Synthesis and morphological characterization of new nanostructures formed by milk whey proteins submitted to a thermal gelation process

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ABSTRACT

β-lactoglobulin (β-Lg) and α-Lactalbumin (α-La) are the two major bovine milk whey proteins. In addition to their high content in essential amino acids, these proteins present several functionalities (small molecules binding ability, emulsifying and gelling properties, among others). Regardless these strongly positive characteristics, the related literature is still poor in studies on the synthesis and characterization of nanostructures constituted specifically by these proteins. In this work, nanostructures formed by β-Lg and α-La were synthesized and characterized using atomic force microscopy. Further investigations aiming to determine the stability and some techno-functional properties of these nanostructures, as well as their potential for controlled release of food antioxidants, are now being pursued by our research team and will be published in due course.

Keywords: α-Lactalbumin, β-lactoglobulin, gelation process, nanostructures, whey proteins.

1 INTRODUCTION

Nanomaterials or nanostructures are those presenting structural features and/or components with at least one dimension at the 10^{-9} of the meter scale (range 1-100 nm). These materials and structures can be formed by minerals, metals, ceramics, polymers and biomolecules. Due to their array at the nanometer level, they often display differentiated properties when compared to their more conventional counterparts, enabling novel applications [1]. Nanotechnology is the ensemble of concepts and methods employed to design, produce and find applications for nanostructured materials. It is a highly interdisciplinary area, encompassing elements from chemistry, physics, biology and engineering. In particular, nanotechnology has become a tool of great importance in food engineering and science [2].

α-Lactalbumin (α-La) and β-lactoglobulin (β-Lg) are the two major milk whey proteins. These proteins have been widely explored with biotechnological purposes because of their various nutritional and technological functionalities. β-Lg is the predominant protein in the whey, representing 58 % of the whey proteins and 10 % of the total milk proteins. This protein is formed by 162 amino acids, possessing a molar mass of about 18 kDa. Its pI is 5.2 and, under physiological conditions (pH ≈ 6.8), it forms non-covalently associated dimers. Each protein molecule has a free thiol group (-SH). Its secondary structure is essentially constituted by eight β-sheets disposed in two perpendicular plans, forming an hydrophobic central crevice. This last is surrounded by a α-helice (Figure 1a) [3]. α-La is the second most abondant protein in the whey, representing approximatelly 13 % of its proteins and 2 % of the total milk proteins. The molecule is constituted by 123 amino acids, with a molar mass of about 14 kDa. Its pI value is 4.6. Under physiological conditions, this protein is present as monomers. Its secondary structure counts four α-helices and two paralell β-sheets. The tertiary structure is stabilized by four dissulfide bonds; but, contrarilly to the β-Lg, the molecule has no free thiol groups (Figure 1b) [4].

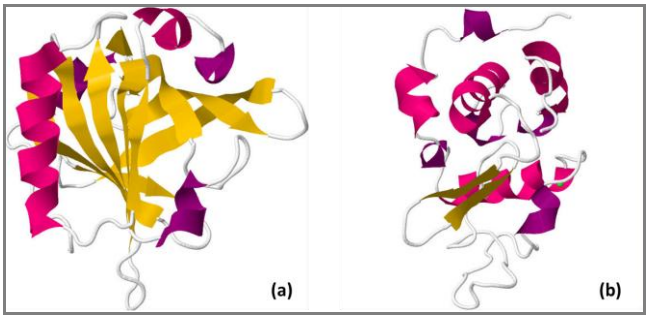

Figure 1: 3D structures of the milk whey proteins (a) β-lactoglobulin [3] and (b) α-lactalbumin [4].

Among the biotechnological applications of these proteins, an important one is their use in the development of matrices for controlled release of bioactive compounds or food aromas and antioxidants [5]. Indeed, thanks to their size and tridimensional structure with different kind of amino acid exposed on their surface, protein nanostructures are able to non-covalently bind bioactive compounds [6-8].

