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Partition of α-lactoalbumin and β-lactoglobulin by cloud point extraction

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ABSTRACT

This work aimed to study the partition of cheese whey proteins α-lactoalbumin and β-lactoglobulin using aqueous two-phase system by applying the cloud point extraction technique. The cloud point temperatures were determined under different concentrations of copolymer and salt. The system providing the best protein separation conditions was 20 mass% of copolymer PE61 and potassium phosphate salt solution of 100 mM, at pH 7. The protein α-lactoalbumin remained preferentially in the aqueous phase and the β-lactoglobulin was transferred to the copolymer phase.

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1. Introduction

Greenish-yellow in color, cheese whey is a cheese by-product, obtained from milk coagulation. The world production of cheese whey per year is estimated as 130 million tons, accounting for around 780,000 tons of proteins. Whey proteins are considered complete proteins, containing all the essential amino acids, vital for the human metabolism. These proteins have a high commercial value and a wide range of functional attributes for nutritional and biological applications [1–3]. In addition, they act as food ingredients and as foaming and emulsifying agents. Because of the differences in their functional and nutritional properties, there has been a growing interest in the separation and fractionation of whey proteins.

The major proteins found in the whey are the α-lactoalbumin (α-la) and the β-lactoglobulin (β-lg). These proteins are responsible for the hydrating, emulsifying, foaming and jelly properties of the whey protein ingredients [4]. The α-la and β-lg presented high nutritional and biological values, as well as commercial importance. Thus, different methods have been used for the separation of these cheese whey proteins such as gel filtration [5], high-resolution chromatography [6], ultra filtration [7] and liquid–liquid extraction [8].

However, the conventional liquid–liquid extraction, in which aqueous solutions and organic solvents are used, is not adequate for separating components of biological origin, such as proteins and cells, due to the low stability of these substances in organic solvents.

A variant of the traditional liquid–liquid extraction using aqueous two-phase system (ATPS) has been successfully applied in the isolation of proteins and other bio-compounds. ATPS has advantages over other purification methods, such as reduced volume, good resolution and yield, high capacity and short processing time. These systems can be formed by adding either two incompatible polymers, such as polyethylene(glycol) (PEG) and dextran or a polymer and a salt, such as PEG and $(NH_4)_2SO_4$, to water. The most common polymer + polymer system is composed of PEG and dextran [9–11]. New polymeric systems containing thermo-separation polymers, as the triblock copolymers, have been developed, and are being used in the purification of different types of biomolecules. The triblock copolymers are synthesized by simultaneous polymerization of more than one type of monomer. The result of this synthesis is called block copolymer if the individual monomers occur as blocks of various sizes in the monomer's copolymer molecule [12–14].

The triblock copolymers of ethylene oxide (EO) and propylene oxide (PO) with $(EO)_x(PO)_y(EO)_x$ structure are attractive model systems for hydrophobic interaction studies and relative progress has been obtained in understanding their properties and structures in solution as a function of temperature. The copolymers EO–PO–EO are classified based on their molar mass range and composition according to the PO/EO ratio. The abbreviations used for these tri-

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Table 1
Triblock copolymers evaluated

Triblock copolymers	Mean molar mass (g/mol)	EO (mass%)	Viscosity (cPs)	Physical form
L35	1900	50	375	Liquid
ULTRARIC PE61	1925	10	285	Liquid
L31	2000	10	325	Liquid
L	2900	40	850	Liquid

block copolymers are the letters L (liquid), S (solid) and F (flake). The first or the two first numbers after the initial letter indicate the molar mass of block PO and the last number indicates the mass fraction of block EO. For example, P 104 and F 108 have the same molar mass PO does (in the order of 3000), but P 104 has 40 mass% of EO and F 108 has 80 mass% of EO [14–16].

An advantage of using the block copolymers in ATPS extraction is the possibility of recycling the polymer after separation without the need of another costly separation method [20]. Thus, in such cases, short processing time and the low temperatures used are also attractive and ideal for maintaining biomolecule stability [11].

