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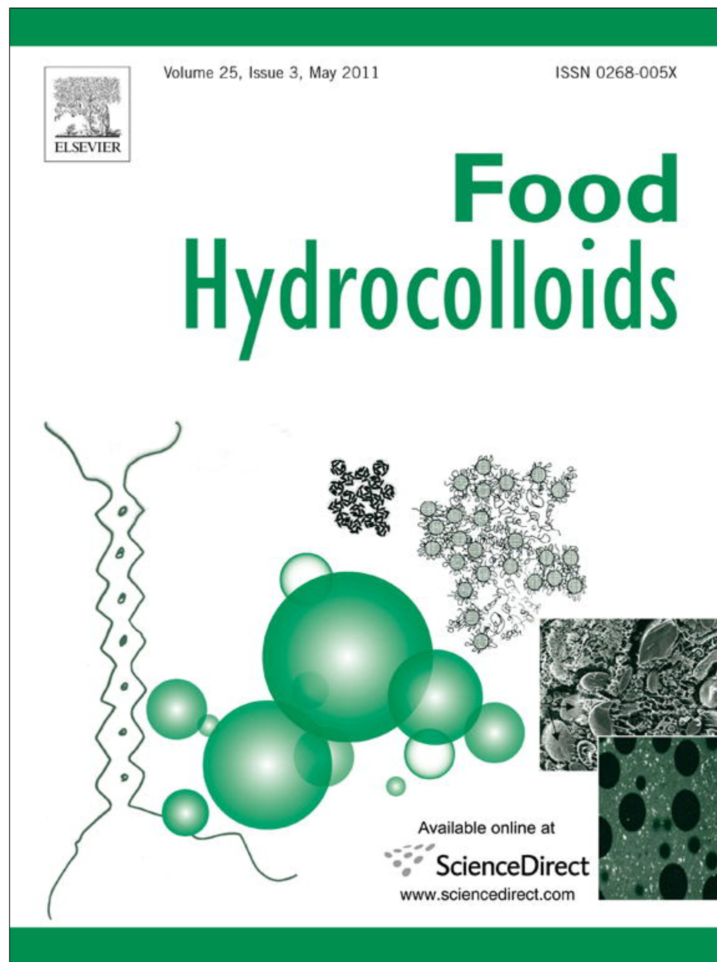

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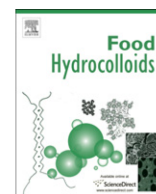
Food Hydrocolloids 25 (2011) 374–380

Contents lists available at ScienceDirect

Food Hydrocolloids

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



Partitioning of α-lactalbumin and β-lactoglobulin in whey protein concentrate/hydroxypropylmethylcellulose aqueous two-phase systems

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ARTICLE INFO

Article history:
Received 23 March 2010
Accepted 2 July 2010

Keywords:
Thermodynamic incompatibility
Protein partitioning
Phase separation
Hydroxypropylmethylcellulose
Whey proteins

ABSTRACT

Whey protein concentrate (WPC) was fractionated by using hydroxypropylmethylcellulose (HPMC) at pH 6.5. Incompatible mixtures with different proportions of HPMC and WPC were prepared. After phase separation, the protein concentration in both phases was determined by the Kjeldahl method and the proportion of each protein by SDS-PAGE combined with image analysis. The results show that the low molecular weight proteins α-lactalbumin (α-lac) and β-lactoglobulin (β-lg) were retained in high proportion in the upper phase (about 90% compared to 64% of WPC). The most efficient condition to fractionate β-lg and α-lac was the phase separation of an incompatible mixed system with a high initial concentration of WPC and a low initial concentration of HPMC i.e., WPC 20%, wt/wt/HPMC 0.5%, w/w. It can be concluded that the thermodynamic incompatibility which arises from mixing WPC with HPMC could be used as a method for fractionation of whey proteins.

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1. Introduction

Whey is the principal by-product of cheese manufacture and represents a valuable source of high quality protein. Whey proteins, well known for their nutritional value and versatile functional properties, are widely utilized in the food industry (González-Martínez et al., 2002). The observed functional properties of whey are the sum of the functionality of individual proteins, being the most important β-lactoglobulin (β-lg), α-lactalbumin (α-lac) and bovine serumalbumin (BSA). These represent 70% of the whole whey proteins and are responsible of the hydration, gelation, emulsifying and foaming properties of whey (Pérez, Wargon, & Pilosof, 2006).

