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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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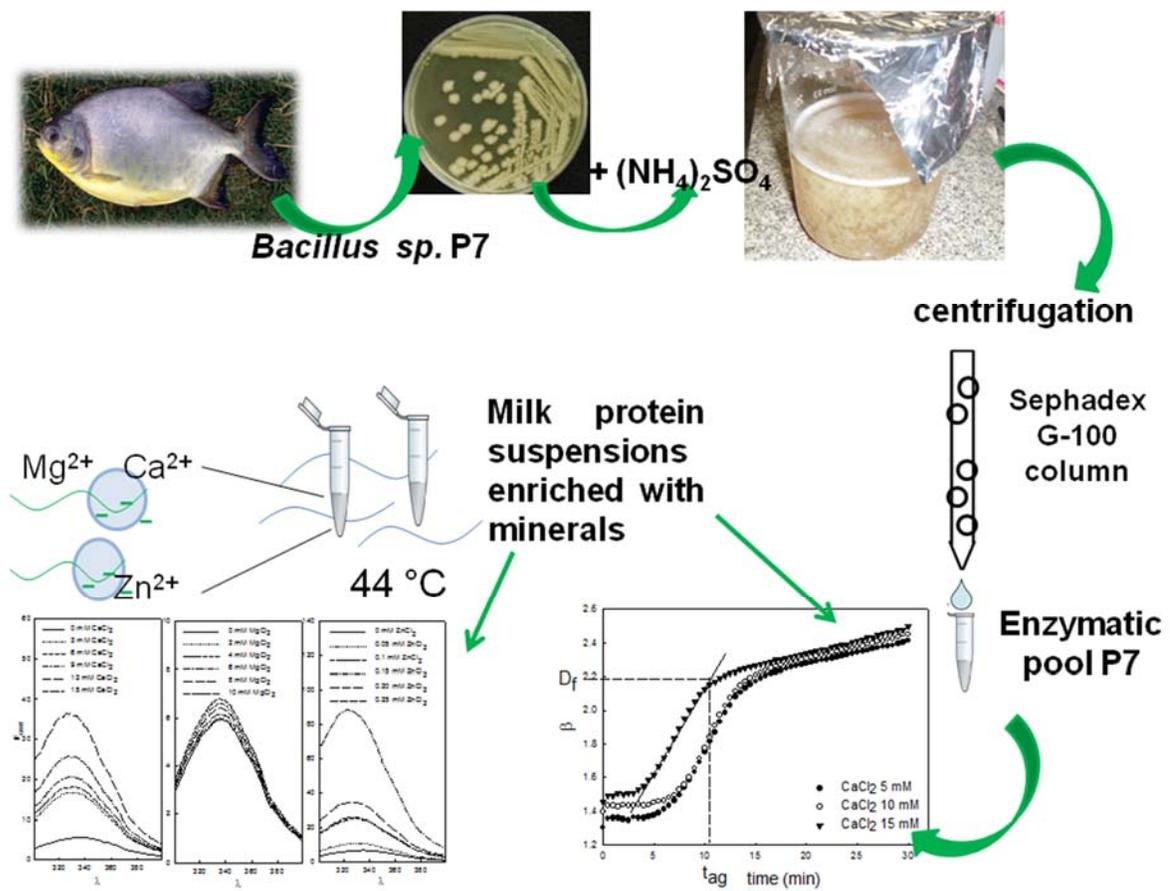
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Graphical abstract

Highlights:

- Screening of the negative casein charges by the cations caused some structural rearrangements
- The largest casein micelles were observed in the presence of zinc
- When Zn^{2+} concentration increased, milk protein aggregation induced by a novel enzyme went faster
- When higher Ca^{2+} and Zn^{2+} concentrations were tested, the gel resulted less compact

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 affect milk protein aggregation kinetics and the structure of the aggregates formed.

Key words: bacterial enzyme, casein micelle aggregation, structural changes, divalent cations

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

4

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 equation (1) [22].

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

where $A_{S,248}$ and $A_{S,256}$ are the absorbance values of the sample at 248 and 256 nm, $A_{T,248,}$ 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 to 15 mM for Ca^{2+}, 2 to 10 mM for Mg^{2+} and 0.05 to 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 equation (2) [23]:

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

where $A(\lambda_{exc})$ and $A(\lambda_{em})$ 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 (equation 3). This parameter indicates the extent of ANS binding [24].

$$S_0 = \partial IF / \partial [protein] \qquad (3)$$

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

7

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:

$$IF^0/IF = (1 + K_D [Q]). \exp(V.N.[Q]/1000) \qquad (4)$$

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*].*N*/1000; therefore, the average number of molecules in the sphere is *V*.[*Q*].*N*/1000 [23].

2.4.   Zeta potential and particle size of milk proteins

Dynamic light scattering is a useful technique to study the average diameter of particles in a suspension [25] and also to monitor electrokinetic behaviour of suspensions by zeta potential (ζ potential) [26]. In this study, measurements were carried out at 44°C using a Nanozetasizer 100 (Horiba Scientific, Edison, United States) equipped with a laser of 532 nm as light source (10 mW). Water viscosity and refraction index values were 0.000891 kg/m·s and 1.33, respectively. ζ potential and particle size were measured in triplicate. Samples were prepared with ultrapure water (Milli-Q). Final milk protein concentration was 0.3 g/L and the pH was adjusted to 7.4. Different cation concentrations were tested (5, 10 and 15 mM for Ca^{2+}; 5 and 10 mM for Mg^{2+}; 0.125 and 0.25 mM for Zn^{2+}).

