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Evaluation of α-Lactoalbumin and β-Lactoglobulin goat milk whey protein partition by aqueous two-phase systems using polymer/maltodextrin

Luziany A.C. Freire, Camila G. Pereira*

Department of Chemical Engineering/Laboratory of Separation Processes in Foods, Federal University of Rio Grande do Norte, Natal, Brazil

ORIGINAL RESEARCH ARTICLE

ABSTRACT

The recovery of biomolecules represents an important field of study in evolution with fundamental application in the advances of biotechnology. In this work, the study of α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) partitioning was carried out using aqueous two-phase systems (formed by polymer/maltodextrin) with different synthetic polymers (polyethylene glycol_PEG, polypropylene glycol_PPG, polyvinylpyrrolidone_PVP) and different molecular weights (from 425 to 40000). The influence of the type, molecular weight and percentage of the polymer used in the system was evaluated in the partition of these proteins. The results indicated that maltodextrin may be a good substitute for dextran in the processes with aqueous two phase aqueous system and it was observed that after the process the proteins tend to remain in the maltodextrin rich phase.

KEYWORDS

α-lactalbumin (α-La); β-lactoglobulin (β-Lg); aqueous two-phase system; separation

1. INTRODUCTION

Proteins play an important role in the organism, either through its metabolic regulating action, or in the immune system, or in its structural action (as a basic constituent for the formation of muscle fibers, hair, bones, teeth and skin). Goat's whey is a by-product of residual cheese production, rich in proteins, however it is usually discarded into the environment. In addition to the high level of BOD (biochemical oxygen demand), this material has a high amount of protein that could be reused. The whey proteins represent about 20% of the proteins present in milk (Haraguchi et al., 2006), with α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) as the majority proteins, responsible for more than 50% of the protein material (Morr and Ha, 1993; Freire, 2015). Thus, separation processes have been studied for the recovery and reuse of this material.

Among the biomolecule separation processes, the use of aqueous two-phase system (ATPS) is well established. The application of ATPS in the recovery of biomolecules has been observed in several studies (Baskaran et al., 2018; Ferreira, et al., 2018, Garcia et al., 2018 ; Song et al., 2018 ; Shad et al., 2018; Pirestani et al., 2018, Du et al., 2019). The main advantages of this process are simplicity, low surface tension facilitating mass transfer between phases, operation occurring at room temperature, rapid and selective separation (Albertsson, 1986; Raja et al., 2011; Ruiz-Ruiz et al., 2012, Phong et al., 2018). ATPS is a liquid-liquid extraction process that uses two aqueous phases for separation. The aqueous phases may be formed by mixing two hydrophilic polymers, or a polymer and an organic or inorganic salt. The formation of these two phases allows the separation of the biomolecule through their affinity with each phase being directly related to the equilibrium conditions. Because it has a high amount of water and the process occurs at room temperature, this process is well indicated for the partitioning of biomolecules by promoting a separation without affecting the biological activity of these

Corresponding author: **Camila G. Pereira**
Tel: +55 08432153773
Fax: +55 08432153773
E. mail: camila@eq.ufrn.br

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biological compounds (Kula et al., 1982; Albertsson, 1986).

The phenomenological process of partitioning of biomolecules in aqueous two - phase aqueous systems is still the subject of many studies. It is understood that the mechanism that governs the partition is a complex phenomenon that involves several intermolecular factors that act in the interaction of the molecules, such as hydrogen bonds, van der Waals forces, hydrophobic interactions, steric effects and ionic interactions of the biomolecules with the phase in which it is contained (Gunduz and Kormaz, 2000). As a matter of fact, among the challenges and future work prospects, the research work in this area is mainly to understand the forces governing to the partition of biomolecules to better predict and optimize the separation process (Phong et al., 2018). Nonetheless, it is known that the effect of these intermolecular factors is incorporated in the variations observed in the partition coefficients. Then, it is usual to evaluate the degree of separation of the biomolecule by the partition coefficient, or distribution coefficient, of that material in each phase, which is determined by the ratio of the biomolecule concentration in the upper and lower phases. The distribution of the protein in ATPS depends on several factors (Baskir et al., 1989), such as system type (polymer/polymer, polymer/salt) (Freire and Pereira, 2016, Freire and Pereira, 2018), type and molecular mass of the polymer, polymer chemical modifications (Sasakawa and Walter, 1972; Cordes et al., 1987), temperature and pH (Lehninger, 1982), types of ions present or added to the systems (ionic strength) (Ryden and Albertsson, 1971), size, charge, surface properties and biomolecule concentration (Axelsson, 1978; Zaslavsky et al., 1983; Bamberger et al., 1985; Abbott et al., 1992).