Whey proteins are usually obtained by a combination of membrane concentration and chromatographic methods (i.e. ionic exchange, affinity and radial flow chromatography) (Cheang & Zydney, 2004). Although, these techniques can effectively be used for protein partitioning, they induce an increase of protein denaturation with the corresponding decrease of the functional properties (Sivars & Tjerneld, 2000).

Partitioning in aqueous two-phase systems (ATPS) is a good alternative method to be employed as a first step purification. Many authors have developed this partitioning technique for the separation and fractionation of proteins, enzymes, viruses, cells and cell organelles between two aqueous polymer phases (Albertsson, Cajarville, Brooks, & Tjerneld, 1987; Johansson & Walter, 2000; Zaslavsky, 1995). ATPS are widely used for separations of biological material, because of their high water content (80–95%) which provides mild extraction conditions. It is important to remember that the high water content in both phases avoids protein denaturation (Christian, Manley-Harris, & Richards, 1998; Rito-Palomares, 2004). In most instances, these systems can be formed by combining two water-soluble polymers that differ in their chemical structure. The most widely used systems for biomaterials separation are dextran/methylcellulose, dextran/ficoll, dextran/polyvinylpyrrolidone (PVP) and guar/amylopectin (Simonet, Garnier, & Doublier, 2000). Moreover, some studies were performed on biphasic systems containing polysaccharides, i.e. guar/amylopectin (Closs, Conde-Petit, Roberts, Tolstoguzov, & Escher, 1999) and polyethylene glycol (PEG)/maltodextrin (da Silva & Meirelles, 2000).

Mixtures of proteins and polysaccharides usually tend to separate in two phases due to thermodynamic incompatibility. This mainly occurs at a pH higher than the isoelectric point of the protein (p*I*) and, in general, above a critical total biopolymer concentration (>4% wt/wt) (Tolstoguzov, 2003). Tolstoguzov and co-workers coined the term membraneless osmosis to describe the process in which proteins are concentrated in a small volume by the addition of polysaccharides (Polyakov, Grinberg, & Tolstoguzov, 1997).

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doi:10.1016/j.foodhyd.2010.07.003

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In a previous work, the phase diagram for the Whey Protein Concentrate (WPC)/Hydroxypropylmethylcellulose (HPMC) system at 25 °C and pH 7 was determined (Pérez et al., 2006). It was shown that the compatibility zone was relatively small and phase separation took place in a broad range of polymer concentrations. The phase separation threshold corresponded to 2% wt/wt of WPC and 3% wt/wt of HPMC. Nevertheless, WPC is a complex mixture of proteins, which may exhibit different degrees of incompatibility with the HPMC. If so, it can be expected a differential partition behaviour of these proteins between the protein-rich and the HPMC-rich phases.

The purpose of the present work was to explore the possibility of a selective separation of the proteins from a typical WPC, in order to develop an alternative method to fractionate the whey proteins.

2. Materials and methods

2.1. Materials

Commercial β-lactoglobulin powder was used as a β-lg control due to the high proportion of this protein in this product. HPMC commercial denomination E50LV (food grade) powder from Dow Chemical Company (Findlay, OH) was kindly supplied by Colorcon SA (Buenos Aires, Argentina). It was used without further purification. According to the supplier, this cellulose derivative has 29.1% methyl groups, 9.2% hydroxypropyl groups and yields a methyl/hydroxypropyl ratio of 3.2. Viscosity, measured on 2% w/v aqueous solution (20 °C) was 41.0 mPa s^{-1}, and its average molecular weight was 18 kDa. Moisture content was 1.6%. WPC80 powder was kindly provided by Milka Frank-Milkaut SA (Santa Fe, Argentina). This product is derived from cheese whey. As stated by the manufacturer, the composition of WPC80 powder was 78.9% protein, 5% lactose, 6% fat, 4.3% ash and 5.6% moisture. All other chemical reagents used were of analytical grade.