If aqueous solutions of these polymers are heated above critical temperature, denoted as their cloud point temperature, their solubility decreases and a system composed of an aqueous phase and a polymeric one is formed [17]. The polymer-rich phase presents small volume composed mostly of the polymer and a low quantity of water. The aqueous phase, in equilibrium with the polymeric rich phase, contains a polymer content close to its critical micellar concentration [18].

The cloud point extraction (CPE) technique is a simple procedure used to extract and concentrate analytes in a single step [19]. The CPE has been applied for the extraction of metal chelates, viruses, herbicides and vitamins [21].

The aim of this work was to study the partition of cheese whey proteins α-la and β-lg, using aqueous biphasic systems formed by copolymers with thermo-separation properties with the cloud point extraction technique.

2. Materials and methods

2.1. Chemicals and materials

The triblock copolymers used (Table 1) were ULTRARIC PE61 from Oxiteno (Brazil); L35, L31 and the copolymer identified in this work by L (code 43544-9, Catalog Handbook of Fine Chemicals Aldrich, 1996–1997), from Aldrich (Germany). The sodium chloride was P.A. grade (F. Maia, Brazil) and acetonitrile was HPLC grade (Vetec, Brazil). The whey protein isolate (WPI) was a gift from Davisco Foods (USA) and the standard proteins α-la and β-lg were purchased from Sigma Chem. Co. (USA).

The salt solutions, at concentrations of 15, 45 and 100 mM, at pH 7, were previously prepared by using monobasic (KH_2PO_4) and dibasic (K_2HPO_4) potassium phosphate salts (Vetec, Brazil). All reagents were used without additional purification.

2.2. Determination of cloud point temperatures

The cloud point temperatures were determined using 5 g of aqueous biphasic systems composed of triblock copolymer (PE61, L31, L35 and L), and monobasic and dibasic potassium phosphate salts. The systems were prepared in glass tubes. The copolymers concentrations in the systems ranged from 5 to 30 mass%, and the concentrations of the salt solutions were of 15, 45 and 100 mM, at pH 7.

Following the addition of the reagents (copolymer and saline solution), the glass tubes were manually stirred, by inversion, for 5 min and then transferred into a thermostat bath. The system temperature was increased at the rate of 0.5 °C/min and the cloud point temperature was recorded as the temperature at which the initial turbidity of the system was observed.

2.3. Partitioning experiments and quantification of α-lactoalbumin and β-lactoglobulin in the phases

Graduated centrifuge tubes were used to prepare 12 g of each system composed of triblock copolymers (L31 and PE61), water, potassium salts and WPI. The copolymers L and L35 were not utilized in the partitioning experiments since they presented higher cloud point temperatures, which might cause protein denaturation.

The aqueous solution containing WPI was prepared at a concentration of 20 mg of WPI/mL. Thus, the α-la and β-lg concentrations in this solution were, respectively, of 1.98 and of 8.41 mg/mL, which correspond to 10.47 mass% of α-la and 44.45 mass% of β-lg, respectively, in the WPI used in our experiments. The total protein content of the WPI quantified by Kjeldahl ($N \times 6.38$) method was of 94.6 mass%.

The copolymers were added at concentrations ranging from 5 to 30 mass% of the total ATPS mass, followed by the addition of salt solution at concentrations of (15, 45 and 100) mM, and finally by the addition of 1 g of the WPI aqueous solution, totaling 144 experiments with two replications. The components were manually agitated, by inversion, for 5 min and then centrifuged (Eppendorf 5804, Germany) at 3640 × *g* for 20 min. After centrifugation, the tubes were kept in a thermostat bath (Tecnal, model TE 184, Brazil) at 10 °C above the cloud point temperatures of the systems, for 16 h, to reach the phase equilibrium.

Aliquots of the upper and lower phases were collected, respectively, with a Pauster pipette and with a long needle syringe. These aliquots were used to determine the protein partitioning coefficient. The phases were separated and stored under refrigeration.

