus facile de penser à cette onde comme une bulle de l'infrarouge qui se retrouve a la surface du cristal. Cette onde transmet quelques microns (0,5 μ - 5 μ) au-delà de la surface du cristal et de l'échantillon.

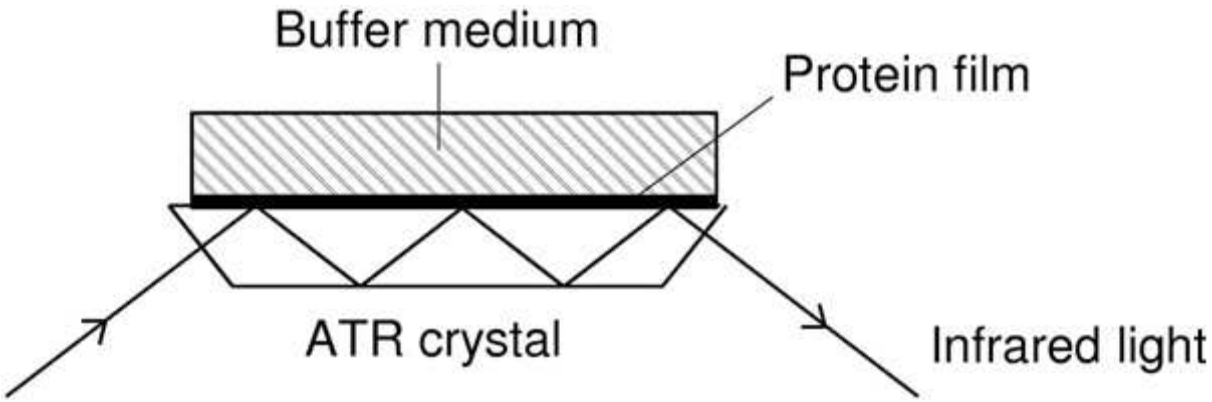


Figure 13: Principe d'un support ATR pour l'analyse des protéines

De nombreux matériels pour les cristaux de l'ATR sont disponibles. Le Séléniure de zinc (ZnSe) et le Germanium sont les plus courants. Le Séléniure de zinc est un matériel relativement peu coûteux et idéal pour l'analyse des liquides et les gels non-abrasifs, c'est en cette matière qu'en la cellule utilisée pendant la thèse. Cependant, il s'avère peu robuste pour une gamme de pH comprise entre 5-9. Ce matériel est fragile et des précautions doivent être prises surtout lors du nettoyage pour éviter des rayures potentielles. L'utilisation d'un tissu non pelucheux est recommandée.

Pour terminer, un thermostat (Rhéostat, Haake DC 30, Allemagne) est couplé à la cellule ATR pour augmenter la température de 30 à 90°C par palier de 2°C par observation (à l'aide du logiciel OPUS (Dynamics protéines O-PRO, Bruker, Karlsruhe, Allemagne).

4.1.3. Acquisition des spectres et premiers traitements

Les analyses FTIR ont été obtenues avec un spectromètre de Tensor 27 mi-FTIR Bruker (Bruker, Karlsruhe, Allemagne) équipé d'une cellule ATR (Réflexion Totale Atténuée) et d'un détecteur MCT (Mercury-Cadmium Telluride). Comme dit précédemment, ce détecteur est refroidi avec de l'azote liquide. La vitesse de balayage est de 20 kHz, 256 scans ont été utilisés à titre de référence et des échantillons entre 4000 et 850 cm^{-1}. La résolution nominale de l'instrument est de 2 cm^{-1}.

Les références ont été enregistrées sur les solutions salines en fonction de leurs concentrations. Puis, 2 mL des dispersions protéiques ont été déposés sur le cristal ATR (ZnSe). Avant la mesure, la dispersion est laissée pendant 5 minutes pour permettre aux protéines de s'adsorber sur le cristal. Ceci a considérablement amélioré le rapport signal/bruit. L'équipement ATR est purgé en continu avec de l'air sec pendant toute la durée des mesures. Trois à cinq expériences distinctes ont été réalisées pour chaque concentration de sel.

Le traitements de tous les résultats a été effectué a l'aide d'un logiciel OPUS (Bruker, Karlsruhe, Allemagne). Tout d'abord, les traces d'eau et de CO_2 sont soustraites des spectres. Ensuite, les spectres sont lissés en utilisant les neufs point Savitsky-Golay. Ils sont finalement coupés entre 1720 et 1580 cm^{-1}, ou bien entre 1200 et 1350 cm^{-1} pour l'analyse des bandes amide I et amide III, respectivement. Pour terminer, une correction de la ligne de base élastique a été réalisée à l'aide de 200 points, une normalisation et une conversion spectrale en unité absorbance.

4.1.4. Curve fitting et procédure de déconvolution

Pour initier la déconvolution spectrale, les spectres dérivés ont été calculés sur des données centrées et normalisées. Ces spectres de dérivée seconde ont été utilisés uniquement pour identifier les positions des pics individuels **(Byler and Susi, 1986, Curley *et al*., 1998, Farrell *et al*., 2001)**

Une fois les positions définies, les spectres ont été traités par un programme de déconvolution (Software Opus). Les aires intégrées de chaque pic ont été calculées et reliées à des structures secondaires caractéristiques (hélice-α, feuillet-ß, coudes, structure desordonnée...). Cette décomposition a permis de déterminer la fraction de divers éléments secondaires de la protéine dans les différents l'environnement ioniques et/ou à différentes températures. Les structures suivantes ont été attribuées: 1700-1660 cm-1, les virages; 1652-1648 cm-1, α-hélice; 1648-1642 cm-1, non ordonnées, et 1640-1620 cm-1, ß-feuille. La même procédure a été suivie pour la région Amide III. Affectations provisoires de Amide III ont également été déjà fait suivant **(Cai and Singh, 1999)** : 1330-1295 cm^{-1}, hélices α; 1295-1270 cm^{-1}, coudes ß; 1270-1250 cm^{-1}, structures désordonnées; and 1250-1220 cm^{-1}, feuillets β.

4.2. Propriétés rhéologiques

4.2.1. Principe de mesure

Dans les mesures d'oscillation dynamique, l'énergie potentielle et l'énergie dissipée en chaleur sont mesurées séparément en tant que module élastique (G') et module visqueux (G"). La mesure est basée sur la déformation de cisaillement imposée à l'échantillon selon l'équation 5 :

$$\gamma = \gamma\, 0 \sin \omega t$$

L'échantillon répond à cette déformation avec une contrainte sinusoïdale dépendante des propriétés de l'échantillon (eq 6):

$$\sigma = \sigma_0 \ sin \ \omega \ t$$

ω: Fréquence angulaire (rad.s^{-1})

γ_0: déformation de cisaillement

σ0: Contrainte de cisaillement (N.m$^{-2)}$

L'ange de perte δ (déphasage) est le rapport viscoélastique, il reflète l'importance relative du caractère élastique ou visqueux du produit eq (7) :

$$\mathrm{tg}\,\delta = \frac{G''}{G'} = \frac{\frac{\sigma_0}{\gamma_0}\sin\delta}{\frac{\sigma_0}{\gamma_0}\cos\delta}$$

Pour un solide élastique la contrainte est en phase avec la déformation, Indépendamment de la fréquence de sollicitation, toute l'énergie absorbée est transférée sous forme d'énergie potentielle $\delta = 0$, mais pour un liquide visqueux newtonien la contrainte est en phase avec la vitesse de déformation $\delta = \frac{\pi}{2}$

Si $0 < \delta < \frac{\pi}{2}$ une partie de l'énergie fournie lors de la déformation est emmagasinée par le produit est l'autre partie est dissipée, ce qui confère à l'échantillon un comportement viscoélastique **(Berland and Launay, 1995)**.

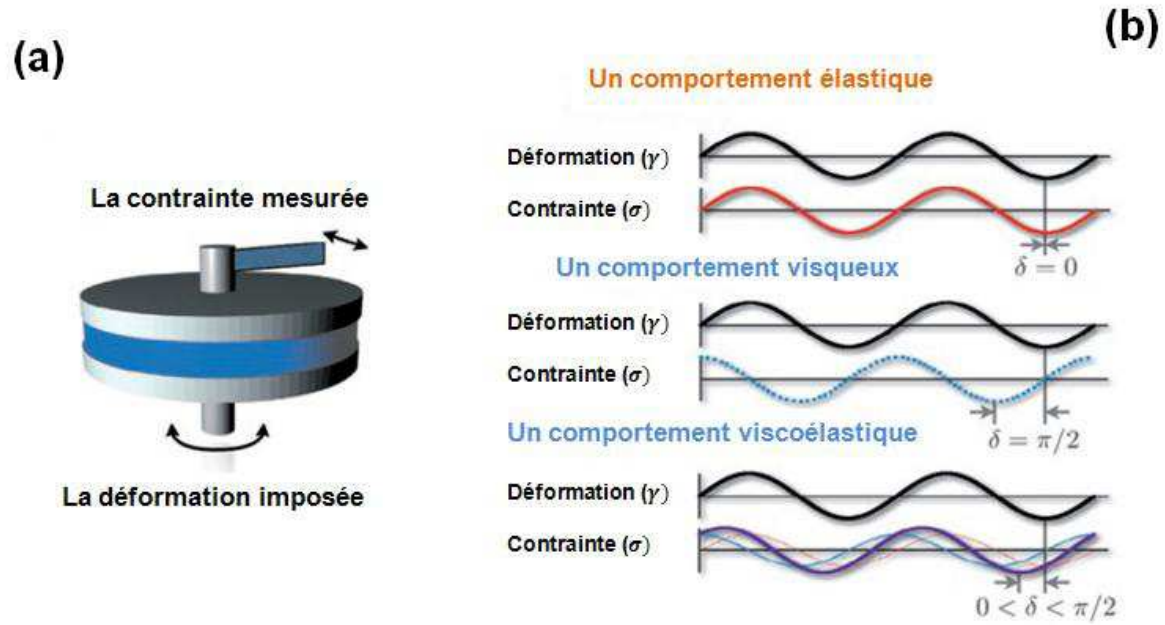


Figure 14: (a) Schéma représentatif d'un échantillon placé entre deux plaques d'un rhéomètre. (b) exemple de réponse par la contrainte à la déformation de cisaillement appliquée à un solide élastique, un liquide visqueux et un matériau viscoélastique.

4.2.2. Mode opératoire

Les analyses rhéologiques pour les dispersions protéiques ont été réalisées à l'aide d'un rhéomètre rotatif Kinexus (Malvern Instruments, KNX 2100, UK). Le rhéomètre est équipé d'un régulateur de température (effet Peltier) permettant l'obtention d'une rampe de température très précise. La formation du gel en fonction du temps et de la température était caractérisée par les déformations oscillatoires (strain 0,01%; 1,0 Hz) du module élastique (G'). La géométrie plate CP2/50 fut désormais utilisée. La température de gélification est définie comme la température à laquelle G' commence à augmenter. Les différentes dispersions protéiques ont été chauffées à des températures de 30°C à 90°C à une vitesse de 1°C.min^{-1}. De l'huile de paraffine a été appliquée à la surface de l'échantillon exposée à l'air afin d'éviter l'évaporation de l'eau lors de la rampe de température **(Michalski *et al*., 2002).**

4.3. Mesures des propriétés thermiques par DSC

La dénaturation thermique des dispersions protéiques est également analysée par microDSC en présence et en absence de sel.

4.3.1. Principe

La calorimétrie différentielle à balayage a été réalisée avec 2 capsules. Une capsule, appelée capsule de référence, contient de l'air ou un tampon et l'autre capsule contient l'échantillon. Son principe est basé sur la différence d'énergie entre la référence et l'échantillon pendant le chauffage, le refroidissement ou l'isotherme. Cette technique donne des résultats sous forme de pics. Sur la base de la différence dans les énergies entre référence et échantillon, la DSC fournit l'enthalpie de réaction. Une valeur positive de l'enthalpie indique une réaction exothermique, une valeur négative indique que la réaction est endothermique. Le type de DSC utilisé dans cette étude est la micro DSC. Cette technique donne des résultats plus sophistiqués.

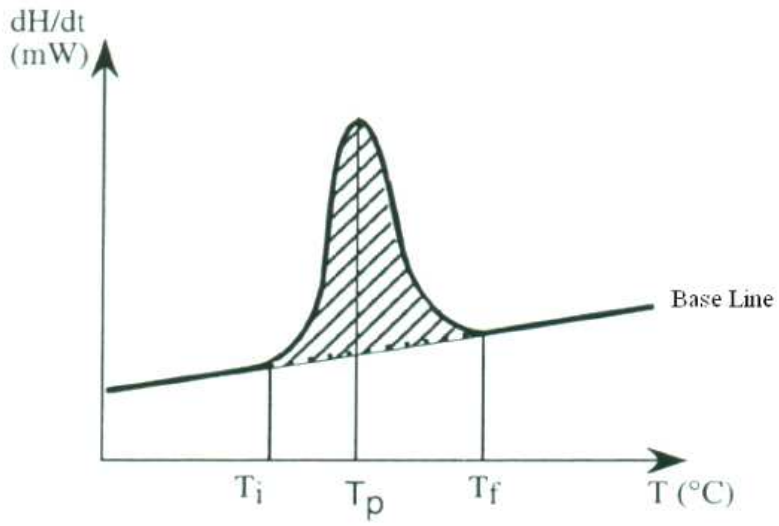


Figure 15: Exemple de thermogramme obtenu par DSC (réaction endothermique)

4.3.2. Protocole expérimental pour la Micro DSC

Les thermogrammes obtenus sur les dispersions protéiques en présence ou en absence de sel ont été étudiés par une microDSC III (Setaram, Caluire, France). Les dispersions protéiques (850 mg) ont été hermétiquement fermées dans des capsules de 1 cm^3 (Hastelloy C276, France). La capsule de référence est remplie d'eau (eau MilliQ) et/ou des différentes solutions salines appropriées. Après équilibrage pendant 10 minutes à 10°C, la température augmente à 90°C à une vitesse de 1,2°C.min^{-1}. Une fois la température de 90°C atteinte, une rampe de refroidissement est mise en place jusqu'à 30°C à une vitesse de 2°C.min^{-1}. Le changement d'enthalpie apparente total (ΔH) a été obtenu en intégrant la zone du thermogramme à l'aide d'une ligne droite entre le début et la température finale de la transition thermique. De plus, les températures de dénaturation (T_D) ont été déterminées à partir de l'intégration des thermogrammes calorimétriques.

5. Analyse statistique des données

Le traitement statistique des données est réalisé à l'aide du logiciel KyPlot version 2.0. Les comparaisons entre les poudres sont réalisées avec un test multiparamétrique. Le niveau de signification est : $^{***}P < 0,001$; $^{**}P < 0,01$; $^{*}P < 0,05$ et $^{NS}P > 0,$

Chapitré IV : Résultats et Discussion

Chapitre IV. I

Réhydratation de poudres de protéines laitières dans des milieux complexes

Caractérisation de la réhydratation de poudres laitières riches en protéines dans différentes solutions salines

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Résumé

La réhydration de poudres de caséine micellaire (NMC) et d'isolats des protéines (WPI) solubles a été suivie dans différents environnements ioniques. Deux solutions sont utilisées pour réhydrater les poudres : une solution de NaCl et de $CaCl_2$. Leur concentrations varient de 0 à 12% (% en poids). Un capteur de turbidité a été utilisé pour suivre in situ dans le réacteur et interpréter les différentes étapes de réhydratation (mouillage, gonflement et dispersion).

Deux comportements ont été observés en fonction de la concentration en sel. Pour les poudres de caséines micellaires, un changement significatif a été observé entre 3 et 6% de NaCl et entre 0,75 et 1,5% de $CaCl_2$. Le premier comportement (à faible concentration en sel), démontre la présence d'un profil classique de réhydratation lente: mouillage rapide, gonflement et dispersion très longue. Dans le deuxième comportement (à forte concentration en sel), l'étape de dispersion est réduite de façon significative indiquant une forte modification dans la conformation protéique.

La réhydratation des protéines soluble est moins influencée par les sels. A des concentrations faibles en sel, un profil type a été observé: mouillage avec formation de grumeaux, pas de gonflement visible suivi d'une dispersion rapide. A des concentrations élevées en sel, une instabilité des valeurs de turbidité ont été observés qui peuvent être expliquées par le déploiement et la dénaturation des protéines.

En outre, les changements dans les structures secondaires des deux protéines sous l'effet des concentrations élevées en sel ont été suivis par spectroscopie infra rouge (FTIR) pour confirmer les différents profils observés.

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Characterization of high-milk-protein powders upon rehydration under various salt concentrations

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ABSTRACT

Rehydration of native micellar casein and native whey isolate protein powders was followed in different ionic environments. Solutions of NaCl and $CaCl_2$ in theconcentration range of 0 to 12% (wt %) were used as rehydration media. The rehydration profiles obtained were interpreted in terms of wetting, swelling, and dispersion stages by using a turbidity method. Two behaviors were observed depending on the salt concentration. For native micellar casein powder, a significant change was observed between 3 and 6% NaCl and between 0.75 and 1.5 % $CaCl_2$. The first behavior (low salt concentration) presents a typical rehydration profile: quick wetting, swelling, and long dispersion stage. The dispersion stage of the second behavior (high salt concentration) was significantly shortened, indicating a strong modification of the protein backbone. The rehydration of whey protein powder was less influenced by salts. At low salt concentrations, a typical profile for whey powders was observed: wetting with lump formation and no swelling followed by a quick dispersion. At high $CaCl_2$ concentrations, no turbidity stabilization was observed, indicating a possible protein unfolding and denaturation. Additionally, the changes in secondary structures of the 2 proteins upon salt increase were followed by Fourier transform infrared spectroscopy and confirmed the different profiles observed.

Key words: ionic environment, milk protein, powder rehydration

RESULTS

Micellar Casein Rehydration in Different Ionic Environments

Micellar Casein Rehydration in Water. The rehydration of 5% native micellar casein in water was followed with a turbidity sensor. The mean profile obtained in water is presented in Figure 1A. The turbidity profile was interpreted in terms of wetting, swelling, dispersion, and total rehydration stages as already described in detail by **Gaiani et al. (2007).** The rehydration profiles show an early wetting stage with a first turbidity peak obtained 10 s after powder addition. The wetting stage was followed by a light turbidity decrease corresponding with the swelling stage 2 min after powder addition. Then, a turbidity increase was observed (i.e., particle dispersion) and was followed by a stabilization of the turbidity 467 min after powder addition. This stabilization was related to the total rehydration stage.

Micellar Casein Rehydration in NaCl Solutions. Two rehydration behaviors were obtained for micellar casein rehydration in NaCl and rehydration times are summarized in **Table 1**. The first behavior was found for casein rehydration in water with up to 3% salt (0, 0.75, 1.5, 2.25, and 3%). For these 5 profiles, it is possible to distinguish the following stages: wetting, swelling, and dispersion **(behavior I in Figure 2).** In comparison with water, the rehydration profiles obtained for 0.75, 1.5, 2.25, and 3% NaCl had longer wetting (33, 37, 40, and 45 s) and swelling (14, 21, 53 and 55 min) stages. In comparison with water, the total rehydration was significantly delayed and was not completed, even after 500 min (no turbidity stabilization). The second behavior **(behavior II in Figure 2)** was obtained for higher salt concentrations (6, 9, and 12%). The wetting stage was still longer than in water (55, 56, and 62 s), but the total rehydration was significantly shortened (around 230 min). For this behavior, the swelling stage was not observed.

Micellar Casein Rehydration in $CaCl_2$ Solutions.Two rehydration behaviors also were established for micellar casein rehydration in $CaCl_2$ **(Table 1).** The first behavior was noticed only in water and 0.75% salt **(behavior I in Figure 2).** For 0.75% salt, a longer wetting stage in comparison with water was observed (around 45 s). The swelling stage also was longer and and the total rehydration was not complete, even after 500 min. The second behavior was observed when the salt concentration was augmented (from 1.5% up to 12%). In these cases, the swelling stage was not observed and the total rehydration was significantly shortened (around 200 min).

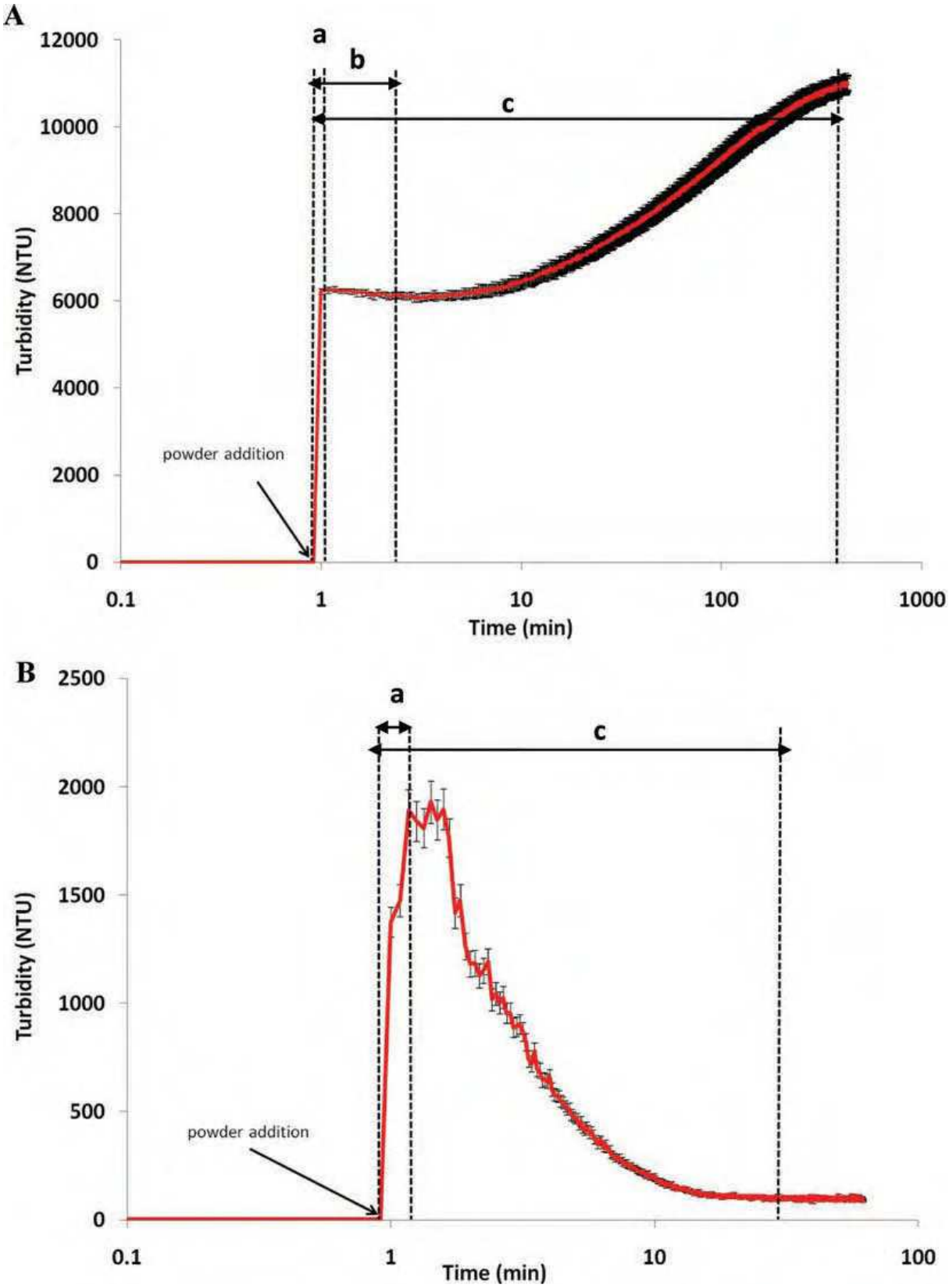

Figure 1. Turbidity profiles (NTU, nephelometric turbidity units) obtained during rehydration of (A) 5% native micellar casein powder and (B) 5% whey protein isolate powder at 24°C in water. The red (gray) profile is the mean of 3 independent analyses (standard deviation in black): (a) wetting time, (b) swelling time, and (c) total rehydration time.

Native Whey Proteins Rehydration in different Ionic Environments

Native Whey Protein Rehydration in Water. The turbidity profile obtained during the rehydration of native whey protein powder in water and NaCl is presented in **Figure 1B**. From this mean profile, the first turbidity peak is obtained 23 seconds after powder addition. No more swelling stages were observed. The total rehydration was observed after 25 min and was related to turbidity stabilization.

Native Whey Protein Rehydration in NaCl Solutions. Almost similar rehydration behaviors (behavior I) were observed for NWI rehydration in NaCl salts solutions upto 12% salts (Figure 3). For NaCl concentration up to 6%, no significant difference observed in the rehydration times (wetting, swelling and total rehydration in **Table 1**). For 9% and 12% NaCl, the wetting time and the total rehydration time are significantly longer.

Native Whey Protein Rehydration in $CaCl_2$ solutions. **Figure 3** represents the two rehydration behaviors obtained in $CaCl_2$ salt solutions during NWI rehydration. The first behavior is observed for NWI rehydrated in water up to 1.5% salt. For these three concentrations, no significant differences were observed between the rehydration times. The turbidity profile **(**behavior I in **Figure 3)** showed an early wetting and a short rehydration time. The swelling time is not observed. On the contrary, for the second rehydration behavior (behavior II in **Figure 3)** the total rehydration is not completed even after 30,000 seconds. The wetting stage is slightly increased from 23 seconds in water to 44 seconds in 12% $CaCl_2$.