2.5.   Colour digital analysis of milk protein suspensions

A simple method that uses a combination of a digital camera, a computer, and the graphics software Photoshop (Adobe Systems Incorporated, San Jose, United States) was

used to measure and analyze the surface colour of food products [27]. Mineral-enriched milk protein suspensions (0.3 g/L protein) were photographed on a matte black background in a wooden box. A digital camera (Canon EOS-Rebel T3) was used in manual mode with a lens aperture at f = 8, time of exposition 1/200, zoom 35 mm, no flash, ISO sensibility 400 and maximum resolution (3648 X 2736 pixels). The images were stored in JPEG and RAW formats.

An IT8 calibration card (Wolf Faust, Frankfurt, Germany) was photographed under the same conditions as the milk protein suspensions and was used to obtain a colour profile. This profile was applied to sample images using Adobe Photoshop. The average values of luminance (L) and the two chromatic components, a (from green to red) and b (from blue to yellow) were obtained (considering the whole sample) from histogram window and were then converted to L*, a* and b* [28]. Whiteness index (WI) was calculated using the equation WI = L* - 3b*, as described Salcedo-Chávez et al. [29].

2.6. Protease production and purification

Bacillus spp. were stored at -20°C in brain heart Infusion (BHI, Oxoid, Basingstoke, England) broth containing 20% (v/v) glycerol (Sigma-Aldrich Co., St Louis, United States) and was propagated twice in fresh BHI medium before use.

The medium used for enzyme production was chicken feather meal broth, which contained 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g KH_2PO_4 (Sigma-Aldrich Co., St Louis, United States), and 10 g feather meal per liter. The pH was adjusted to 8.0 before autoclaving. The strain was inoculated and incubated at 30°C for 48 h with shaking at 180 rpm. The culture was centrifuged at 10,000 *g* for 20 min at 4°C and the supernatant was partially purified using the protocol: (i) protease concentration with ammonium sulfate (0-60 % saturation) (Sigma-Aldrich Co., St Louis, United States) and (ii) gel-filtration chromatography on a Sephadex G-100 column (Pharmacia Biotech, Uppsala, Sweden) [5,

30, 31]. The partially purified protease preparation (enzymatic pool P7) was used in the kinetic studies of milk clotting activity.

2.7. Enzymatic activity

Enzymatic activity was determined using azocasein (Sigma-Aldrich Co., St Louis, United States) as previously described [32]. The reaction mixture contained 100 µL of enzyme preparation and 300 µL of 10 g/L azocasein in 10 mM Tris buffer, pH 7.4. The mixture was incubated for 30 min at 44°C, and the reaction was stopped by the addition of 10% w/v trichloroacetic acid (Cicarelli SRL, San Lorenzo, Argentina). After centrifugation at 10,000 g for 5 min, 800 µL of the supernatant were added to 200 µL of 1.8 M NaOH (Cicarelli SRL, San Lorenzo, Argentina) and absorbance was determined at 420 nm. One unit of enzymatic activity was the amount of enzyme which caused a change of absorbance of 0.01 at the assayed conditions [5, 30].

2.8. Milk protein aggregation induced by hydrolysis with P7

Milk protein aggregation, induced by P7, was followed by monitoring the dependence of turbidity (τ) on the wavelength (λ). τ was measured as absorbance in the 400 to 600 nm range, where the absorption due to protein chromophores is negligible. Milk protein suspensions (0.3 g/L) were prepared at pH 7.4 and enriched with cations. After the addition of P7, absortion spectra were obtained every 30 s until a maximum and constant absorbance value (A) was reached, indicating the formation of a protein network. From these spectra, β parameter was calculated applying equation (5):

$$\beta = 4.2 + \partial \, logA/\partial \, log\lambda \qquad (5)$$

in which, $\partial logA/\partial log\lambda$ represents the slope of the linear plot log A versus log λ. This parameter is related to the mean size changes during the aggregation process. From β vs. time plots, fractal dimension (D_f), related to the degree of compactness of the aggregates,

and aggregation time (t_{ag}) were estimated as β and time values when the protein network is formed [33].

τ was measured as absorbance by pouring 3 mL of the different samples into a 1 cm cuvette in a jacketed cuvette holder maintained at 44°C. In all the experiments in which absorbance was measured, a diode array spectrophotometer (SPEKOL 1200, Analytikjena, Belgium) was used.

A three level factorial experimental design was carried out. The different cation concentrations assayed were considered as independent factors. D_f and t_{ag} were considered as response variables and depending on the ANOVA results, significant factors were determined.

2.9. Statistic analysis

Data were reported as mean values ± standard deviations for all data points. All the experiments were carried out in at least duplicate. ANOVA was used to determine significant differences ($p < 0.05$) between variables. In the correlation analysis, the strength of the linear relationship between variables was calculated using the Pearson coefficient of correlation r. The software used for data processing were Design Expert 6.0 (Stat-Ease Inc., Minneapolis, United States) and SigmaPlot 12 (Systat Software Inc., California, United States) trial versions both.

3. Results and discussion

3.1. Structural changes of milk proteins

3.1.1. Spectrofluorimetric study

The fluorescence spectrum of Trp is highly sensitive to the hydrophobicity and to the polarizability of its environments. Monitoring Trp fluorescence is a convenient method for detecting structural alterations of its immediate environment in proteins. The emission

spectra of 0.1 g/L milk protein suspensions with different Ca^{2+} and Zn^{2+} concentrations are shown in Fig. 1.

The cations assayed caused an increase in the IF. This indicates a decrease in non-radiative decay processes. In almost all conditions tested the increase in IF values was proportional to the cation concentration. Only in the presence of Mg^{2+} the IF increased up to 4 mM and then decreased (data not shown). In the presence of Zn^{2+}, the highest variations of IF were obtained. No shifts of the maximum emission wavelength (330 nm) were observed.

