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Application of the response surface methodology for optimization of whey protein partitioning in PEG/phosphate aqueous two-phase system

Lizzy Ayra Pereira Alcântara [a], Luis Antonio Minim [a,*], Valéria Paula Rodrigues Minim [a], Renata Cristina Ferreira Bonomo [b], Luis Henrique Mendes da Silva [c], Maria do Carmo Hespanhol da Silva [c]

[a] Department of Food Technology, Federal University of Viçosa, Av. P. H. Rolfs - Campus, 36570-000 Viçosa, MG, Brazil
[b] Department of Animal and Rural Technology, State University of Southwest of Bahia, 45700-000 Itapetinga, BA, Brazil
[c] Department of Chemistry, Federal University of Viçosa, 36570-000 Viçosa, MG, Brazil

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ABSTRACT

In order to develop a new strategy for β-lactoglobulin (β-lg) removal from whey protein, partitioning of α-lactalbumin (α-la), β-lg and glycomacropeptide (Gmp) was studied using aqueous two phase systems (ATPS). A system composed of 13% (w/w) polyethylene glycol (PEG, average molar mass 2000 g/mol) and 13% (w/w) potassium phosphate was used at 25 °C. A central composite rotatable design (CCRD) associated to the response surface methodology (RSM) was applied to investigate the effects of NaCl concentration and pH on the partition of these proteins. It was found that α-la and Gmp partitioned to the top phase rich in PEG, whereas β-lg partitioned to the bottom phase rich in salt. According to the RSM, optimal conditions for β-lg removal where found where pH was equal to 6.7 and salt concentration was 0.35 mol/L. Under these conditions, the partition coefficient K_α was 0.48 and K_{Gmp} was 0.92. On the other hand, the partition coefficient K_β was only 0.01. In such conditions β-lg preferentially concentrates in the bottom phase, while the top phase exclusively contains the proteins α-la and Gmp. Fractionation of the proteins from fresh whey was performed in a three stage cross-flow extraction system. The extraction yield for β-lg in the bottom phase was 97.3%, while the yields for α-la and Gmp in the top phase were 81.1% and 97.8%, respectively.

1. Introduction

Milk whey represents a complex and heterogeneous proteins mixture, known for its wide biological, nutritional and technological applications in food products. Enriched α-lactoalbumin (α-la) fractions may be used as ingredients for infants or in hypoallergenic food formulations, while enriched glycomacropeptide (Gmp) fractions may be used in diets for patients suffering from hepatic diseases or phenylketonuria [1–3]. β-Lactoglobulin (β-lg) has excellent heat- and cold-set gelation characteristics as well as an excellent amino acids profile. The β-lg enriched fraction is often applied in areas where water binding and gelation are required [4–6]. However, some individuals, mainly children, show allergenic reactions to β-lg. Such proteins can be totally or partially hydrolyzed in order to produce infant or hypoallergenic formulations [7,8].

Several methods have been used to fractionate or remove β-lg from whey, such as ion exchange chromatography [9], precipitation [10,11] and ultrafiltration [12]. However, chromatographic methods present problems related to adsorbent regeneration and high costs. Although techniques using membranes are considered clean methods, ultrafiltration is not able to efficiently separate whey proteins, since α-la and β-lg present similar molar mass and isoelectric points. Selective precipitation has been investigated by some authors [10,11], however this method presents some disadvantages including biological activity loss and low purity.

Liquid–liquid extraction using aqueous two phase systems (ATPS) has been widely applied for the extraction and purification of many biological products [13–16]. Since this process is cost-effective and its scale-up is very easy and reliable, ATPS have become a very attractive process for many industrial applications [17–19]. The high water content of the system ensures molecular stability of proteins throughout the extraction process [19]. Moreover, extraction using ATPS is suitable for continuous purification processes using equipment traditionally applied in conventional liquid–liquid extraction [20].

In order to develop a new strategy for β-lg removal from whey proteins, in this work the partitioning of α-la, β-lg and Gmp was studied in ATPS formed by polyethylene glycol and potassium phosphate and the effects of pH and NaCl concentration were

* Corresponding author. Tel.: +55 31 3899 1617; fax: +55 31 3899 2208.
E-mail address: lminim@ufv.br (L.A. Minim).

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investigated. A central composite rotatable design (CCRD) with the response surface methodology (RSM) was used for system optimization and analysis of the factors affecting the partitioning process.

