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Determination of hydro-colloidal characteristics of milk protein aggregates using Asymmetrical Flow Field-Flow Fractionation coupled with Multiangle Laser Light Scattering and Differential Refractometer (AF4-MALLS-DRi)

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ABSTRACT

During heat treatment, whey proteins (WP) are denatured and form different kinds of whey protein aggregates (WPA) depending on the physico-chemical conditions. In the presence of casein micelles, mixed aggregates (MA), resulting from the interaction of κ-casein and WP, are formed. The aim of this study is to understand the effect of pH on the WPA structure and the impact of the casein/whey proteins (Cas/WP) ratio on MA morphology. In this work, WP solutions were heated at pH 5.8 or 7 to produce WPA. On the other hand, aqueous mixtures containing three Cas/WP ratios (82/18, 56/44 and 30/70) were heated at 80 °C during 1 h at pH 6.3 to obtain MA. Milk protein aggregates were analyzed by Transmission Electron Microscopy (TEM) and Asymmetrical Flow Field-Flow Fractionation coupled with Multiangle Laser Light Scattering and Differential Refractometer (AF4-MALLS-DRi). Dense and spherical WPA were formed at pH 5.8 whereas branched and fractal aggregates were obtained at pH 7. Regarding MA, results suggested that they were mainly produced with the 82/18 Cas/WP ratio whereas a majority of WPA was obtained with the 30/70 ratio. At intermediate ratio, the mixture was composed of small MA and WPA that did not interact with κ-casein. Moreover, WP seem to interact preferentially with larger casein micelles. Thereby, AF4-MALLS-DRi proved to be a powerfull technique to characterize the complex structure of milk protein aggregates and an interesting alternative to size exclusion chromatography especially for MA and casein micelles which interact with the stationary phase and are retained in the column.

1. Introduction

Milk proteins are widely used in food formulation due to their high nutritional and functional properties as gelling, foaming or emulsifying agent. They are divided in two fractions according to their solubility at pH 4.6: caseins (Cas) are precipitating and whey proteins (WP) remained soluble. Casein micelles are assigned to spherical particles composed of four proteins (α_{S1}-, α_{S2}-, β and κ-caseins) which interact with each other. The casein cohesion is insured by the presence of calcium phosphate nanoclusters inside the entity to form casein micelles. The structure of the casein micelles is stabilized by an outer layer made of the hydrophilic C-terminal part of κ-caseins and form remarkably stable colloïdal suspensions (Bouchoux, Gésan-Guiziou, Pérez, & Cabane, 2010; Bouchoux et al., 2009; Eigel et al., 1984; McMahon & Brown, 1984; Payens, 1966). However, their structure can be dissociated under certain conditions of pH and temperature (Anema & Klostermeyer, 1997; Anema & Li, 2000; Holt & Horne, 1996; Holt, 1992). Whey proteins are mainly composed of β-lactoglobulin, α-lactalbumin, Bovine Serum Albumin (BSA) and immunoglobulins (McSweeney & Fox, 2003; Mulvihill & Donovan, 1987). WP are heat sensitive and heating a WP solution above 70 °C induces their

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unfolding leading to the formation of whey protein aggregates (WPA) of different sizes and shapes depending on the experimental conditions. Thereby, dense and spherical aggregates are formed at pH close to their isoelectric point whereas branched and fractal aggregates are obtained at neutral pH (Jung, Savin, Pouzot, Schmitt, & Mezzenga, 2008).

During heat treatment of milk, whey proteins are denatured and can interact with casein micelles to form mixed aggregates (MA). Under heating, κ-caseins, which are positioned at the surface of the casein micelle, and whey proteins are able to associate via hydrophobic interactions and disulfide bonds (Haque & Kinsella, 1988; Jang & Swaisgood, 1990; Sawyer, 1969). The structure of MA depends on the experimental conditions and on the Cas/WP ratio (Anema & Li, 2003; Long, Van Winkle, & Gould, 1963). Dynamic Light Scattering (DLS) was used to characterize WPA and MA but this method is not really adapted for polydisperse samples and non-spherical objects (Elofsson, Dejmek, & Paulsson, 1996; Hoffman, Roefs, Verheul, Van Mil, & De Kruif, 1996; Surel et al., 2014). Polydisperse samples require first to separate the different entities present in the mixture. Size exclusion chromatography allows to fractionated hydro-colloids according to the differences in excluded volumes for different fractions distributed inside and outside a well-defined pore-matrix. Size Exclusion Chromatography coupled with Multiangle Laser Light Scattering (SEC-MALLS) was shown to be an appropriate technique to determine molar mass and radius of gyration of WPA (De la Fuente, Singh, & Hemar, 2002; Schokker, Singh, & Creamer, 2000). However, SEC-MALLS is inappropriate to characterize MA and casein micelles that interact with the solid phase of SEC column and are retained in the system (Almlöf, Larsson-Raźnikiewicz, Lindqvist, & Munyua, 1977; Guyomarc'h, Violleau, Surel, & Famelart, 2010; Kang, Moon, & Lee, 2011). Moreover, MA and casein micelles are too large to be separated with size exclusion chromatography.