When protein fluorophores are exposed to the solvent, they are more sensitive to the interaction with other molecules that can deactivate the excited state. These results, however, demonstrate just the opposite: some structural changes move exposed Trp residues to a more hydrophobic surrounding and the IF that was extinct before, is now emitted. It may be proposed that the presence of these cations is inducing structural rearrangements in the milk proteins that modify the accessibility of the solvent to the Trp residues. The effect is more pronounced in the case of Zn^{2+}, followed by Ca^{2+} and being almost negligible in the case of Mg^{2+}.

All the fluorescence spectra obtained exhibit the emission maximum at 330 nm and a shoulder at 360 nm. These spectra indicate that milk proteins have two Trp populations: one exposed to the solvent ($\lambda_{emission}$ 360 nm) and the other one inside the tertiary protein structure ($\lambda_{emission}$ 330 nm) [23]. By analyzing the variation of the IF_{330nm}/IF_{360nm} ratio in the presence of metallic cations, it would be possible to study structural changes caused by the transition of a Trp residue from a polar solvent to a non-polar one (when IF_{330nm}/IF_{360nm} increase) or the opposite (when IF_{330nm}/IF_{360nm} decrease). The IF_{330nm}/IF_{360nm} ratio increased with Ca^{2+} and Zn^{2+} concentration (Pearson correlation coefficient > 0,810; p value < 0,05). These results are also indicating that the increase in the concentration of Ca^{2+} and Zn^{2+} produces the increase in the hidrophobicity of the environment of the Trp. However, no

correlation between IF_{330nm}/IF_{360nm} ratio and Mg^{2+} concentration was observed (Pearson correlation coefficient = - 0.371, p value= 0.468). This could be suggesting that the dipolar moment in the microenvironment of the tryptophan is not being affected by the presence of Mg^{2+}.

Caseins, which are the major protein constituent of mammalian milk, have net negative charge in solution at neutral pH. Cations would electrostatically interact with caseins, inducing structural rearrangement of the protein chains with the incorporation of Trp residues into a more hydrophobic environment. This behaviour was also observed by Chakraborty & Basak, when they studied the interaction of caseins with aluminium and zinc [21].

3.1.2. Effect of cations on ANS binding to milk proteins

The relative S_0 values of milk protein suspensions were calculated as the ratio between S_0 of each sample and S_0 in the absence of cations (Fig.2).

All metallic cations assayed caused significant variations of the relative S_0 values. The presence of Ca^{2+} and Mg^{2+} enhanced relative S_0 values up to a given salt concentration (9 mM and 6 mM, respectively), above which relative S_0 values decreased. In the presence of Zn^{2+}, relative S_0 values increased as the cation concentration increased.

Higher relative S_0 values indicate a higher quantity of fluorescent probe bound to the protein. This suggests a decrease in the net negative charge of the caseins in the presence of cations, which promotes casein-ANS interaction. The decrease in the ANS binding ability at higher Ca^{2+} and Mg^{2+} concentrations could be related to the charge screening effect due to the high ionic strength [4]. On the other hand, higher relative S_0 values obtained in the presence of Zn^{2+} can be related to the large affinity of this cation to caseins [4, 20, 34].

3.1.3. Fluorescence quenching using acrylamide

The Stern Volmer plots for the quenching experiments with magnesium are shown in Fig. 3. Nonlinear plots were obtained.

This apparent static component is due to the quencher being adjacent to the fluorophore at the moment of excitation. These closely spaced fluorophore–quencher pairs are immediately quenched, and thus appear to be complexes. This type of apparent static quenching is usually interpreted in terms of a "sphere of action" [23].

Experimental data were analyzed using equation (4). The K_D values decreased in the presence of cations. This could be explained by the fact that acrylamide does not penetrate the hydrophobic regions of proteins [23]. When the rearrangement caused by the cations turns the environment of some Trp residues more hydrophobic, quenching by acrylamide is avoided. The trend was similar to that obtained for relative S_0 values: Ca^{2+} and Mg^{2+} have an effect on K_D and S_0 (a decrease in the first and an increase in the second one) up to a given metal concentration (9 mM and 6 mM, respectively) above which both parameters returned to the initial value. This means that the extent of quenching decreases at low Ca^{2+} and Mg^{2+} concentrations (up to 9 mM and 6 mM, respectively) and increases at higher cation concentrations. In the presence of Zn^{2+}, K_D values decrease as cation concentration increases.

All the fluorescence results can be explained by possible protein structure rearrangements. Caseins, the major proteins of mammalian milk, adopt extended conformational states at neutral pH, thus most of their Trp residues are exposed to the solvent and accessible to quenching (high K_D value). According to the results, the decrease in the net negative charge of casein micelles in the presence of cations induces structural rearrangements that change the environments of some Trp residues to more hydrophobic ones, thus promoting a decrease in the quenching extent (K_D values). These more hydrophobic environments, where Trp residues are protected from quenching are called "Trp envelopes". As cation concentration increases, caseins and casein micelles

14

begin to aggregate thus maintaining the "Trp envelopes" exposed to the solvent (K_D values increase again). These structural rearrangements also explain the changes in relative S_0 values: as caseins aggregate, a less superficial area is exposed to interact with ANS.

Apparently, the aggregation of caseins caused by Zn^{2+} occurs in a different way, in which Trp residues are less accessible to quenching and thus, quenching extent decreases with cation concentration.

3.1.4.   Zeta potential and average size of casein micelles

Table 1 gives the average diameter and ζ potential determined of the casein micelles. The average diameter of casein micelles increased in the presence of the assayed cations, which agrees with fluorescence results that suggested a more aggregated state for casein micelles in the presence of Ca^{2+}, Mg^{2+} and Zn^{2+}, with Trp disposed in more hydrophobic environments. The screening of the casein negative groups (Asp, Glu, Ser-P) by cations caused structural rearrangements in protein chains and, above a given cation concentration, protein aggregation ocurred.