It is expected that nanostructures obtained with these proteins display improveed technological applications compared with the individual protein molecules.

As several food proteins, α-La and β-Lg can undergo gelation when submitted to specific thermal treatments. The thermally-induced gelation of proteins encompasses the following events:

(1) conformational changes, leading to the exposition of hydrophobic amino acid residues which are buried in the non-denatured protein;
(2) aggregation of protein molecules, due to the hydrophobic interactions formed between the exposed hydrophobic residues;
(3) stiffening of the aggregates, due to the formation of disulfide (S-S) bonds between cysteine residues and, finally;
(4) improvement of the gel elasticity, due to the formation of H bonds involving side chains of polar residues and the backbone amino (-NH) and carbonyl (-C=O) groups.

While the three first events are triggered by the heat in the first step of the process, the fourth one occurs during subsequent cooling second step [9].

Some authors have recently reported the synthesis of nanostructures involving milk proteins, alone or combined with other materials, such as polysaccharides and metal oxides [10-12]. However, to our knowledge, the literature lacks works adressing the synthesis of nanostructures formed specifically by the two major proteins from the milk whey, i.e., α-La and β-Lg. Hence, in the present study, the feasibility of nanostructures formed by α-La and β-Lg in aqueous media was investigated. The nanostructures formation was induced by applying a thermal gelation process. The obtained structures were characterized using atomic force microscopy.

2 MATERIALS AND METHODS

2.1 Materials

The proteins α-lactalbumin (90.0 % of purity; protein = 97.0 %, water = 5.5 %, ash = 3.0 %, fat = 0.5 % and lactose < 0.5 %) and β-lactoglobulin (93.6 % of purity; protein = 93.6 %, water = 5.0 %, ash = 1.8 %, fat = 0.3 % and lactose < 0.5 %) were a kind gift of Davisco Food International, Inc. (Eden Prairie, MN, USA). The proteins were directly used in the experiments, without further purification. Water was distilled and deionized (Milli-Q; 18.2 MΩ·cm). All the acids and alkalis employed were of pro analysis grade**.**

2.2 ITC experiments

Isothermal Titration Calorimetry (ITC) was performed in duplicate in order to determine the thermodynamic parameters of the supramolecular interaction, including the stoichiometry ratio of α-La and β-Lg. The solutions were prepared by dissolution of the proteins in Milli-Q® water. Experiments were carried out, at 25 °C, after the electrical and chemical calibration of the equipment in amicrocalorimeter (NanoITC 2G TA Instruments, USA). The final ITC curve was fitted using the independent model supplied by the NanoAnalyze software. Each titration experiment consisted of successive injections (5 μL) of a 10 $mmol.L^{-1}$ β-Lg aqueous solution in a 1 $mmol.L^{-1}$ α-La aqueous solution (cell volume = 1.2 mL). The first injection of 1 μL was discarded to eliminate diffusion effects of the syringe material into the calorimetric cell. The final curve was obtained from the subtraction of the β-Lg aqueous solution in water and the dilution process of α-La with water.

2.3 Synthesis of α-La/β-Lg nanostructures

Aqueous solutions of α-La and β-Lg (2 $mg.mL^{-1}$) were prepared and their pH was adjusted to the desired value (model pH 212, Hanna Instruments, USA). The solutions were then mixed so that the molar ratio β-Lg:α-La was 2:1, according to their interaction stoichiometry determined by the preliminary ITC experiments. The resulting mixtures were heated to 80 ^{o}C (Tecnal TE-184, Brazil) for 30 minutes. After the heating, the mixture was cooled to 5 °C during 60 minutes. In each case, mixtures of (β-Lg + α-La), at the 2:1 ratio and pH equal to that of the synthesis media, without any thermal treatment and kept at ambient temperature, were taken as control.