Secondary Structure of Milk Proteins in different Ionic Environments

Native Micellar Casein. **Figure 4A** shows the original FTIR spectrum (from 1200 to 1700 cm^{-1}) of native micellar casein totally rehydrated (overnight) in water and in 12% salt. The amide I peak is easily identifiable and centers on 1641 cm^{-1}. The amide I region (1700-1600 cm^{-1}) is principally associated with the stretching vibrations of peptide carbonyl groups. The amide II peak, centered on 1550 cm^{-1} (1600-1700 cm^{-1}) is mainly N-H bending and C-N stretching modes. The amide III region (1350-1200 cm^{-1}) presents relatively weak signals but may be easily resolved and is better defined than Amide I and II regions.

No differences were observed in the Amide II shape for casein rehydrated in water and in high ionic environment. On the contrary, Amide I and Amide III regions presented different shapes in water and in 12% salt. For casein rehydrated in NaCl solutions (**Figure 4A**), the Amide I center is significantly shifted from 1641 to 1635 cm^{-1} and the amide III principal peak is also shifted from lower wavenumbers (1249 to 1247 cm^{-1}). Casein rehydrated in $CaCl_2$ presents similar shifts. For all rehydration media (each concentration and each salt), the only differences observed are peak shifts more important when the salt concentration increase.

Table 1. Comparison of the rehydration times obtained for casein and whey proteins powders in the different media in comparison with NMC and NWI rehydrated in water[1].

powder	salt	Concentration (%)	wetting time (s)	swelling time (s)	total rehydration time (s)	rehydration behavior
NMC	NaCl	0	10	120	28 000	I
		0.75	33*	880***	nr	
		1.5	37*	1233***	nr	
		2.25	40*	1405***	nr	
		3	45*	3295***	nr	
		6	55**	no	14 291**	II
		9	56**	no	13 100**	
		12	62**	no	12 999**	
	$CaCl_2$	0	10	120	28 000	I
		0.75	45*	3410***	nr	
NMC		1.5	48*	no	15 690**	II
		2.25	50*	no	11 212***	
		3	50*	no	11 320***	
		6	51*	no	11 561***	
		9	49*	no	12 888***	
		12	50*	no	11 901***	
NWI	NaCl	0	23	no	1 525	I
		0.75	25^{ns}	no	$1\ 616^{ns}$	
		1.5	24^{ns}	no	$1\ 623^{ns}$	
		2.25	31^{ns}	no	1 981*	
		3	35^{ns}	no	$1\ 613^{ns}$	
		6	38^{ns}	no	$1\ 599^{ns}$	
		9	41*	no	1 678*	
		12	45*	no	1 821*	
NWI	$CaCl_2$	0	23	no	1 525	I
		0.75	35^{ns}	no	$1\ 497^{ns}$	
		1.5	34^{ns}	no	$1\ 560^{ns}$	
		2.25	36^{ns}	no	nr	II
		3	35^{ns}	no	nr	
		6	41*	no	nr	
		9	44*	no	nr	
		12	44*	no	nr	

[1]NR = not rehydrated after 500 and 100 min for casein and whey, respectively; NO = not observed.

$^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{NS}P > 0.05$

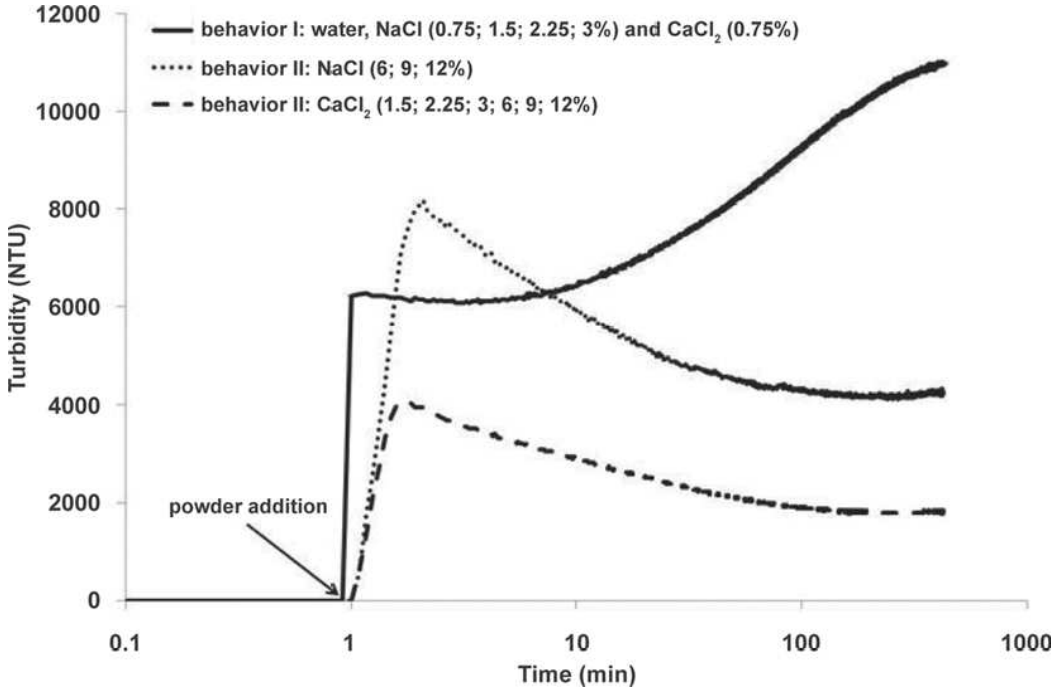

Figure 2. Turbidity profiles (NTU, nephelometric turbidity units) obtained during rehydration of 5% native micellar casein powder at 24°C for 1,000 min in different ionic environments (mean of 3 independent analyses). Behavior I: wetting stage, swelling stage, and long total rehydrationstage. Behavior II: wetting stage, no swelling stage, and short total rehydration stage.

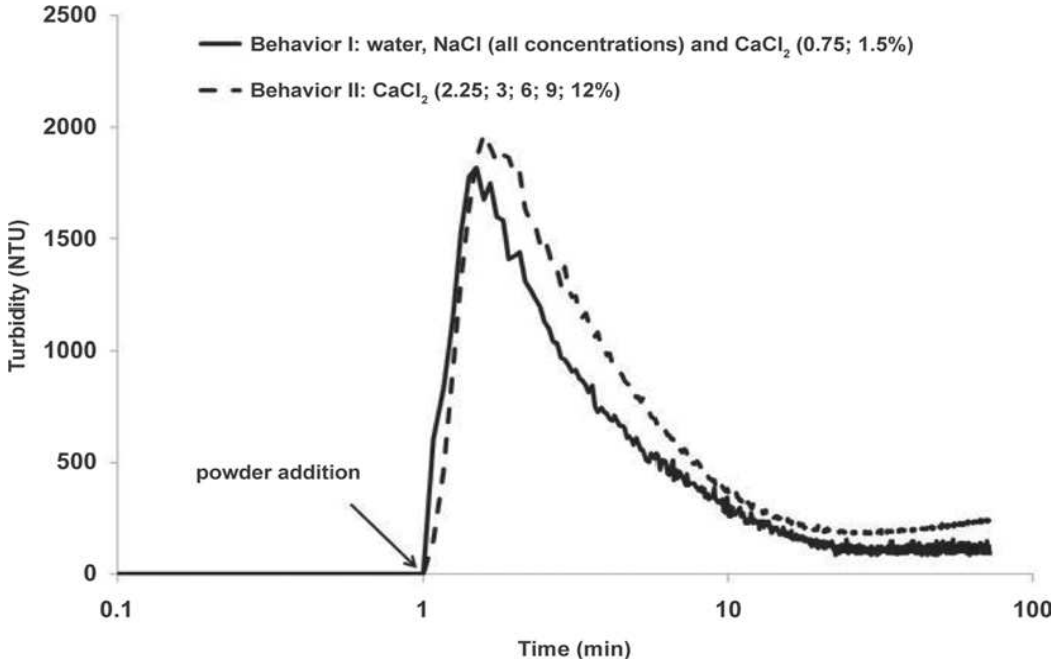

Figure 3. Turbidity profiles (NTU, nephelometric turbidity units) obtained during rehydration of 5% native whey protein isolate powderat 24°C for 1,000 min in different ionic environments (mean of 3 independent analyses). Behavior I: wetting stage, no swelling stage, and quick total rehydration stage. Behavior II: wetting stage, no swelling stage, and rehydration stage not observed.

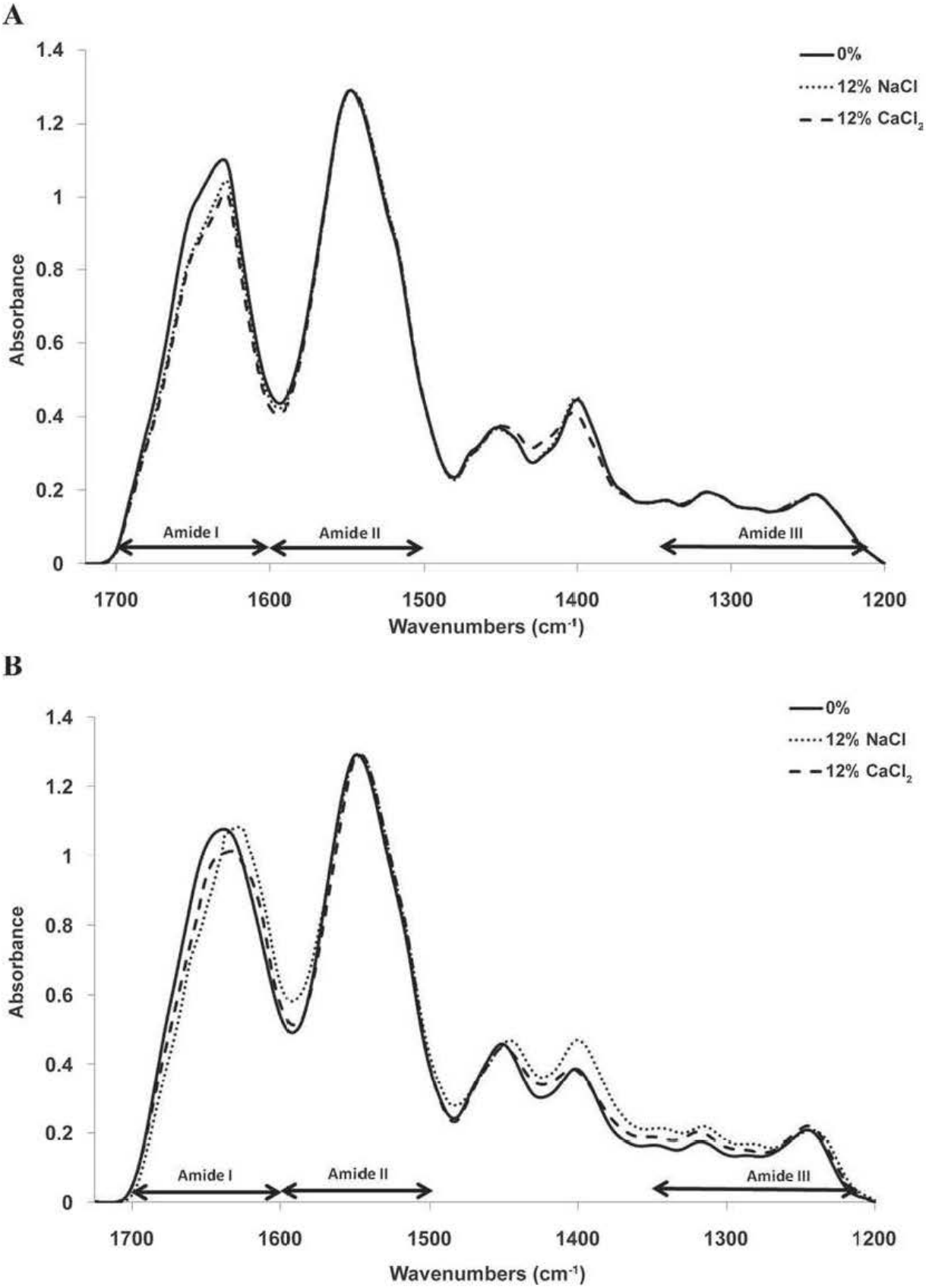

Figure 4. Original Fourier transform infrared spectrum of (A) native micellar casein and (B) native whey protein isolate rehydrated in NaCl and in $CaCl_2$ solutions.

Native Whey Proteins Isolate. **Figures 4B** shows the original FTIR spectrum of whey protein isolate powders rehydrated in water and in 12% salt. The amide I peak is center on 1630 cm^{-1} whatever the salt media. The amide II peak, centered on 1548 cm^{-1} (1600-1700 cm^{-1}) and the amide III region (1350-1200 cm^{-1}) are only slightly influenced by the salt concentration. Amide I region presented very different shapes in water and in 12% salt. For NWI rehydrated in NaCl solutions, the Amide I center is slightly shifted towards lower wavenumbers (1630 to 1628 cm^{-1}). The NWI rehydrated in $CaCl_2$ presents the same shifts. On average, these shifts amounted to about 2 cm^{-1} for each media. In conclusion, for all rehydration media (each concentration and each salt), the only significant differences observed were thar Amide I peaks shifted to lower wavenumbers and Amide I peaks intensity less important when the salt concentration increased.

DISCUSSION

Rehydration of Micellar Casein

In agreement with **Gaiani et al., (2005, 2007)**, the total rehydration of micellar casein in water is time consuming with a quick wetting stage and a very long dispersion stage. The rehydration time obtain in this study (467 min) is in accord with that reported by **Gaiani et al., (2007)**. They found a total rehydration time of 566 min for nonagglomerated micellar casein powders. Milk protein concentrate powders also are characterized by a low solubility index. **Mimouni et al. (2009)** reported that a low solubility index for milk protein concentrate powders at room temperature was the result of slow dissolution kinetics rather than the existence of a large amount of insoluble material in the rehydrated powder. In other words, a significant fraction of powder material remains undissolved after a reasonable time of reconstitution at room temperature **(Havea, 2006).**

According to these authors, 2 overlapping steps are involved in the rehydration process: the disruption of agglomerated particles into primary particles and, concurrently, the release of material from the powder particles into neighboring aqueous phase. The nature of the insoluble material also has been characterized. It mainly consists of casein micelles linked together by fibril-like protein structures (although their nature has not been clearly established). These linkages seem to involve hydrophobic interactions and nonmicellar caseins dissociated from the micelles during milk protein concentrate powders manufacture **(Anema et al., 2006; Havea, 2006).** Consequently, micellar casein powders are generally considered to be poorly soluble powders for which rehydration of micelles is a time-consuming process **(Jost, 1993).**

In NaCl and $CaCl_2$, 2 rehydration behaviors were observed. The first was similar to the profile obtained in water but with a time delay: presence of the wetting stage, swelling stage and total rehydration. According to previous studies, dispersion of micelles into NaCl not only

increases micellar rehydration **(Grufferty and Fox., 1985),** but also lead to solubilzation of calcium and phosphate **(Famelart et al., 1999).** Moreover, it is well-known that increase in ionic strength cause decrease in activity coefficients of the diffusible ions and consequently increases in the dissociation of ion pairs so hydration of casein micelles is increased because of these changes. Even if salts are hygroscopic and may increase the micelle hydration, no amelioration of the rehydration stages in comparison with water is observed in this study.

The second rehydration behavior was observed from 6% and 1.5% for NaCl and $CaCl_2$ respectively. This behavior was different from that seen with water. The total rehydration was significantly shortened and the swelling stage was absent. As has already been observed for powders with quick rehydration times (**Gaiani et al., 2007**), the swelling stage may be mixed up with the dispersion stage. Our study confirms the findings of **Huppertz and Fox (2006).** They reported that addition of NaCl induces changes in physico-chemical stability of casein micelles by decreasing negative charge on the micelles. In agreement with **Schuck et al. (2002),** the addition of NaCl (420 m*M*) improved the reconstitution time

These authors observed an infinite reconstitution period for $CaCl_2$ addition at 222 m*M*. **Philippe et al. (2005),** revealed that addition of divalent cations ($CaCl_2$) into milk system cause strong modification of casein micelles may decrease micelle hydration. According to **Famelart et al. (1999),** casein solubilization and hydration showed slight biphasic changes with $CaCl_2$ addition. Negative charges of casein are reduced by binding of calcium between 0-0.1 M $CaCl_2$. In conclusion, this second behavior may be the result of a change on micellar structure due to high salt environment. This change may occur between 3 and 6% (517 and 1,034 m*M*) in NaCl and between 0.75 and 1.5% (67 and 135 m*M*) in $CaCl_2$ solutions.

This change in micellar structure was also observed by FTIR (**Figures 4**). Conformational changes of the casein backbone may be induced by Ca^{2+} binding to the side chain **(Curley et al., 1998)**. The Ca^{2+} binding involved serine phosphate and carboxyl groups of glutamate and aspartate residues corresponding to the side chain vibration (1,600-1,610 cm^{-1}). Hence, the shift observed toward lower wavenumbers corresponding to the side chain vibration may be due to ion binding. Similar FTIR spectra were observed for casein rehydrated in NaCl.

Rehydration of Whey Proteins. The NWI powder demonstrated rapid dispersion properties after wetting but was found to have poor wettability in agreement with **Gaiani et al. (2005, 2007)**. The turbidity instability observed at the beginning of the profile could be due the lump formation flowing past the sensor as noticed by **Freudig et al. (1999)**. **Kinsella, (1984**) has established that globular proteins bind less water than intact casein micelles. This may explain why the swelling stage was not present or noticeable for whey proteins. An electrophoresis study

carried out by Anema *et al*. (2006) indicated that caseins were the insoluble proteins, whereas the whey proteins showed early and strong soluble behavior

The first rehydration behavior was observed for NWI rehydration in water, for all NaCl solutions and up to 1.5% for $CaCl_2$. All these profiles showed poor wettability, no swelling and rapid dispersion. An increased of NaCl from 0 to 12% showed no significant effect on the turbidity profile. Globular proteins are shaped in a compact form at a low ionic strength due to selective binding of anion by protein cations. However, at higher ionic strength, 2 factors, (i.e., screening of electric charge by electrolyte and association between protein molecules) may result in precipitate formation that may lead to early hydration **(Kinsella, 1982).**

The second important and observable aspect was that both cationic salts (NaCl and CaCl2) at low concentration show similar behavior and nonsignificant effects on rehydration times of powders.Our results supports studies of **Simons et al. (2002) and Mercadé-Prieto et al. (2007)** who reported that calcium salts acts similarly to sodium in screening charges but because Ca^{2+} binds especially to the carboxylates so, much lower concentration are required than Na^{+} to yield same screening effect. Moreover, monovalent cations do not play any significant role in dissolution of NWI.

The second rehydration behavior is only observed from 2.25% to 12% $CaCl_2$. The wetting stage is shortly elongated, the swelling stage is still absent and the total rehydration is significantly prolonged. **Ju and Kilara (1998)** also observed that a variation in $CaCl_2$ concentration (from 5 to 50 m*M*) greatly affect the denaturation behavior of WPI solutions , resulting in turbidity increase. The turbidity increase at the end of the profile may be due to protein unfolding/denaturation rather than powder rehydration.

According to earlier studies **(Kuhn and Foegeding, 1991; Caussin et al., 2003)** divalent cations may affect more significantly electrostatic interaction between charged protein molecules than monovalent cations. In this study, divalent cations present also a more significant effect on whey protein system than monovalent cations. The turbidity increased at the end of the rehydration profile may be attributed to intermolecular association of Ca^{2+} with whey protein. As a result, small aggregates may be formed and detected by the turbidity sensor. The $CaCl_2$ was more effective at screening electrostatic interactions than NaCl due to higher valency of counter ions and because calcium ion can form ion bridges between negatively charged protein molecules. This whole phenomenon may influence the rehydration behavior of NWI powder. These findings are in agreement with **Kuhn and Foegeding (1991) and Bryant and McClements (2000).**

From the FTIR spectra, it could be noticed that spectra corresponding to whey proteins differed in intensity with addition of salt. It would be of interest to evaluate quantitatively

changes in structures (α-helix, turns, irregular, and β-sheet) by curve fitting and tentative assignments as already accomplished by **Curley et al. (1998)** on micellar casein. This work will be done in a future study. Similar results were obtained for β-lactoglobulin rehydrated first at pH 2 and 7 and then heated at 85°C **(Schmitt et al., 2009).** It was concluded that the main consequence at these pH levels was protein denaturation and not aggregation. One hypothesis is that in a highly ionic environment, NWI may be more prone to denaturation and less to aggregation. For instance, **Schmitt et al. (2009)** demonstrated that the relative sensitivity of band attributed to β-sheet and unordered/α-helix secondary structure was a clear indicator of differences in the denaturation/aggregation balance. This point will need to be studied in detail in further studies.

CONCLUSIONS

The interpretation of the turbidity profiles was quite helpful to better understand the rehydration stages of native micellar casein and native whey proteins isolate powders in increasing salt environments. We observed two distinct behaviors depending on the salt type and concentration. $CaCl_2$ seemed to act in a manner similarl to NaCl, but a lower concentration was required to destructure (casein) and unfold (whey). Nevertheless, multi-scale characterization will be required to investigate more precisely the structure of the 2 proteins in these environments. This will be done in a future paper by coupling transmission electron microscopy, dynamic light scattering, reverse phase HPLC and FTIR curve fitting.

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Relations entre les proprietes morphologiques et la rehydratation des poudres de lait

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Résumé

Les propriétés de réhydratation des poudres alimentaires dépendent d'une part de leur nature chimique et d'autre part de leurs caractéristiques morphologiques comme leur taille, leur forme ou encore leur état de surface.

L'objecif de ce travail était double. Le premier était d'étudier l'influence des caractéristiques morphologiques de cinq différentes poudres laitières sur leurs propriétés fonctionnelles comme l'aptitude au mouillage, la solubilité et la dispersibilité (définies par les norme FIL/IDF ou les méthodes Niro Atomizer). Le second était de voir, en utilisant le logiciel Unscrambler, si les propriétés de réhydratation pouvaient être prédites uniquement en fonction de la taille et de la morphologie des particules sans passer par les normes (FIL, ADMI, Niro).

L'évaluation des caractéristiques morphologiques a été effectuéee par un nouvel appareil QICPIC. Cet appareil a permis de caractériser très précisément la taille et la forme des particules. Les paramètres morphologiques étudiés étaient la convexité, la sphéricité et le diamètre de sphère équivalente. Le traitement des données et la prédiction a été réalisé par le logiciel Unscramber.

Cette étude a montré une corrélation entre les caracteristiques morphologiques des particules et les propriétés d'hydratation.

Mots-clés : poudres laitières, propriétés de réhydratation, prédiction, morphologie, sphéricité

1. Introduction

Depuis quelques années, les poudres laitières sont devenues des ingrédients de plus en plus utilisés dans l'industrie alimentaire aussi bien du point de vue nutritif que techno-fonctionnel. Les avantages qu'elles proposent par rapport au lait liquide sont nombreux. Leur conservation par exemple est plus longue; principalement grâce à une plus basse activité de l'eau qui ne permet pas la multiplication des bactéries. La plupart des poudres alimentaires doivent être dissoutes avant leur utilisation c'est pourquoi la réhydratation est leur propriété fonctionnelle la plus importante. Il est généralement admis que le processus de réhydratation se déroule en différentes étapes **(Schubert, 1980, 1993 ; Freudig *et al*. 1999)** qui comprennent successivement: le mouillage, la coulabilité, la dispersion et enfin la dissolution des particules. Du point de vu des normes, il est possible d'évaluer le mouillage qui est la première étape du processus de réhydratation et qui reflète l'aptitude d'une poudre à s'immerger après avoir été déposée à la surface de l'eau. On teste également la solubilité, c'est à dire l'aptitude d'une poudre à se solubiliser après une série de centrifugations définies. Enfin, la dispersibilité représente l'aptitude d'une poudre à se briser en particules pouvant passer à travers un tamis dont le diamètre des pores est défini. En fonction des propriétés physiques et chimiques des poudres, chaque étape peut prendre plus ou moins de temps dans le procédé de réhydratation **(Gaiani *et al*. 2005, 2006).**

Les propriétés de réhydratation des poudres laitières dépendent d'une part de leur nature chimique et physique et d'autre part de leurs caractéristiques morphologiques comme leur taille et leur forme. Ces dernières ont été très peu étudiées, alors que l'influence de la composition et de la structure ont été largement apprécié **(Gaiani, 2006).** L'agglomération des poudres laitières joue un rôle primordial pour l'amélioration des propriétés de réhydratation et de stockage **(Bylund, 2003).** Comme L'objectif de ce travail, tout d'abord nous avons voulu étudier l'influence des caractéristiques morphologiques de différentes poudres laitières sur les propriétés de réhydratation (mouillage, solubilité et dispersibilité). En effet, jusqu'à présent seule la microscopie (très couteuse en terme de temps et de mise en œuvre) suivie d'une analyse d'image permettait d'obtenir les facteurs de forme. Actuellement, avec l'appareil Quicpic, il est possible d'obtenir ces facteurs en quelques minutes pour un très grand nombre de particules. L'analyse des images et la microscopie des particules est la technique la plus souvent utilisée pour caractériser la forme des particules **(Bao *et al.* 2004 ; Li *et al,* 2005)** mais le niveau de l'automation pour l'analyse des images est bas, la technique est compliquée. De plus, le nombre des particules analysées est relativement faible, ce qui provoque des erreurs statistiques significatives.