2. Materials and methods

2.1. Materials

The proteins α-la and β-lg were purchased from Sigma Chem. Co. (St. Louis, USA). Gmp was supplied by Davisco Foods International (MN, USA), with purity greater than 83%. Polyethylene glycol (PEG, average molar mass of 2000 g/mol) was purchased from Fluka Chem. Co. (USA). Potassium phosphate (monobasic and dibasic) and sodium chloride (99%) were purchased from Sigma (USA). Whey was obtained from the local dairy industry and was previously centrifuged in order to remove fat globules and other particles. All other reagents were of analytical grade.

2.2. Partition experiments

Stock solutions of PEG (50% w/w), and proteins (50 mg/mL) were previously prepared, and the pH of the solutions was adjusted to the desired condition with NaOH (1 M) or HCl (1 M) (pHmeter HI 221, HANNA instruments). Potassium phosphate (20% w/w) was prepared and the pH was adjusted to the desired condition using proper amounts of the dibasic and monobasic forms of the salt. Systems of 25 g were prepared in 50 mL centrifuge tubes by weighting proper amounts of water, PEG and potassium phosphate (analytical balance AUX220, Shimadzu, Japan) in order to achieve the concentrations defined in the experimental design. Sodium chloride was added to the system as a dry component. The tubes were agitated in a vortex shaker for 2 min and then centrifuged at 2000 × *g* for 20 min (Eppendorf 5804, Germany) and left in an incubator (Tecnal TE-184, Brazil) for 16 h to reach the equilibrium. After this period, the separated phases were immediately collected from the tubes using syringes.

Partition experiments were performed in 15 mL centrifuge tubes, using 2.5 mL of the pre-equilibrated top and bottom phases. From the protein stock solution, 50 μL were added to the tubes, which were mixed in a vortex shaker for 2 min and then centrifuged at 2000 × *g* for 20 min. Subsequently the tubes were incubated for 16 h under constant temperature (25 °C) to reach equilibrium. Samples from the top and bottom phases were immediately collected with syringes and the proteins (α-la, β-lg and Gmp) were quantified according to the Bradford method [21]. To avoid interference of the phase components, samples were analyzed against blanks containing the same phase composition, but without proteins. Absorbance was measured at 595 nm in a UV–vis spectrophotometer (Biomate 3, Thermo Scientific, USA), and the analytical curves were constructed using standard solutions of α-la, β-lg and Gmp at concentrations ranging from 0 to 1 mg/mL. The partition coefficient (K) was determined according to Eq. (1).

$$K = \frac{C_T}{C_B} \tag{1}$$

where C_T and C_B are the protein concentrations in the top and bottom phases, respectively.

Extraction experiments using fresh whey were conducted in 50 mL centrifuge tubes, simulating a three-stage cross-flow extractor. Whey was added to the salt phase and mixed for 5 min with the PEG phase, then centrifuged for 20 min (2000 × *g*) and left in an incubator for 4 h at constant temperature (25 °C). All experiments were performed with three repetitions.

Protein quantification in the samples taken during the extraction experiments was performed by high performance liquid chromatography (HPLC) using a liquid chromatograph (Shimadzu, Japan) with a diode array detector (Shimadzu, Japan), at a wavelength set at 280 nm. A reverse phase column (C18 Shim-pack VP-ODS, 250 mm × 4.6 mm, Shimadzu) was employed for chromatographic separation, with the mobile phase composed of a 0.15 mol/L NaCl pH 2.5 aqueous solution (solution A), and pure acetonitrile (solution B), at the flow rate of 1 mL/min. Samples of the top and bottom phases were diluted accordingly and filtered through an acetate cellulose membrane (0.22 μm). A volume of 20 μL was directly injected into the chromatograph and the proteins were eluted from the column using a gradient method [13].

Table 1
Factors and levels of the CCRD for the partition coefficients of α-la, β-lg and Gmp.

Factors	Levels				
	$-\sqrt{2}$	−1	0	+1	$+\sqrt{2}$
pH (X_1)	5.29	5.5	6.0	6.5	6.71
C_{Salt} (mol/L) (X_2)	0.159	0.2	0.3	0.4	0.441

2.3. Experimental design and regression analysis

The partitioning conditions of α-la, β-lg and Gmp were optimized using the response surface methodology. RSM includes factorial designs and regression analysis [22], and it is used to evaluate the relative significance of several influencing factors even in the presence of complex interactions.

To explore the effect of the different variables on the response in the region of investigation, a rotatable central composite design was constructed with two variables and three levels. The factors considered to affect the proteins partitioning in the ATPS systems were pH (X_1) and NaCl concentration (X_2), with temperature maintained constant at 25 °C, and PEG and phosphate concentrations fixed at 13% (w/w) (see Table 1).

