Food Chemistry 136 (2013) 266–272

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Effects of solvent and alkaline earth metals on the heat-induced precipitation process of sodium caseinate

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ARTICLE INFO

Article history:
Received 4 May 2012
Received in revised form 27 July 2012
Accepted 30 July 2012
Available online 8 August 2012

Keywords:
Caseinate
Fluorescence
Precipitation temperature
Deuterium oxide
Divalent ions

ABSTRACT

The precipitation temperatures of sodium caseinate in H_2O and D_2O in the presence of Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} were investigated through fluorescence, turbidity and conductivity experiments.

As for the ability of the divalent cations (1–17.5 mM) to induce the precipitation process in H_2O, the sequence $Ba^{2+} \geqslant Ca^{2+} > Mg^{2+} > Sr^{2+}$ was found. Remarkably, while at low salt concentrations (<10 mM) precipitation temperatures (T_{Ps}) were found to change significantly depending on the specific cation, at higher concentrations (>10 mM) the differences among the different cations were greatly reduced. By fitting these results with a modified Jones–Dole equation, we confirmed that the less hydrated ions possess a greater capacity to induce precipitation.

In D_2O, the order of ion ability to induce caseinate precipitation was $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+}$. The different hydrophobicity between D_2O and H_2O was shown to affect significantly the T_{Ps} of caseinate in the presence of calcium, strontium and barium.

1. Introduction

The functionality of a protein depends on the specific features of both the polypeptide and of the solvent. Proteins fold into characteristic and functional three-dimensional structures that are determined by the specific interactions between the chemical functionalities of their amino acid sequence and the surrounding solvent. Positively and negatively charged amino acids can attract each other and contribute to the overall protein conformation, while the non polar amino acids have a strong tendency to associate with one another. Such an effect in proteins gives rise to the formation of hydrophobic cavities and contributes to the stabilization of the protein structure. The extent of these interactions depends on the protein type, pH and ionic strength of the dispersion, on the nature and concentration of the ionic species that are dissolved in the medium, as well as on the solvent characteristics.

The influence of salts on macromolecular aggregates has been reported in several papers (Baldwin, 1996; Cuomo, Palazzo, Ceglie, & Lopez, 2009; Curtis, Prausnitz, & Blanch, 1998; Kunz, Lo Nostro, & Ninham, 2004; Lawal, Afolabi, Adebowale, Ogunsanwo, & Bankole, 2005; Lo Nostro, Peruzzi, Severi, Ninham, & Baglioni, 2010; Lo Nostro et al., 2006; Voinescu et al., 2006; Zhao, 2005). While the origin of many specific ion effects is still debated, it is widely accepted that the mechanism by which salts destabilize proteins is relevant to the suppression of aggregation. A fundamental parameter for the formation of ion pairs in solution is ion hydration, which in turn depends on the ions' charge density (Collins, 1997). In summary, as demonstrated by Collins, small ions of high charge density are strongly hydrated (kosmotropes) whereas large monovalent ions of low charge density are weakly hydrated (chaotropes).

On the other hand, it is well known that the affinity of ions for proteins depends on the presence of the binding sites that can be made more or less accessible by modulating the hydrophobic–hydrophilic balance of the protein interactions. A suitable strategy for altering this intricate equilibrium is the use of deuterium oxide (D_2O). In fact, it has been demonstrated for a number of proteins that aggregation phenomena are greatly influenced by replacing H_2O with D_2O. For example, the deuterated solvent stabilized the oligomeric form of halophilic malate dehydrogenase, (Bonnete, Madern, & Zaccai, 1994) and it also affects the stability of some proteins such as β-lactoglobulin (Verheul, Roefs, & de Kruif, 1998). D_2O is able to exert the above mentioned effects by enhancing the self-association of several proteins (Chakrabarti, Kim, Gupta, Barton, & Himes, 1999). Although the mechanism of protein assembly in D_2O is not well understood, it is thought to be due to the enhancement of hydrophobic interactions and hydrogen bonding which are much stronger in D_2O than in H_2O. Presumably, in deuterium oxide there is a greater entropic effect and consequently an enhancement of the hydrophobic interactions (Kresheck, Schneider, & Scheraga, 1965; Parker & Clarke, 1997).

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http://dx.doi.org/10.1016/j.foodchem.2012.07.117

Keeping in mind these arguments, the concomitant effect of the salts and of the solvent represent the crucial issue for intra- and inter-association phenomena that occur in protein solution systems. Many proteins are involved in specific ion interactions that are strongly related to their functionality and stability. Among these, casein represents a good candidate for an investigation of the influence of salt type and solvent characteristics on protein functionality.

Caseins are present in milk as large colloidal particles (casein micelles) held together by electrostatic and hydrophobic interactions (Fox & Brodkorb, 2008; Phadungath, 2005). The presence of calcium and phosphate ions is necessary for micelle integrity (Alvarez, Risso, Gatti, Burgos, & Sala, 2007; Guo, Campbell, Chen, Lenhoff, & Velev, 2003; Waugh, 1961) and the stability of casein micelles is affected by pH, ionic strength and temperature (HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008; Liu & Guo, 2008). Casein is made up of four main components known as α_{s1}, α_{s2}, β and κ-casein; these protein fractions are phosphorylated on specific serine residues and contain 8, 9–11, 5 and 1 phosphate groups, respectively (Farrell et al., 2004). The presence of anionic phosphoserine and other anionic amino acid residues such as carboxylate moieties of aspartic and glutamic acids make caseins available for cation binding (Byler & Farrell, 1989). The prediction of the effect induced by the addition of different cations is not easy since many factors, such as the kind of cations and their concentration, affect the association between the protein and the cations. The association that has been studied the most is the one with calcium ions. Calcium ions generate a number of important effects on casein solubility and on colloidal stability. Another important divalent cation, albeit contained in milk to a lesser extent, is magnesium. Several authors report differences in the association behaviour of calcium and magnesium with caseins (de la Fuente, Montes, Guerrero, & Juárez, 2003; Kull, Nylander, Tiberg, & Wahlgren, 1997; Nylander, Tiberg, & Wahlgren, 1999).