2.4 Force Atomic Microscopy (AFM) analyses

For the AFM analyses, samples were prepared by naturally drying for 1 h a drop of nanostructure solution on a freshly cleaved mica surface at room temperature (25 ºC ± 2 ºC). Images were captured by a scanning probe equipment NTEGRA Probe Nanolaboratory (Molecular Devices and Tools for NanoTechnology, NT-MDT, Russian) under semi-contact mode, with a silicon cantilever of 125 μm and an E-type vertical engage piezoelectric scanner. Data were treated using the WSXM5.0 Develop 1.3 – Image Browser software.

3 RESULTS AND DISCUSSION

3.1 ITC experiments

The thermodynamic parameters ($\Delta H°$, $\Delta G°$ and $T.\Delta S°$) of the α-La and β-Lg interaction were determinated by means of Isothermal Titration Calorimetry (ITC), prior to the β-Lg/α-La nanostructures synthesis. In addition to the thermodynamic parameters, the ITC experiments were useful to get insights about the β-Lg/α-La stoichiometry supramolecular structure, since the preliminary tested molar ratio at 1:1 was not successful to obtain nanostructures (data not shown).

The ITC titration curves are depicted in Figure 2a, in which one can observe that both α-La dilution and the titration of β-Lg in water did not present heating flow;

however, when β-Lg is titrated in α-La an exothermic process is observed. Figure 2b shows the final ITC titration curve of β-Lg (10 mmol.L^{-1}) in α-La (1 mmol.L^{-1}), after subtracting of the titration of water in α-La and the dilution of β-Lg aqueous solution in water, as well as the non-linear fitting.

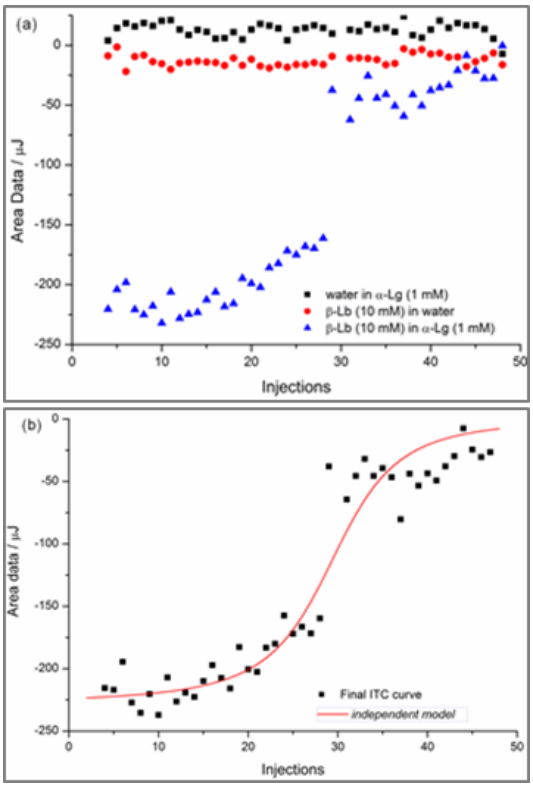

Figure 2. (a) ITC curves of water in α-La (at 1 mmol.L^{-1}), β-Lg (at 10 mmol.L^{-1}) in water and β-Lg in α-La (b) final ITC curve and independent model fitting.

This adjust allowed to determinate the stoichiometry coefficient (n = 1.7), which can correspond to a 2:1 molar ratio of β-Lg in α-La. Since then, this molar β-Lg:α-La ratio = 2:1 was used in the nanostructures synthesis (as described in section 2.3).