2. Matériels et Méthodes

2.1 Préparation des échantillons

Cinq différentes poudres laitières ont été étudiées : caséine micellaire native (NMC), protéines solubles du lait (WPI), poudre de lait entier, poudre de lait demi-écrémé et poudre de lait écrémé. Pour chaque poudre, 7 tailles de particules sont obtenues par tamisage. Au total, 35 poudres sont donc analysées avec des diamètres variant de 50 à 380 µm.

2.2 Analyses physico-chimiques

2.2.1. Détermination de la mouillabilité

Le mouillage par immersion est la première étape du processus de réhydratation d'une poudre laitière. Il reflète l'aptitude d'une poudre à s'immerger après avoir été déposée à la surface de l'eau. La mouillabilité peut être mesurée selon la méthode de Niro Atomiser (1978) ou de la FIL. Selon ces normes, 1g de poudre est déposé dans un bécher rempli d'eau distillée à température ambiante. Le temps au bout duquel toutes les particules sont mouillées est exprimé en secondes. Industriellement, une poudre dont le temps de mouillage est supérieur à 30 minutes est considérée comme non mouillable. On effectue trois répétitions pour chaque échantillon et on note la moyenne correspondante des 3 valeurs obtenues.

2.2.2. Détermination de la solubilité

La mesure de la solubilité est un critère essentiel dans le contrôle de la qualité d'une poudre laitière qui donne une idée de l'aptitude à la réhydratation, mais elle caractérise surtout la quantité de poudre qui ne sera pas dissoute. La détermination de la solubilité est effectuée selon la méthode gravimétrique américaine modifiée (A.D.M.I).

2.2.3. Détermination de la dispersibilité

La dispersibilité d'une poudre dans l'eau est mesurée par les normes FIL (1985) ou par la méthode de Niro Atomizer (1978). D'après ces méthodes, la dispersibilité est le critère le plus important pour évaluer le caractère « instantané » d'une poudre de lait car elle dépend à la fois de la solubilité, de la mouillabilité et du gonflement des poudres.

2.3. Analyse de taille et de forme

2.3.1 Principe du QicPic

Pour caractériser la poudre on a besoin de déterminer ses facteurs de forme et sa distribution de taille. Le nouvel appareil QicPic (Sympatec Inc. Claustrale-Zellerfeld, Germany) permet

d'effectuer rapidement des analyses granulométriques (mesure de la taille des particules), ainsi que des analyses morpho métriques (forme des particules).

LIXELL est un système très flexible qui peut être adapté à l'analyse des dispersions, des émulsions. Ce système permet un analyse d'image pour des tailles de particules variant de 2 µm à 1700 µm et une analyse de taille par diffraction laser pour des particules de 0.1 µm à 1750 µm. Sa construction de base comporte une cellule de débitmètre avec un connecteur d'admission et un de sortie. L'analyse se fait sur plus de 90 000 particules et permet la détermination de la taille et de la forme sur un grand nombre de particules (analyses représentatives). Des cuvettes avec des longueurs de chemins optiques variables existent. Au laboratoire nous sommes équipés de cuvettes de 0.2 mm (adaptés aux milieux turbides comme des gouttelettes d'eau/huile, du lait etc.) et de 1 mm (particules de 3 à 500 µm).

2.3.2 Analyse de la taille des particules

L'appareil peut mesurer trois types de diamètres :

1) **diamètres de sphère équivalente** : Il s'agit du diamètre du cercle qui aurait la même surface que la surface apparente de la particule.

2) **diamètres de féret** : Il s'agit de la distance entre deux tangentes au contour apparent de la particule menées parallèlement à une direction fixe.

3) **diamètre de Martin** : C'est la longueur d'une corde divisant la surface apparente de la particule en deux aires égales.

2.3.4. Mode opératoire pour l'analyse Qicpic

On récupère 0,25 g de poudre et on les disperse dans 0,75 ml d'éthanol pur et anhydre. On effectue 3 répétitions avec une durée de mesure 60 sec. Pendant 60 secondes, l'appareil compte les particules, mesure les diamètres, les formes et enregistre une vidéo. Les paramètres étudiés sont le diamètre de sphère équivalente (facteur de taille), la convexité et la sphéricité (facteurs de forme).

3. Résultats et Discussion

3.1. Influence de la taille des particules et de nature des poudres sur les propriétés de réhydratation

3.1.1. Le mouillage

Nous avons fait varier les tailles des particules de 40 à 430 µm. Ces résultats sont représentés sur la **Figure 1** qui montre que le comportement des différentes poudres ne suit pas la même loi en fonction de la taille des particules.

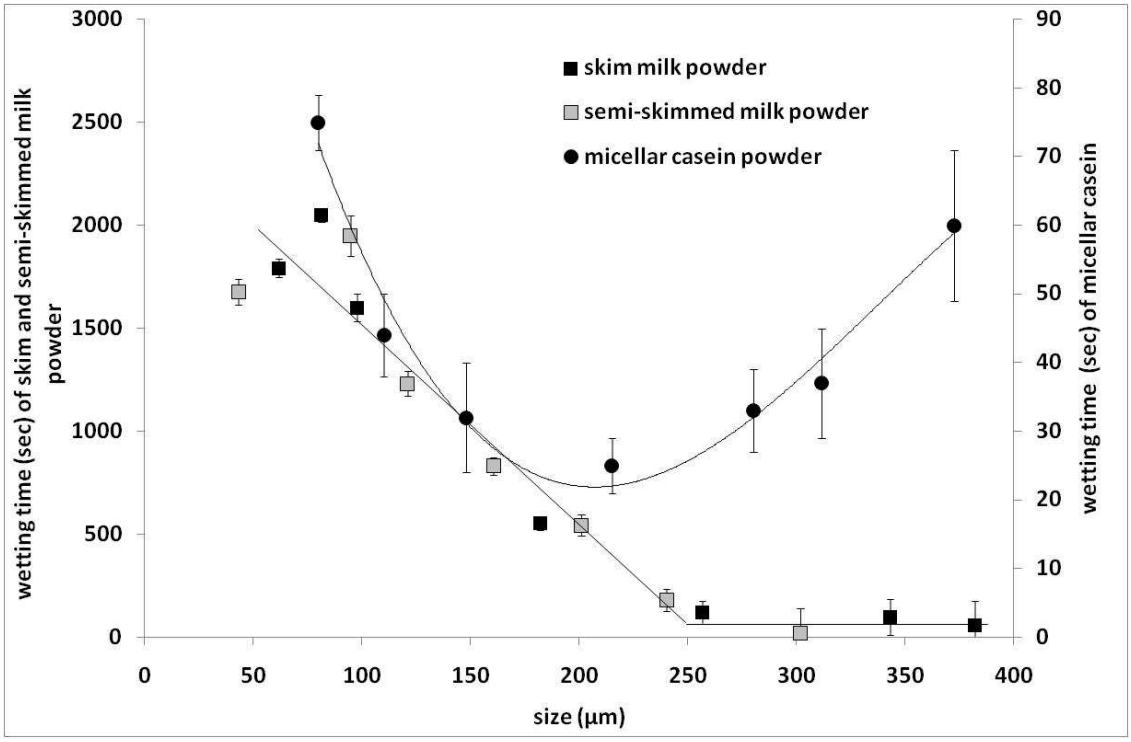

Figure 1 : Influence de la taille des particules sur le temps de mouillage

Tout d'abord, les résultats pour les poudres de lait entier et les poudres de protéines solubles ne sont pas présentés, car ces poudres sont non mouillables (> 3000 secondes) quelle que soit la taille des particules. Pour la caséine micellaire, une taille moyenne de 215 µm permet d'obtenir un temps optimal de mouillage (le plus court). Une relation différente est observée dans le cas des poudre de lait écrémé et demi-écrémé. Ainsi, plus la taille augmente plus la mouillabilité s'améliore (plus le temps de mouillage diminue). Ainsi, au dessus de 250 µm ces poudres se mouillent très rapidement. On voit donc que non seulement la taille des particules mais également la nature des poudres va fortement influencer leur comportement de mouillage.

On peut supposer que les poudres de protéines solubles et les poudres de lait entier ne sont pas mouillables à cause de l'organisation des composants à la surface qui empêche la pénétration de l'eau à l'intérieur des particules. Dans le cas du lait entier, la matière grasse libre qui se trouve en surface des particules peut créer une barrière hydrophobe et entraver leur mouillabilité. Pour les protéines solubles, c'est la présence d'une haute valeur protéique sur la surface, qui pourrait causer la faible mouillabilité de la poudre **(Kim *et al.*, 2002)**. Il a également été démontré que de la matière grasse résiduelle se trouvait en surface de ces poudres de protéines soluble empêchant leur mouillage **(Gaiani, 2006).** Pour les poudres contenant du lactose (composé hygroscopique), et moins de matière grasse (composé hydrophobe), le

mouillage sera favorisé. C'est le cas des poudres de lait écrémé et de lait demi-écrémé. La caséine micellaire présente un comportement intermédiaire, ou la structure des particules (porosité, forme…) doit également influencer le mouillage; d'où une courbe en « cloche ».

3.1.2 La solubilité

La composition biochimique de la poudre influence également sa solubilité. Ainsi, des poudres à forte teneur en lactose présentent peu d'insolubles **(Schuck *et al.*, 1994 ; 1994a).** Comme la capacité de solubilité d'un constituant dépend de l'affinité de ce constituant vis à vis de l'eau, un composé hygroscopique va permettre un transfert d'eau facilité aussi bien pendant le séchage (de l'intérieur vers l'extérieur de la particule) que pendant la réhydratation (de l'extérieur vers l'intérieur de la particule). Par contre, la cristallisation du lactose va diminuer la solubilité **(Yetismeyen et Deveci, 2000).** Une concentration en caséine élevée va également diminuer la solubilité des poudres **(Mistry et Hassan, 1991 ; Schuck, 1998).** Pour ces poudres, l'indice de solubilité sera faible et d'environ 70% **(Pierre *et al.*, 1992 ; Schuck *et al.*, 1994 ; 1994a).** Nos résultats confirment ces observations (**Figure 2**) pour la poudre de caséine. Dans notre étude, le pourcentage est bien inférieur à celui trouvé par Schuck *et al.*, 1994. Ce pourcentage est inférieur à 20% quelle que soit la taille des particules. On peut expliquer ce résultat par l'âge de la poudre de caséine micellaire. Cette poudre a été fabriquée il y a plus d'un an et la caséine micellaire est connue pour présenter des problèmes d'insoluble en vieillissant.

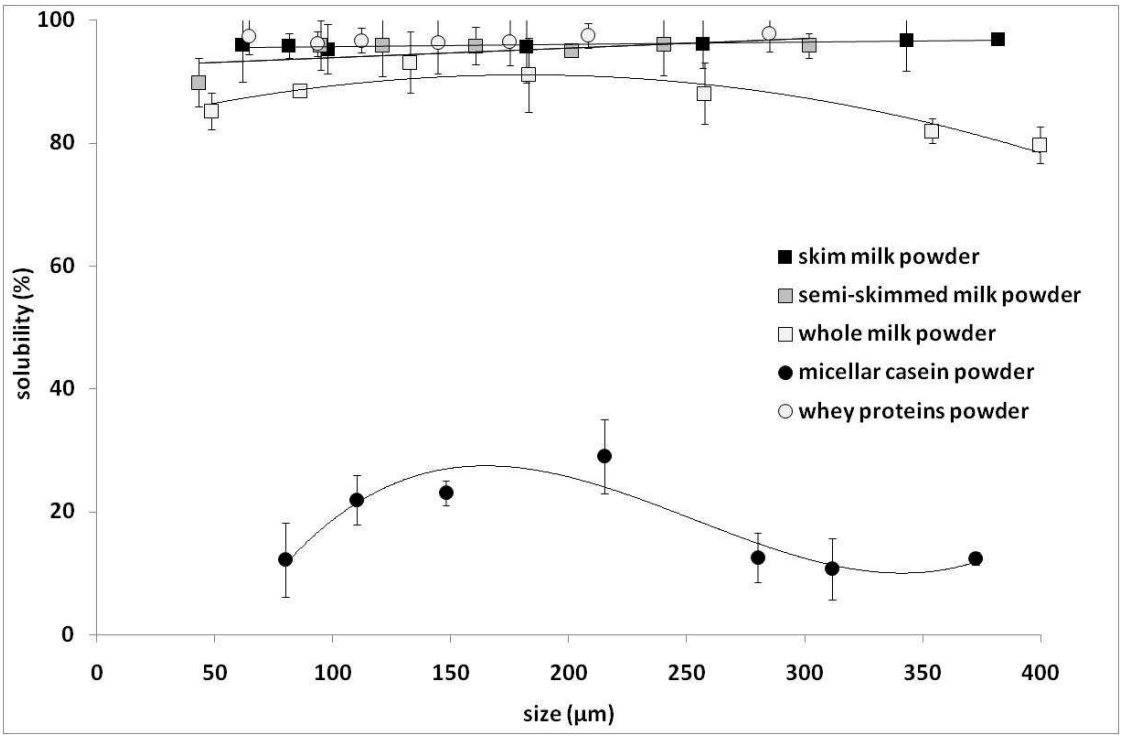

Figure 2 : Influence de la taille des particules sur l'index de solubilité

3.1.3 La dispersibilité

Le comportement des poudres de lait entier, de lait demi-écrémé et de lait écrémé par rapport à la dispersibilité n'est pas linéaire. On observe une courbe en cloche (**Figure 3**). Pour une taille de particule identique, les poudres pauvres en matière grasse sont plus dispersibles: lait écrémé (90,1 8%), lait demi-écrémé (81,36 %), lait entier (80,3 %). La quantité de matière grasse est donc un important facteur; surtout si elle se trouve en surface. La présence de matière grasse en surface de la poudre peut entraîner une diminution de la mouillabilité mais également de la dispersibilité **(Kim *et al.*, 2002).** Selon les normes de la Fédération Internationale de Laiterie (F.I.L.), une poudre est dite '' instantanée'' si la dispersibilité est d'au moins 85 % (pour le lait entier) et de 90% (pour le lait écrémé).

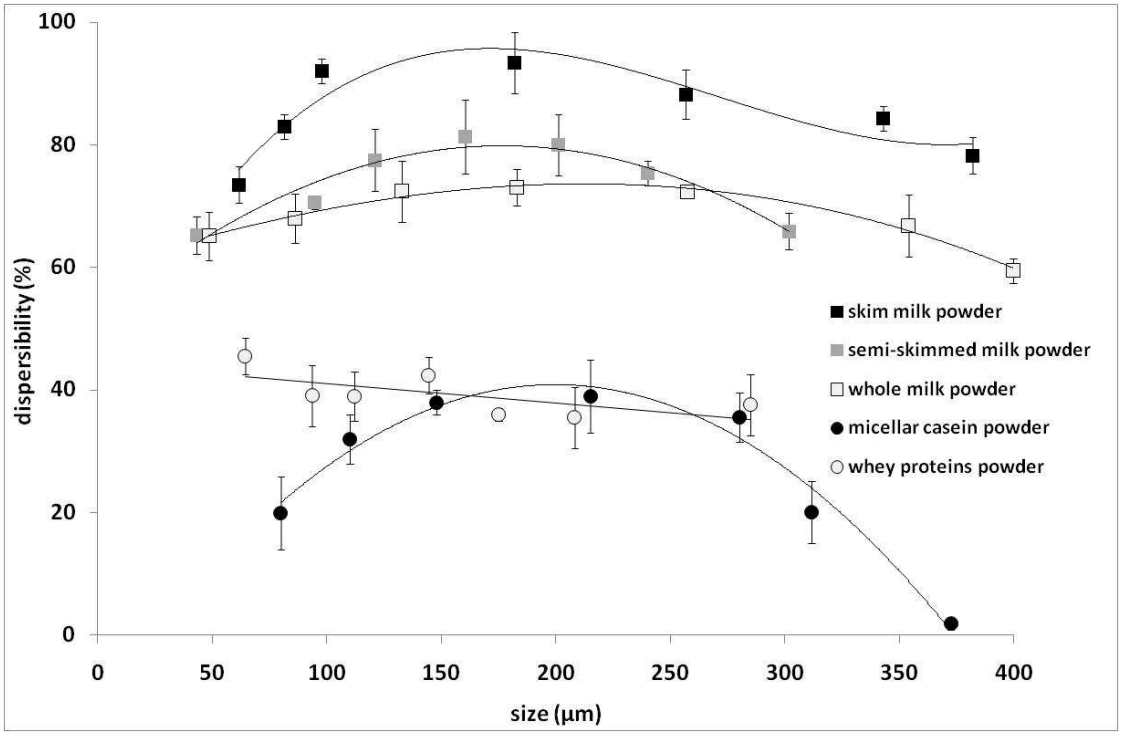

Figure 3. Influence de la taille des particules sur la dispersibilité

Les poudres de caséine micellaire et les poudres de protéines solubles présentent des dispersibilités inférieures. Une courbe en cloche est observée pour la caséine alors qu'une tendance linéaire est observée pour les protéines solubles. Néanmoins, une taille moyenne des particules de 130 µm à 250 µm semble présenter un optimum de dispersibilité.

3.2. Influence de la morphologie et de la nature des poudres sur les propriétés de réhydratation

Cette partie du rapport va plus se focaliser sur la morphologie des particules. La sphéricité et la convexité ont été déterminées avec l'aide de l'appareil Qicpic. Le second objectif de cette étude est donc de voir si ces paramètres influencent eux aussi sur les propriétés de réhydratation et dans l'idéal s'il est possible de prédire le comportement (de réhydratation) des poudres à partir de ces valeurs.

3.2.1 Relation entre taille des particules et morphologie

Pour toutes les particules (nature), la tendance est exactement la même. On observe qu'une augmentation de taille des particules va entraîner une diminution de la convexité et de la sphéricité comme présenté sur la **Figure 4**. Comme la tendance est la même, nous n'avons présenté qu'une seule figure représentative de l'ensemble de résultats.

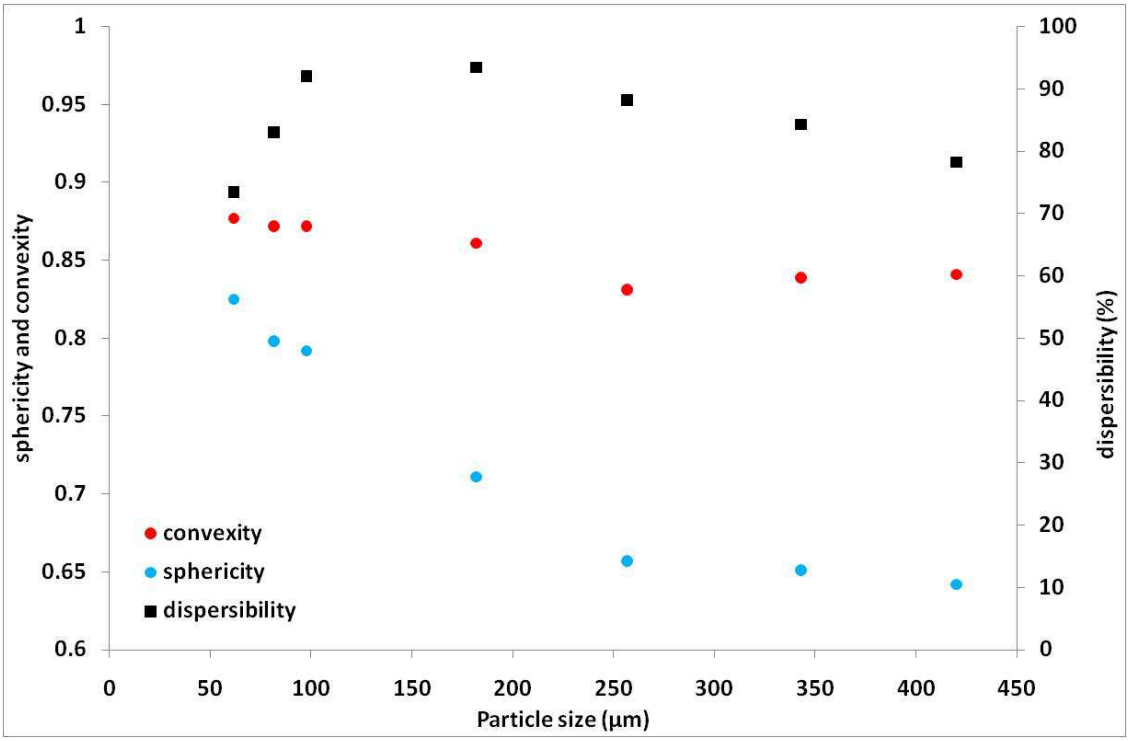

Figure 4. Influence de la taille des particules sur la sphericity et convexity

CONCLUSION

Ce travail a permis de travailler sur deux axes. Dans un premier temps, les relations entre taille des particules et les propriétés de réhydratation des poudres ont été approfondies. Ces résultats ne sont pas exceptionnels, et sont déjà plus ou moins connus et reconnus dans la communauté scientifique. Ils permettent toutefois de poser les bases de notre étude.

La seconde partie de l'étude est plus originale. De nombreuses études plus complexes vont donc découler de ce travail. J'ai également réussit à établir une corrélation entre forme des particules et propriétés d'hydratation. Ainsi, aucune étude dans le domaine des poudres alimentaire ne s'est encore intéressée à cette relation entre morphologie et propriétés de mouillage. Pour la mouillabilité, il sera nécessaire de tester d'autres poudres pour arriver à un modèle correct. On peut également imaginer les améliorer en rajoutant des poudres (autre nature, autre taille, autre morphologie…) pour affiner les valeurs de prédictions. Pour conclure, le laboratoire possède maintenant une base de donnée et des modèles intéressants qu'il sera possible de valoriser auprès des industriels du secteur.

Chapitre IV. II

Caractérisation multi-niveau des solutions protéiques réhydratées

Caractérisation multi-échelle de dispersions protéines contenant des caséines micellaires dans des milieux salins

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Résumé

Les dispersions de caséines micellaires ont été étudiées à une concentration constante en protéine de 5% (p/v), dans un environnement élevé en sel, allant de 0% à 12%. Les dispersions ont été caractérisées par différentes techniques biophysiques: morphologie et taille des micelles, ainsi que le contenu en protéines dans le surnageant après centrifugation. Les changements dans les structures secondaires de la protéine lors de l'augmentation des proportions en sel ont été suivies par spectroscopie infra rouge (FTIR).

Pour la première fois, l'estimation des éléments de la structure secondaire (structures irrégulières, feuillets β, hélices α et coudes) par la bande amide I et III ont été corrélé. Dans l'eau les dispersions ont été caractérisées par microscopie électronique à transmission (TEM) par une forme sphérique entre 100 et 200 nm. L'augmentation des concentrations en sel provoque une déstabilisation de la micelle, suivie de la formation de submicelles plus ou moins agrégées. La taille du nouvel édifice était presque semblable à la micelle native.

Les résultats obtenus par TEM ont été confirmés par DLS. En effet, la taille moyenne des micelles des caséines est comprise entre 150 et 180 nm. A chaque augmentation en sel, la FTIR a révélé une augmentation des structures irrégulières et une diminution concomitante des feuillets ß. Le pourcentage en structures secondaire obtenues à partir des bandes Amide I et Amide III est presque semblable.

L'utilisation de ces techniques multi-échelles aboutit à une meilleure compréhension de l'édifice micellaire dans un environnement élevé en sel. A des concentrations dépassant 3% de NaCl, une bonne corrélation a été observée entre la déstabilisation de l'édifice micellaire, les modifications de la structure secondaire caséines et la répartition des caséines entre surnageant et culot après ultracentrifugation

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Multiscale Characterization of Casein Micelles Under NaCl Range Conditions

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ABSTRACT

Micellar casein (**MC**) dispersions were studied at a constant protein concentration of 5 wt % in high NaCl environment. The micellar edifices were characterized as to their morphology, size, and content of proteins in the supernatant after ultracentrifugation. Additionally, changes in secondary structures of the protein upon salt increase were followed by Fourier Transform Infrared Spectroscopy (**FTIR**). For the first time, the estimations of secondary structural elements (irregular, ß-sheet, α-helix and turn) from Amide III assignments were correlated with results from Amide I. Casein micelles dispersions in water were characterized by Transmission Electron Microscopy (**TEM**) by a spherical shape and a size between 100 and 200 nm. A salt increase resulted to a destabilization of the micelle and the formation of mini-micelles more or less aggregated. The size of the new edifice was almost similar to the native micelle. These TEM observations were confirmed by a constant casein micelle hydrodynamic diameter determined by Dynamic Light Scattering (**DLS**) and ranging between 150 and 180 nm. Upon salt increase, FTIR revealed an increase in irregular structures and a concurrent decrease in ß-sheet structures. Secondary structural elements percentages were almost similar from Amide I and Amide III. The use of these multiscale techniques led to a better understanding of the micellar edifice under high salt environment. Around 3% NaCl addition, a good correlation was observed between destabilization of the micellar edifice, modifications of the caseins secondary structure and repartition of caseins between supernatant and pellet after ultracentrifugation.

Keywords: casein micelle, ionic environment, microscopy, FTIR, salt.