The α-la and β-lg content in the phases were determined by high performance liquid chromatography-reverse phase (HPLC-RP), using a Shimadzu chromatograph (LC-10 VP model). All solutions and samples were filtered through cellulose acetate membranes of 0.2 μm. Samples of 20 μL were injected automatically into a reverse phase column (CLC ODS-C18-Shimadzu) of 250 mm × 4.6 mm, coupled to a guard column (CLCG-ODS-Shimadzu) of 10 mm × 4.6 mm, both balanced with sodium chloride solution of 0.15 M, pH 2.5 (solution A) and acetonitrile (solution B). The column was eluted with a gradient formed by solutions A and B as listed in Table 2, at a rate of 1 mL/min. The run time was of 33 min and the detection occurred at 210 nm using a diode array detector (SPD-M10AVP-Shimadzu), with the column being kept at 40 °C. All analyses were performed in duplicate. The analytical curves obtained to calculate the protein concentrations were constructed using solutions of the standard proteins α-la and β-lg at concentrations ranging from 0.02 to 3.0 mg/mL.

Table 2
Gradient used for the chromatographic analyses by HPLC-RP

Time (min)	Concentration of A (%) (NaCl 0.15 M; pH 2.5)	Concentration of B (%) (acetonitrile)
0–3	85	15
3–7	64	36
7–20	55	45
20–24	55	45
24–33	100	0

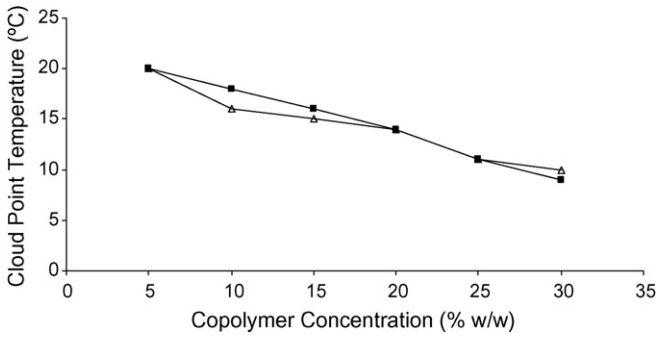

Fig. 1. Influence of the concentration of copolymers L31 (■) and PE61 (△) on the cloud point temperature for systems containing potassium phosphate salt, 15 mM, at pH 7.

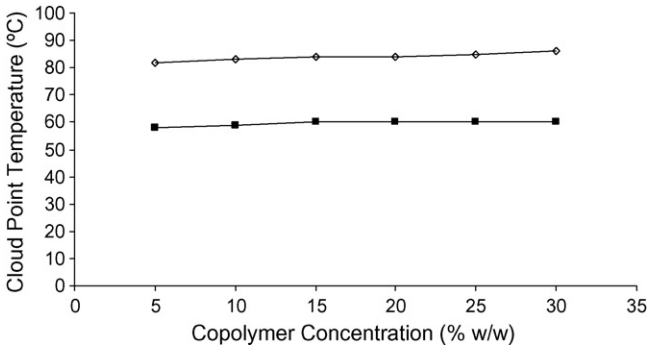

Fig. 2. Influence of the concentration of copolymers L35 (◊) and L (■) on cloud point temperature for systems containing potassium phosphate salt solution, 15 mM, at pH 7.

The partitioning coefficient (k) was defined as the ratio between the concentration of the protein (α-la or β-lg) in the water-rich upper phase (C_1) and the protein concentration in the polymer-rich lower phase (C_2), $k = C_1/C_2$. The calculation of the α-la and β-lg content in the lower phase was obtained by the difference between the protein mass added to the systems and the protein mass quantified in the upper phases.

3. Results and discussion

3.1. Influence of copolymer concentration on cloud point temperature

The molecular mechanism of the cloud point phenomenon is not totally understood though different interpretations have been reported based on the ethylene oxide dehydration, hydrogen interactions between oxygen of EO and water molecules, and EO polar and non-polar conformations [22]. It is possible to manipulate the cloud point of a polymeric solution by adding salt to the system, by changing the molar mass of the copolymer, the ratio between EO and PO or by changing the copolymer concentration [17].