The obtained experimental data were analyzed by the response surface regression procedure using the second order polynomial equation (Eq. (2)):

$$K_i = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \tag{2}$$

where K_i is the predicted response, β_0, β_i, β_{ii} and β_{ij} are regression coefficients for the intercept, linear, quadratic and interaction effects, respectively and X_i and X_j are the coded independent variables. An analysis of variance (ANOVA) for the models was performed and the model significance was examined via Fisher's statistical test (F-test) by determining significant differences between sources of variation in experimental results, i.e. the significance of the regression (SOR), the lack of fit (LOF), and the coefficient of multiple determination (R^2). Parameters with less than 95% significance ($p > 0.05$) were excluded and added to the error term. All statistical analyses were performed using the SAS v.9 software.

3. Results and discussion

3.1. Optimization of the partition coefficients

Results from the partitioning study are presented in Table 2. It is observed that the partition coefficient of β-lg was roughly 0.02, meaning that this protein will strongly transfer to the bottom phase. Although the partition coefficients of α-la and Gmp were lower than 1, they were much greater than the partition coefficient of β-lg, indicating that this protein could be isolated in the studied ATPS. Boaglio et al. [23] reported partition coefficients of 0.4 and 0.6 for β-lg and α-la, respectively, in a system composed of PEG (molar mass of 1500) and sodium citrate at pH 6.2 and 20 °C. The

Table 2
Partition coefficient for each experimental condition according to the central composite rotatable design.

Assay	Coded variables		$K_{\alpha\text{-}la}$	$K_{\beta\text{-}lg}$	K_{Gmp}
	X_1	X_2			
1	$-\sqrt{2}$	0	0.399	0.021	0.488
2	−1	−1	0.407	0.024	0.565
3	−1	+1	0.373	0.024	0.498
4	0	$-\sqrt{2}$	0.427	0.025	0.578
5	0	$+\sqrt{2}$	0.334	0.019	0.479
6	+1	−1	0.429	0.020	0.854
7	+1	+1	0.416	0.011	0.788
8	$+\sqrt{2}$	0	0.482	0.011	0.915
9	0	0	0.355	0.021	0.734
10	0	0	0.358	0.019	0.786
11	0	0	0.342	0.022	0.782
12	0	0	0.344	0.018	0.770

system and conditions investigated in this work certainly present greater selectivity.

The effect of salt concentration and pH on the partitioning of α-la, β-lg and Gmp for the studied system was assessed in Figs. 1 and 2. As the system pH was increased, K_α and K_{Gmp} increased while K_β decreased. These results suggest that pH is determinant in the separation of these proteins. It can also be observed that salt concentration negatively affects the partition coefficient of the proteins, although its effect appears to be irrelevant in higher pH values, which is in accordance with the results of Rodrigues et al. [24].

Based on a simple model derived from the Flory–Huggins theory [25], partition can be governed in terms of enthalpic and entropic contributions. In the absence of enthalpic effects, the solute will diffuse to a phase that contains a greater number of molecules per unit volume. In the ATPS studied in this work the salt phase is that containing a greater number density and thus the proteins should have partitioned to the bottom phase rich in phosphate. According to the experimental results of this study (Table 2), β-lg partitioned strongly to the bottom phase (K_β value is around 0.02) and thus we can infer that its partition is significantly governed by entropy. α-La and Gmp proteins (K_α of roughly 0.4 and K_{Gmp} of approximately 0.8) preferentially partitioned to the bottom phase, however their partition coefficients are much larger than that of K_β. According to

Table 3
Analysis of variance performed for the α-la, β-lg and Gmp partition coefficients.

Protein	Source	SS[a]	DF[b]	MS[c]	*F*-value	*p*
α-la	X_1	0.0042	1	0.0042	66.02	0.0039*
	X_2	0.0040	1	0.0040	63.32	0.0041*
	X_1^2	0.0115	1	0.0115	182.04	0.0009*
	Lack of fit	0.0027	5	0.00054	8.58	0.0535
	Pure error	0.00015	3	0.00005		
	Total	0.02255	11			
β-lg	X_1	0.00012	1	0.00012	44.36	0.0003*
	X_2	0.00004	1	0.00004	14.36	0.0068*
	X_1^2	0.00002	1	0.00002	8.46	0.0227*
	$X_1 \times X_2$	0.00002	1	0.00002	6.76	0.0354*
	Lack of fit	1.2E−5	4	3.0E−6	1.12	0.4811*
	Pure error	7.85E−6	3	2.62E−6		
	Total	0.0002	11			
Gmp	X_1	0.1748	1	0.1748	123.58	0.0001*
	X_2	0.0093	1	0.0093	6.57	0.0335*
	X_2^2	0.0679	1	0.0679	48.01	0.0001*
	Lack of fit	0.0097	5	0.00194	3.50	0.1658
	Pure error	0.0017	3	0.00057		
	Total	0.2634	11			

[a] SS: sum of squares.
[b] DF: degree of freedom.
[c] MS: mean square.
* 95%.