Abbreviations:
MC Micellar casein
DLS Dynamic Light Scattering
FTIR Fourier Transform Infrared Spectroscopy
SEM Scanning Electron Microscopy
TEM Transmission Electron Microscopy

RESULTS

Secondary structure estimation of casein micelle in different ionic environment. Second derivative spectra were calculated from Amide I, II and III bands. The frequencies of the bands obtained were used to define peaks positions during the curve fitting procedure. The curve fitting procedure was already described in detail in the material and method part. **Figure 1A** present the Amide I and II regions fitted with a Gaussian line shape function; peaks position being defined from the second derivative spectrum. During the iteration process the peaks positions were allow to shift, anyway the positions were not or only slightly changed (less than 2 cm^{-1}). Optimal fits were obtained when the root mean square was in the range of 10^{-4}. The same procedure was followed for the Amide III region. The fits for Amide III region are presented in **Figure IB**. Peak assignments of Amide I region were made using the results of former studies collected for globular proteins **(Byler and Susi., 1986)** and caseins **(Malin et al., 2001; Curley et al., 1998; Farrel et al., 1996 & 2001; Hoagland et al., 2001).**

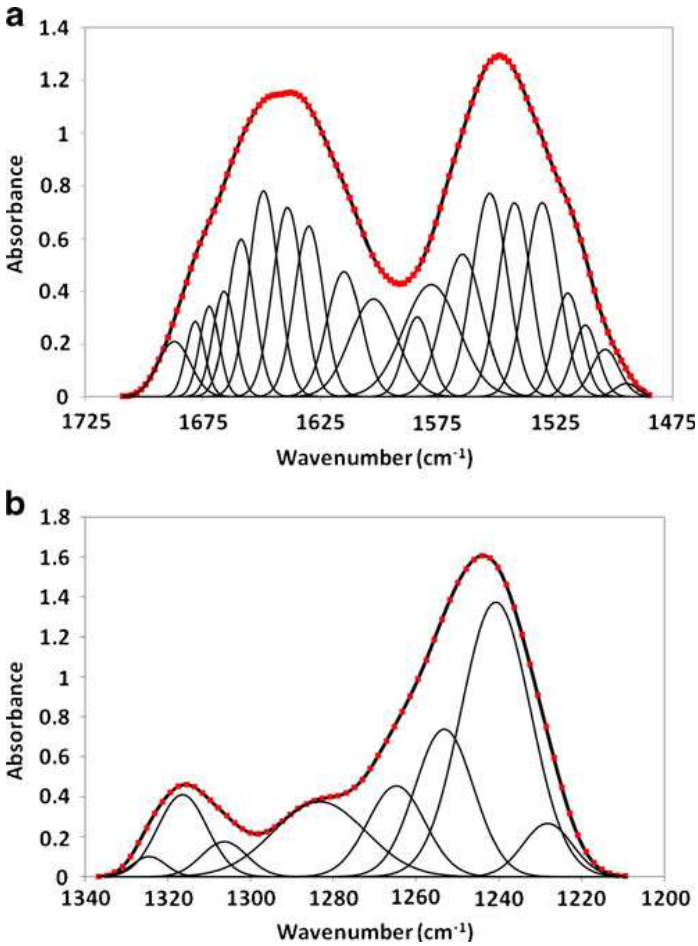

Fig. 1 FTIR spectra of casein micelle in water. Amide I and II regions(a) and Amide III region (b) were fitted at peak positions obtained from second-derivative spectra followed by Gaussian curve fitting. Red dots represent experimental data; black curves represent individual Gaussian components and their sum

The following structures were assigned: 1700-1660 cm^{-1}, turns; 1652-1648 cm^{-1}, α-helix; 1648-1642 cm^{-1}, irregular structures; and 1640-1620 cm^{-1}, ß-sheet. Peaks assignments were not performed for the amide II region. This region was only conserved to provide a better fit of the amide I, principally in the valley around 1590 cm-1 between the two peaks **(Curley et al., 1998)** (**Figure 1A**). Tentative assignments of Amide III were also already done by some authors **(Cai and Singh., 1998)** : 1330-1295 cm^{-1}, α-helix; 1295-1270 cm^{-1}, ß-turns; 1270-1250 cm^{-1}, irregular structures; and 1250-1220 cm^{-1}, ß-sheet.

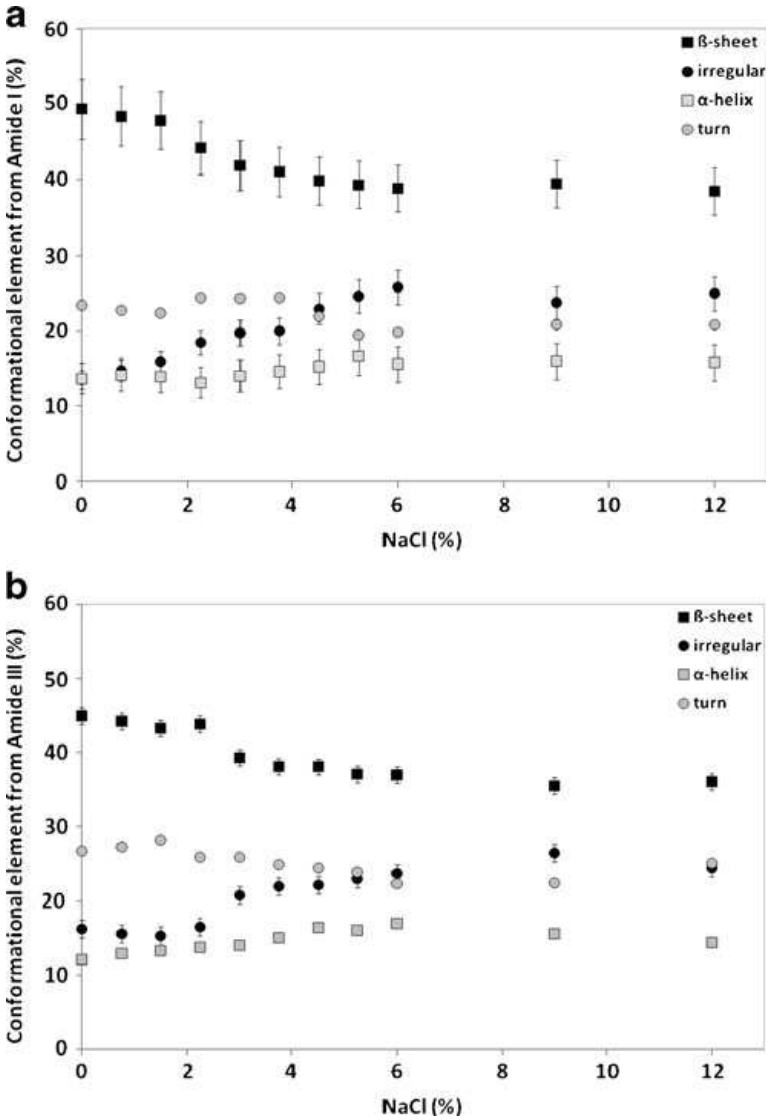

Fig. 2 Structural components (ß-sheet, Irregular, Turn and α-helix) percentage peaks areas obtained for casein micelle dispersions in different NaCl environment measured from Amide I (a) and Amide III (b) (mean of three independent analyses)

Table 1. Variation of the hydrodynamic diameters and ratio of the peaks amplitude (peak 1 / peak 2) with added NaCl concentration.

NaCl concentration (%)	1st peak Hydrodynamic diameter (nm)	2nd peak Hydrodynamic diameter (nm)	Ratio of intensity (1st peak / 2nd peak)
0.00	153 ± 2.9	46 ± 3.5	19.6
0.75	177 ^{ns} ± 5.3	36 ^{ns} ± 7.3	3.4**
1.50	182 ^{ns} ± 8.2	38 ^{ns} ± 9	3.5**
2.25	180 ^{ns} ± 14.5	37 ^{ns} ± 9.2	3.9**
3.00	175 ^{ns} ± 22.0	33 ^{ns} ± 14.1	3.8**
3.75	165 ^{ns} ± 22.7	37 ^{ns} ± 12.1	4.4**
4.50	162 ^{ns} ± 18.8	30 ^{ns} ± 10.2	2.3**
5.25	175 ^{ns} ± 13.3	35 ^{ns} ± 19.7	1.1***
6.00	161 ^{ns} ± 4.6	31^{ns} ± 2	1.2***
9.00	157 ^{ns} ± 29.8	34 ^{ns} ± 1	1.0***
12.00	149 ^{ns} ± 14.3	34 ^{ns} ± 4	1.2***

$^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{ns}P > 0.05$

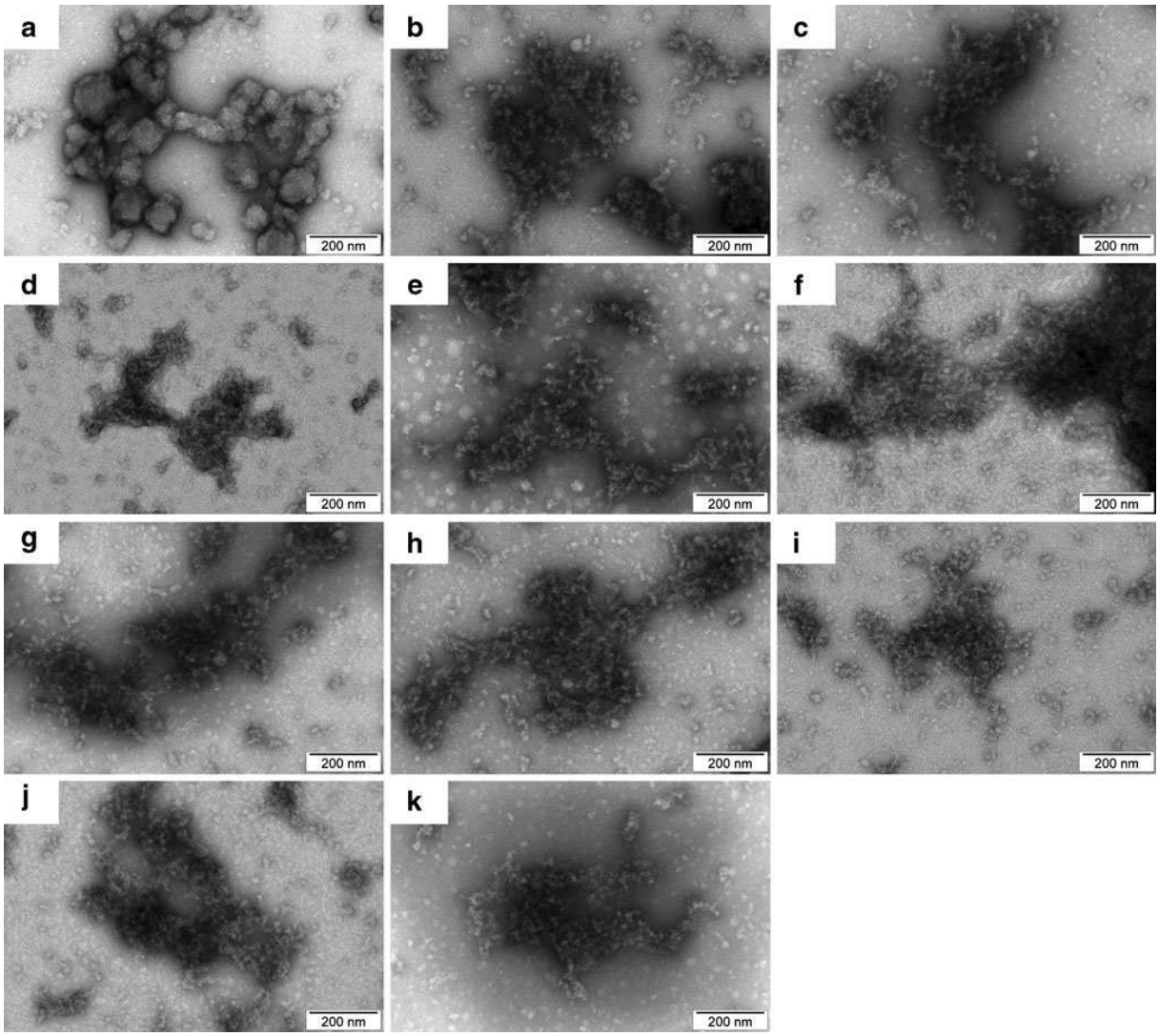

Fig. 3 Transmission electron micrographs of casein micelles in different NaCl environment. a 0%; b 0.75; c: 1.5; d: 2.25; e: 3.0; f:3.75; g: 4.5; h: 5.25; i: 6.0; j: 9.0 and k: 12.0 % NaCl. Imaging was at 50,000× magnification. Each image is representative of at least 6 images observed. Scale bar=200 nm.

Distribution of structural components. The calculated areas in ß-sheet, Irregular, Turn and α-helix are presented in **Figures 2A** and **B** for amide I and amide III respectively. These areas calculated from the fitted spectra are considered to be proportional to the conformational elements that were assigned. The objective was to use the Amide III as a complementary method to Amide I analysis for protein structure. Similar tendencies are observed in **Figure 2A** (Amide I) and **Figure 2B** (amide III). A ß-sheet decrease is noticed when the ionic strength increase; the beginning of the decrease being between 1.5 and 2.25% NaCl for Amide I and 2.25 and 3.0% NaCl for Amide III. Concurrently the Irregular structures increased at the same salt concentration whereas the α-helix and turns percentages are mostly constant. Even if the trend is the same for both bands (Amide I and III), the percentages of conformational elements are not identical. For example in water, the distribution in ß-sheet, Irregular, Turn and α-helix structures

obtained are respectively around 50, 13, 24 and 13% (Amide I) and 46, 16, 26 and 12% (Amide III).

Influence of various ranges of NaCl on size and morphology of micellar casein dispersions. 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 1**. Two populations are systematically observed. A first hydrodynamic diameter 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. Upon addition of NaCl (ranging from 0.75% to 12%), no significant differences was observed for each Dh; the particle size ranging between 149 and 182 nm for the first peak and between 31 and 46 nm for the second peak. However, significant differences were observed in peaks intensity ratio. The first peak decreases while the second peak increases when the salt percentage increase (**Table 1**).

The morphological characteristics of MC under NaCl environment were examined using a TEM (50,000× magnification). Negative staining TEM images of micellar casein in water are shown in **Figure 3A**. This Figure revealed intact and well separated roughly spherical nanoparticles structures in the size ranges from 100 to 200 nm. Addition of NaCl (0.75 to 12%) resulted in the dissociation of micelles into smaller particles (**Figures 3B** to **K**). These sub-micelles around 20 nm radius existed on their own. In other instances, the particles were found to be clumped together or in a bead like formation. All observations via TEM confirm the particle size measurements by DLS.

Influence of various ranges of NaCl on caseins distribution in the rehydrated media. **Figure 4** illustrates the evaluation of nitrogen contents in supernatants, carried out by Kjeldahl method. A significant decrease in nitrogen content in ultracentrifuge supernatants was observed with NaCl increase. For example, addition of NaCl (from 129 to 2068 mmol.kg-1) lead to an average nitrogen decrease from 7% to 51% correspondingly. These modifications may be related to the presence of more proteins in the pellet corresponding to the micelles.

Table 2. Comparison bewteen predictions of secondary elements percentages (α-helix, β-sheet, turns and irregular structures) measured by FTIR with literature.

Nature of casein	α-helix	β-sheet	turns	irregular	References
β-casein	29	34	32	4	Kumosinski *et al*., 1996
κ-casein	17	35	25	23	Farrell *et al*., 2001
αs_1-casein	30	25	39	11	Farrell *et al*., 1996
αs_2-casein Amide I	32	27	31	9	Farrell *et al*., 2001
αs_2-casein Amide III	29	37	34	34	Farrell *et al*., 2001
casein micelle Amide I	25	30	36	9	Kumosinski *et al*., 1993
This study Amide I casein micelle	12	45	26	16	-
This study Amide IIIcasein micelle	14	49	23	14	-

DISCUSSION

Secondary structural elements associated with changes in NaCl environment. In caseins, a controversy exists over the existence of a secondary structure. Some authors developed the concept of caseins as rheomorphic proteins which suggests that caseins are dynamic in nature and their conformation adapts to their environment **(Horne, 2002).** Furthermore, bovine casein has never been crystallized and there is little information on its structure to date. In addition, caseins, could present unique structural components (large loop for example) that are not contained in databases **(Kumosinski et al., 1997).** Nevertheless, tentative assignments of secondary elements were already done by some authors (**Curley et al., 1998; Farrel et al., 1996).**

First, 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 analyzed. A 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 and higher concentrations. An effort was also made to compare Amide I and Amide III spectrums. Assignments of secondary structure elements to whole casein and various individual caseins have been compared in **Table 2**.

Naively, one may have thought that the same molecule is doing vibrating; bending, stretching and so all studies might have given the same results. For example, the percentages of α-helix assigned for casein micelle varied from 12% in this work to 25% in another study **(Curley et al., 1998)**. Concurrently, the irregular structures varied from 16% to 9% respectively. These differences are mainly due to different spectrum treatments. Indeed, some authors underline the somewhat arbitrary nature of the assignment of components contributing to spectral bands **(Horne., 2002).** Nevertheless; some information was collected by many researchers through both Raman and infrared spectroscopy with which our data correlates fairly well **(Byler and Susi., 1986; Curley et al., 1998; Bylet et al., 1988).** 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. The exact frequency of Amide I band depends on the nature of hydrogen bonding between C=O and N-H moieties **(Ngarize et al., 2004; Panick et al., 1999).** This band suffers principally from an unstructured spectral contour and an overlap of the random coil and α-helix bands **(Cai and Singh., 1999).**

Consequently, the standard deviation presented in **Figure 2A** was high for Amide I results. On the opposite, Amide III region which is predominantly due to the in-phase combination of N-H in plane bending and C-N stretching vibrations, was already found very sensitive to the protein secondary structure **(Cai and Singh., 1999; Cai and Singh., 2004; Anderle and Mendelsohn., 1987).** 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 (**Figure 1B**) in comparison with the Amide I (**Figure 1A**).

Casein micelle size, morphology and repartition associated with changes in NaCl environment. The first peak measured by DLS was easily attributed to casein micelle. Indeed, the hydrodynamic diameter measured in water (around 150 nm) is in agreement with values determined by others **(Le Tran., 2008).** Upon addition of NaCl, no significant changes in micellar size were reported. Indeed, it was demonstrated in concentrated and unconcentrated milk that the average particle size of casein micellar remain unchanged during salt addition **(Huppertz and Fox., 2006; Gaucheron, 2005).** In general, it is well known that addition of sodium chloride to milk systems induces an increase in the solubility of calcium phosphate salts **(Walstra, 2003).**

According to **Huppertz and Fox, (2006),** there is a loss of Ca^{2+} from the micelles upon addition of NaCl, furthermore the amount of calcium bound to αs1-casein **(Dalgleish and Parker., 1980**) or β-casein (**Parker and Dalgleish., 1981)** decreases with increasing ionic strength. Accordingly, addition of NaCl may be at the origin of a dissociation of calcium bridges that holds the casein molecules together and also increase an ionic pressure inside the micelle. These effects could lead to a less connected network.[53] As a consequence of these changes, the hydration of casein micelles is increased, even if their sizes stay constant **(Gaucheron, 2005).**

Aggregation is strongly influenced by attraction and repulsion forces, which are dependent on pH, nature of solvent, ionic strength, temperature, charge or concentration of the protein **(McClements, 2002).** The extent of NaCl used in our study (129 to 2068 mmol.kg-1) is largely enough to cause disintegration of casein micelles by inducing solubilization of micellar calcium, and leading to aggregation of sub-micelles. These assumptions are confirmed by TEM images (**Figure 3**). According to **Unterhaslberger et al., (2006)** when low amount of NaCl are added (< 60 mmol.kg-1), insufficient number of chloride ions may screen the positive charge of protein so that the preferential hydration forces are prevalent. When higher amount of NaCl are considered, more chloride ions could bind to the protein surface so that the preferential hydration by water is overcome, leading to the observed salting-out effect. Another study **(Horne and Davidson, 1986)** 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 our case, TEM micrographs present interesting complementary information. But illustration of casein micelles by TEM is a real challenge because the fixation and staining steps can introduce artifacts **(McMahon, 1998; Auty et al., 2005; Marchin et al., 2007).** In addition, TEM observations include a dilution step and it was demonstrated that casein micelle may tend to be quite "unstable" at low concentrations **(Shukla et al., 2009).** Without salt, casein micelles seem to be compact and spherical with a size around 100-200 nm corresponding to the micelle size measured by DLS. When salt is added, the micelles changed in lumpy shaped, disintegrated, constellated aggregates of sub-micelles (around 20 nm). These sub-micelles could correspond in size to the second peak observed by DLS (with a Dh of about 20 to 30 nm).

Moreover, the ratio between the intensity of the micelle peak and sub-micelle peak tend to decrease significantly meaning an increase of sub-micelles with NaCl increase (**Table 1**). These results confirm previous studies in which small aggregates of casein molecules have been observed in addition to large micelles **(Chu et al., 1995; Panouillé et al., 2004**). An hydrodynamic radius of about 20 nm was also observed in bovine casein systems and in the presence of monovalent salt **(HadjSadok et al., 2008; Panouillé et al., 2004**).

In that case, an additional population of larger particles with a hydrodynamic diameter between 100 nm and 200 nm was observed that strongly correlates with our results. Aggregates of similar size are also found in pure κ-casein and β-casein solutions **(Rollema, 1992; Morris, 2002)** and these structures were named as minimicelles (20 nm in diameter) **(Müller-Buschbaum et al., 2007).** On **Figure 3A**, the micellar surface may suggest that the interior of the micelle could be readily accessible from its exterior. To summarize, TEM micrographs very correctly complete our findings of DLS, and depicts the morphological and structural modification of casein micelles and furthermore, appearance of sub-micelles in NaCl medium.

Another evidence may be proposed in relation with the diminution of protein percentage in the ultracentrifugal supernatant (**Figure 4**). Indeed, in MC dispersions different types of macromolecules coexist with casein micelles: minimicelles, residual serum proteins, serum caseins, proteose-peptone, peptides **(Bouchoux et al., 2009**). In the absence of NaCl, supernatant of MC dispersions contain a small quantity of free proteins. This quantity is about 14% of total casein content and in agreement with **Philippe et al., (2005).** During NaCl increased, a part of serum caseins (mainly αs1- and ß-caseins) that are non bound into micelles and present in the supernatant may entry into the porous structure of micelle casein. This decrease in protein was already reported in some studies **(Le Ray., 1998; Philippe et al., (2005).**

Correlation between MC secondary structure, size and morphological descriptors. A correlation between the modification of MC secondary structures and a decreasing trend in percentage of supernatant protein was found. Indeed, the strong decrease of proteins in the supernatant from 3% NaCl correlates quite well with the protein structural modification obtained by FTIR: ß-sheet decrease and irregular structure increase (around 3% salt). 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 (**Bouchoux et al., 2009; Stothart and Cebula, 1982).** 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 TEM micrographs and DLS). These sub-micelles partially constituted by serum casein may be moved from the ultracentrifuged supernatant to the ultracentrifuged pellet causing a decrease of nitrogen in supernatant.

The formation of casein micelle at two temperatures was already studied **(Curley et al., 1998).** At 37°C and with Ca^{2+}, the casein micelles were totally reformed and the Amide I was slightly moved to higher energies (greater wave numbers). On the opposite, at 15°C, the micelle formation was incomplete with a great number of sub-micelles and the Amide I was moved to lower energies. This shift was also observed with the same conditions (protein, temperature and salt environment). We may suppose that the modifications of secondary structures observed

during salt increased correspond to the disintegration of the micelle in submicelles. Concurrently, the relative number of free macromolecules (casein micelle/serum casein) may not be the same allowing the differences observed by Kjeldahl. Moreover, **Kumosinski et al., (1993)** reported a disruption of internal hydrogen bonds in Oxytocin by addition of 1 M NaCl causing a β-sheet decrease and thus a predomination of denatured structure.

CONCLUSION

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 well correlated with TEM observations. 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. The protein repartition in pellet and supernatant after ultracentrifugation yielded more support on the migration of serum casein and/or submicelles in the micelle. For the first time, the Amide III band was compared to the Amide I band in whole caseins.

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.Further investigation will be required to determine more precisely the percentages of the different casein molecules (αs1-, αs2-, β- and κ-casein) in the ultracentrifuge supernatant under NaCl increased. In addition it might be interesting to study more deeply the secondary structural assignments of micellar casein under salt environment. This work will be carried out soon via Circular Dichroism.

Caractérisation multi-échelle des isolats de protéines solubles dans des environnements elevés NaCl

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Résumé

L'objectif de cette étude est d'examiner l'influence de la concentration élevée en sel sur la structure secondaire et l'hydrophobicité de dispensions d'isolats de protéines solubles. Les dispersions ont été étudiées à des concentrations constantes en protéines (5% p/v) dans un environnement en NaCl allant de 0% à 6%.

Les dispensions protéiques sont caractérisées par leur couleur, leur hydrophobicité de surface. En plus, les changements dans la structure secondaire sous l'influence des concentrations croissantes en sel ont été suivis par spectroscopie infra rouge (FTIR).

L'estimation des éléments de la structure secondaire est corrélée aux résultats obtenus à partir de l'amide I. En augmentant les proportions en sel, aucune différence dans la structure secondaire n'a été constatée à l'exception de l'hydrophobicité de surface liée au nombre de sites hydrophobes disponibles. En parallèle, des différences de couleur (ΔE) relatives à l'évolution des dispersions protéiques contenant des concentrations variables en NaCl a augmenté d'une manière significative.