The largest casein micelles obtained with Zn^{2+} agrees with the most hydrophobic Trp environments obtained by fluorescence measurements (section 3.1.1).

In Table 1 the variation of ζ potential with cation concentration is shown. The initial ζ potential of casein micelles was around -25 mV at the pH assayed. Ca^{2+} and Mg^{2+} cations caused a marked displacement of ζ potential to less negative values. With Zn^{2+}, the smallest ζ potential variation was found, which is in agreement with literature [20]. The lower the casein negative charge, the higher the cation concentration, which is consistent with the average diameter results. As the ζ potential values become more positive, there will be no force to prevent micelle aggregation, resulting in flocculation.

3.2.   Colour digital analysis of milk protein suspensions

Addition of Ca^{2+}, Mg^{2+} and Zn^{2+} induced an increase in L* parameter and a decrease in a* and b* components. Calculation of the whiteness index (WI) revealed an

increase in the 'perceived' whiteness of milk protein suspensions in the presence of all the studied cations. However, the WI seems to be proportional to Ca^{2+} concentration but not to Mg^{2+} o Zn^{2+} concentrations (Table 2).

These results show that the presence of the studied ions affect more the lightness than the chromatic parameters, in agreement with results obtained elsewhere [20]. The slight effect of these ions on chromatic parameters is in agreement with the absence of color of these ions in aqueous solution. The increase in the L* and WI agrees with the previous assay that shows that the casein micelles have a bigger size in the presence of cations, which also produce an increase in the turbidity of the suspension. The higher L* and WI values in the presence of Zn^{2+} would be related to the more screened state of the negative casein charges. However, this was refuted by the ζ potential results. It seems that, at the conditions in which the experiments were carried out, the affinity of each cation for some groups in the milk proteins played a more important role than electrostatic interaction. Actually, it has been reported that the order of association of the cations assayed with caseins is: $Zn^{2+} > Ca^{2+} > Mg^{2+}$ [4, 20]. The typical Zn^{2+} interacting ligands in proteins are histidine, aspartate or glutamate, and cysteine [35]. These amino acids are present in all milk proteins in different extents and could be responsible for the high Zn^{2+} association observed.

3.3. The effect of added cations on enzymatic activity

The effect of cations on the proteolytic activity of P7 was investigated. Results are shown in Table 3. Two of the assayed cations, Ca^{2+} and Mg^{2+}, stimulated P7 activity while Zn^{2+} had little effect on it.

The increase in the enzymatic activity could depend on the formation of ionic bridges that stabilize the enzyme active site. On the other hand, the minor inhibitory effect of 0.250 mM Zn^{2+} would be related, not only to the low cation concentration assayed, but

also to the formation of bridges between $Zn(OH)^+$ and Zn^{2+} ions at the enzyme active site, as reported for some metalloproteases [5].

This study characterizes the enzymatic clotting kinetics of casein micelles with added cations and presents the possibility that quicker clotting kinetics might be due to faster enzymatic activity or more aggregated initial states.

3.4.　　Evaluation of the aggregation process induced by P7

The variation of β (related to the mean size of casein micelles) with time during the hydrolysis of milk protein suspensions by P7 is shown in Fig. 4. Same curves were obtained with $CaCl_2$, $MgCl_2$ and $ZnCl_2$, however, for practical reasons, only results in the presence of different $CaCl_2$ concentration are shown. These curves showed that P7 has a different clotting mechanism from that of standard Hansen's liquid rennet [36]. Three stages could be identified in Fig. 4: the first one in which β did not vary significantly with time which could be related to minimum average size variation of casein micelles during the first stage of hydrolysis and therefore not detectable by this technique. During the second step, when casein micelle colloidal stability is lost, β increased sharply during the aggregation process. Finally, after reaching t_{ag}, β continued to increase slowly. The D_f and t_{ag} values were determined as shown in Fig. 4.

Milk clotting by chymosin involves the cleavage of κ-casein (κ-CN) at Phe 105-Met 106 which renders the casein micelles unstable and eventually causes aggregation, increasing β. A possible explanation could be that the cleavage site of the enzymatic pool P7 is different from that of chymosin. Another explanation could be that proteolysis of κ-CN, and probably other caseins, continues after casein micelle destabilization.

Before the addition of P7, at time = 0, β showed higher values as the cation concentration increases, in agreement with the results in section 3.1.4 and therefore validating the good correlation between β and the average size of casein micelles. The second stage began earlier at the highest $CaCl_2$ concentration (15 mM). During this stage,

the average size of the casein aggregates at a given time were larger at the highest $CaCl_2$ concentration, as expected. Both results are related to the larger average casein micelle size observed in this study (section 3.1.4). Positive cation charges screen the negative casein charges, allowing casein micelles to form bigger aggregates.

The t_{ag} and D_f values obtained at all the conditions assayed are included as supplementary data. Mesh plots for the effect of the presence of different cation concentrations on t_{ag} and D_f are shown in Fig. 5.

Only Zn^{2+} concentrations were significant on t_{ag} (p value= 0.0009), results of ANOVA are included as supplementary data. When cation concentration increased, t_{ag} values decreased (Fig. 5 a). This could be related to the fact that in the presence of Zn^{2+}, the biggest casein micelles were observed. The aggregation of these bigger casein micelles took less time than the aggregation of the small ones.

Equation 6 describes, and predicts, the behaviour of t_{ag} in the presence of different $ZnCl_2$ concentrations:

$$t_{ag}= 17.95 - 37.63 * [ZnCl_2] \qquad (6)$$

The effect of cations on P7 activity appears not to have an important role on t_{ag} since the decrease in this parameter was independent of the active or inhibitor effect carried out by each cation. However, the particular interaction between Zn^{2+} and milk proteins seems to have more influence on the way in which aggregation takes place. As mentioned above, Zn^{2+} could interact with histidine, aspartate or glutamate, and cysteine, amino acids present in milk proteins.