Polymer/polymer systems have great applicability in the protein partition. Some studies have been reported using these systems, where satisfactory results have been obtained. The partition of 15 pure proteins, including β-Lg, was also evaluated in ATPS formed by PEG 8000 and dextran at pH 7.4 (Ferreira et al., 2014). A higher partition coefficient for ATPS formed by 6.05% PEG 8000 and 12.33% dextran, without addition of salts. In all the cases studied, β-Lg was predominantly concentrated in the dextran rich phase. Another study performed the partitioning of pure proteins α1-antitrypsin and human albumin into PEG and dextran systems (Nucci et al., 2001). In this, ATPS formed by 5.8% PEG 8000 and 8.4% dextran 500 showed a decrease in the partition coefficient of α1-antitrypsin proteins and human albumin at pH 4.0 to 6.0 and an increase in the partition coefficient at pH 6.0 to 8.0. The study also showed a decrease in the partition coefficient of both proteins when higher molecular weight polymers.

Due to the high cost of dextran, new studies have been evaluated to allow their replacement. Maltodextrin is a polysaccharide obtained by the enzymatic partial hydrolysis of the starch, and is in the form of a fine, water-soluble white powder. It has been used in food products as infant formulations, dietary products, beverages, flavor encapsulator, chocolate and derivatives, baking and confectionery, and frozen desserts (Silva, 2000). In the case of application for use in ATPSs, maltodextrin appeared as an alternative to replace dextran due to its low cost, being US$ 0.74/g the cost of maltodextrin and US$ 1365.8/g the cost of dextran (Sigma-Aldrich, 2015). In addition, due to the similarity in polydispersity of maltodextrin to dextran, maltodextrin has been seen as a viable substitute for dextran (Silva, 2000).

The partition of the bovine serum albumin, α-La and β-Lg proteins was evaluated in ATPSs using PEG / maltodextrin showed satisfactory results in the partition of these proteins (Silva and Meirelles, 2000). These studies indicate the various possibilities in applying ATPSs using polymer/polymer in protein separation. However, to date this evaluation has been performed considering systems containing proteins in their pure forms. Studies of partition of proteins using milk whey in-nature and goat milk whey considering maltodextrin in the bioseparation were not found in literature. Thus, the objective of this work was to evaluate the use of ATPS formed by polymer/maltodextrin, in the separation of proteins from real medium (goat's milk whey). In this study, the influence of the type, molecular weight and percentage of the polymer in the partition of these proteins was evaluated.

2. MATERIALS AND METHODS

2.1. Materials and reagents

The goat milk whey was supplied by Association of Small Ranchers of the Angicos wilderness (APASA). It was characterized to be comprised of 0.65 ± 0.02 mg/mL of α-La and 3.19 ± 0.09 mg/mL of β-Lg (A+B). The polymers utilized were: a) PEG of molecular weight 1500 Daltons (Impex - Lot 35263-D), 4000 (Synth -

Lot 152264), 8000 (Sigma - Lot 120M0004V), b) PPG of molecular weight 425 (Sigma – Lots 01817PUV), 2000 (Sigma - Lots BCBJ2891V), 4000 (Sigma – Lot MKBK1954K) and c) PVP of molecular weight 3500 (Acros Organisc - Lot A0316503), 10000 (Sigma - Lot SLBH8882V), 40000 (Sigma – Lot SLBF0853V). The maltodextrin of molecular weight 4004 (Lorenz, lot QP 398) was supplied by Cia Lorenz 2001.