Multiscale Characterization of Whey Protein Isolates Under High NaCl Environments

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ABSTRACT:

The objective of this study was to examine the influence of high concentration of NaCl on the secondary structure, hydrophobicity and opacity of Whey Protein Isolates (WPI) dispersions. Dispersions were studied at a constant protein concentration (5%) in high NaCl environments (from 0 to 6%). Protein dispersions were characterized as to their color and protein surface hydrophobicity. Additionally, changes in secondary structures upon salt increase were followed by Fourier Transform Infrared Spectroscopy (FTIR). The estimations of secondary structural elements from Amide III assignments were correlated to results obtained from Amide I. Upon salt increase, no differences in secondary structure were observed whereas the protein surface hydrophobicity related to the number of hydrophobic sites available decreased. Concurrently, color differences (ΔE) related to evolution of the protein dispersions containing variable NaCl concentration increased significantly.

RESULTS

Determination of secondary structure of protein dispersions in variable NaCl environments

***Amide I and III shape*. Figure 1(A)** shows an example of whey spectra in water for Amide I. An absorbance maxima was observed at 1648 cm^{-1} with a slight shoulder at 1653 cm^{-1}, indicating a predominantly β-sheet structure with presence of α-helix **(Hamada et al., 1995; Dong et al., 1998)**. **Figure 1(B)** shows the Amide III spectra of whey protein in water. Similar characteristics were observed with a maxima observed at 1243 cm^{-1} indicating the predominance of β-sheet structure. A second peak was observed at 1315 cm^{-1} signalling the presence of α-helix.

***Curve fitting and peak assignments*.** Second derivative spectra were calculated from Amide I and amide III bands. The frequencies of the bands obtained were used to define peaks positions during the curve fitting procedure. During the iteration process, the peaks positions were allow to shift. Anyway the positions were only slightly changed (less than 1.5 cm^{-1}). Optimal fits were obtained when the root mean square was in the range of 10^{-4}. **Figures 1(A) and 1(B)** present the Amide I and Amide III regions fitted with a Gaussian line shape function. Peak assignments of Amide I region (**Fig. 1A**) were made using the results of former studies collected for globular proteins **(Byler and Susi., 1986).** The following structures were assigned: 1700-1660 cm^{-1}, turns; 1652-1648 cm^{-1}, α-helix; 1648-1642 cm^{-1}, unordered; and 1640-1620 cm^{-1}, ß-sheet. The same procedure was followed for the Amide III region (**Fig. 1B**). Tentative assignments of Amide III were also already done following **Cai and Singh (1999).** : 1330-1295 cm^{-1}, α-helix; 1295-1270 cm^{-1}, ß-turns; 1270-1250 cm^{-1}, unordered; and 1250-1220 cm^{-1}, ß-sheet.

Quantitative analysis. Areas calculated from the fitted spectra are considered to be proportional to the conformational elements that were assigned and are presented in **Table 1**. The Amide I band was deconvoluted in nine components (**Fig. 1A**). The β-sheet structures were related to three bands. The band at 1634 cm^{-1} is known to be strongly characteristic of amide groups involved in the extended β-sheet structure **(Byler and Susi., 1986)** while bands at 1616 and 1627 cm^{-1} exhibited low frequency β-sheets. The low frequency band 1616 cm^{-1} may be caused by aromatic side chains of the individual amino acids **(Susi and Byler., 1988)** and do not well contribute to the secondary structure. The band at 1627 cm^{-1} is also assigned to β-sheet structure and as evidenced by its low wave numbers position was assigned to the formation of intermolecular β-sheet structure. The unordered structures were assigned to two bands (1641 and 1648 cm^{-1}) and α-helix structures to the 1656 cm^{-1} band. Finally, the presence of 1666, 1681 and 1692 cm^{-1} bands was related to β-turn structures.

The Amide III band was also deconvoluted in nine components (**Fig. 1B)**. The maximum absorbance for β-sheets (four bands) and α-helix (three bands) is around 1241 cm^{-1} and 1324 cm^{-1} respectively. The wavenumbers at 1261 and 1284 cm^{-1} show unordered and β-turn structures.

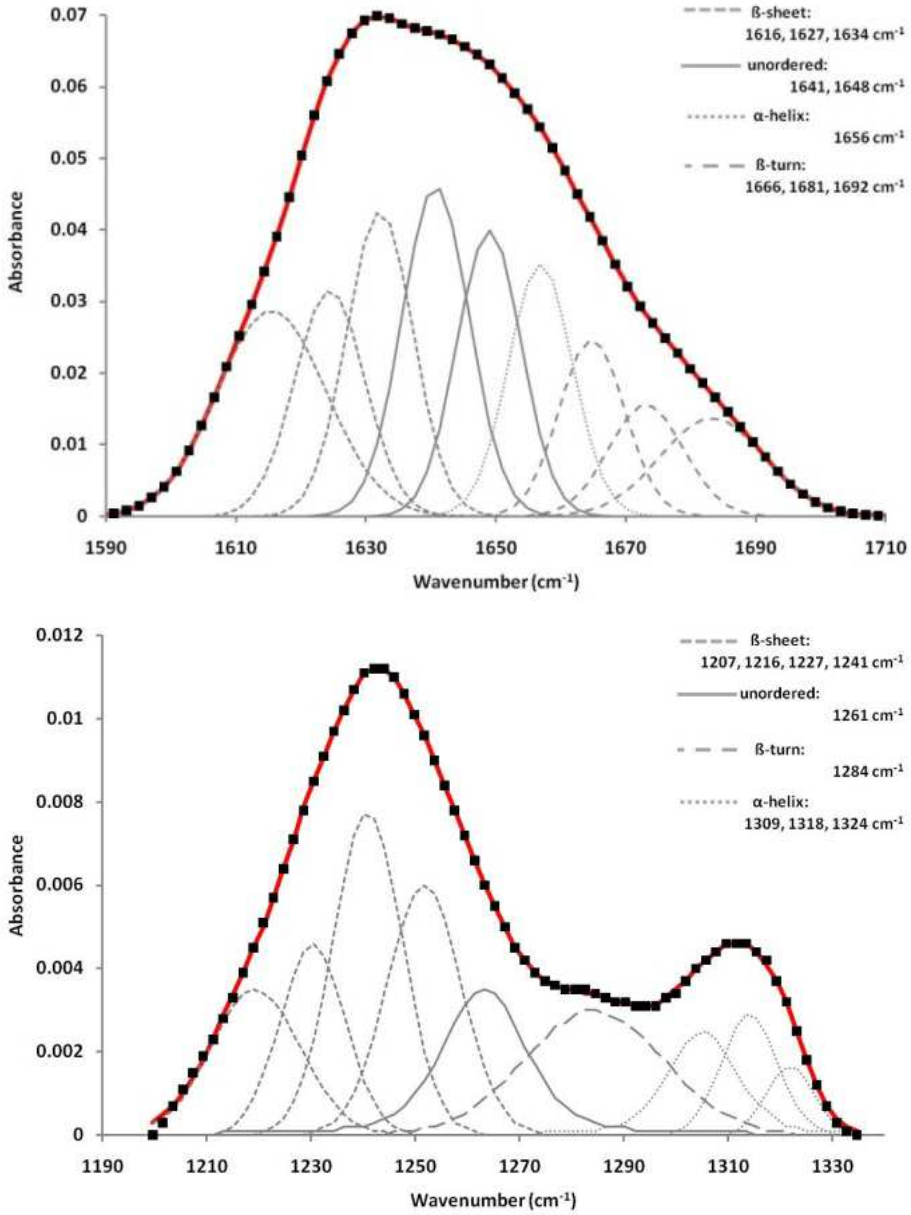

Fig. 1 Example of FTIR spectra obtained for whey proteins dispersion in water (5% wt). Amide I (A) and Amide III (B) regions were fitted at peak positions obtained from second-derivative spectra followed by Gaussian curve fitting. Black dots represent experimental data; red curves represent the sum of the individual Gaussian components (grey curves).

From Amide I and III, no significant differences in structural percentages were noticed upon NaCl increase (**Table 1**). Nevertheless, differences were observed between Amide I and III results for the same salt concentration. For example in water, the distribution in α-helix, ß-sheet, ß-turn and unordered structures obtained are respectively around 13, 46, 21, 19 % for Amide I

and 17, 48, 16, 18 % for Amide III. Nevertheless, these results were consistent with those found in literature **(Kumosinski and Farrell., 1993; Dong et al., 1998).**

Table 1. Structural component (ß-sheet, unordered, α-helix and ß-turn) percentage areas obtained for native whey proteins dispersions in different NaCl environments measured from Amide I and Amide III (mean of three independent analyses).

	Structural component (%)	NaCl concentration (%)								
		0	0.75	1.5	2.25	3	3.75	4.5	5.25	6
from Amide I	ß-sheet	46.2±1.2	46.1±1.0	45.9±0.9	45.7±1.5	45.9±2.1	46.0±1.7	45.7±1.5	45.4±2.1	46.1±1.4
	unordered	19.4±0.8	19.2±1.3	19.2±1.3	18.3±1.2	19.0±1.7	19.2±1.5	18.7±1.2	19.2±2.1	19.2±2.2
	α-helix	13.1±0.9	13.1±1.5	13.1±0.9	13.2±1.1	13.1±1.8	13.2±1.4	13.2±1.3	12.7±1.8	12.8±2.1
	ß-turn	21.3±1.5	21.5±1.5	21.8±1.0	22.7±0.6	22.0±1.6	21.7±1.0	22.4±1.4	22.7±1.8	21.9±1.9
from Amide III	ß-sheet	48.3±2.2	49.2±1.1	50.8±1.1	50.4±1.0	50.4±1.1	51.8±0.8	49.3±1.5	55.7±1.3	54.2±1.4
	unordered	18.1±1.2	17.0±1.0	15.8±1.4	15.0±1.1	15.1±0.9	15.1±1.0	16.6±1.1	12.3±1.2	13.3±0.4
	α-helix	17.2±1.1	18.1±0.8	16.4±0.8	17.8±0.8	18.2±0.9	16.7±0.9	16.0±1.3	17.0±0.5	17.2±0.7
	ß-turn	16.4±07	15.6±0.7	17.0±0.7	16.8±0.7	16.3±0.7	16.4±1.2	18.1±0.3	16.0±0.4	15.3±0.5

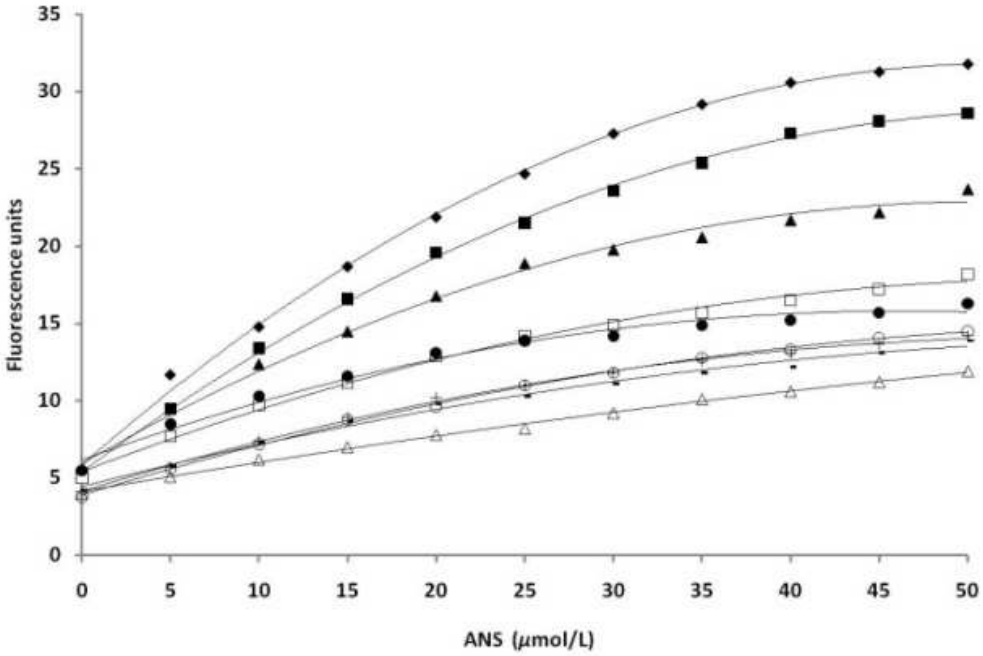

Fig 2. Spectrofluorimetric titration curves of whey proteins dispersions in variable NaCl concentrations: 0% (♦), 0.75% (■), 1.50% (▲), 2.25% (□), 3% (●), 3.75% (○), 4.50% (+) 5.25% (-) and 6% ( ). △

Determination of protein surface hydrophobicity in variable NaCl environments

Fig. 2 shows a representative set of titration curves obtained from WPI dispersions in variable NaCl solutions. Analyses of binding data allow the determination of values of F_{max} at saturating ANS concentration. F_{max} is determined by the asymptotic value of the titration curve. The K_d of the fluorescent ANS-protein complex is determined as the ANS concentration required to obtain half the value of F_{max}. From these two values, the PSH was calculated and was related

to the average stinginess of binding the fluorescent marker to available hydrophobic sites of protein **(Moro et al., 2001; Schmitt et al., 2009).**

The dependence of PSH upon variable NaCl concentrations is reported in **Fig. 3**. When increasing the NaCl concentration, the results are indicative of a decreasing trend of PSH values. The maximum value of PSH was observed around 1.6 (for 0% NaCl) and the lowest around 0.3 at 6% NaCl. This trend is related to a decreasing protein surface hydrophobicity.

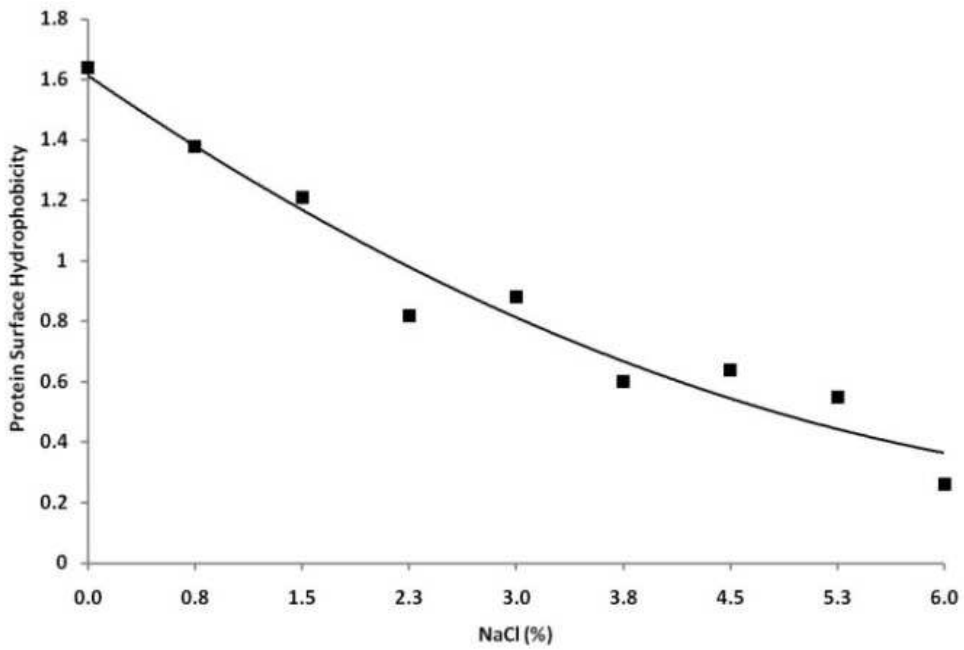

Fig 3: Protein surface hydrophobicity as a function of whey proteins dispersions in variable NaCl concentrations (from 0 to 6%)

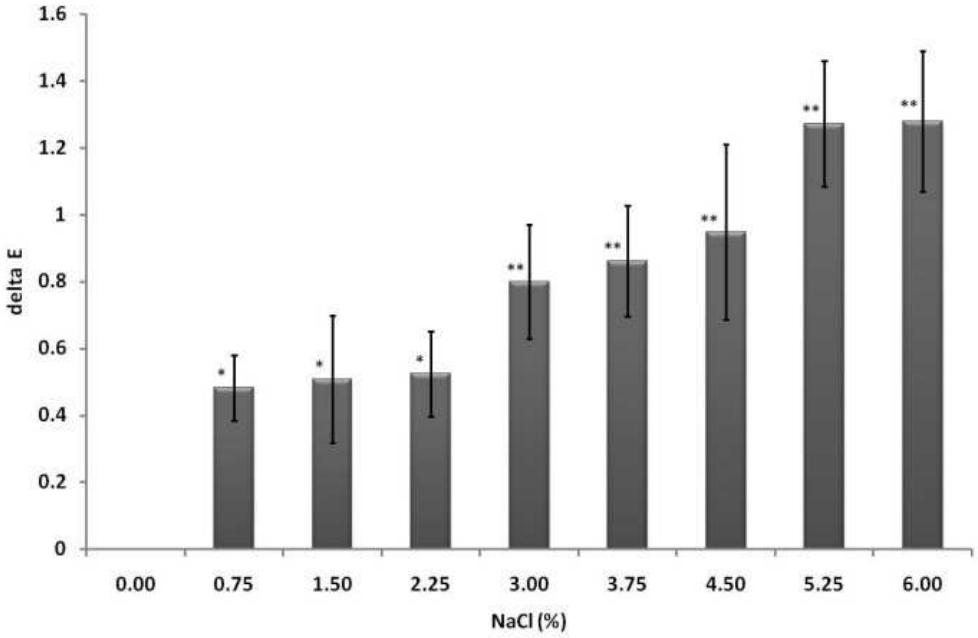

Fig 4: Colour differences (ΔE) evolution of the protein dispersions containing variable NaCl concentrations (mean of three independent analyses). Comparisons were performed between ΔE in water and in NaCl solutions $^{**}P < 0.01$, $^{*}P < 0.05$ and $^{ns}P > 0.05$.

Determination of Protein Opacity in variable NaCl environments

Color differences (ΔE) of the variable dispersions are presented in **Fig. 4**. A decrease in L*, an increase in b*, and no significant changes in a* values were noticed with salt increased (data not shown). These three colorimetric parameters represent measures of lightness, redness and yellowness respectively. From these parameters, ΔE was calculated from equation 1 as described in chapter 3 section 3.2.1 of this thesis. Significant variations were observed when comparing the color of the dispersion in water with the color of dispersions with salt increase (**Fig. 4**). The ΔE was significantly augmented from 0 to 1.6. At high salt concentration (6% or 1034 mol.kg^{-1}), the ΔE was not increased anymore. This stabilization may suggest that the whey proteins aggregates have attained a constant light-scattering structure in which particles have dimensions in several micrometers.

DISCUSSION

Secondary structure of whey proteins isolates with salt augmentation

Up to now, amide I spectral region was the most commonly used band because of its very strong signal but some recent results indicate that amide III region gives a better prediction for protein secondary structures than amide I **(Cai and Singh., 1999; Hussain et al., 2011)**. This is because amide I band suffers from an unstructured spectral contour and an overlap of the random coil and α-helix bands **(Cai and Singh., 1999)**. In this work, the distributions of components within classes of structures are reproducible and the tendencies are exactly the same whatever the band analyzed. At room temperature and pH between 6.5 to 6.7, the secondary

structure of whey proteins was not appreciably altered by NaCl (129 to 2068 mmol.kg^{-1}). Similar effect of NaCl on β-La secondary structure has also been reported **(Verheul et al., 1998).**

Surface hydrophobicity and opacity of whey proteins isolates with salt augmentation

A significant decrease in the PSH with NaCl addition was observed and related to a lower availability of hydrophobic groups certainly due to the formation of large aggregates. The evolution of PSH ratio with salt increase was characteristic of the presence of less hydrophobic sites available to ANS marker. This may be related to the fact that hydrophobic groups are buried inside the proteins native structure so with the salt induced aggregation it is possible that their access to ANS probe is limited **(Moro et al., 2001)**. Another explanation may be an interaction between hydrophobic sites of the proteins during salt aggregation and a reduction of the number of available ANS binding sites **(Schmitt et al., 2007)**. Concurrently, the formation of aggregates is responsible of the ΔE increased due to more light scattered.

CONCLUSION

This study revealed structural details of whey proteins dispersions upon high NaCl environments. Under NaCl increase, denser structures appeared. Concurrently, FTIR investigations did not highlight any redistribution of components areas. Even if the secondary structure was not influenced by the environment, differences were observed concerning the protein surface hydrophobicity and opacity. Nevertheless, further research is required to evaluate how the combined effect of heat and ionic strength could improve the functional properties of whey protein dispersions used as structuring agents.

Caractérisation morphologique des agrégats des protéines sériques induits par l'environnement salin

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Résumé

Les sels monovalents comme le NaCl sont connus pour leur rôle dans la modification de la structure des protéines laitières. Dans cette étude, des dispersions de protéines solubles sont étudiées à des concentrations constantes en protéines (5% p /v) et à des concentrations variables en sel (0% à 6%).

La morphologie et la taille des protéines et/ou aggrégats sont caractérisés par microscopie électronique à transmission et diffusion dynamique de lumière. Dans de l'eau, les images de TEM ont révélé que les agrégats de protéines étaient de forme sphérique avec deux populations d'environ 6 et 70 nm. La taille de ces édifices a été confirmée par diffusion dynamique et statique de la lumière.

L'ajout de sel dans le milieu de réhydratation conduit à une agrégation des protéines et à la formation des grumeaux plus denses observés par les deux techniques. Ces résultats ont une importance sur le plan technologique et industriel.

Morphological characterization of salt induced whey protein aggregated species

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ABSTRACT :

Monovalent salts like NaCl have characteristic role in modifying the structure of milk proteins. In this study, whey protein isolate (**WPI**) dispersions were studied at a constant protein concentration (5%) but in varying NaCl concentrations (from 0 to 6%). Dispersions were characterized as to their morphology and size using Transmission Electron Microscopy (**TEM**) and light scatterings techniques respectively. TEM images revealed that protein aggregates were spherical in shape with two populations around 6 and 70 nm. The size of these edifices was further confirmed by Dynamic Light Scattering (**DLS**) and static light scattering **(SLS)**. Salt addition resulted in an aggregation of the proteins and the formation of clumpy denser aggregates as observed by both techniques. These results have a strong technological and industrial importance regarding rehydration and structure dependent functionalities of whey proteins at multiscale level.

RESULTS

Morphological characterizations were done by TEM with negative staining (**Fig. 1)**. For WPI dispersions in water, well separated roughly spherical nanoparticles structures presenting a particle size below 70 nm are observed. At a relatively low salt concentration of 0.75% (129 mmol.kg^{-1}), small clumpy aggregates are noticed in addition to the well separated spherical nanoparticles. When the NaCl concentration was increased up to 1.5 and 2.25% (258 and 387 mmol.kg^{-1}), the aggregates appeared to be denser and larger in size. Larger size aggregates were formed. Finally, particles aggregation clearly appeared in higher ionic environments of 3 to 6% (517 to 1034 mmol.kg^{-1}). A change in the structure of protein aggregates was observed; the aggregates being bigger and denser.

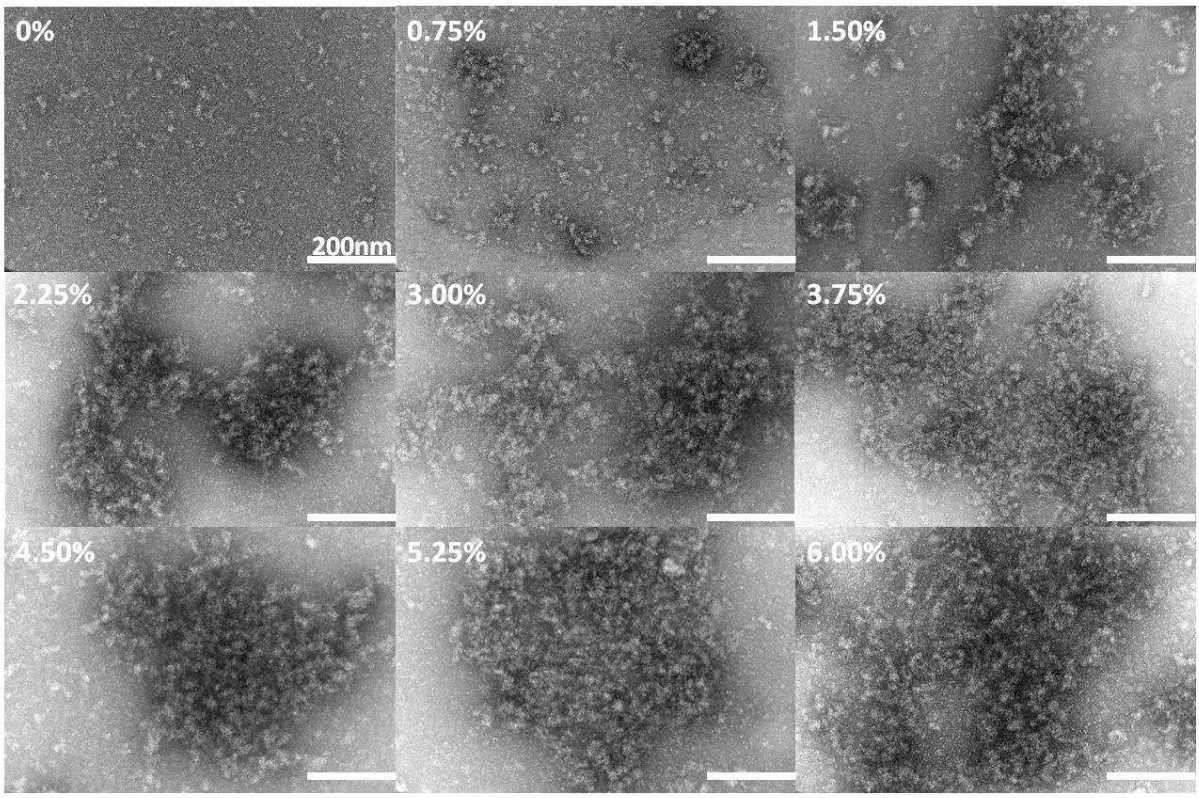

Figure 1. Transmission electron micrographs of whey proteins dispersions in variable NaCl environments ranging from 0 to 6%. Imaging was performed at 50.000x magnification (scale bar = 200 nm). Each image is representative of at least 6 images observed.