Additionally to the coefficient stoichiometry, the thermodynamic properties of the supramolecular interaction between these proteins were obtained: $\Delta G°$ = -25.8 kJmol^{-1}, $\Delta H°$ = -4.6 kJmol^{-1}, T.$\Delta S°$ = 21.2 kJmol^{-1} and K = 32,950 M^{-1}. These results demonstrated that the interaction between α-La and β-Lg is spontaneous and is driven by both enthalpy and entropy. The declivity of the final ITC curve, Figure 2b, demonstrated the existence of a strong interaction between the species, which was confirmed by the calculated equilibrium constant (K = 32,950 M^{-1}). This constant binding are in accordance with other proteins system [13], however, higher the other supramolecular systems reported in the literature [14, 15].

3.2 Force Atomic Microscopy (AFM) analyses

The morphology and the size of the synthesized nanostructures were evaluated by AFM imaging. This technique allows obtaining three dimensional images evidencing topological surface details of a few nanometers. The size and shape of nanostructures changed in function of the pH of the medium synthesis.

Nanostructures formed at pH = 5 exhibited average height ≈ 240 nm. For these particles, AFM images are shown in Figure 2-A1 and the corresponding topological profile is shown in Figure 2-A2. In this case, as the pH value of the medium synthesis was close to the pI of the proteins (pI ≈ 4.6 – 5.2), largest nanostructures were formed, indicating a more favourable aggregation of the proteins, as expected. The surface of these nanostructures presented some roughness.

At pH values out of the proteins pI range, the sizes of the synthesized particles were considerably smaller. For particles obtained with the pH of the medium = 7.3, AFM images and the corresponding topological profile are given in Figures 2-B1 and 2-B2, respectively. These nanostructures presented average height ≈ 25 nm. Another noticeable difference is that the nanostructures surfaces, in this case, were smooth and more regular.

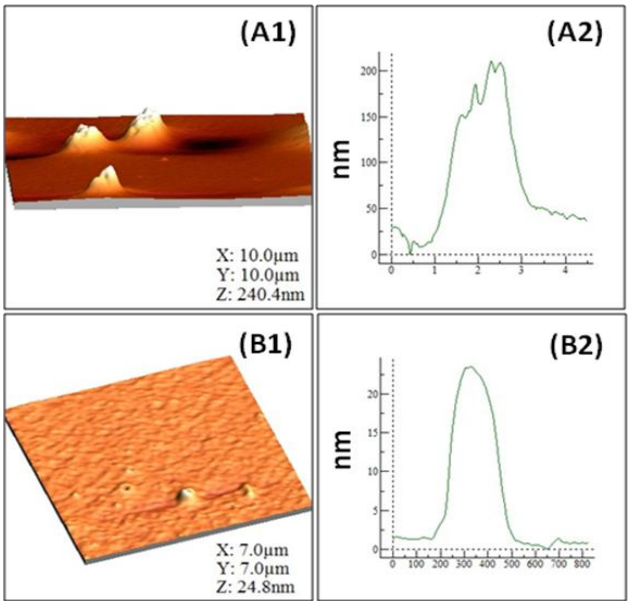

Figure 2. Atomic force microscopy results for (A1) nanostructures formed at pH near 5.0 and (A2) its topological profile; (B1) nanostructures formed at pH 7.3 and (B2) its topological profile.

4 CONCLUSIONS AND PERSPECTIVES

In this work, nanostructures formed by the whey proteins β-Lg and α-La were successfully obtained by a thermal gelation process (80 ºC / 30 minutes, followed by cooling to 5 ºC / 60 minutes) applied in an aqueous mixture

of these two proteins with β-Lg:α-La molar ratio = 2:1. At pH = 5, which is close to the pI of the proteins (pI ≈ 4.6 – 5.2), the nanostructures formed were 10-fold larger than those formed at pH = 7.3. Indeed, at pH = 7.3, the proteins are negatively charged, difficulting their aggregation.

These nanostructures are expect to be useful in enhancing the texture and techno-functional properties of food formulations, in producing active packaging food or cosmetic products, or still in creating matrices for the controlled release of preservatives and antioxidants in food or beverage products.

Further studies aiming to characterize the stability and the techno-functional properties of these new nanostructures are now in progress in our lab.

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