2.2. Partition of proteins

The ATPSs formed by polymer/polymer were established using the methodology described by Silva and Meirelles (2000). These systems were prepared from stock solutions of the polymers and maltodextrin with the masses of the components used for each ATPs being defined by weighing the stock solutions to the desired concentration. For the protein separation, 1 g of goat's milk in-nature was applied to each system, followed by deionized water. The system components were placed in centrifuge tubes and shaken for 10 min, then centrifuged (Macro Evlab, model ev:025, Londrina Brazil) at 2900 rpm for 40 min and placed in a thermostatic bath (Quimis, Q215M2, Diadema, Brazil) for 5 h to ensure equilibrium of system. Subsequently, The samples were filtered through cellulose acetate membrane with a porosity of 0.45 μm using acetonitrile (Sigma, Lot SHBD1824V, Purity 99.9%) and trifluoroacetic acid (Sigma-Lot BCBM0756V, Purity 99%), and degassed in an ultrasonic bath for 30 min.

A factorial design with 3^3 + 2 central points was used, considering the type of polymer (PPG, PEG, PVP), molecular weight, and percentage of the polymer as variables of the process. Equilibrium condition of the ATPSs was carried out considering the systems at 25°C with pH 7.0.

2.3. Quantification of Proteins

After the partition, the quantification of α-La and β-Lg present in the superior phase, rich in polymer, was done by high performance liquid chromatography (Shimadzu, model Prominence, Kyoto, Japan series) containing a ternary pumping system (LC-20AT), detector per diode array (SPD-M20A), column oven (CTO-20A), autosampler (SIL-20AHT) and interface (CBM-20A). LC solution data were analyzed using an acquisition software, version 1.25 and Rigaku data treatment. The chromatographic separation happened in reversed phase, the column and parameters used were similar to that presented in Buffoni et al (2011), a Jupiter C4 column (2504.6 mm, 300 Å pore size, 5 μm particle size, Phenomenex) was used. The oven temperature was 30 °C, injection volume equal to 20 μL and mobile phase flow equal to 1 mL/min. The eluent was monitored using a detector per diode array at 205 nm. The eluents solutions and elution gradient utilized were similar to that described by Enne et al. (2005), where the mobile phase (A) was composed of water of HPLC grade containing 0.1% of trifluoroacetic acid, and mobile phase (B) was composed of Acetonitrile containing 0.1% of trifluoroacetic acid. The elution gradient was 35% B from 0 to 1 min, 35% - 38% B from 1 to 8 min, 38% - 42% B from 8 to 16 min, 42% - 46% B from 16 to 22 min, 46% - 90% B from 22 to 24 minutes, 90% B from 24 to 25 min, 90% - 35% B from 25 to 30 min, 35% from 30 to 35 min. Due to the high viscosity of the maltodextrin phase, the concentrations of α-La and β-Lg proteins at this stage were determined through mass balance.

2.4. Separation Process Evaluation

The partition process of the proteins was characterized in terms of volume ratio, partition coefficient and selectivity as follows.

For the determination of the volume ratio of the phases, the biphasic aqueous systems were prepared in graduated centrifuge tubes. The volume ratio between the phases (V_r) was calculated by equation (1):

$$V_r = V_{UP}/V_{LP} \quad (1)$$

where V_{UP} is the volume of the upper phase and V_{LP} is the volume of the lower phase.

The partition coefficient (K_i) was calculated considering the ratio between the protein concentration in the upper phase (C_{UP}) and the concentration of the same protein in the lower phase (C_{LP}), according to equation (2):

$$K_i = C_{UP}/C_{LP} \quad (2)$$

The selectivity (S) was determined using the ratio between the partition coefficients of α-La and β-Lg in the two equilibrium phases:

$$S = K_{\alpha La}/K_{\beta Lg} \quad (3)$$

Table 1. Partition coefficients of α-La and β-Lg from goat's milk whey in-nature for systems composed of polymer-maltodextrin.