Particles sizes were determined by DLS and/or SLS in order to measure the hydrodynamic and/or particles diameters (**Table 1**). The distributions obtained in water were bimodal. Indeed, two populations were systematically observed. The first peak at 6 nm was significantly less represented than the second peak at 69 nm. Similar size was observed by the analysis of a significant number (more than 10 images) of particles from TEM images (≈ 70 nm). For 0.75 % (129 mmol.kg^{-1}) salt, similar hydrodynamic diameters were measured. Nevertheless, the 2nd peak (74 nm) was more important and represented more than 90% of the total particles. For

1.5 % (258 mmol.kg^{-1}), salt, a combination of two techniques (DLS and SLS) were used. The choice of the technique depends mainly on the particle size range. Aggregates larger than 500 nm could not be correctly measured by DLS.

Table 1. Particles size (in nanometer) obtained with static light scattering (SLS), dynamic light scattering (DLS) and/or TEM images (mean of three independent measures).

	Hydrodynamic diameter determined with DLS			Particle diameter determined with SLS		Particle diameters from TEM images[1]
NaCl (%)	**1st peak (nm)**	**2nd peak (nm)**	**3rd peak (nm)**	**1st peak (nm)**	**2nd peak (nm)**	
0.00	5.8 ± 0.2	69.4 ± 5.9	-			≈ 70
0.75	6.9 ± 1.6	73.9 ± 21.1	-			≈ 100
1.50	-	52.6 ± 16.3	316.7 ± 11.3	420 ± 8	35560 ± 10	≈ 480
2.25				420 ± 6	35560 ± 13	> 720
3.00				420 ± 5	35560 ± 5	>1000
3.75				420 ± 5	35560 ± 21	>1000
4.50				360 ± 26	35560 ± 2	>1000
5.25				420 ± 5	41430 ± 23	>1000
6.00				420 ± 4	41430 ± 26	>1000

[1] mean of at least 10 particles observed in three different TEM images.
grey cases: not determined due to inadequate equipment

Conversely, aggregates under 200 nm were not correctly evaluated by SLS. As a consequence, the DLS technique was still possible for 1.5 % salt (258 mmol.kg^{-1}), but not for higher concentrations where SLS was more appropriated. At 1.5 % salt (258 mmol.kg^{-1}), the smaller particles corresponding to individual proteins (around 6 nm) were not detected anymore. The intermediates particles around 70 nm were still observed and a third new peak was measured around 320 nm. Two populations were observed with SLS. The first one was similar to those found by DLS. The second one (around 35 µm) was related to important aggregates impossible to detect by DLS. Up to 2.25 % salt (387 mmol.kg^{-1}), similar particle sizes were found by SLS. The first population was related to small aggregates (420 nm) already noticed for lower salt concentrations. The second and most important population was related to large aggregates (around 35 µm).

DISCUSSION

In water. Concomitant observations were obtained from TEM and size analyses. Well separated roughly spherical nanoparticles (around 70 nm) were observed by TEM and also measured by DLS. Another population was also found by DLS with a hydrodynamic diameter around 6 nm. These small particles were not visible on TEM images certainly due to the magnification used and may correspond to individual whey proteins. These two hydrodynamic diameters are in agreement with values determined by **(Martinez et al., 2009)** and **(Huppertz, 2007)** for β-Lg alone (corresponding to the first peak) and whey proteins (corresponding to the second peak). The lack of aggregation without salt could be related to the presence of substantial negative charge. Hence, strong electrostatic repulsions between molecules prevent them from coming into close proximity as reported by **Baussay et al. (2004)** for β-Lg.

In low NaCl environment (< 200 mmol.kg^{-1}). Low salt concentrations, around 0.75% (129 mmol.kg^{-1}), resulted in the formation of small clumpy aggregates (TEM). Nevertheless, the hydrodynamic diameters were not significantly different to those found in water. These discordances may be correlated to the important standard deviation found for the hydrodynamic diameter and to the different dilutions performed for each experiment (TEM or DLS analyses). Aggregation is known to be strongly influenced by attractive and repulsive forces. The major forces favoring protein aggregation are hydrophobic and van der Walls forces; whereas electrostatic and hydration repulsions oppose this phenomenon. It seems that WPI started to aggregate above a critical salt concentration.

According to **Unterhaslberger et al. (2006)** when low amounts of NaCl are added (< 60 mmol.kg^{-1} or 0.33%), insufficient number of chloride ions may screen the positive charge of protein so that the preferential hydration forces are prevalent. In our case (129 mmol.kg^{-1} or 0.75%), the electrostatic repulsion between proteins molecules may be screened sufficiently, more chloride ions could bind to the protein surface so that the preferential hydration by water is overcome. This may enable the protein molecules to approach together closely enough to aggregate.

*In high NaCl environment (> 200 mmol.kg^{-1}).*Increasing NaCl up to 400 mmol.kg^{-1} or 2.3 % resulted in an acceleration of WPI aggregation and the formation of denser aggregates. DLS and SLS measurements were in agreement with TEM images and the presence of aggregates were detected. At higher salt concentrations, from 3 to 6% salt (517-1034 mol.kg^{-1}), the electrostatic repulsions between protein molecules were screened more efficiently i.e., the solution ionic strength is high enough to screen electrostatic interactions. These networks consisted of aggregated protein particles that are typically between 0.1 µm and 10 µm in diameter, hence forming a large number of aggregated protein particles within each particle,

associated into particulate structures that have relatively large sizes **(Barbut, 1995; Verheul and Roefs., 1998; Verheul et al., 1998).**

It's worth noting that contrast between particles and background in the TEM images was higher at these concentrations depicting a denser structure of WPI aggregates. Finally, these results were in agreement with those reported by **Pouzot et al. (2004)** and **Schmitt et al. (2009)** for β-Lg alone. Local densification of the protein aggregates was depicted and significant structural changes **(Schmitt et al., 2007)** related to augmentation of protein aggregates size in salt aqueous environment were observed.

CONCLUSION

This study revealed structural detailed characterization of whey proteins dispersions upon high NaCl environments. These dispersions in water were composed of well separated proteins with a diameter around 70 nm well correlated with TEM observations. Moreover, aggregates with different morphologies may be produced for different applications in protein based foods by proper selection of the process conditions. In addition, it might be interesting to study more deeply the effect of WPI protein concentration, salt content/type, and protein composition (e.g., presence of α-lactalbumin or bovine serum albumin) on physicochemical properties in order to approach conditions that might correspond to commercial whey protein isolates. Finally, further work will be carried out to investigate the functionality of the whey protein isolates under combined effect of heat and salt variables that could be used as building blocks for the self assembly of biomaterials while studies are still in progress to reveal the impact of this morphological behavior of salt induced aggregates on secondary or tertiary structure of protein and will be discussed in a separate article.

Chapitre IV. III

Propriétés fonctionnelles des solutions protéiques réhydratées

Propriétés fonctionnelles de dispersions protéiques laitières: effet combiné de l'environnement ionique et de la température

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Résumé

Des isolats de protéines solubles à 5% P/V (pH=6,5), avec plus au moins de NaCl (de 0 à 500 mmol.kg^{-1}) sont soumis à un traitement thermique dynamique (rampes de température allant de 30 à 90°C). Les propriétés rhéologiques, la taille des protéines, la morphologie, la dénaturation thermique et l'hydrophobicité de surface des dispersions protéiques ont été caractérisés.

A une faible force ionique (< 200 mmol.kg^{-1}), le gel obtenu devient élastique et atteint une forte rigidité. Une concentration en sel supérieure conduit à la formation d'un gel plus faible. Aucun gel n'a été formé en absence de sel. L'autre paramètre influencé par la force ionique est la température de gélification. Ainsi l'accroissement de la température de dénaturation et la stabilité thermique ont été observés par calorimétrie différentielle à balayage (DSC).

En outre, le traitement thermique et l'augmentation de la concentration en sel provoque des changements dans la structure secondaire des protéines. Ces changements ont été suivis par spectroscopie infra rouge (FTIR). Les éléments de structure secondaire obtenus à partir de la bande amide I ont été corrélés avec ceux de la bande Amide III. En absence de chauffage, aucune différence dans la structure secondaire n'a été constatée lors de chaque augmentation en sel, alors que lors du chauffage seul, les données FTIR ont révélé une augmentation des feuillets ß intermoléculaire au détriment des structures désordonnées et des coudes sans que les hélices α ne soient affectées.

Cependant, l'addition de NaCl ainsi que le traitement thermique dynamique des dispersions ont montré un effet stabilisateur sur les éléments de structure secondaire des bandes amide I et III. Les dispersions des protéiques dans l'eau ont également été caractérisées par microscopie électronique en transmission par une forme sphérique avec deux populations (6 et 70 nm). L'augmentation du pourcentage en sel seul abouti à la formation d'agrégats plus denses. Sous l'effet de la combinaison sel/chaleur, une transition à partir d'agrégats protéiques sphériques / agrégats des protéines compact aux formes linéaires a été observée. La taille importante de ces édifices a été confirmée par des techniques microscopiques et par diffusion dynamique de la lumière.

WHEY PROTEINS UNDER HEAT TREATMENTAND SALT

Combined Effect of Heat Treatment and Ionic Strength on the Functionality of Whey Proteins

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ABSTRACT

A 5% w/v whey proteins isolate (**WPI**) dispersion (pH 6.5), with more or less NaCl was submitted to dynamic heat treatment. Protein dispersions were characterized as to their rheological properties, particles size, morphology, denaturation temperatures and protein surface hydrophobicity. At low ionic strength (< 200 $mmol.kg^{-1}$), gel elastic modulus increased and strongest gel stiffness was achieved. Higher salt concentrations lead to a weaker gel while no gels at all were formed without salt. The gelation temperature was also influenced by ionic strength and an increase in denaturation temperature and thermal stability was also observed by using Differential Scanning Calorimetry (**DSC**). Additionally, heat induced changes in secondary structures upon salt augmentation were followed by Fourier Transform Infrared Spectroscopy (**FTIR**). Secondary structural elements estimations obtained from Amide I assignments were correlated with those from Amide III. Upon salt increase, no differences in secondary structure were observed without heating whereas upon heating and without salt increase, the FTIR data revealed an increase in intermolecular β-sheets at the cost of β-turns and random coils with no change in α-helical structures. However, NaCl addition along with dynamic heat treatment of WPI dispersion showed a stabilizing effect on the secondary structural elements of both Amide I and Amide III bands. WPI dispersions in water were also characterized by Transmission Electron Microscopy (**TEM**) by a spherical shape with two populations (6 and 70 nm). Salt increase alone resulted in the formation of denser aggregates whereas a transition from spherical/compact protein aggregates to linear ones was observed due to combined salt/heat effect. The important size of these edifices was confirmed by microscopy and light scattering techniques. Moreover, protein surface hydrophobicity (**PSH**) related to the number of hydrophobic sites available decreased significantly. Finally, experimental results demonstrated the strong interaction between ionic strength and dynamic thermal treatment on protein functional properties and their careful adjustment could enable food industry to effectively utilize WPI as gelling agent.

Key Words: whey protein, ionic strength, conformational change, temperature.

RESULTS

Particle Size of WPI Dispersions

Particles sizes were determined by DLS and/or SLS in order to measure the hydrodynamic and/or particles diameters **(Table 1).** The choice of the technique (DLS or SLS) depends mainly on the particle size range. Aggregates larger than 500 nm could not be correctly measured by DLS. Conversely, aggregates under 200 nm were not correctly evaluated by SLS. The distributions obtained in water at 30°C were bimodal. Indeed, two populations were systematically observed. The first peak at 6 nm was significantly less represented than the second peak at 69 nm. Similar size was also observed by the analysis of a significant number of particles from TEM images (≈ 70 nm). For 0.50 % salt (86 $mmol.kg^{-1}$), similar hydrodynamic diameters were measured. Nevertheless, the 2nd peak (72 nm) was more important and represented more than 90% of the total particles. Concurrently, the major 2nd peak exhibited a hydrodynamic diameter of 160 and 316 nm for 1% (172 $mmol.kg^{-1}$) and 1.5% salt (258 $mmol.kg^{-1}$) respectively. As a consequence, the DLS technique was still possible until 1.5% salt (258 $mmol.kg^{-1}$) but not for higher concentrations where SLS was more appropriated. For higher salt concentrations, similar particle sizes presenting two populations were found by SLS. The first population was related to small aggregates (420 nm) already noticed by DLS for lower salt concentrations. The second and most important population was related to large aggregates (around 35 µm) impossible to detect by DLS. These statements are reinforced with the images of TEM.

Particle sizes of heated WPI dispersions were also measured. Distributions were systematically monomodal whatever the ionic environment. In water, a hydrodynamic diameter of 263 nm was observed that corresponds fairly well with TEM images. With salt addition, a significant shift towards smaller sizes (from 246 to 85 nm) was observed showing a decreasing trend of hydrodynamic diameters after heating.

Table 1. Particles size (in nm) obtained by static light scattering (SLS), dynamic light scattering (DLS) and/or from TEM images analysis (mean of three independent measures).

	At 30°C					At 90°C	
	Hydrodynamic diameter (DLS)[1]		**Particle size (SLS)[1]**		**Particle size (TEM)[1,2]**	**Hydrodynamic diameter (DLS)[1]**	**Particle size (TEM)[1,2]**
NaCl (%)	1st peak (nm)	2nd peak (nm)	1st peak (nm)	2nd peak (nm)			
0	5.8 ± 0.2	69.4 ± 5.9			≈ 70	263 ± 1.5	≈ 255
0.50	11.3* ± 0.8	72.0^{ns} ± 2.6			≈ 85^{ns}	246^{ns} ± 1.9	≈ 228^{ns}
1.00	26.6** ± 3.8	161.4* ± 14.8			≈ 148**	182* ± 1.6*	≈ 115*
1.50	52.6***± 16.3	316.7** ± 11.3			≈ 480**	118** ± 2.7	≈ 100**
2.00			420***± 6	35560***± 13	≈ 660***	101** ± 1.3	≈ 100**
2.50			420*** ± 9	35560***± 7	>1000***	88** ± 6.2	≈ 100**
3.00			420*** ± 5	35560***± 5	>1000***	85** ± 8.8	≈ 100**

[1] DLS: Dynamic Light Scattering; SLS: Static Light Scattering; TEM: Transmission Electron Microscopy
[2] mean of at least 10 particles observed in three different TEM images.
grey cases: not determined due to equipment limitation
Comparison between whey dispersion in water (control) and other concentration: ***P < 0.001, **P < 0.01, *P < 0.05 and $_{ns}$P > 0.05.

Morphological characterization of WPI dispersions

The specific and combined effect of salt and heat on the morphology of WPI dispersions is highlighted in the TEM images presented in **Figures 1.**

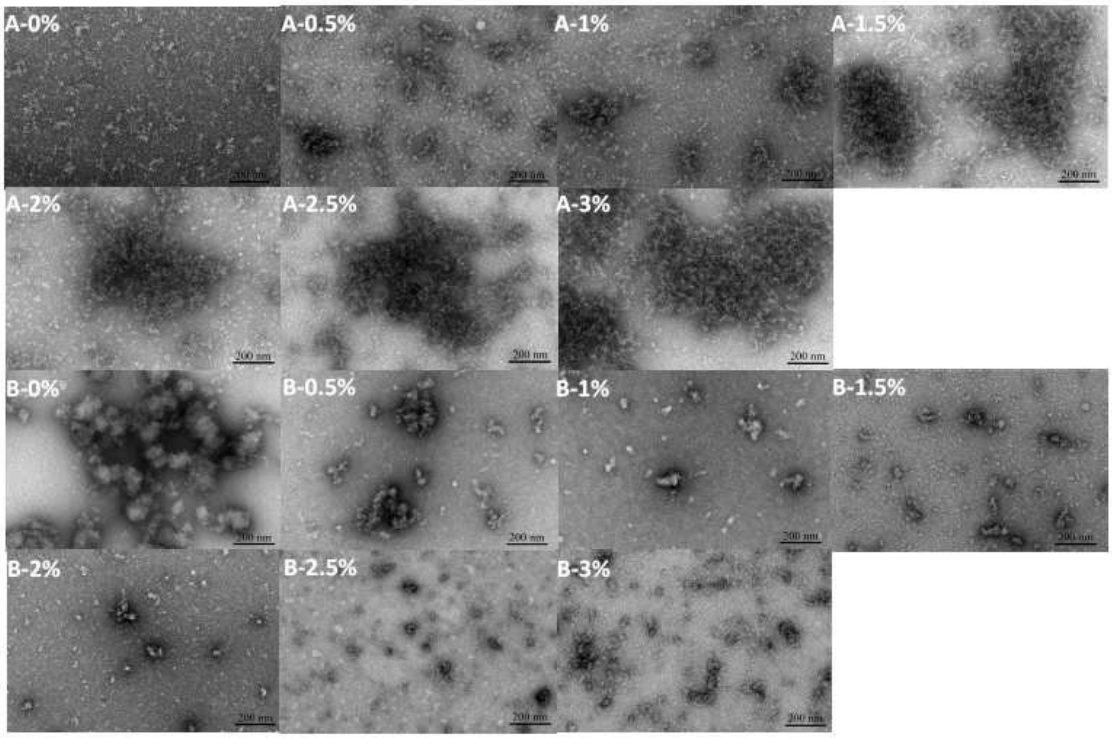

Figure 1. Transmission Electron Micrographs of WPI dispersions after heated at 30°C (A) and 90°C (B) and in variable NaCl environments (0; 0.5; 1; 1.5; 2; 2.5 and 3% salt). Imaging was performed at 24,000x magnification (scale bar = 200 nm). Each image is representative of at least 6 images observed.

Without salt and at 30°C, well separated roughly spherical nanoparticles structures presenting particle size below 70 nm was observed **(Fig 1A-0%)**. At relatively low salt concentration (86 mmol.kg^{-1}), small clumpy aggregates are noticed in addition to the well separated spherical nanoparticles **(Fig 1A-0.5%)**. When the NaCl concentration was increased up to 258 mmol.kg^{-1}, the aggregates were quite clearer, denser and larger **(Fig 1A-1% and 1A-1.5%)**. Finally, particles aggregation clearly appeared in higher ionic environments (344 to 517 mmol.kg^{-1}). A change in the structure of protein aggregates was observed; the aggregates being bigger and denser **(Fig 1A-2% to 1A-3%)**.

Figures 1B show the result heat (90°C) on WPI dispersions. Without adding salt, compact/spherical aggregates take the form of white masses due to the negative staining method employed (**Fig 1B-0%)**. Images also depict regions of excess stain originating from

negative staining preparation method. These structures present a particle size around 260 nm. With addition of NaCl at relatively low concentration (86 mmol.kg^{-1}) rather thinner and denser aggregates are observed (**Fig 1B-0.5%)**. Upon increasing salt concentration to 1.50% (258 mmol.kg^{-1}), these spherical aggregates tend to be smaller and well dispersed (**Fig 1B-1% and 1B-1.5%)**. Finally at higher environments of 2 to 3% (344 to 517 mmol.kg^{-}1), particles were characterized by a length less than 100 nm with a less numerous number and more irregular in form **(Fig 1A-2% to 1A-3%)**.

Rheological and Thermal Properties of WPI Dispersions

In **Figure 2**, WPI dispersions under variable NaCl ranges are plotted against the elastic modulus as a function of a heat ramp (30-90°C). A change in the visoelastic properties of the dispersions was observed when subjected to thermal treatment.

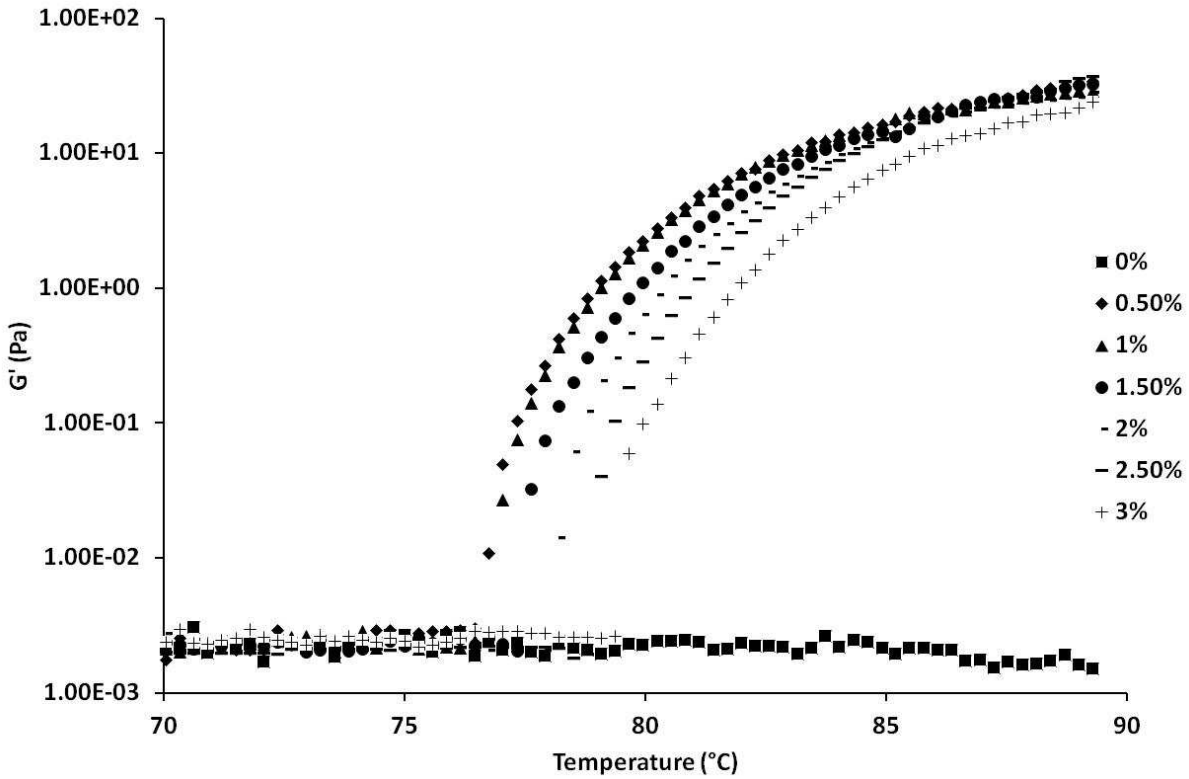

Figure 2. Determination of rheological behavior (G'- elastic component) of whey proteins dispersions in variable NaCl environments from 30 to 90°C. Each curve is representative of three independent experiments.

Without the addition of salt, the WPI dispersions showed non-gelling behavior, presenting low values of G' throughout heating and holding a translucent appearance. In the presence of NaCl, the values of G' increased strongly between 75 and 80°C, showing a visoelastic behavior and a plateau curve convincingly indicating an inflection point as temperature approached 88°C, demonstrating the formation of a continuous protein network.

Furthermore, with addition of 0.50% NaCl a sharp increase in modulus was observed at 76°C while this behavior was noted at 80°C for 3% NaCl showing a significant increase in the gelling temperature point.

Effect of NaCl addition on the thermal properties of WPI dispersion was examined by µDSC **(Figure 3)**. In addition, their corresponding values of denaturation temperature are shown in **Figure 4**.

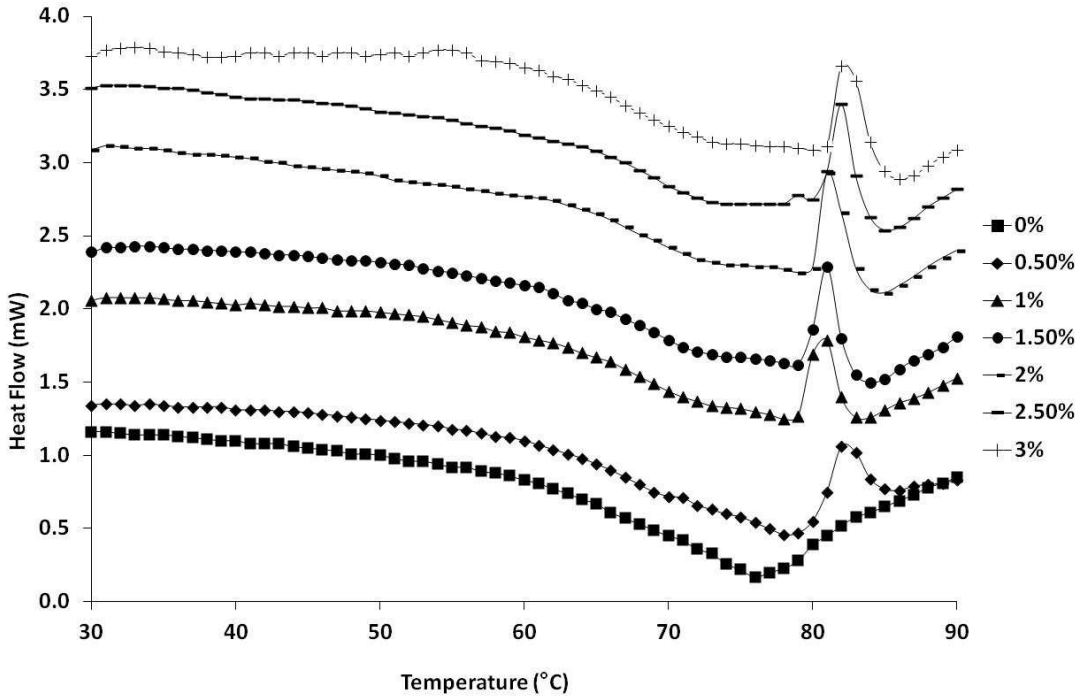

Figure 3. Thermograms of whey proteins dispersions in variable NaCl environments (from 0 to 3%). Each curve is representative of three independent experiments.