The cloud point temperatures determined in this work had a precision of 1 °C for the copolymers.

Fig. 1 shows the tendency of reduction of the cloud point temperatures for the copolymers L31 and PE61 with a copolymer concentration increase. For example, in the system containing 15 mM potassium phosphate solution, the increase of the copolymer L31 content from 5 up to 30 mass% led to the reduction of the cloud point temperature from 20 to 9 °C, respectively. This fact may be explained by an increase of the interactions between the copolymer molecules and their consequent aggregation, leading to the phase separation [17,23–24].

The determination of cloud point temperatures in the partition of the bromelain enzyme using ATPS formed by triblock copolymers EO–PO–EO showed the cloud point temperature decreased from 32 °C to 18 °C when the copolymer concentration increased from 5 to 30 mass%, respectively. The systems were formed by copolymers with 10 mass% of EO, mean molar mass of 1100 g/mol and potassium phosphate salt solution of 15 mM at pH 6 [17].

Fig. 2 presents the cloud point temperature behavior of the copolymers L35 and L, with the EO contents of 50 and 40 mass%, respectively. These cloud point temperatures were higher than the temperatures of the copolymers L31 and PE61, and also provided constant temperature values with the copolymer addition. Cloud point temperatures for the systems formed by copolymer L and potassium phosphate salt solution of 15 mM were at 58 °C for polymer concentrations of 5 mass% and at 60 °C for polymer concentration of 30 mass%.

The PO blocks are hydrophobic and the EO blocks are hydrophilic, thus, copolymers with high EO concentration dissolve more easily in water. This is likely due to the degree of hydrogen interactions between the copolymer molecules [17].

3.2. Influence of salt concentration on cloud point temperature

Fig. 3 shows the temperature reduction of the solutions containing potassium phosphate salt solution of 100 mM compared to that of 45 mM and 15 mM. Cloud point temperatures of systems formed with salt solution at concentrations of (15, 45 and 100) mM and 5 mass% of the L31 and PE61 copolymers were, respectively, at 20, 19 and 18 °C and at 20, 18 and 16 °C. These results agree with that observed by [17] and [18] who also found a small variation in the cloud point temperature as function of salt content. The cloud point temperatures of the copolymers L31, PE61, L35 and L, at different salt concentrations, followed the same tendency (data not shown).

The decrease of the cloud point temperature due to the electrolyte addition is attributed to the ability of some ions in modifying the structure of the water and to their competition with the copolymer molecules, influencing the hydration of EO and PO blocks [25–27]. Also, since phosphate salts are hydrophilic, this may present a salting-out effect [17].

Bahadur et al. [28] reported the decrease of the cloud point of the copolymer F68 after the addition of KF 1 M into the copolymer aqueous solution. Jain et al. [29] also observed a linear decrease of the cloud point temperatures for copolymer P 65 at 1% (w/v) as NaCl concentration increased from 0 up to 2 M.

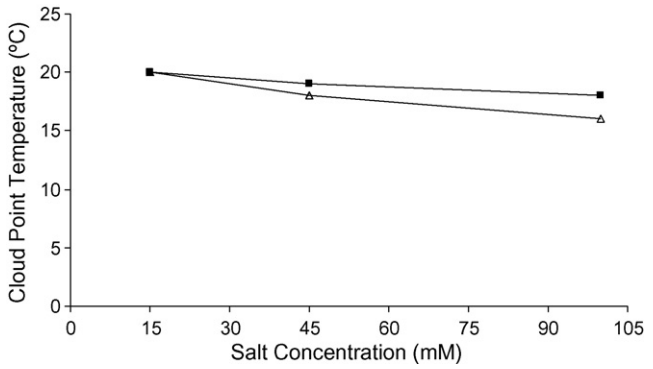

Fig. 3. Influence of potassium phosphate salt concentrations on the cloud point temperature for the copolymers L31 (■) and PE61 (△) in solution with 5 mass% of copolymer, at pH 7.