	Polymer (%)	Maltodextrin (%)	H_2O (%)	V_r	$K_{\alpha\text{-La}}$	$K_{\beta\text{-Lg}}$
PPG 425	22.03	29.10	48.87	1.1 ± 0.1	0.023	0.011
	24.99	30.95	44.06	1.0 ± 0.1	0.010	0.002
	28.00	31.99	40.01	0.8 ± 0.1	0.005	*
PPG 2000	10.73	33.40	55.87	0.2 ± 0.0	**	**
	20.05	29.68	50.27	0.4 ± 0.0	**	**
	27.84	32.05	40.11	0.6 ± 0.1	**	**
PPG 4000	21.61	28.49	49.90	0.4 ± 0.0	**	**
	24.70	30.60	44.70	0.4 ± 0.0	**	**
	27.86	31.81	40.33	0.5 ± 0.1	**	**
PEG 1500	10.93	33.83	55.24	1.3 ± 0.1	0.034	0.025
	12.55	36.13	51.32	1.3 ± 0.1	0.030	0.009
	13.75	43.15	43.10	1.0 ± 0.1	0.025	0.003
PEG 4000	11.00	34.96	54.04	1.1 ± 0.1	0.017	0.0028
	12.57	36.10	51.33	0.7 ± 0.1	0.0148 ± 0.0002	0.0027 ± 0.0001
	13.82	43.10	43.08	1.1 ± 0.1	0.013	0.0022
PEG 8000	11.01	34.96	54.03	1.0 ± 0.1	0.007	*
	13.24	36.73	50.03	1.1 ± 0.1	0.005	*
	14.93	38.58	46.49	1.1 ± 0.1	0.004	*
PVP 3500	11.04	33.62	55.34	***	***	***
	20.05	29.89	50.06	***	***	***
	28.11	24.07	47.82	***	***	***
PVP 10000	20.20	19.49	60.31	***	***	***
	25.03	19.93	55.04	***	***	***
	28.17	24.01	47.82	***	***	***
PVP 40000	10.84	33.50	55.66	***	***	***
	28.14	24.04	47.82	***	***	***
	29.91	22.10	47.99	***	***	***

* β-Lg did not partition, it concentrated on the lower phase rich in maltodextrin.
** The analysis could not be performed due to the high viscosity of the solutions in both phases.
*** The systems under the conditions studied did not form two phases.

3. RESULTS AND DISCUSSION

The composition of the ATPSs, as well as the volume ratio and the partition coefficients of the α-La and β-Lg proteins from the goat's milk whey in-nature for the polymer/maltodextrin systems are presented in Table 1.

According to the results presented in Table 1, it can be observed that in all systems studied using polymer/maltodextrin, the partition coefficients of α-La and β-Lg were less than 1, and therefore both proteins were concentrated in the lower phase, which is rich in maltodextrin. Nucci et al. (2001), in their study using the PEG and dextran system, reported a higher concentration of pure proteins α1-antitrypsin

and human albumin in the dextran-rich phase. In another study, Ferreira et al. (2014) evaluated the partition of 15 pure proteins into ATPS formed by PEG and dextran and found that the majority of the proteins were concentrated in the dextran rich phase, including β-Lg, and only 3 proteins (α-chymotrypsinogen, papain and subtilisin A) had a partition coefficient greater than 1. Walter and Johansson (1986) also reported that in PEG and dextran systems, proteins generally tend to partition in the dextran-rich lower phase, thus having a partition coefficient less than 1. Although the distribution coefficients obtained in the present study were lower than 1, all the above mentioned studies evaluated the application of the ATPSs to the partition of pure proteins using a polymer and dextran. Silva and Meirelles (2000) studied the partition of pure bovine serum albumin, α-La and β-Lg applying ATPS using PEG with different molecular weights (1450, 8000, 10000) and maltodextrin (2000, 4000) and obtained satisfactory results on the partition. The majority of the systems formed had a distribution coefficient less than 1, the system formed by PEG 1450 and maltodextrin 4000 being the one with the best distribution coefficients (Kα-La > 4.0). Then, based on the studies of Silva and Meirelles (2000) it is observed that the change from dextran to maltodextrin is a good alternative in the separation of proteins.