The endothermic transition peaks indicated that structural changes were associated with temperature. Denaturation temperature of WPI dispersion without salt was 76.4°C which shifted towards higher temperature 78.4°C at 0.50% salt. Increasing NaCl concentration to 3% enhanced the denaturation temperature up to 80.2°C.

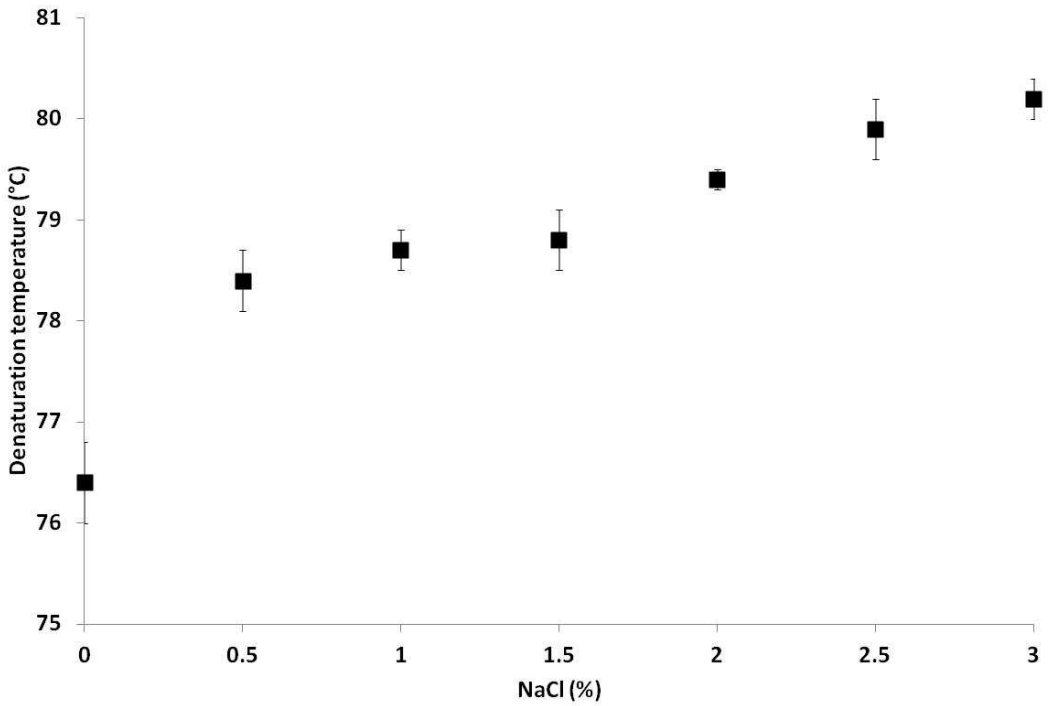

Figure 4. Denaturation temperature calculated from thermograms for WPI dispersions at different NaCl environments (mean of 3 experiments).

Protein Hydrophobicity

Analysis of the emission spectra (data not shown) of ANS-WPI dispersions in variable NaCl solutions with or without heating revealed a representative set of titration curves. Investigation of binding data allows the determination of values of F_{max} at saturating ANS concentration. F_{max} is determined by the asymptotic value of the titration curve. The K_d of the fluorescent ANS-protein complex is determined as the ANS concentration required to obtain half the value of F_{max}. From these two values, the PSH was calculated and indicates the binding average of the fluorescent marker to available hydrophobic sites of protein **(Schmitt et al., 2009)**. The dependence of PSH upon variable NaCl concentrations for heated and unheated samples is reported in **Figure 5.** When increasing the NaCl concentration and/or heat treatment, the results are indicative of a decreasing trend for PSH values. The maximum values of PSH were observed around 1.6 and 0.40 (0% NaCl) whereas the lowest were found around 0.8 and 0.17 (3% NaCl) for unheated and heated dispersions respectively.

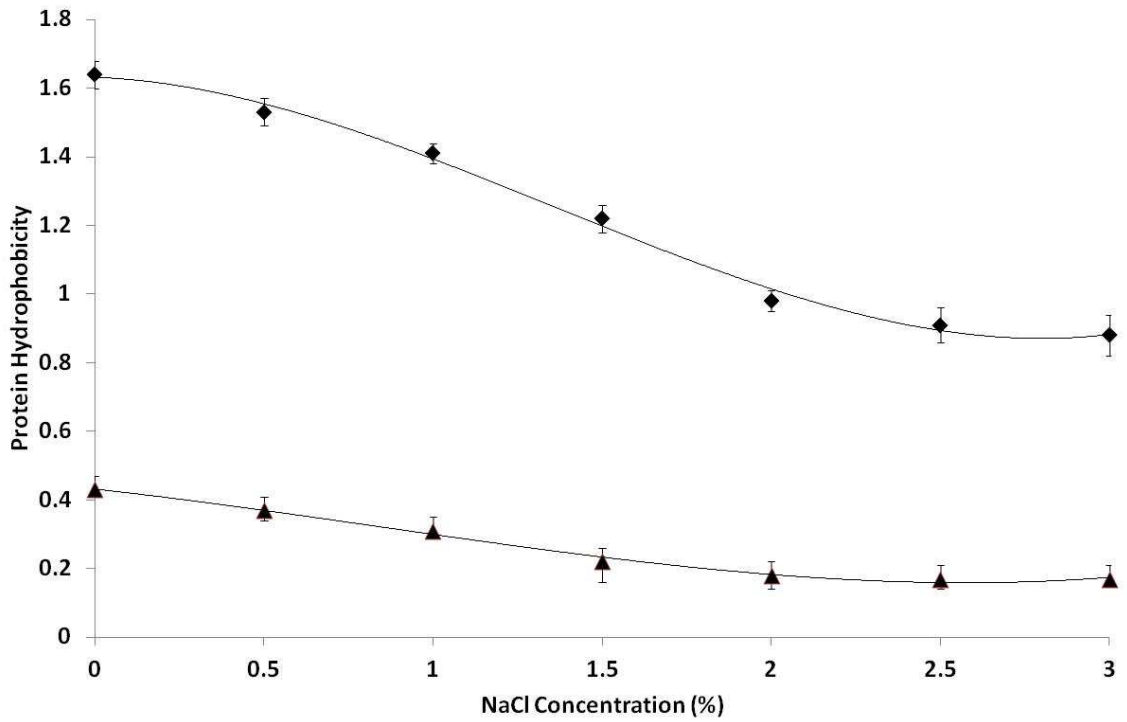

Figure 5. PSH as a function of NaCl concentrations for WPI dispersions at 30 (▲) and 90 °C (♦) (mean of three Independent analyses).

Secondary Structure of WPI dispersions

Curve fitting and peak assignments. Second derivative spectra were calculated from Amide I and amide III bands. The frequencies of the bands obtained were used to define peaks positions during the curve fitting procedure. During the iteration process, the peaks positions were allow to shift. Anyway the positions were only slightly changed (less than 1.5 cm^{-1}). Optimal fits were obtained when the root mean square was in the range of 10^{-4}. **Figures 6A** and **B** present an example of the Amide I and Amide III regions fitted with a Gaussian line shape function for WPI in water at 30°C. Peak assignments of Amide I region were made using the results of former studies collected for globular proteins **(Byler and Susi, 1986)**. The following structures were assigned: 1700-1660 cm^{-1}, turns; 1652-1648 cm^{-1}, α-helix; 1648-1642 cm^{-1}, unordered; and 1640-1620 cm^{-1}, ß-sheet. The same procedure was followed for the Amide III region. Tentative assignments of Amide III were also already done following **(Cai and Singh, 1999):** 1330-1295 cm^{-1}, α-helix; 1295-1270 cm^{-1}, ß-turns; 1270-1250 cm^{-1}, unordered; and 1250-1220 cm^{-1}, ß-sheet.

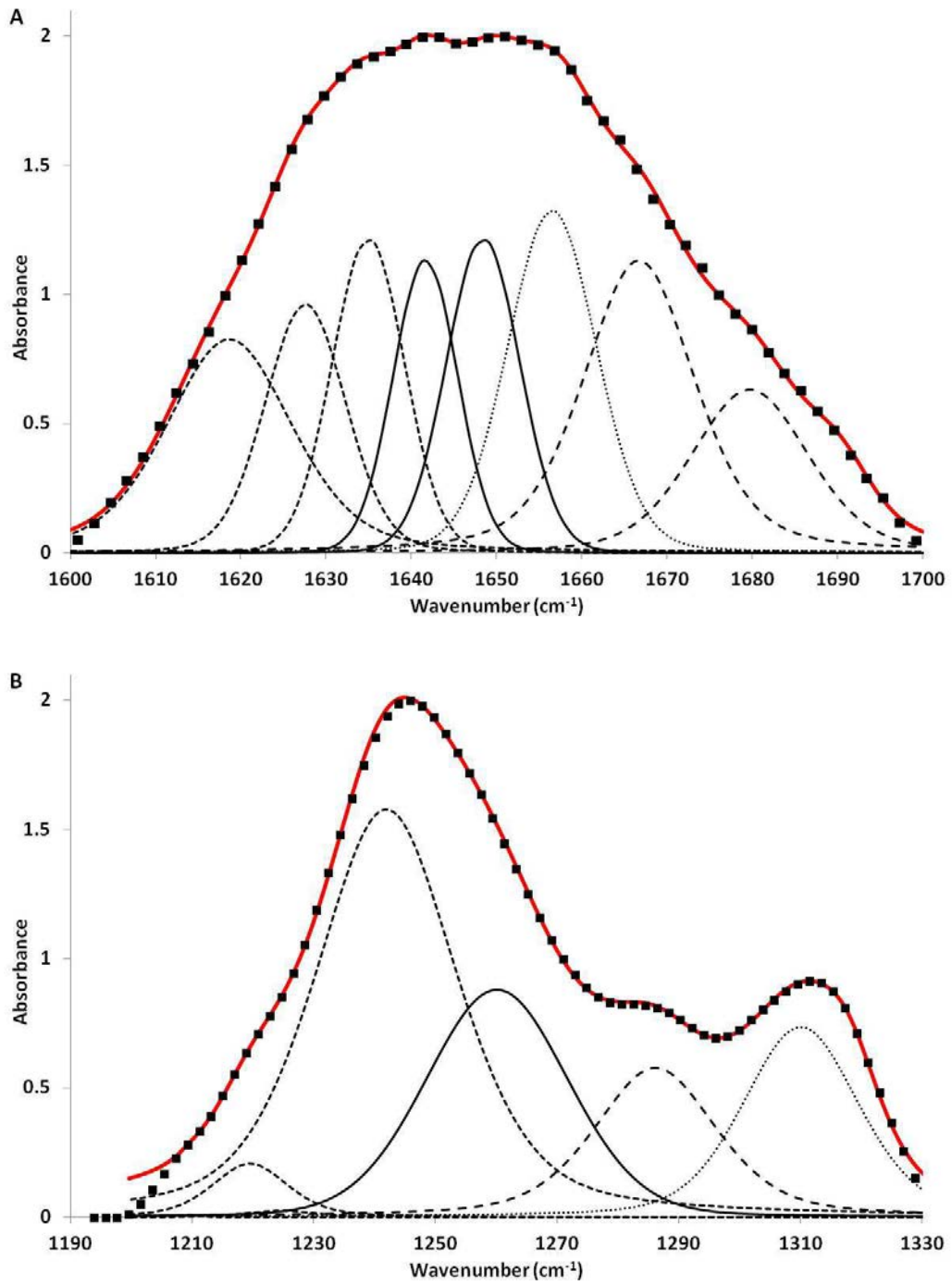

Figure 6. Example of FTIR spectra obtained for whey proteins dispersions in water at 90°C. Amide I (A) and Amide III (B) region were fitted at peak positions obtained from second-derivative spectra followed by Gaussian curve fitting. Black dots represent experimental data; red curves represent the sum of the individual Gaussian components (ß-sheet: ....., Unordered: ———, α-helix:..... and ß-turn: – – – –).

The Amide I band was deconvoluted in eight peaks (**Figure 6A**). The β-sheet structures were related to three bands. The band at 1634 cm^{-1} is known to be strongly characteristic of amide groups involved in the extended β-sheet structure **(Byler and Susi, 1986)** while bands at 1616 and 1626 cm^{-1} exhibited low frequency β-sheets. The low frequency band 1616 cm^{-1} may be caused by aromatic side chains (C=C stretching vibrations) of the individual amino acids **(Susi and Byler, 1988)** and do not well contribute to the secondary structure.

The relative intensity of band at 1634 cm^{-1} is the strongest even though α-helix/random coils and turns are detectable. The band at 1626 cm^{-1} is also assigned to β-sheet structure and as evidenced by its low wave numbers position was assigned to the formation of intermolecular β-sheet structure. The unordered structures were assigned to two bands (1641 and 1647 cm^{-1}) and α-helix structures to the 1655 cm^{-1} band. Finally, the presence of 1666 and 1681 bands were related to β-turn structures. The Amide III band was deconvoluted in five peaks (**Figure 6B**). The maximum absorbance for β-sheets (two bands) and α-helix (one band) is around 1241 cm^{-1} and 1318 cm^{-1} respectively. The wavenumbers at 1261 and 1284 cm^{-1} show unordered and β-turn structures.

Quantitative analysis. Areas calculated from the fitted spectra are considered to be proportional to the conformational elements that were assigned and are presented in **Table 2**. From Amide I and III, no significant differences in structural percentages were noticed during NaCl increase for WPI dispersions without heating. Nevertheless, differences were observed between Amide I and III results upon heating for the same salt concentration. For example, in water, the distribution in α-helix, ß-sheet, ß-turn and unordered structures obtained are around 22, 40, 16, 22% at 30°C and 20, 62, 7, 11% at 90°C for Amide I. For Amide III, the percentages obtained are 18, 45, 15, 23% and 13, 71, 5, 10% for 30 and 90°C respectively (**Table 2**). Nevertheless, these results were consistent with those find in literature for globular proteins **(Panick et al., 1999)**.

Table 2. Structural component (ß-sheet, unordered, α-helix and ß-turn) percentage areas obtained for native whey proteins dispersions in different NaCl environments measured from Amide I and Amide III at 30° C and 90° C (mean of three independent analyses).

	Structural Component (%)	NaCl concentration (%)											
		0	0.50	1.00	1.50	2.00	3.00	0	0.50	1.00	1.50	2.00	3.00
		At 30°C						At 90°C					
from Amide I	ß-sheet	40.4	41.6^{ns}	38.9*	41.6^{ns}	41.6^{ns}	42.6^{ns}	62.3	59.7^{ns}	47.3*	44.7***	47.6*	47.6*
	unordered	21.6	21.5^{ns}	23.5^{ns}	24.1	24.3^{ns}	22.4^{ns}	10.6	8.0^{ns}	13.4*	14.0*	15.2**	14.8**
	ß-turn	16.0	14.9^{ns}	17.8^{ns}	14.5^{ns}	14.4^{ns}	14.5^{ns}	7.4	10.4^{ns}	14.9*	15.3**	18.7**	17.8**
	α-helix	22.1	22.1^{ns}	19.8	19.8	19.8^{ns}	20.4^{ns}	19.7	22.0^{ns}	24.5^{ns}	26.0^{ns}	18.5^{ns}	19.9^{ns}
from Amide III	ß-sheet	44.9	46.3^{ns}	45.3^{ns}	43.2^{ns}	45.8^{ns}	48.4*	71.1	58.7**	61.0*	65.1*	55.7**	53.0***
	unordered	22.5	20.0^{ns}	17.2*	22.4^{ns}	21.4^{ns}	18.4*	10.3	13.2*	13.9*	12.7*	17.4***	18.0***
	ß-turn	14.7	18.0*	19.7*	16.6^{ns}	16.4^{ns}	16.3^{ns}	5.3	17.5**	17.3**	12.3*	18.0**	17.8**
	α-helix	17.8	15.7*	17.8^{ns}	17.7^{ns}	16.3^{ns}	16.9^{ns}	13.3	10.7^{ns}	7.8*	9.8^{ns}	8.9^{ns}	11.2^{ns}

Comparison between whey dispersion in water (control) and other concentration: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ and $_{ns}P > 0.05$.

DISCUSSION

WPI dispersions size and morphology with combined effect of NaCl and heat

Dispersions in water. Concomitant observations were obtained from TEM images and size analyses. Well separated roughly spherical nanoparticles (around 70 nm) were observed by TEM and measured by DLS. Another population was also found by DLS with a hydrodynamic diameter around 6 nm. These small particles were not visible on TEM images certainly due to the magnification used and may correspond to individual whey proteins. These two hydrodynamic diameters are in agreement with values determined by **(Martinez et al., 2009, Schmitt et al., 2007)** for β-La alone (corresponding to the first peak) and serum heat-induced milk proteins (corresponding to the second peak). The lack of aggregation can be related to the presence of substantial negative charge. Hence, strong electrostatic repulsions between molecules may prevent them from coming into close proximity **(Baussay et al., 2004)**. While for heated dispersions in water, hydrodynamic diameter value obtained by DLS was in agreement with values reported by others **(Schmitt et al., 2011)**. This also coincides well with the particle size determination made from TEM images and assuming a complete protein denaturation and formation of clumps of big soluble spherical aggregates.

Dispersions in low NaCl environments (< 200 mmol.kg^{-1}). The addition of sodium chloride in the system impacted the size and morphology of the WPI aggregates. Low salt concentrations (86 mmol.kg^{-1}) resulted in the formation of small clumpy aggregates (TEM images). Nevertheless, the hydrodynamic diameters were not significantly different to those found in water. These discordances may be correlated to the important standard deviation found for the hydrodynamic diameter. Aggregation is known to be strongly influenced by attractive and repulsive forces. The major forces favoring protein aggregation are hydrophobic and van der Walls forces; whereas electrostatic and hydration repulsions oppose this phenomenon. It seems that WPI started to aggregate above a critical salt concentration.

Similarly, **(Unterhaslberger et al., 2006)** had already shown that when low amount of NaCl are added (< 60 mmol.kg^{-1}), insufficient number of chloride ions may screen the positive charge of protein so that the preferential hydration forces are prevalent. In our concentrations, the electrostatic repulsion between proteins molecules may be screened sufficiently, more chloride ions could bind to the protein surface so that the preferential hydration by water is overcome leading to aggregation. It appeared also that at low ionic strength the primary aggregates associates mainly head to tail (**Pouzot et al., 2005)**. This enables the protein molecules to approach closely enough together to form clumpy aggregates. Conversely, the hydrodynamic and particle diameters observed by DLS/TEM of the all heat treated samples

with or without salt present a monomodal distribution showing decreasing trend and totally differs from particle size distribution of non heat dispersions.

This phenomenon is a characteristic of an ongoing protein denaturation **(Schokker et al., 1999)**. Actually, these findings can be explained by the second effect of ionic strength, i.e. salting-out effect of salts. At NaCl concentrations (≤ 200 mmol.kg^{-1}), more chloride ions bind to protein surface so that preferential hydration by water is overcome leading to the observed denaturation and further protein aggregation coagulation phenomenon. Some other findings **(Nicorescu et al., 2008, Unterhaslberger et al., 2006)** also support our results although it seems that it widely depends on co-solute, concentration, chemical environment as well as the technological and operating conditions.

Dispersions in high NaCl environments (> 200 mmol.kg^{-1}). Increasing NaCl up to 258 mmol.kg^{-1} resulted in an acceleration of WPI aggregation and the formation of denser aggregates. DLS and SLS measurements were in agreement with TEM images and the presence of aggregates were detected. From 258 to 517 mmol.kg^{-1}, the electrostatic repulsions between protein molecules were screened more efficiently i.e., the ionic strength is high enough to screen electrostatic interactions. These networks consisted of aggregated protein particles that are typically between 0.1µm and 10µm diameter, hence forming a large number of aggregated protein particles within each particle, associated into particulate structures that have relatively large sizes **(Langton and Hermansson, 1996, Verheul et al., 1998)**.

It's worth noting that contrast between particles and background in the TEM images was higher at these concentrations depicting a denser structure of WPI aggregates. Such local densification and branching association of the protein aggregates in salt aqueous environment was reported for others for whey proteins **(Pouzot et al., 2004)**. Upon heating the dispersions in higher salt concentrations (258-517 mmol.kg^{-1}), particle sizes measured by DLS and TEM are well correlated and showed similar decreasing trend. As already discussed, the likely explanation of smaller particles could be a combined salt/heat induced denaturation process leading into solid aggregated gels at higher salt concentrations. Moreover, the size and microstructure of particles was insensitive to higher salt concentration (> 200 mmol.kg^{-1}). The transition from spherical/compact protein aggregates to linear ones upon salt/heat increase has already been reported for WPI dispersions (1% wt) at 85°C **(Schmitt et al., 2007)**.

Rheological and thermal properties of WPI dispersions

Gelling properties of WPI dispersions are significantly influenced by the concentration of NaCl present in solution (**Mulvihill and Ennis, 2003**). The lack of gelation in the absence of salt occurs because protein molecules have substantial negative charges, hence there is a

relatively strong electrostatic repulsion between unfolded protein molecules which prevents them from coming into close proximity **(Bryant and McClements, 1998)**. It is virtually impossible to induce gelation by denatured proteins, in itself, as individual molecules must associate with one another to form a continuous network **(Mulvihill et al., 1990)**. In presence of NaCl, the G' increase is logical, as salt addition leads to a reduced intermolecular repulsion of unfolded protein molecules. Thus it results in an increase in both the protein-protein interactions and their aggregation capacity, which finally contributes to a strong three dimensional protein network which contribute to a gel **(Lorenzen and Schrader, 2006)**.

It is noteworthy that higher G' values and maximum gel strength was observed at low NaCl concentrations. These results can be explained by the screening of negative charges carried by unfolded proteins, which in turn allowed an increase in strong protein-protein interactions in this salt range. A considerable increase in the gel firmness and stabilizing effect in the presence of 0.2 M of NaCl was confirmed by others **(Bertrand and Turgeon, 2007)**. Nevertheless, the addition of higher concentration of NaCl (> 200 mmol.kg^{-}1) has shown negative influence on gel formation/firmness by decreasing protein unfolding **(Boye et al., 1995)** and increasing protein-solvent interactions (**Sun and Arntfield, 2011**). At higher NaCl ranges (> 200 mmol.kg^{-1}), **(Chantrapornchai and McClements, 2002)** observed a decrease in G' due by either an increase in size of insoluble heat induced aggregates or a decrease in the number of protein-protein contact.

By increasing the ionic strength, a significant increase in denaturation temperature for WPI dispersions was observed. Such shift in denaturation temperature was due to structural rearrangements induced by NaCl. Identical increase in thermal stability has also been observed for whey proteins **(Nicorescu et al., 2008)** and β- Lg **(Haug et al., 2009)**. WPI dispersions are composed of 2 major proteins β-lactoglobulin and α-lactalbumin presenting denaturation temperatures around 76°C and 67°C respectively **(Ju et al., 1999)**. These temperatures are normally pH dependent. For example, the temperature of denaturation increased (85°C) for β-Lg **(Boye et al., 1997)** and decreased (58°C) for α-La **(Bernal and Jelen, 1984)** by lowering their pH. In our case, the pH of all systems remains stable. The shifts observed in denaturation (µDSC) and gelling (rheometer) temperatures for WPI dispersions are well corroborated and showed that an effect of combined heat treatment and ionic strength may become a rate limiting factor for their final functional properties.

Protein surface hydrophobicity index (PSH)

The significant decrease in the PSH index with NaCl addition could be related to a lower availability of hydrophobic groups certainly due to the formation of large aggregates. This evolution was characteristic of the presence of less hydrophobic sites available to ANS

marker. This may be related to the fact that hydrophobic groups are buried inside the native structure of WPI molecules so with the salt induced aggregation it is possible that their access to ANS probe is limited **(Moro et al., 2001)**. Another explanation may be an interaction between hydrophobic sites of the proteins during salt induces aggregation and a reduction of the number of available ANS binding sites **(Schmitt et al., 2007)**.

Similarly, the PSH decrease observed for heated WPI dispersions may be due to ongoing denaturation-aggregation phenomenon and structural collapse due to high temperature resulting in limited availability of ANS binding sites and in turn insoluble aggregates with decreased surface hydrophobicity. Some authors observed also a decrease of the PSH of whey proteins when increasing the temperature from 75 to 80°C **(Carbonaro et al., 1996)**. It's worth noting that although there is gradual decrease in PSH values upon addition of NaCl under heated and unheated conditions but from 1.5% NaCl, this decrement is limited and coincide well with TEM micrographs and data summarized.