Table 3
Best conditions for the separation between the proteins α-la and β-lg in systems formed by copolymers L31 and PE61

Copolymer	Proteins	Recovered proteins in the upper phase (%)	Partition coefficient (k)
L31	α-Lactoalbumin	99.37 ± 0.10	157.73
	β-Lactoglobulin	51.18 ± 1.97	1.05
PE61	α-Lactoalbumin	98.76 ± 0.20	79.65
	β-Lactoglobulin	49.45 ± 0.18	0.98

3.3. Influence of EO content on cloud point temperature

Fig. 2 presents the cloud point temperature increase of the copolymer solutions with the increase of the copolymer EO content. The copolymers concentrations ranged from 5 up to 30 mass%. For a copolymer concentration of 5 mass% and a salt solution of 15 mM at pH 7, the cloud point temperature was 58 °C for L copolymer (40 mass% of EO and mean molar mass 2900 g/mol), and 82 °C for L35 copolymer (50 mass% of EO and mean molar mass 1900 g/mol).

EO content affected the copolymer solubility variation rate. The rate increases as the relative ratio of block EO increases, and decreases as the molar mass of polymers increases for copolymers with the same PO/EO composition ratio. This is likely the result of the degree of hydrogen interaction between the copolymer molecules, modifying the copolymers' physical form (liquid for low molar mass, low EO content; solid for high molar mass, high EO content) [16].

Xiuli et al. [27] also observed the increase of the cloud point temperature for copolymers with similar molar masses with the increase of the EO content. These authors reported cloud point values of 71.5 and 38 °C, respectively, for the triblock copolymer $(EO)_{10}(PO)_{16}(EO)_{10}$ and for the triblock copolymer $(EO)_1(PO)_{17}(EO)_1$, at concentration of 1 mass%.

3.4. Partition of α-lactoalbumin and β-lactoglobulin

The results obtained for partitioning of the proteins α-la and β-lg refer to the copolymers L31 and PE61 since they presented low cloud point temperatures, ideal for biomolecules. Both contain 10 mass% of EO, but with viscosities of 285 cPs for PE61 and of 325 cPs for L31. The other copolymers were not utilized since they presented higher cloud point temperatures (Fig. 2), which might cause protein denaturation, especially for α-la, known to be susceptible to denaturation by heat at 65.2 °C and pH 6 [30].

Protein content in the lower phase was difficult to quantify by HPLC-RP due to the high viscosity and low solubility in water of the bottom phase at room temperature.

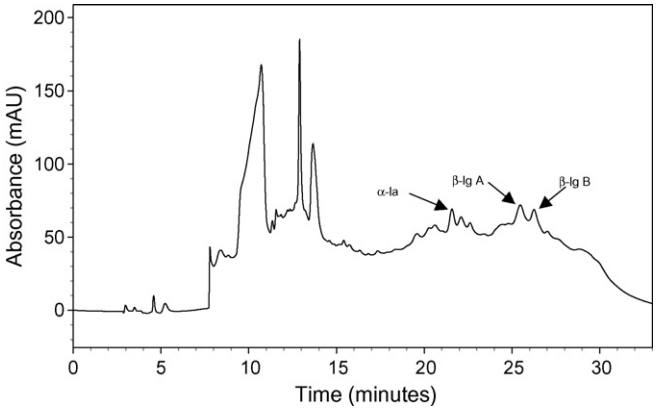

Fig. 4. Lower phase chromatogram of a system formed by WPI, 25 mass% of L31 copolymer and salt solution of 45 mM, at pH 7.

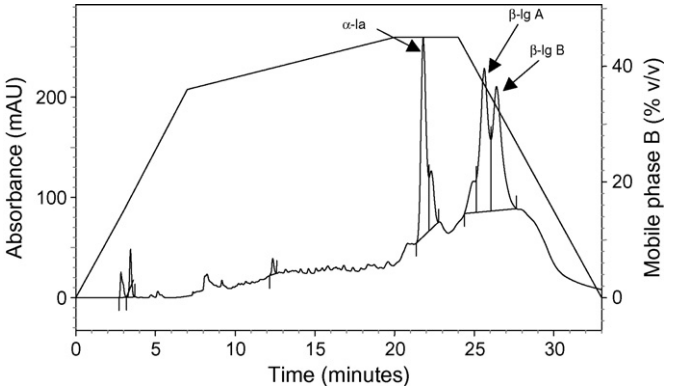

Fig. 5. Upper phase chromatogram of a system formed by WPI, 30 mass% of L31 copolymer and salt solution of 45 mM, at pH 7 and 17 °C.