Upon evaluating Table 1, it was observed that ATPS with 10.93% of PEG-1500 and 33.83% of maltodextrin, using goat's milk whey in-nature, presented a partition coefficient of α-La and β-Lg ($K_{\alpha\text{-La}}$ = 0.034 and $K_{\beta\text{-Lg}}$ = 0.025) lower than those reported by Silva and Meirelles (2000) on the partition of pure α-La and β-Lg proteins using 10.96% PEG 1450 and 33.82 % maltodextrin 4000 ($K_{\alpha\text{-La}}$ = 4.75 and $K_{\beta\text{-Lg}}$ = 0.95). However, as previously mentioned, all these results presented in the literature used proteins in their pure form. Thus, the differences observed between the present study and the study by Silva and Meirelles (2000) are fully justified since in the study of Silva and Meirelles (2000) the pure α-La and β-Lg proteins of bovine origin were used and in the present study the goat milk whey in-nature was used in each system. And also, beside the difference of raw materials (pure protein and goat milk whey in-nature), Silva and Meirelles (2000) used around 50 mg for each protein in their experiments and in our work only 1 g of serum, containing approximately 0.65 mg of α-La and 3.10 mg of β-Lg, was used, i.e., the amount of proteins used in our experiments was much lower than the amount used in the study by Silva and Meirelles(2000).

In addition to the water present in the serum (91.81%, Freire, 2015), this dairy product contains other components that may interfere with the partitioning of α-La and β-Lg proteins, such as sugars, lipids, salts, vitamins, minerals and other compounds (Huffman, 1996). Direct use of the serum in polymer/ maltodextrin ATPS provided a distribution coefficient of less than 1, showing that the evaluated proteins tend to be in the maltodextrin-rich phase. This indicates that the presence of other compounds present in the real serum mixture affects the separation process of this system, preventing the partition of the proteins in the two phases.

3.1. Effect of polymer type

The type of polymer used in ATPSs had an influence on the partitioning of α-La and β-Lg proteins from goat's milk whey in-nature. In the systems formed by PPG 425 the separation of both α-La and β-Lg proteins occurred, except for the system composed of 28% PPG 425 and 31.99% maltodextrin, where β-Lg was concentrated in the rich phase in maltodextrin.

In the systems formed by PPG 2000 and 4000 it was not possible to carry out the analyzes since the solutions of the two phases presented high viscosity. In the systems formed by PEG 1500, 4000 and 8000, it was possible to separate the proteins, except for β-Lg in the systems formed by PEG 8000, and the best results were obtained for PEG 1500. Finally, systems formed by PVP (3500, 10000 and 40000) and maltodextrin, in the compositions studied, did not form two phases.

3.2. Effect of molecular weight

The molecular weight effect of the polymers was evaluated based on the systems formed by PEG and maltodextrin. According to Table 1, the increase in the molecular weight of the PEG caused a decrease in the partition coefficient of the α-La and β-Lg proteins. Silva and Meirelles (2000) observed the same behavior for α-La and albumin proteins using PEG and maltodextrin. In a study performed for the albumin partition, Forciniti et al. (1991) analyzed the effect of the molecular weight of the polymer using the PEG/ dextran system on protein separation and also reported that increasing the molecular weight of the polymer promoted the decrease of the partition coefficient. Nucci et al. (2001) evaluated the partition of α1-antitrypsin and human albumin in PEG and dextran systems and observed that with increasing PEG molecular mass both proteins were predominantly concentrated in the

lower phase, rich in dextran. Walter and Johansson (1986) reported that in general, there is a tendency for the molecular weight decrease of the polymer at a given stage to increase the concentration of the biomaterial at that stage. Therefore, the data presented in this study are consistent with the literature.