The study of parameters and forces involved in the casein precipitation process in the presence of divalent ions is highly relevant for technological applications (Raikos, 2010). Sodium caseinate is commonly used as an ingredient in a wide range of formulated food emulsions (Dickinson, 2009; Maroziene & de Kruif, 2000) owing to its physicochemical, nutritional and functional properties (Raouche, Dobenesque, Bot, Lagaude, & Marchesseau, 2009). Derived from the casein micellar fraction, it lacks calcium and phosphate and associates in solution as aggregates ranging from monomers up to small complexes of about 500–800 kD. In the presence of calcium ions, sodium caseinate forms larger particles based on precipitates of α_s- and β-caseins stabilized by κ-casein (Parker, Donato, & Dalgleish, 2005) in order to shield the hydrophobic parts of casein molecules from water.

Caseins are defined as native unfolded proteins characterised by an increase in their structural complexity in response to extreme environmental changes, such as high temperature or extreme pH (Uversky, 2002). Hence, the effects induced by exposure of caseins and caseinates to temperature treatments have been under intensive investigation for many years (Le Ray et al., 1998; Ono, Yoshida, Tanaami, & Ohkosi, 1999). On this basis, the study of temperature-induced changes in the physicochemical properties of these proteins can elucidate the general mechanisms responsible for the structural stability and functionality of peptides, highly relevant from a colloidal chemistry perspective.

In a recent paper, we demonstrated the difference in sodium caseinate precipitation temperatures induced by calcium and magnesium in H_2O and D_2O (Cuomo, Ceglie, & Lopez, 2011). In this study, we extended our previous investigation into the divalent ions series Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+}. While the ability of calcium and magnesium to induce precipitation is well known, there are few data in the literature for strontium and barium (Zhang & Aoki, 1995). Protein precipitation was assessed by following the decrease of casein intrinsic fluorescence. The effect of the salt concentrations on this process was also analysed in detail. Additionally, the fluorescence data were correlated with turbidity experiments. The role of divalent cations, the microenvironment (H_2O and D_2O) and the relative induced precipitation temperature (T_P) of sodium caseinate were compared and discussed.

2. Materials and methods

Sodium caseinate, magnesium chloride, calcium chloride, strontium chloride, barium chloride and deuterium oxide were purchased from Sigma–Aldrich. The samples were prepared with a fixed sodium caseinate concentration (4 mg/mL) dispersed in a 0.1 M sodium chloride solution in water. Suitable amounts of salts were then added to the sodium caseinate solution. All the solutions were prepared with ultrapure water. Corresponding solutions were also prepared in deuterium oxide.

Steady state fluorescence spectroscopy measurements were performed with a Varian Eclipse spectrofluorimeter equipped with a Peltier element in a 1 cm quartz cuvette at 25 °C. The emission intensities were collected at 338 nm with excitation at 290 nm; the excitation and the emission slits were of 5 nm and the PMT was set at 550 Volts. The temperature scan rate was 1 °C/min. All the numerical fitting procedures were performed running the Origin software (Origin Lab Corporation, USA).

The precipitation temperature (T_P) of caseinate is defined as the temperature value that causes the sharp formation of bigger aggregates (that precipitate with a slow process) and was calculated by means of T-scan experiments, as previously described (Cuomo et al., 2011; Palazzo, Lopez, & Mallardi, 2010). Briefly, the fluorescence of tryptophan (λ_{max} = 338 nm) was used as a signal to follow the protein fluorescence (Vivian & Callis, 2001) while the temperature was increased continuously at a fixed scan rate. A simple model was applied to the experimental fluorescence data and the data were reported by means of Eq. (1).

$$\chi_S = \frac{I_f - (a' + b'T)}{(a + bT) - (a' + b'T)} \quad (1)$$

Eq. (1) describes the experimental change of χ_S (mole fraction of the suspended casein fraction) by means of fluorescence (I_f) as a function of the temperature. T_P values were calculated in the salt concentration range 1–17.5 mM for all the alkaline earth metals used.

Turbidity analyses were carried out by means of spectrophotometric measurements in the 800–200 nm range, under shearing conditions at 25 °C, with a Cary 100 Bio UV/Vis multicell spectrophotometer Varian using quartz cells with a path length of 1 cm.

Conductivity measurements were performed with a CDM230 conductivity meter (Radiometer Analytical) equipped with a two pole conductivity cell tailored for small volumes (CDC749; cell constant 1.84 cm^{-1}).

3. Results

The specific precipitation temperatures T_p were calculated by following the mole fractions of the soluble protein in H_2O and D_2O as a function of temperature (Cuomo et al., 2011). The caseinate concentration was fixed at 4 mg/mL in 0.1 M NaCl in order to avoid association phenomena between the suspended particles (Pitkowski, Nicolai, & Durand, 2009) and thus increase the stability of the caseinate solution. In fact, in the absence of NaCl, the T_p values were found to be considerably lower for all the salts tested (data not shown). In Fig. 1, the T-scan experiments performed on samples suspended in H_2O (upper panel) or D_2O (lower panel) in the presence of