Chen [26], the presence of tryptophan on the molecular surface of α-la (its content is about 5.2 mol% of the total amino acid residues) may contribute to hydrophobic interaction of this protein with PEG. In the case of Gmp which has a relatively large number of branched chain amino acids (isoleucine and valine) resulting in its hydrophobic character, hydrophobic interaction with PEG can also occur. Consequently, the greater partition coefficients of these two proteins compared to that of β-lg may be resultant from hydrophobic forces acting to transfer α-la and Gmp to the upper phase.

Statistical analysis of protein partitioning using a full quadratic model was tested by the Fisher's *F*-test for analysis of variance (ANOVA) and the results are presented in Table 3. It was observed that the factors X_1 and X_2 significantly affected protein partitioning ($p < 0.05$). Moreover, the quadratic effect of factor X_1 was significant for α-la and β-lg, while the quadratic effect of X_2 was significant

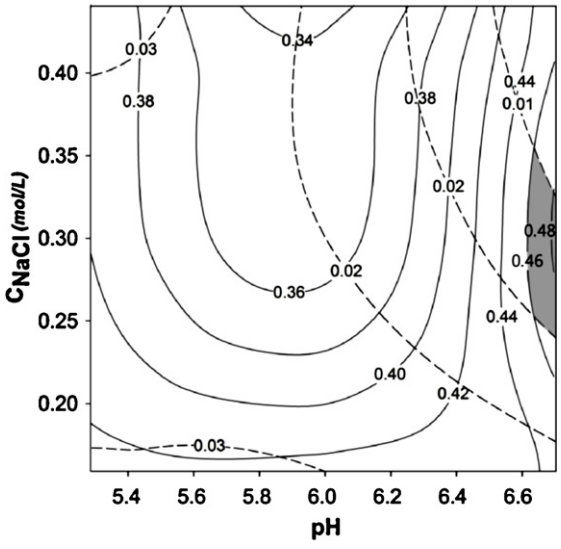

Fig. 1. Effect of pH and NaCl concentration (mol/L) on the partition coefficient of α-la (—) and β-lg (- - -) in PEG/Potassium phosphate systems.

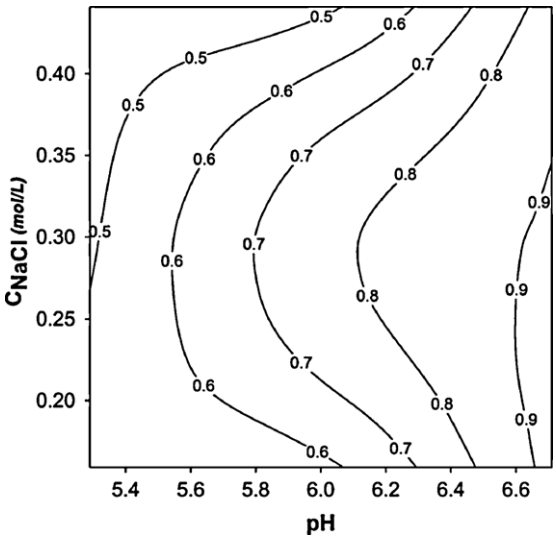

Fig. 2. Effect of pH and NaCl concentration (mol/L) on the partition coefficient of Gmp in PEG/potassium phosphate systems.