Secondary structure of whey proteins isolates with salt/heat augmentation

Up to now, amide I spectral region was the most commonly used band because of its very strong signal but some recent results indicate that amide III region gives a better prediction for protein secondary structures than amide I **(Cai and Singh, 2004, Hussain et al., 2011a).** This is because amide I band suffers from an unstructured spectral contour and an overlap of the random coil and α-helix bands **(Cai and Singh, 2004)**. In this work, the distributions of components within classes of structures are reproducible and the tendencies are exactly the same whatever the band analyzed. At room temperature (and pH around 6.6), the conformation (ß-sheet, unordered, ß-turn and α-helix) of whey proteins was not appreciably altered by NaCl (from 86 to 517 $mmol.kg^{-1}$). These results are corroborated by **Boye et al., 1996)**. Similar effect of NaCl on β-Lg secondary structure has been reported by others **(Kavanagh et al., 2000, Verheul et al., 1998)**. Upon heat treatment without salt, ß-sheets structures increased primarily at the cost of ß-turn and unordered structures. This ongoing denaturation/aggregation process at elevated temperatures was also observed during rheological behavior study of WPI dispersion as discussed earlier. These results are in agreement with those of (**Panick et al., 1999)** for β- Lg studied by spectroscopic and scattering techniques.

Conversely, these results differs from those of **(Zhu and Damodaran, 1994)** who described a progressive decrease in ß-sheets along an increase in unordered structures while studying heat induced conformational changes in whey protein isolates with different heating times. This conflict may be attributed to the method adopted for heating proteins. Moreover, a salt increase (86 to 517 $mmol.kg^{-1}$) tend to illustrate a stabilizing effect of NaCl **(Boye et al., 1996, Verheul et al., 1998)** suggesting an increase in thermal stability of WPI dispersions

followed by a decrease in percentages of secondary structural elements. This increase in thermal stability was also observed by DSC analysis showing that higher temperatures may be required to disrupt the native proteins structure at higher ionic strength.

CONCLUSIONS

This work demonstrated that whey protein gelation properties depend on both salt and how heat treatment is applied. Experimental data showed that during heat treatment and at low ionic strength, gel elastic modulus increased and strongest gel stiffness was achieved. Higher salt concentrations lead to a weaker gel while no gels at all were formed without salt. Additionally, NaCl addition along with dynamic heat treatment showed a stabilizing effect on the secondary structural elements of both Amide I and III bands and higher denaturation temperatures. A morphological investigation showed a transition from spherical/compact protein aggregates to linear ones due to combined salt/heat effect. Moreover, by combining the results of DLS, TEM with those of PSH one may conclude that the number of hydrophobic sites available decreased significantly because of the formation of denser aggregates. For the first time, a combination of techniques with additional work on fundamental aspects of whey proteins was presented. These results add additional value and could validate other studies that independently made such observations.

Careful adjustment of heat and NaCl concentration are necessary to effectively utilize whey protein isolate as a functional additive. Nevertheless, further work is required to understand sequential events occurring during salt and temperature-induced unfolding and aggregation process of whey proteins. It will be done in a future paper by use of FTIR combined with 2D correlation spectroscopy.

Chapitré V : Conclusion générale et Perspectives

Conclusions

The main objectives of this thesis were to study and improve our understanding of mechanisms of protein-salt interactions and linkage between milk proteins powders and milk proteins dispersions (5% w/v) mainly NMC and WPI under various ranges of ionic environments. Generally, this study could be classified into three important stages. Our strategy has initially been limited to investigate the influence of the ionic environments (distilled water, NaCl solution and $CaCl_2$ solution) on milk proteins powders rehydration profiles. The second phase of this study includes the characterization of milk proteins dispersions in an aqueous ionic environment regarding changes in the protein: secondary structure (ß-sheet, α-helix, ß-turns and unordered structures), size, morphological features and surface hydrophobicity. In the last part, the functionality of whey proteins was studied by the combined effect of heat and different ratios of salts. For this purpose, the study was exclusively focalized in an aqueous ionic medium composed of NaCl at different temperatures (30 - 90°C).

The results obtained from the first part (rehydration) showed two distinct behaviors depending on the salt type and concentration. The rehydration profiles obtained were interpreted in term of wetting, swelling and dispersion stages by using a reactor equipped with captors. For native micellar casein powder, a significant change was observed between 3 and 6% NaCl and between 0.75 and 1.5% $CaCl_2$. The first behavior (low salt concentration) presents a typical rehydration profile: quick wetting, swelling and long dispersion stage. On the opposite, the dispersion stage of the second behavior (high salt concentration) was significantly shortened indicating a strong modification of the protein backbone. The rehydration of whey proteins powder was less influenced by salts and their concentration. At low salt concentrations, a typical profile for whey powders was observed: wetting with lump formation and no swelling followed by a quick dispersion. At high salt concentrations, no turbidity stabilization was observed indicating a possible protein unfolding and denaturation. $CaCl_2$ seems to act similarly as NaCl but lower concentrations were required to de structure (casein) and unfold (whey).

In the second part of the thesis, casein and whey dispersions under high NaCl environments were characterized at various structural levels; including principally a study of molecular changes occurring upon salt increase and aggregate formation and to investigate such aggregates formed for their morphology and particle size by means of FTIR, TEM, and DLS with other complimentary techniques.

For the NMC dispersions, the micellar edifices were characterized as to their morphology, size, and content of proteins in the supernatant after ultracentrifugation. Additionally, changes in secondary structures of the protein upon salt increase were followed by Fourier Transform Infrared Spectroscopy (FTIR). These dispersions in water were composed of spherical micelles

with an average size around 150 nm well correlated with TEM observations. Under NaCl increase, this open-hydrated micellar structure may be disintegrated in sub-micelles around 20-30 nm more or less aggregated. The preliminary, FTIR investigation highlights a significant redistribution of component areas. The protein repartition in pellet and supernatant after ultracentrifugation yielded more support on the migration of serum casein and/or submicelles in the micelle. For the first time, the estimations of secondary structural elements (irregular, ß-sheet, α-helix and turn) from Amide III assignments were correlated with results from Amide I. Around 3% NaCl addition, a good correlation was observed between destabilization of the micellar edifice, modifications of the caseins secondary structure and repartition of caseins between supernatant and pellet after ultracentrifugation.

For whey proteins dispersions, structural details were revealed under high NaCl range (0-6%). These dispersions in water were composed of well separated proteins by a spherical shape with two populations around 6 and 70 nm well correlated with TEM observations. Salt increase resulted in an aggregation of the proteins and the formation of denser aggregates. Concurrently, FTIR investigations did not highlight any redistribution of components areas. Even if the secondary structure was not influenced by the environment, differences were observed concerning the protein surface hydrophobicity and opacity.

Finally, the last phase of the study deals with the investigation of the behavior leading up to heat/salt induced gelation of WPI (5% w/v) as a function of salt concentrations (0-3%) and temperatures range (30 to 90°C). This work demonstrated that whey protein gelation properties depend on both salt and how heat treatment is applied. Experimental data showed that during heat treatment and at low ionic strength, gel elastic modulus increased and strongest gel firmness was achieved. Higher salt concentrations lead to a weaker gel while no gels at all were formed without salt. Additionally, NaCl addition along with dynamic heat treatment of WPI dispersion showed a stabilizing effect on the secondary structural elements of both Amide I and III bands and higher denaturation temperatures observed by FTIR and DSC respectively. A size and morphological investigation showed a transition from spherical/compact protein aggregates to linear ones due to combined salt/heat effect. Moreover, by combining the results of DLS, TEM with those of PSH one may conclude that the number of hydrophobic sites available decreased significantly because of the formation of denser aggregates.

Perspectives.

Because of important use of milk proteins in industry and fundamental research projects worldwide, there is ample justification in continuing research for improvement in its physicochemical and functional properties of rehydration with respect of microenvironments (pH, ionic strength, temperature etc). This is underscored by the urgent requisite to develop

standard methodology for evaluating the fundamental research aspects like rehydration process, structural modification, particle sizes, morphology along with surface hydrophobicity and opacity. The results obtained could be fairly used for correlating these properties with specific commercial uses of proteins, which may encourage substantial utilization of milk proteins.

Moreover, such informations are necessary to expedite appropriate methods of processing in order to control compositional variability, extent of aggregation and denaturation, and possible structural modification of protein by different microenvironments. However, it is necessary to investigate the actual mechanism and its order associated each type of added salts and their concentration on the quality of final product produced from milk proteins. More research is necessary for a better understanding of the mechanism involved and to find a way to manufacture milk proteins powders with better functional properties and nutrional values.

Examples of prospectives and recommendations for further research may include.

- A parallel approach of meso-modeling will be developed using Dissipative Particle Dynamic Method (Materials studios) and expertise in this regard is already present in LIBio. This method allows to study the effect of various environmental parameters (pH, temperature, ionic strength, shear stress, water activity..) on the mechanisms of rehydration, multilevel characterization and functionality of milk proteins dispersed in simple (water), ionic aqueous solutions (different salt with different concentrations) and complex medium (milk)
- A more sophisticated and comparative technological based on rehydration profiles study could be carry out by using Qicpic analyzer (Sympatec GmbH, Clausthal-Zellerfeld, Germany) now available in our laboratory. As compared to Turbitory Sensor, this tool is also suitable for powders in suspension and provides data on particle size and shape in dynamic mode.
- This study (rehydration properties studies, characterization of proteins and investigation of their functionality) could be further proceed with other environmental factors like modification of pH ranges. In current study, we worked with constant pH of 6.5 ± 1.
- Investigation of the functional properties of milk proteins powdr isolated by different methods e.g. Bovine serum albumin, Whey protein concentrates.
- Determination of usefulness of milk proteins especially whey proteins for blending with and/or complementing other food proteins for new food product .
- New spectroscopic methods (2 D correlation spectroscopy, circular dichroism (CD) and other microscopic (AFM, CLSM) and complementary techniques could be used for further study.

- Study of salt induced cold gelation phenomenon of various milk proteins fractions and elucidation of their structural, morphological, and functional properties under effect of various microenvironments.

Conclusion

Le but de cette thèse était d'étudier et de mieux comprendre les mécanismes d'interaction entre protéines laitières (caséines micellaires natives NMC et protéines de lactosérum natives WPI) sous forme de poudres dispersées (5% p/v) dans des milieux fortement ionisés par des sels (NaCl, CaCl2).

D'une manière générale notre étude a été organisée en trois parties. Premièrement, nous avons étudié l'influence des milieux ioniques (eau distillé, solution de NaCl, solution de CaCl2) sur la réhydratation de protéines laitières. Dans une seconde partie nous avons caractérisé les dispersions en milieux ionisés par les études respectives de la structure secondaire des protéines, de la taille et de la morphologie des particules en cours de dispersion et de l'hydrophobicité des protéines. Dans la troisième et dernière partie nous nous sommes intéressés aux propriétés fonctionnelles des protéines du lactosérum par l'effet combiné de la température (30 – 90°C) et de la concentration en NaCl (0,75 – 3%).

La réhydratation des protéines du lait a montré deux comportements distincts en fonction du type de sel d'une part (NaCl, $CaCl_2$) et de la concentration d'autre part (0 – 12%). Un réacteur équipé d'un turbidimètre à permis d'étudier les étapes de mouillage de gonflement et de dispersion et ainsi d'obtenir des profils de réhydratation des poudres. Pour la caséine micellaire native un changement significatif a été observé entre 3 et 6% de NaCl et entre 0,75 et 1,5% de $CaCl_2$. Pour de faibles concentrations quelque soit le sel utilisé le profil de réhydratation présente un mouillage rapide, une étape de gonflement des particules et une dispersion longue. Pour de plus fortes concentrations en sels cette étape de dispersion est fortement réduite, il a été montré que durant cette étape il y avait modifications de la structure secondaire de protéines observées par FTIR.

La réhydratation des poudres de protéines de lactosérum est moins influencée par la nature du sel et sa concentration. À de faibles concentrations en sel, un profil type est obtenu avec une étape de mouillage et formation de grumeaux, pas de gonflement et une dispersion rapide. À des concentrations élevées en sel, le signal turbidimétrique n'est pas stable ce qui est probablement du à une dénaturation et a un redéploiement des protéines.

Globalement il a été montré que les 2 sels ont le même effet sur la réhydratation des protéines, néanmoins $CaCl_2$ semble agir à de plus faibles concentration sur la dénaturation des protéines laitières.

Dans la deuxième partie de la thèse des dispersions de poudres de caséine et de lactosérum dans des solutions de NaCl ont été caractérisés. Dans cette partie nous avons mis l'accent sur l'étude des changements moléculaires (FTIR) et la formation d'agrégats liés à l'augmentation de la concentration en sel. Ces agrégats sont caractérisés par l'étude de leurs

tailles et de leurs formes (TEM, DLS, SLS) avec d'autres techniques (spectrofluorimètre, calorimètre).

Pour les dispersions NMC, les édifices micellaires ont été caractérisés par leurs morphologies, leurs tailles, et leurs teneurs en protéines dans le surnageant après ultracentrifugation. Les modifications dans les structures secondaires de la protéine lors de l'augmentation de la concentration en sel ont été suivies par spectroscopie à transformée de Fourier (FTIR). Ces dispersions dans l'eau sont composées de micelles sphériques avec une taille moyenne de l'ordre 150 nm (DLS et TEM). Avec l'ajout de NaCl et augmentation de la concentration on observe une ouverture de la structure micellaire avec libération de sub-micelles d'une taille d'environ 20-30 nm qui sont plus ou moins agrégées. Une analyse par ATR-FTIR montre une importante redistribution des zones qui composent la structure micellaire. La répartition des protéines dans le culot et le surnageant après ultracentrifugation montre une migration des protéines sous forme de sub-micelles du culot vers le surnageant. Il a été montré ainsi pour la première fois que des éléments de la structure secondaire (feuillet ß, hélice α, …) de l'amide III ont pu être corrélés avec les résultats de l'Amide I. L'addition de NaCl à 3%, montre qu'il existe une corrélation entre la déstabilisation de l'édifice micellaire, les modifications de la structure secondaire des caséines et la répartition des caséines dans le surnageant et le culot après ultracentrifugation.

Pour des dispersions de protéines de lactosérum, des modifications de structure ont été observées avec NaCl pour des concentrations allant de 0,75 à 6%. Dans l'eau seule ces dispersions sont composées de protéines ayant une forme sphérique avec deux populations aux environs de 6 et 70 nm (TEM, DLS, SLS). L'augmentation de la concentration en NaCl conduit à une agrégation des protéines. Parallèlement, l'exploitation des spectres FTIR ne montrent pas de différences concernant la structure secondaire des protéines, néanmoins des mesures par spectrofluorimètre et par colorimétrie montrent des disparités sur l'hydrophobicité et l'opacité du milieu.

Enfin, la dernière phase de l'étude porte sur l'étude des propriétés fonctionnelles des WPI (5% p/v) en fonction de la température (30 – 90°C) et de la concentration en NaCl (0 – 3%). Ce travail montre la gélification des WPI en fonction de la concentration en sel et de la température. Les données expérimentales montrent que pendant le traitement thermique et à faible force ionique (< à 1,5% en NaCl), le module élastique G' et la fermeté du gel augmente. Pour des concentrations supérieures en sel (> à 1,5% en NaCl) la fermeté du gel est plus faible.

L'ajout de NaCl associé avec le traitement thermique de la dispersion de WPI montre un effet stabilisateur sur les structures secondaires des bandes Amide I et III et la température de dénaturation augmente, ceci est observé par FTIR et DSC. Les analyses de taille et de

morphologie permettent d'observer le passage d'une forme sphérique à une forme allongée en fonction de la température et de la concentration en sel. En outre, en combinant les résultats de DLS, TEM avec ceux de PSH, on peut conclure que le nombre de sites hydrophobes disponibles a diminué de façon significative en raison de la formation d'agrégats plus denses.

Perspectives

En raison de l'utilisation importante de protéines de lait dans l'industrie et des projets de recherche fondamentaux à travers le monde, il est important de poursuivre les recherches pour l'amélioration des propriétés physico-chimiques et fonctionnelles de la mise en suspension de ces poudres dans des solutions qui ont différents pH, force ionique, température, etc. Cela passe par la nécessité d'élaborer des méthodologies standards pour évaluer les aspects fondamentaux comme le processus de réhydratation, les modifications de structures, les tailles et la morphologie de particules ainsi que l'hydrophobicité de surface et l'opacité. Les résultats obtenus pourraient ainsi être corrélés aux propriétés d'usage afin de favoriser leurs utilisations commerciales.

De telles informations sont nécessaires pour optimiser les méthodologies afin de contrôler la variabilité de la composition des matières premières, quantifier l'agrégation et la dénaturation et observer les modifications de structure des protéines en fonction de leurs environnements. Cependant, il est nécessaire d'analyser les différents mécanismes (agrégation, dénaturation, …) et leurs chronologies en fonction du sel et de sa concentration sur la qualité du produit fini et en fonction du type de protéines utilisées. De nombreux travaux sont encore nécessaires pour une meilleure compréhension des mécanismes impliqués afin d'optimiser les fabrications de poudres de protéines laitières ayant encore de meilleures propriétés fonctionnelles et nutritionnelles.

Les perspectives et recommandations de ce travail peuvent être les suivantes :

- Développer une approche parallèle de méso-modélisation en utilisant la méthode « des particules Dissipative dynamique » (Logiciel Matérials studios). Le Laboratoire à déjà une expertise dans ce domaine. Cette méthode pourrait permettre d'étudier l'effet des paramètres environnementaux (pH, température, force ionique, contrainte de cisaillement, activité de l'eau ..) sur les mécanismes de la réhydratation, la caractérisation et la fonctionnalité multi-niveaux de protéines de lait dispersés dans un milieu simple (eau), ionique (sels différents avec différentes concentrations) ou complexe (lait)
- Une étude comparative de la réhydratation mettant en œuvre le granulomorphomètre Qicpic (Sympatec GmbH, Clausthal-Zellerfeld, Allemagne) et le capteur turbidimétrique pourait être effectuée. Ces outils sont bien adaptés pour la caractérisation des poudres en suspension et fournit des données sur la taille et la forme des particules en mode

dynamique. Des millions de particules sont ainsi instantanément évaluées, caractérisées et traitées.

- Ce travail (propriétés de réhydratation, caractérisation des protéines et étude de leurs fonctionnalités) peut être également complété par d'autres facteurs environnementaux comme la modification du pH. Dans notre étude nous avons travaillé avec un pH constant de 6,5 ± 1.
- L'étude des propriétés fonctionnelles d'autres fractions de protéines laitières comme par exemple, bovine serum albumin et whey protein concentrates peuvent également être étudiées.
- Etudier des mélanges de protéines laitières en association avec d'autres ingrédients alimentaires dans le cadre d'un nouveau produit formulé.
- De nouvelles méthodes instrumentales comme la 2D correlation spectroscopy et circular dichroism (CD), d'autres méthodes (AFM, CLSM) microscopiques pourraient être utilisées afin d'obtenir des informations complémentaires.
- Etudier l'influence de l'incorporation de sels après le processus de dénaturation par la chaleur.

Chapitré VI : Références

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Annexes

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

Raza Hussain, Claire Gaiani, and Joël Scher

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, β-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,

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Structuration mechanism of β-lactoglobulin – acacia gum assemblies in presence of quercetin

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ABSTRACT

The interactions of β-lactoglobulin (BLG) with total Acacia gum (TAG) in presence of quercetin have been investigated in aqueous solutions at pH 4.2 and 25 °C. Isothermal titration calorimetry (ITC) has been used to determine the type and magnitude of the energies involved in the complexation process. Dynamic light scattering (DLS), electrophoretic mobility (μ_E), turbidity measurements (τ), optical microscopy and Fourier transform infrared spectroscopy in total attenuated reflection mode (ATR-FTIR) were used as complementary methods to better understand the sum of complicated phenomena at the origin of thermodynamic behaviour.

The first structuring stage was characterized by an exothermic signal and was mainly controlled by favourable enthalpy changes due to electrostatic interactions between biopolymers. The second structuring stage was largely endothermic and more entropy driven, probably due to the release of small counterions from the electrical double layer and hydrophobic contribution to the binding process, implying the release of water molecules. The population distribution of the different species in solution and their size were determined through DLS. Dispersion turbidity of particles markedly increased and reached a maximum at 0.013 TAGQ:BLG molar ratio corresponding to the appearance of coacervates. Above TAGQ:BLG molar ratio of 0.015, dispersions turbidity decreased, which might be due to an excess of negative charges onto particles as revealed by electrophoretic mobility measurements. FTIR experiments indicated that BLG–TAG interactions, in presence or in absence of quercetin, induced a change in the α-helical structure of BLG. The results also showed significant loss in β-sheets indicating a change in the environment of BLG hydrophobic amino acids and the formation of protein–flavonoid complexes stabilized by hydrophobic associations.

The results presented in this study should provide information about thermodynamic mechanisms of TAG–BLG binding processes in presence of an antioxidant, quercetin and will facilitate the application of the formed supramolecular assemblies as functional ingredients in food and non food systems.

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1. Introduction

Protein–polysaccharide complexes mostly originate from electrostatic interactions between oppositely charged macromolecules. These interactions induce the formation of various supramolecular entities such as complexes, aggregated complexes and coacervates. The nature of protein–polysaccharide complexes is influenced by entropic factors, such as structure, and molecular weight of biopolymers. The complexes are also influenced by enthalpic forces, which are regulated by the nature and density of charge on the biopolymers and the protein to polysaccharide molar ratio (Girard, Turgeon, & Gauthier, 2003; Schmitt, Sanchez, Désobry-Banon, & Hardy, 1998; Turgeon, Schmitt, & Sanchez, 2007). Proteins and polysaccharides constitute major compounds in biological systems. They are widely present in living organisms and can be associated in order to maintain cell integrity or cell division (Menger, 2002). Formation of protein–polysaccharide complexes can potentially lead to different functional properties, compared to the two biopolymers taken alone enabling their used in several industrial applications such as protein purification (Montilla et al., 2007), micro- and nano-encapsulation process (Xing, Cheng, Yi, & Ma, 2005), texturization (Schmitt et al., 1998) and interface stabilisation (Noel et al., 2007).

It is well known that the driving force leading to the formation of protein–polysaccharide complexes is the decrease of the total electrostatic free energy of the mixture (de Vries & Cohen Stuart,

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Morphological descriptors and colour as a tool to better understand rehydration properties of dairy powders

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ABSTRACT

The influence of particle size and shape on the rehydration properties of dairy powders has been investigated on five different milk powders more or less agglomerated (a total of thirty-five powders were studied). Morphological descriptors (size, sphericity and convexity) were followed by a Qicpic™ laser diffraction analyzer. Concurrently, the wettability, solubility and dispersibility for each powder were measured. A correlation among particle size and rehydration aptitude was highlighted. Principal component analysis of the rehydration data permits to differentiate powders according to their nature (with colour measurements) but also to their size and morphology. Then, by the use of the shape descriptors, prediction models for dispersibility and solubility were successfully developed and optimized whereas reliable wettability predictions remained unattainable. Results suggested that the size associated with the shape descriptors could be important factors influencing milk powders rehydration properties.

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1. Introduction

Rehydration is an essential quality characteristic of a dairy powder, as most powders are dissolved before use. Consequently, their ability for an easy rehydration is one the most important requirement for the food industry. The rehydration process mainly consists of three different stages (Freudig, Hogekamp, & Schubert, 1999; Schubert, 1993). First, the wettability is the ability to absorb water and can be determined by the IDF (1979). Second, the dispersibility is defined as the ability to break down into particles passing through a defined sieve of 210 μm (IDF, 1979). Third, the solubility is the ability of powder to be solubilised after a series of centrifugations (IDF, 2005). The length of each phase in the total rehydration process depends on the powder physical and chemical properties (Gaiani et al., 2006b; Gaiani, Schuck, Scher, Desobry, & Banon, 2007). The rehydration properties of food powders rely on their chemical composition as well as on their physical characteristics (size, density, shape, etc.). The influence of the structure (density, porosity, size) (Gaiani et al., 2009; Mimouni, Deeth, Whittaker, Gidley, & Bhandari, 2010), the chemical and the surface composition (Gaiani et al., 2006a) on rehydration properties have been broadly studied.

In contrast, less attention has been given to the particle shape (Langrish, Marquez, & Kota, 2006; Perea et al., 2009). One of the reasons could be the lack of technologies for a reliable quantitative particle shape determination. Shape analyses were usually performed by image processing techniques obtained by confocal laser scanning microscopy (CLSM) and/or scanning electron microscopy (SEM) (Du & Sun, 2004). In this instance, image analyses were frequently used in the past to characterize the particle shape, primarily for mineral powders (Chau, Bruckard, Koh, & Nguyen, 2009; Yekeler, Ulusoy, & Hicyilmaz, 2004). However, the data treatment was too long and non-representative due to the poor rank of automation. This made analyses time-consuming and tiresome with a high level of significant statistical errors (Yu & Hancock, 2008). Nowadays, laser diffraction systems coupled with image processing have been developed to allow the on-line size and shape measurement of several kinds of particles (Nalluri et al., 2010; Yu & Hancock, 2008). Particle shape can also be determined by qualitative terms. Some of these shape descriptors are the sphericity, which is the ratio of the perimeter of the equivalent circle to the real perimeter of the particle, the convexity describing the compactness of a particle or also the Feret diameter (Du & Sun, 2004). The latter representing the common base of a set of diameters derived from the distance of two tangents to the outline of the particle in a well-defined orientation.

In the literature, few studies have been carried out on particle morphology of food powders, contrary to mineral powders which have been abundantly studied (Chau et al., 2009; Tellez-Medina, Byrne, Fitzpatrick, Catak, & Cronin, 2010; Ulusoy, 2008).

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