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Milk protein suspensions enriched with three essential minerals: Physicochemical characterization and aggregation induced by a novel enzymatic pool

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ABSTRACT

Structural changes of casein micelles and their aggregation induced by a novel enzymatic pool isolated from *Bacillus* spp. in the presence of calcium, magnesium or zinc were investigated. The effect of cations on milk protein structure was studied using fluorescence and dynamic light scattering. In the presence of cations, milk protein structure rearrangements and larger casein micelle size were observed. The interaction of milk proteins with zinc appears to be of a different nature than that with calcium or magnesium. Under the experimental conditions assayed, the affinity of each cation for some groups present in milk proteins seems to play an important role, besides electrostatic interaction. On the other hand, the lowest aggregation times were achieved at the highest calcium and zinc concentrations (15 mM and 0.25 mM, respectively). The study found that the faster the aggregation of casein micelles, the less compact the gel matrix obtained. Cation concentrations affected milk protein aggregation kinetics and the structure of the aggregates formed.

1. Introduction

Nowadays, production of mineral-supplemented foods is an important strategy to prevent cation deficiencies. Milk and milk-based products are good candidates for mineral fortification, not only due to their worldwide consumption by all groups at risk of deficiency, but also because of their high nutritional value, the buffering effect on digestion and absorption processes, and the positive effects on growth [1]. In order to really improve mineral intakes, it is important to determine cation bioavailability, which is considerably affected by the physicochemical characteristics of the medium [2,3]. The ability of caseins to bind cations also depends on pH, ionic strength, temperature and the amount of phosphate in the solution, among others [4].

Two important points to take into account in the production of fortified foods are (a) what mineral compound is best to use and (b) how much ion is necessary to add so that there is sufficient concentration in the food product. In the United States, minerals used for food fortification are classified by the Food and Drug Administration as generally recognized as safe (GRAS), e.g., calcium chloride, magnesium chloride and zinc chloride.

An enzymatic pool, P7, produced by keratinolytic *Bacillus* spp. isolated from the intestine of the Amazon basin fish, *Piaractus mesopotamicus*, was characterized and results of inhibition studies and zymogram analysis suggested that P7 consist primarily of serine proteases [5]. The bioactivity of caseinate hydrolysates obtained with P7 has also been investigated [6,7] and P7 has been shown to exhibit milk clotting activity [8]. Although it is well known that aspartic proteases are the most suitable enzymes used as milk clotting agents for the manufacture of cheese [9,10], some serine proteases from different sources have been used recently as natural rennets [11–15]. Therefore, it is important to study casein

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micelle coagulation by P7 in the presence of different mineral salts of interest to the dairy industry.

The cations assayed are nutritionally essential. Ca^{2+} and Mg^{2+} are classified as main elements while Zn^{2+} is a trace element. According to the dietary reference intake (DRI), main elements are essential for humans in concentrations >50 mg/day, while trace elements are essential in concentrations <50 mg/day [16]. Different concentrations of Ca^{2+} (between 3 and 15 mM), Mg^{2+} (between 2 and 10 mM) and Zn^{2+} (0.05 and 0.25 mM) were chosen according to the mineral concentrations of the milk samples used to produce dairy products [1,17–19].

The aim of this work was to study the effect of three cations, Ca^{2+}, Mg^{2+} and Zn^{2+}, on the physicochemical properties of milk suspensions and to determine how these cations affect milk protein aggregation induced by P7. Although there are several studies on mineral-enriched casein suspensions [20,21], it is important to study the effect of the addition of divalent cations on the physicochemical characteristics of milk under optimal milk clotting conditions of P7 for potential future application of this enzyme in the dairy industry.

2. Materials and methods

2.1. Reconstituted bovine skim milk

Skim milk powder (Milkaut, Franck, Argentina) was reconstituted at 10% w/v in 5 mM $CaCl_2$ (Cicarelli SRL, San Lorenzo, Argentina) and stored at 4 °C. Subsequent dilutions, to avoid inner filter in light scattering and florescence experiments, were carried out using 10 mM Tris–HCl buffer pH 7.4 (Sigma–Aldrich Co., St. Louis, United States). Sodium azide (Mallinckrodt Baker Inc., Phillipsburg, United States) 0.01% w/v was added as preservative. The dispersion was stirred for about 1 h at 25 °C before each experiment to allow equilibration.