D_f was significantly affected by Ca^{2+} and Zn^{2+} (p value= 0.0176), results of the ANOVA test are included as supplementary data. Higher cation concentrations caused lower D_f values (Fig. 5 b). According to the above results, when the aggregation process was slower, the gel matrix would carry out restructuration with formation of new interactions. Therefore, the network became more compact (higher D_f).

18

The next equation describes and predicts the behaviour of D_f in the presence of different $ZnCl_2$ and $CaCl_2$ concentrations:

$$D_f = 2.39 - 0.04*[CaCl_2] + 0.21*[ZnCl_2] + 2.09*10^{-3}*[CaCl_2]^2 -$$

$$2.85*[ZnCl_2]^2 + 0.04*[CaCl_2]*[ZnCl_2] \quad (7)$$

The cation effect interactions could be related to the exchange of calcium by zinc in the cation binding sites of caseins as was suggested by Pomatowski et al. [34].

At the third stage of aggregation, the increasing values of β parameter with time could be related to the fact that microbial coagulants show higher proteolytic activity than animal ones [37]. Thus, protein degradation products could be forming aggregates, different to casein micelle aggregates, which became bigger as proteolysis took place.

4. Conclusions

The influence of three divalent cations on casein micelle structure and milk clotting activity of an enzymatic pool isolated from *Bacillus* spp., under the optimum milk clotting conditions of P7, was studied. According to florescence results, at neutral pH, the screening of the negative casein charges by the cations caused some structural rearrangements that moved Trp residues to a more hydrophobic environment, called "Trp envelopes". As cation concentration increased, caseins and casein micelles began to aggregate with "Trp envelopes" exposed to the solvent. Increase in the size of casein micelles was also confirmed by dynamic light scattering. The largest casein micelles were observed in the presence of zinc. Therefore, under the conditions assayed, the association of cations to specific ligands of milk proteins played a more important role than electrostatic interaction.

On the other hand, the effects of the presence of the three cations on aggregation time and the degree of compactness of the obtained clots, after casein micelle destabilization by P7, were determined. Only calcium and zinc showed correlation with these parameters. The higher the Zn^{2+} concentration, the faster the milk protein

aggregation (lower t_{ag}). When higher Ca^{2+} and Zn^{2+} concentrations were tested, the gel resulted less compact (lower D_f). The effect of cations on P7 activity appears not to have a significant role on aggregation time since the decrease of this parameter was independent of the active or inhibitor effect carried out by each cation.

Therefore, this study indicates that, depending on Ca^{2+} and Zn^{2+} concentrations used, it could be possible to obtain clots of different characteristics using a bacterial protease.

Acknowledgments

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Figure captions:

Figure 1. Effect of (a) $CaCl_2$ and (b) $ZnCl_2$ concentrations on emission spectra of milk protein suspensions. Samples containing 0.1 g/L milk protein were incubated in 10 mM Tris-HCl buffer pH 7.4 at 44ºC; λ_{exc}= 280 nm.

Figure 2. Effect of $CaCl_2$ (white bars), $MgCl_2$ (grey bars) and $ZnCl_2$ (black bars) concentration on relative S_0 values of milk protein suspensions. Samples containing 0.1 g/L milk protein were incubated in 10 mM Tris-HCl buffer pH 7.4 at 44ºC in the presence of 40 µM ANS.

Figure 3. Stern Volmer plots resulted from the quenching by acrylamide of milk protein suspensions enriched with different $MgCl_2$ concentrations. Samples containing 0.1 g/L milk protein were incubated in 10 mM Tris-HCl buffer pH 7.4 at 44ºC.

Figure 4. Changes of the β parameter as function of time induced by P7 in the presence of different $CaCl_2$ concentrations (mM): (●) 2.5, (○) 7.5 and (▼) 12.5. Milk protein concentration 0.3 g/L in 10 mM Tris-HCl buffer, pH 7.4, temperature 44 ºC.