The presence of surfactants can interfere in analyses performed using a UV detector due to the overlapping of the solute signal. This problem could be solved by diluting the surfactant-rich phase with an organic solvent before the sample injection [31,32].

To carry out the protein quantification in the ATPS lower phase, acetonitrile and ethyl alcohol were used as solvents to dilute the phases, however the α-la and β-lg peaks did not present adequate resolution for a correct determination of their concentrations (Fig. 4). Thus, the calculation of the α-la and β-lg content in the lower phase was obtained by the difference between the protein mass added to the systems and the protein mass quantified in the upper phases of the systems.

Selection of the best ATPS to separate the α-la and β-lg was performed by evaluating the protein partitioning coefficients. The system considered the most effective in protein separation was the one presenting the highest partition coefficient for α-la and a lower partition coefficient for β-lg, thus allowing a better separation between the two in the system phases.

Table 3 presents the best results of the partition coefficient and the percentage of protein recovered in the superior phase for each system. The most favorable conditions for the α-la and β-lg separation using L31 copolymer was using 30 mass% of copolymer and

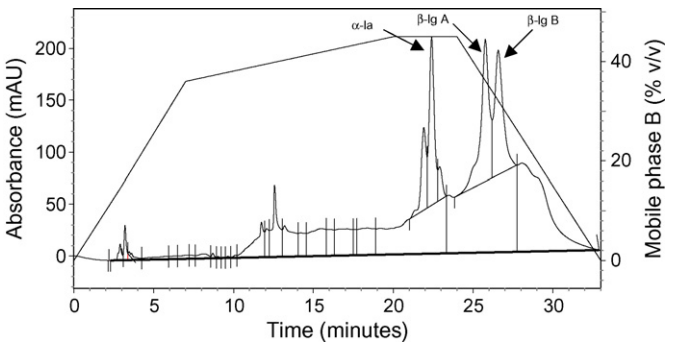

Fig. 6. Upper phase chromatogram of a system formed by WPI, 20 mass% of PE61 copolymer and salt solution, 100 mM, at pH 7 and 20 °C.

potassium phosphate salt solution of 45 mM at pH 7 and 17 °C and for PE61 copolymer was using 20 mass% of copolymer and potassium phosphate salt solution of 100 mM at pH 7 and 20 °C). The protein α-la remained majority concentrated in the upper phase (aqueous phase), while protein β-lg migrated for the inferior phase at about 50%. Since β-lg is more hydrophobic than α-la [33], a greater interaction of β-lg with the copolymeric phase is likely to occur, due to the larger number of hydrophobic groups of the copolymeric, increasing the number and intensity of interactions between hydrophobic groups. The upper phase chromatograms referring to these results are shown in Figs. 5 and 6.

4. Conclusions

The composition and solution concentration of the copolymers EO–PO–EO as well as the concentration of potassium phosphate solutions, influenced the cloud point temperature. In all conditions evaluated in the present work, cloud point temperature ranged approximately from 5 °C up to values around 86 °C. Thus, triblock polymers of the EO–PO–EO type can be used in the extraction and partition of biomolecules under specific conditions.

The protein α-la remained concentrated in the upper phase (aqueous phase) of the systems, while protein β-lg was distributed between the two phases. The copolymers PE61 and L31 presented similar efficiency in the α-la and β-lg partitioning but PE61 was considered more appropriate because of its lower consumption when compared to L31. Among the different systems tested with copolymer PE61, the preferred was the 20 mass% of copolymer and potassium phosphate salt solution of 100 mM at pH 7.

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