2.1.1. Determination of milk protein content

Milk protein content was determined by the Kuaye's method based on the capacity of strong alkaline solutions to change the spectrum of the amino acid tyrosine (Tyr) to higher wavelength values in the UV region. In the range between 248 and 256 nm, the absorbance is a linear function of the wavelength and the slope coefficient is directly proportional to the protein concentration. Thus, measurements of absorbance at two wavelengths were used to estimate the protein content using Eq. (1) [22].

$$[\text{Protein}](\text{g/L}) = \frac{(A_{S,248} - A_{S,256})f_S}{(A_{T,248} - A_{T,256})f_T} \quad (1)$$

where $A_{S,248}$ and $A_{S,256}$ are the absorbance values of the sample at 248 and 256 nm, respectively, $A_{T,248}$, and $A_{T,256}$ refers to the absorbance values for the standard protein at the same wavelengths, f_S and f_T are the dilution factors of the sample and the standard protein, respectively.

2.2. Mineral solutions

Stock solutions of $CaCl_2.2H_2O$ (50 mM), $MgCl_2$ (50 mM) and $ZnCl_2$ (10 mM) were prepared by dissolution of the solid drugs (Cicarelli SRL, San Lorenzo, Argentina) in distilled water. Complete dissolution of $ZnCl_2$ was achieved by addition of 0.1 M HCl (Merck, Darmstadt, Germany) drops.

Suspensions of casein micelles were enriched with the cations at different final concentrations: up to 15 mM for $CaCl_2$, 10 mM for $MgCl_2$ and 0.250 mM for $ZnCl_2$.

2.3. Spectrofluorometric study

2.3.1. Protein intrinsic fluorescence

Among the aromatic amino acids, tryptophan (Trp) is the only one whose emitted fluorescence intensity (IF) depends on polarity and/or local environment [23]. Therefore, the study of changes in the emitted IF of milk suspensions can be useful to determine structural changes in milk proteins. Fluorescence spectra of milk protein suspensions were recorded between 300 and 400 nm using an excitation wavelength (λ_{exc}) of 280 nm.

The protein concentration of the samples was 0.1 g/L and pH was 7.4. Cations were added in different proportions (3–15 mM for Ca^{2+}, 2–10 mM for Mg^{2+} and 0.05–0.25 mM for Zn^{2+}). All IF measurements were performed at least in duplicate to ensure reliability of data on an Aminco-Bowman spectrofluorometer (Edison, United States) using a 1 cm quartz cuvette. The temperature was controlled at 44 °C, the optimal temperature for milk protein hydrolysis by P7 [6].

To correct the attenuation due to absorption of the incident light or absorption of emitted light, the absorbance spectra of milk protein suspensions were obtained between 280 and 400 nm. Corrected IF (IF_{corr}) was obtained applying Eq. (2) [23]:

$$IF_{corr} = IF \times 10^{0.5[A(\lambda_{exc})+A(\lambda_{em})]} \quad (2)$$

where A (λ_{exc}) and A (λ_{em}) correspond to the absorbance values of the sample at both the excitation and emission wavelengths, respectively.

2.3.2. Effect of cations on 8-anilinonaphthalene-1-sulfonate binding to milk proteins

The fluorescent probe 8-anilinonaphthalene-1-sulfonate (ANS) (Sigma–Aldrich Co., St. Louis, United States) is known to bind to hydrophobic surfaces of proteins and is often used to monitor changes in their tertiary structure [21]. Such binding leads to a dramatic increase in IF and exposed hydrophobic surface areas may be quantitatively determined. Solutions of ANS (40 μM) were titrated with 0.1 g/L milk protein suspensions enriched with the cations at pH 7.4. The IF of ANS was recorded at 484 nm using an excitation wavelength of 380 nm. Temperature was controlled at 44 °C with a thermostatically-controlled water bath.

The S_0 parameter was calculated from the slope of the plot IF versus protein concentration (Eq. (3)). This parameter indicates the extent of ANS binding [24].

$$S_0 = \frac{\partial \text{IF}}{\partial [\text{protein}]} \quad (3)$$

S_0 values obtained under the different conditions assayed were compared.

2.3.3. Fluorescence quenching using acrylamide

Acrylamide is a collisional quencher of the IF emitted by Trp residues in proteins. Fluorescence quenching of 0.1 g/L milk protein suspensions fortified with cations was studied at pH 7.4 and 44 °C. A stock solution of 4 M acrylamide (Fluka, Buchs, Switzerland) was used as quencher. A decrease in IF after the addition of acrylamide was registered. Results were interpreted in terms of the "sphere of action" model. Using the modified Stern–Volmer equation:

$$\frac{IF^0}{IF} = (1 + K_D[Q]) \times \exp\left(\frac{V \times N \times [Q]}{1000}\right) \quad (4)$$

where IF^0 and IF are the fluorescence intensities in the absence and presence of quencher, respectively, K_D is the dynamic quencher constant and V is the volume of the sphere within which the probability of quenching is unity, which is only slightly larger than the sum of the radii of the fluorophore and quencher. The average concentration in molecules/cm^3 is given by $[Q] \times N/1000$; therefore,