Figure 5. Synergistic effect of different $CaCl_2$ and $ZnCl_2$ concentrations on (a) milk protein t_{ag} and (b) on the D_f of the obtained clots using P7. Samples contained 0.3 g/L milk protein incubated in 10 mM Tris-HCl buffer pH 7.4 at 44 ºC.
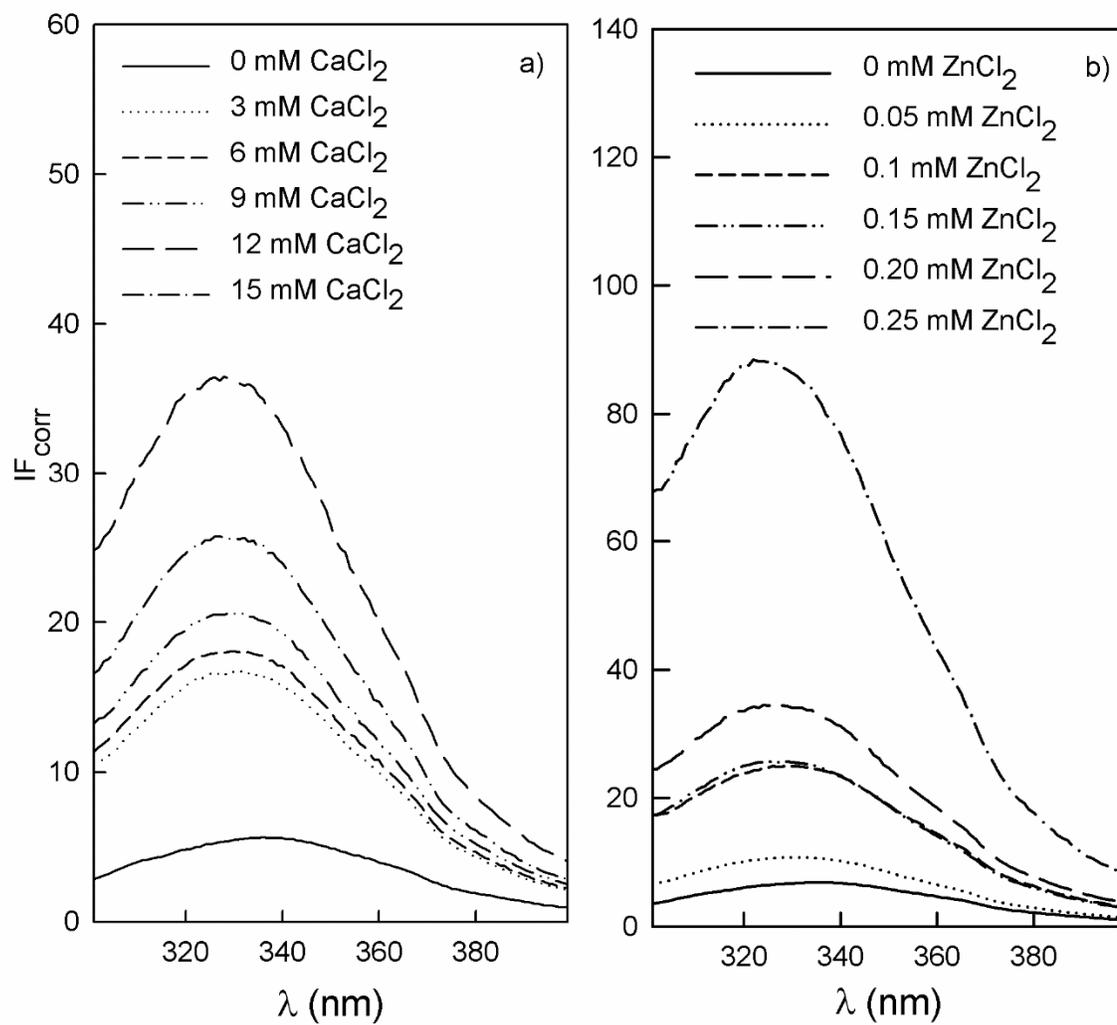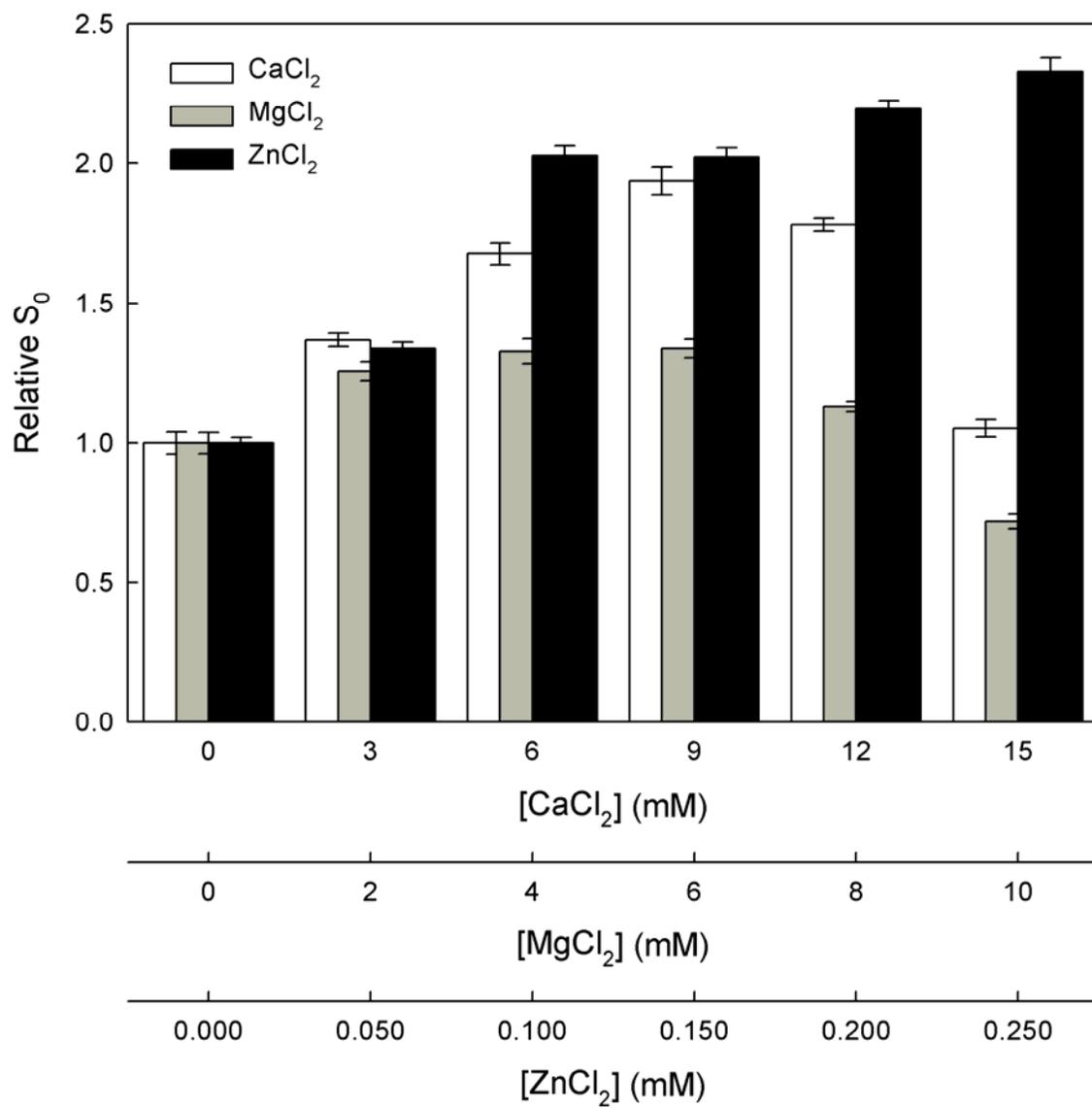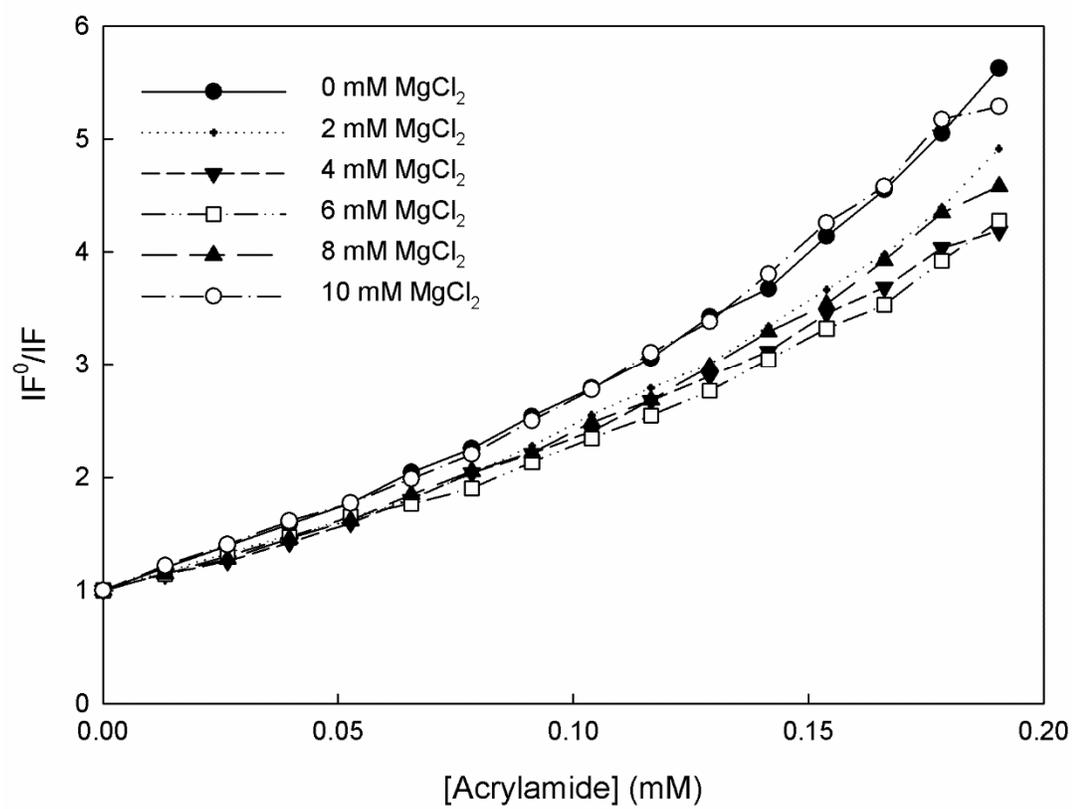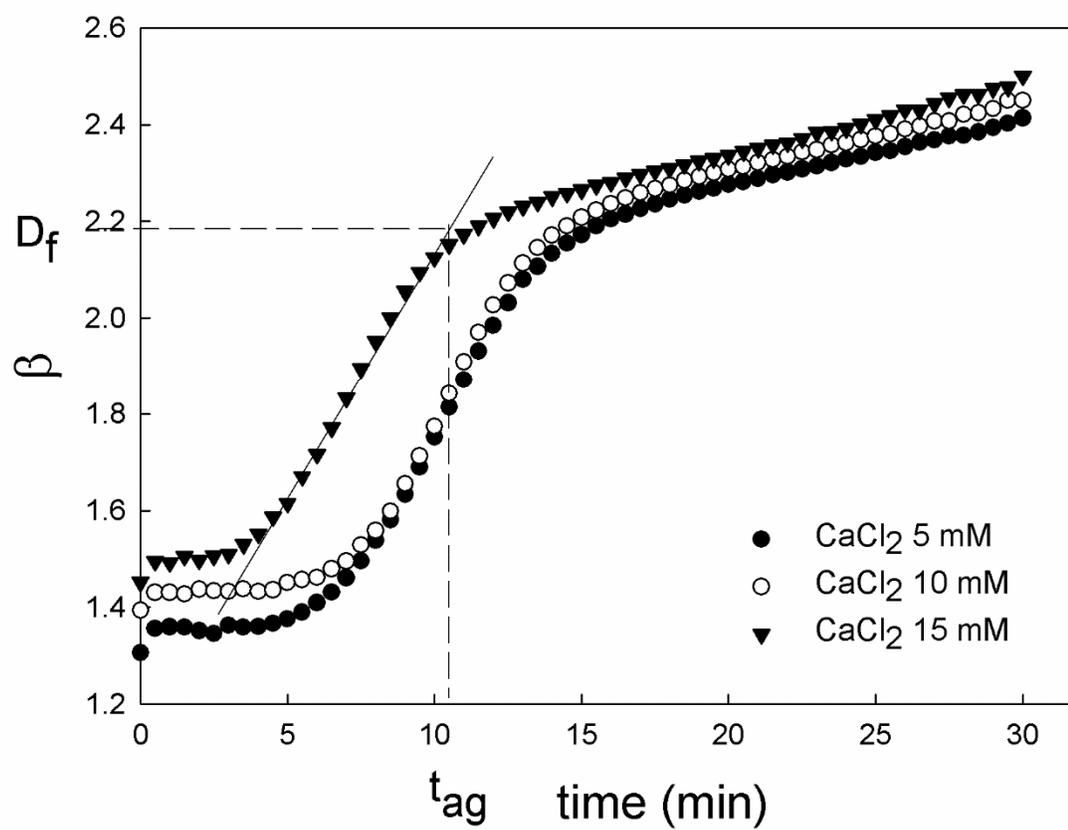