2.2. Preparation of stock solutions

Stock solutions of commercial β-lactoglobulin (10% wt/v), WPC80 (40% wt/wt) and HPMC (4% wt/wt) were prepared in distilled water. The powders were weighed in an analytical balance (VXI, Model 60/220, PCE Group Ibérica S.L., Tobarra, Spain). For β-lactoglobulin and WPC80 solutions preparation, each powder was dispersed in the water at room temperature (25 °C) and stirred for 4 h (magnetic stirred model C-MAG HS7, IKA®, Wilmington, NC) to allow the complete dissolution of the powders. After preparation, these solutions were stored overnight at 4 °C. HPMC solution was prepared by dispersing the powder in the water at 85 °C and cooled down to room temperature (25 °C). Then, the solution was stored at 4 °C for 24 h to achieve the maximum polysaccharide hydration. Prior to the experiments, the stock solutions were heated to room temperature (25 °C) using a thermostatic bath (model MASSON II, Vicking SRL, Buenos Aires, Argentina).

2.3. Preparation of aqueous biphasic systems

Biphasic systems (total weight 10 g) were prepared by mixing appropriate amounts of stock solutions of WPC80 and HPMC, in order to obtain mixed systems with WPC 8–20% (wt/wt) and HPMC 0.5 or 2% (wt/wt). Systems were mixed using a magnetic stirrer within an equilibrium cell with external temperature (25.0 ± 0.1 °C) control from a thermostatic bath (Polystat, Model 12108-15, Cole-Parmer Instrument Co., Vernon Hills, IL) for 2 h to allow a homogeneous distribution of the components. The pH of mixtures was 6.5 (pH Meter, Model A920, Orion Research Inc., Beverly, MA). Complete phase separation of mixtures was achieved by centrifugation in 15 ml graduated plastic tubes at 13,000 *g* for 2 h in a thermostated centrifuge at 25 °C (Eppendorf, Model 5810 R, Eppendorf AG, Hamburg, Germany). After centrifugation, the tubes were placed into the thermostatic bath at 25 °C for 10 h to equilibrate the segregated phases. The volumes of upper phase (V_U) and lower phase (V_L) were visually determined. Then, both phases were separated by removal of the upper phase with a pipette, and weighed in an analytical balance (VXI, Model 60/220, PCE Group Ibérica S.L., Tobarra, Spain). The complete assay was performed at least in triplicate. Mean and standard deviation for the volume and weight were reported.

2.4. Determination of the protein and polysaccharide content in both phases

After isolation, each phase was homogenized for 5 min in a vortex. Then, samples of approximately 0.5 g were taken to determine the biopolymers content. The protein concentration was determined by Kjeldahl method as outlined in AOAC (1995) in a Kjeldahl automatic distillation unit (model Kjeltec Auto 1030 Analizer, TECATOR AB, Höganäs, Sweden).

The polysaccharide concentration in the lower (Y_L) and upper (Y_S) phases, were calculated using the following equation,

$$Y_{L,U} = [-(\alpha Y_0 + Y_0)/(\alpha X_0 + X_0)]\alpha X_{L,U} + (\alpha Y_0 + Y_0) \quad (1)$$

it derives from a tie-line length solution used by Pérez et al. (2006) to develop the WPC/HPCM phase diagram. This equation constitutes the straight segment passing through initial concentration coordinates of the mixture (X_0, Y_0), and the coordinates defining the upper phase composition (X_U, Y_U) and the lower phase composition (X_L, Y_L). α is defined as the volume ratio of phases (V_L/V_U) (Fig. 1). The biopolymers concentration average and standard deviation were reported. All concentrations were expressed as % wt/wt.

2.5. SDS polyacrylamide gel electrophoresis

Samples of WPC80, commercial β-lactoglobulin and each one of the separated phases were subjected to SDS-PAGE electrophoresis