3.3. Effect of polymer percentage

Evaluating Table 1, for the systems formed by PEG and maltodextrin, it is observed that the increase of the percentage of the polymer in all the systems studied caused a decrease in the partition coefficient of α-La and β-Lg proteins, with the exception of β-Lg that did not partition in the system formed by PEG 8000. Walter and Johansson (1986) reported that generally in systems formed by PEG and dextran, the higher the concentration of the polymer, the more proteins are concentrated in the dextran rich phase, and therefore the lower the partition coefficient of these proteins. Albertsson (1986) explains that by increasing the concentration of the polymer causes the protein partition coefficient to decrease because of the effect of excluded volume. Therefore, the results presented are consistent with the literature.

In terms of selectivity, different effect was verified. Figure 1 presents the selectivity found for the ATPS formed by PEG and maltodextrin of 1500 and 4000 of molecular weight and it comparison with partition coefficient of α-La.

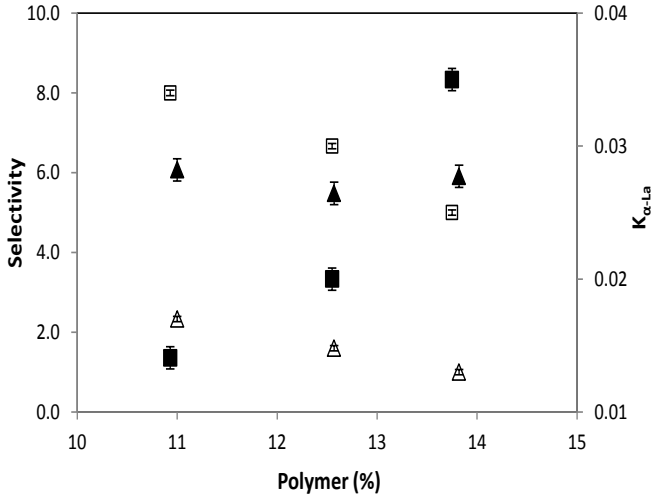

Figure 1. Effect of percentage of the polymer in ATPS PEG/maltodextrin: (■□) PEG 1500 and (▲Δ) PEG 4000. Full symbol: selectivity, and empty symbol: partition coefficient (Kα-La). Data presented with the experimental error bar.

According to the results presented in Figure 1, it is observed that when PEG 4000 was used in the ATPS, the selectivity almost did not change, with a value around 5.8. On the other hand, for the ATPS PEG 1500/maltodextrin was used, the selectivity increased with the percentage of this polymer in the mixture, with higher values of selectivity found when ATPS formed by PEG 1500 13.75 %, and maltodextrin 43.15% was used (S =8.33).

4. CONCLUSIONS

This work evaluated the use of polymer/maltodextrin ATPS in the partition of α-La and β-Lg proteins from goat's milk whey in-nature. It was found that in all conditions studied the partition coefficients were less than 1, indicating that the proteins tend to stay in the maltodextrin-rich phase. The study also showed that results presented with pure proteins can present different results from real systems, due to the concentration of proteins in the systems, and the presence of other compounds that can affect the phase equilibrium. The PEG-maltodextrin system presented better results, with Kα-La = 0.034 and Kβ-Lg = 0.025 for ATPS with 10.93% of PEG-1500 and 33.83% of maltodextrin. The results further indicated that the increase of molecular mass of the polymer and the higher percentage of molecular mass in the ATPSs decrease the coefficient of partition of the proteins. Although the results show a tendency for the proteins to stay in the maltodextrin phase, the results obtained in this work were satisfactory and demonstrated the great potential of ATPSs in the partition of α-La and β-Lg proteins using natural sources such as goat's milk whey in-nature.

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