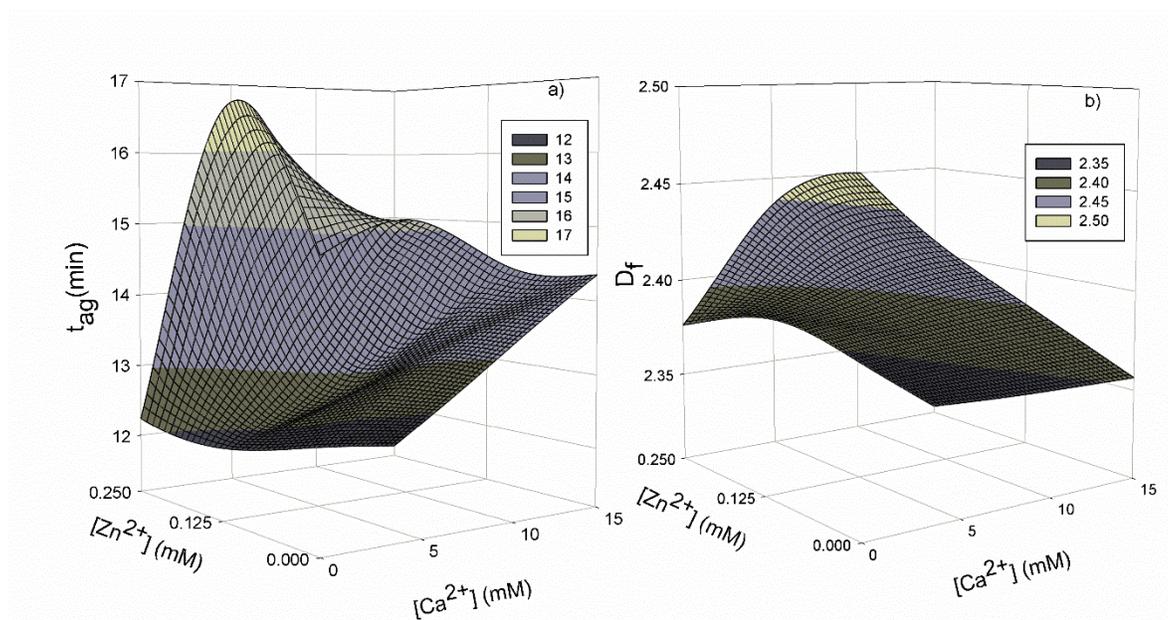Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Table 1. Effect of different $CaCl_2$, $MgCl_2$ and $ZnCl_2$ concentrations on the average diameter and zeta potential of casein micelle. Samples containing 0.3 g/L milk protein were incubated in 10 mM Tris-HCl buffer pH 7.4 at 44°C.