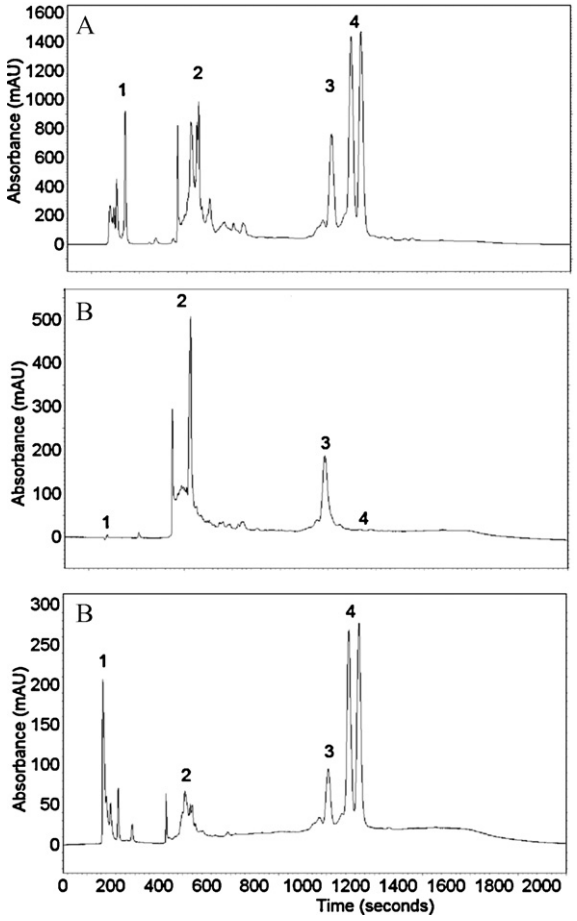

Fig. 3. Chromatogram of fresh whey (A), PEG phase after extraction (B), and salt phase after extraction (C). Chromatographic peaks are as follows: bovine serum albumin (1), glycomacropeptide (2), α-lactoalbumin (3), and β-lactoglobulin (4).

only for Gmp. Interaction between X_1 and X_2 was only significant for β-lg.

The partition coefficient (K) could be explained by the following second-order polynomial equations (Eqs. (3)–(5)).

$$K_\alpha = 6.117 - 1.942X_1 - 0.223X_2 + 0.166X_1^2 \quad (3)$$

$$K_\beta = -0.2749 + 0.0957X_1 + 0.2386X_2 - 0.0075X_1^2 - 0.0435X_1X_2 \quad (4)$$

$$K_{Gmp} = -1.826 + 0.2957X_1 + 5.715X_2 - 10.093X_2^2 \quad (5)$$

The coefficients of determination (R^2) were found to be 0.87, 0.91, and 0.96, for α-la, β-lg and Gmp, respectively; and the lack of fit was non-significant. These results showed the suitability of the model for suitable representation of the real relationship among the studied factors.

Considering the partition coefficient of β-lg, a great stationary area is observed (Fig. 1) corresponding to its maximum value, and as it moves to regions of higher pH and salt concentration, its partition coefficient decreases while the partition coefficient of α-la increases. The partition coefficient of Gmp also increased as observed in Fig. 2. The hachured area depicted in Fig. 1 shows an optimal region for protein fractionation, with the highest partition coefficients for α-la and Gmp and the lowest for β-lg. Optimal pH and salt concentration values determined were 6.7 and 0.35 mol/L, respectively, resulting in maximum partition coefficients for α-la and Gmp (0.48 and 0.92, respectively) and a minimum partition coefficient for β-lg of 0.01. Under such conditions β-lg preferentially concentrates in the bottom phase. The top phase will exclusively contain both α-la and Gmp, and be free of β-lg. Thus, the results confirm the applicability of the studied system for separation of whey proteins.

Fractionation of the proteins α-la, Gmp and β-lg from fresh whey was carried out in a three stage cross-flow extractor, based on the optimal conditions defined previously (pH 6.7 and NaCl 0.35 mol/L) at 25 °C and PEG and phosphate concentration of 13% (w/w). The extraction yield, defined as the ratio of the protein concentration in a phase where it is concentrated and the total protein added to the extractor, was calculated for β-lg in the bottom phase as being 97.3 ± 0.03%, while yields for α-la and Gmp in the top phase were 81.1 ± 0.15% and 97.8 ± 0.04%, respectively. Fig. 3 shows the chromatograms of the feed containing fresh whey before extraction and the PEG and phosphate phases after extraction, respectively. In Fig. 3B, it can be seen that no β-lg was detected in the top phase, while this protein was present in the feed (Fig. 3A) and in the salt phase of the last stage (Fig. 3C) where it was concentrated. Consequently, the proposed system presented the desired characteristic to produce a PEG phase free of β-lg.

4. Conclusion

This work studied the partitioning behavior of the main whey proteins β-lg, α-la and Gmp in ATPS composed of PEG and phosphate, with the objective of developing a strategy for production of a β-lg free fraction of whey protein, and concomitantly another β-lg rich fraction. The present investigation showed that this protein probably partitioned to the phosphate phase due to strong entropic effects. For the proteins α-la and Gmp, hydrophobic interactions with PEG maybe responsible to their significantly higher partition coefficients when compared to β-lg The RSM was effective in determining an optimal region for proteins fractionation. Results presented in this work confirmed the feasibility of the liquid–liquid extraction system composed of PEG 2000 and potassium phosphate to fractionate the proteins from fresh whey, since α-la and Gmp were recovered in the polymer-rich phase and β-lg recovered in the salt-rich phase.

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