Asymmetrical Flow Field-Flow Fractionation (AF4) is a liquid-based chromatography working as well for homogeneous systems as for heterogeneous systems. It was developed by Wahlund and Giddings (1987) and allows to fractionate and characterize a large range of particles based on their translational diffusive mobility (Myers, 1997; Yohannes, 2007). This technique has not been widely used to characterize food proteins (Nilsson, 2013), whereas AF4 was described as a softer method compared to the SEC due to the absence of structural changes of milk proteins aggregates during fractionation (Glantz, Håkansson, Lindmark Månsson, Paulsson, & Nilsson, 2010; Kang et al., 2011; Reschiglian et al., 2005). Kang et al. (2011) indicated that AF4 showed better resolution than SEC for components of high molar mass whereas SEC was able to fractionate proteins of lower molar mass. However, contrary to SEC, only AF4 was efficient to separate two proteins even with small molar mass difference and to fractionate whey protein aggregates present at low concentration (Kang et al., 2011). Thereby, Guyomarc'h et al. (2010) demonstrated that aggregates in heated skim milk are efficiently characterized using Asymmetrical Flow Field-Flow Fractionation coupled with Multiangle Laser Light Scattering (AF4-MALLS).

The aim of the present work was to characterize different types of milk proteins aggregates using Asymmetrical Flow Field-Flow Fractionation coupled with Multiangle Laser Light Scattering and Differential Refractometer (AF4-MALLS-DRi). Microgels (dense and spherical object (Schmitt et al., 2009)) and fractals aggregates (branched and autosimilar structure (Nicolai, Britten, & Schmitt, 2011)) were obtained by heating a whey protein solution at pH 5.8 and 7 respectively. In order to determine the best conditions to produce mixed aggregates (MA), mixtures of whey proteins and casein micelles were heated at three different Cas/WP ratios (82/18; 56/44 and 30/70) and at pH 6.3. Aggregate morphology was also observed using transmission electron microscopy (TEM) in order to corroborate the different results.

2. Materials and methods

2.1. Raw materials

Whey protein isolate (WPI), casein powder and milk permeate were kindly supplied by local dairy companies (confidential origin). WPI was composed of 91.1% of proteins in which were included 12% of casein materials. Casein micelles from skimmed milk were concentrated by ultrafiltration (membrane cut-off of 150 kDa) and further purified by diafiltration. The concentrate was lyophilised and the casein powder was grounded to produce a pure casein powder which was considered for this study. Milk permeate powder was produced by ultrafiltration of skimmed milk using membrane cut-off of 10 kDa and was used for this work as a source of milk soluble minerals.

2.1.1. Fractal aggregates

WPI powder was dissolved in milli-Q water under magnetic stirring overnight at 4 °C to reach a concentration of 85 $g.L^{-1}$. The solution was diluted to 50 $g.L^{-1}$ with distilled water and protein concentration was checked using a UV 1800-spectrophotometer (Shimadzu, Kyoto, Japan) at 278 nm with an extinction coefficient of 1.046 L mol^{-1} cm^{-1} (Mahmoudi, Mehalebi, Nicolai, Durand, & Riaublanc, 2007). To promote protein aggregation, 45 mM NaCl was added and the solution was adjusted to pH 7 with 6 M sodium hydroxide. A volume of 200 mL of sample was placed in a hot water bath at 80 °C during 2 h and cooled down to room temperature after heat treatment. To avoid microbial development, 0.02 w/v% sodium azide was added and the solution of fractal aggregates was stored during few days (less than one week) at 4 °C before use.

2.1.2. Microgel aggregates

WPI solution was prepared at 40 $g.L^{-1}$ as previously described in the part 2.1.1. except the pH was adjusted to 5.8 with 6 M hydrochloric acid. Protein solution was heated at 85 °C during 15 min in a water bath and cooled down to room temperature. Sodium azide (0.02 w/v%) was added and the solution of microgel aggregates was stored few days (less than one week) at 4 °C before characterization.

2.1.3. Mixed aggregates (MA)

WPI was dissolved at 50 $g.L^{-1}$ in a 5.6 w/w% aqueous milk permeate solution under stirring overnight at 4 °C. Casein powder was separately dispersed at 50 $g.L^{-1}$ in the same milk permeate solution under stirring at 20 °C during 30 min. Hydrochloric acid (6 M) was added to the casein dispersion to reach pH 6.3. Then, the dispersion was maintained under magnetic stirring during 45 min at 60 °C and finally overnight at 20 °C in order to correctly hydrate the powder. WPI solution and casein dispersion were mixed to obtain Cas/WP mixtures with three different Cas/WP ratios (82/18 (corresponding to the milk ratio); 56/44; 30/70) at a constant total protein concentration of 50 $g.L^{-1}$. Because of the presence of a high amount of whey proteins in the mixture at 30/70 Cas/WP ratio, the total protein concentration was diluted to 30 $g.L^{-1}$ with the aqueous milk permeate solution to avoid gelation during heat treatment. The Cas/WP mixtures were adjusted at pH 6.3 and then heated in a hot water bath at 80 °C for 1 h. Mixed aggregates solutions were cooled down to room temperature. After adding 0.02 w/v% sodium azide, the MA solutions were stored few days (less than one week) at 4 °C. On the other hand, WP solutions prepared by dissolving WPI powder in the aqueous milk permeate solution at the same WP concentrations and at the same pH than in Cas/WP