Cation	[Cation] (mM)	Average diameter (nm)	ζ potential (mV)
	-	121 ± 2	- 26 ± 2
	5	206 ± 3	- 12 ± 2
Ca^{2+}	10	198 ± 5	- 5 ± 2
	15	193 ± 4	- 4 ± 2
	5	166 ± 6	- 18 ± 3
Mg^{2+}	10	168 ± 4	- 16 ± 3
	0.125	220 ± 2	- 26 ± 3
Zn^{2+}	0.250	206 ± 3	- 24 ± 3

Table 2. A comparison of colorimetric measurements of milk protein suspensions enriched with different CaCl$_2$, MgCl$_2$ and ZnCl$_2$ concentrations. Samples containing 0.3 g/L milk protein were incubated in 10 mM Tris-HCl buffer pH 7.4 at 44°C before taking the pictures.

Cation	[Cation] (mM)	L*	a*	b*	WI
	-	17.5 ± 0.2	1.68 ± 0.05	-4.1 ± 0.2	29.8 ± 0.8
Ca^{2+}	5	33.35 ± 0.03	-0.36 ± 0.08	-6.5 ± 0.3	52.8 ± 0.9
	10	56.64 ± 0.08	-0.9 ± 0.2	-7.1 ± 0.1	77.9 ± 0.4
	15	64.39 ± 0.07	-0.6 ± 0.2	-6.0 ± 0.2	82.4 ± 0.7
Mg^{2+}	5	24.0 ± 0.2	0.5 ± 0.4	-5.3 ± 0.2	39.9 ± 0.8
	10	21.00 ± 0.06	1.0 ± 0.1	-6.1 ± 0.2	39.3 ± 0.7
Zn^{2+}	0.125	66.5 ± 0.8	-2.2 ± 0.2	-8.8 ± 0.3	93 ± 2
	0.250	69.1 ± 0.8	-1.2 ± 0.2	-7.7 ± 0.2	92 ± 1

Table 3. Effect of different $CaCl_2$, $MgCl_2$ and $ZnCl_2$ concentrations on P7 protease activity.

The enzyme was incubated in 10 mM Tris-HCl buffer pH 7.4 at 44 ºC.

Cation	[Cation] (mM)	Relative protease activity (%)
	-	100.0 ± 0.1
Ca^{2+}	5	106.6 ± 0.2
	10	104.5 ± 0.4
Mg^{2+}	5	102.2 ± 0.3
	10	101.9 ± 0.4
Zn^{2+}	0.125	100.0 ± 0.2
	0.250	98.7 ± 0.2