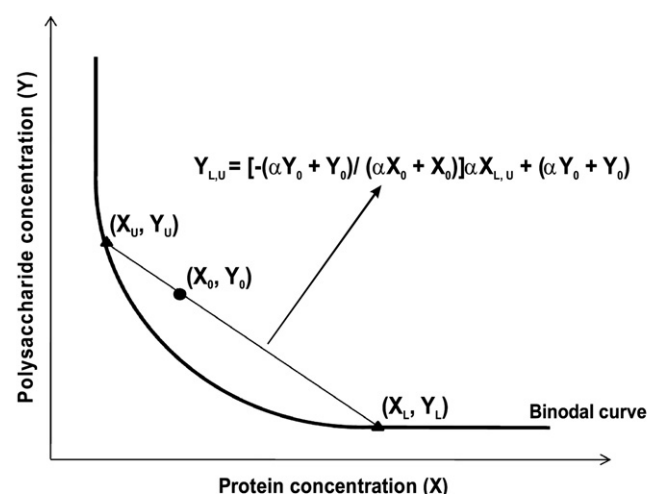

Fig. 1. Outlining simplified of the procedure used to calculate the polysaccharide concentration in the segregated phases. The figure shows a schematic protein/polysaccharide phase diagram, where the binodal curve denotes the limit between the compatibility zone (one-phase mixed systems) and the incompatibility zone (two-phase mixed systems). The tie-line constitutes the straight segment which pass through initial concentration coordinates of a mixture (X_0, Y_0) (●), and the coordinates defining the segregated phases compositions: (X_U, Y_U) and (X_L, Y_L) for the upper lower phases, respectively (▲). Arrow indicates the mathematical expression of this segment which was used to calculate the polysaccharide concentration in the lower (Y_L) and upper (Y_S) phases (α is the volume ratio of phases, V_L/V_U).

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in order to evaluate their protein composition. SDS-PAGE was carried out according to Laemmli (1970) under non-reducing conditions using a separating gel containing 12.5% of acrylamide in a electrophoresis unit (model Mini-Proteans II, Bio-Rad Laboratories Inc., Hercules, CA). The gel was subsequently stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories Inc.).

2.6. Protein quantification in the segregated phases by image analysis

The electrophoresis gels were scanned using a Bio-Rad model GS-800 scanner (Bio-Rad Laboratories Inc.) at 600-dpi resolution. The scanned images were stored in .tif format to be processed. Image process was performed over six scanned images of each gel with Image Pro Plus v 6.0 software (Media Cybernetics Inc., Bethesda, MD) and Matlab R2009a software (The MathWorks Inc., Natick, MA). Then, the processed images were analyzed using Quantity One v22 software (Bio-Rad Laboratories Inc.). First, the traces of six replicates had been aligned using the Compare Lanes software tool in order to determine the accuracy of the image scanning. Second, the molecular weights were determined comparing the relative front (Rf) values of each band with those of the protein standard using the Standards software tool. Finally, protein quantification was made by the volume method. It involves drawing an object around each band of interest. The volume represents the total signal intensity inside a defined boundary drawn (sum of the intensities of the pixels within the volume boundary × pixel area, expressed in OD × mm^2). It is a measurement of the protein amount in the band. Thus, the proportion of the different proteins in WPC80, commercial β-lactoglobulin and each segregated phase was calculated by the following relationship,

$$P_i = \mathrm{BIV}/\mathrm{TBV} \quad (2)$$

where P_i is the protein proportion, BIV is the band volume of the interest protein, and TBV is the total bands volume in the corresponding lane. Moreover, the concentration of the different proteins was calculated using the following modified equation developed by Reed and Park (2008),

$$\mathrm{PC}_i = P_i \times P_{\mathrm{Kj}} \quad (3)$$

where PC_i is the protenin concentration in the corresponding sample, P_i is the protein proportion, and P_{Kj} is the total protein concentration determined by Kjeldahl. PC_i and P_i values were used for further calculations of partition coefficients and protein recovery, respectively.

2.7. Determination of partition coefficient and protein recovery for β-lg and α-lac in the segregated phases

The partitioning of a protein in an aqueous two-phase system can be described by the partition coefficient *K*, which is defined as $K = C_{\mathrm{UP}}/C_{\mathrm{LP}}$, where C_{UP} and C_{LP} are the equilibrium protein concentrations in the upper and lower phases, respectively (Albertsson et al., 1987). Thus, partition coefficient for each protein (K_i) was calculated as the ratio between $\mathrm{PC}_i^{\mathrm{UP}}$ and $\mathrm{PC}_i^{\mathrm{LP}}$.

The protein recovery was calculated in the upper phase using the following equation,

$$Y_i = \left(\mathrm{wp}_{\mathrm{UP}} P_i^{\mathrm{UP}} / \mathrm{wp}_0 P_i^{\mathrm{WPC80}}\right) \times 100 \quad (4)$$

where Y_i is the protein recovery percentage, P_i^{UP} and P_i^{WPC80} are the protein proportions in the upper phase and WPC80, respectively; $\mathrm{wp}_{\mathrm{UP}}$ is the total upper phase weight protein, and wp_0 is the total weight protein present in the initial mixed system.

The values calculated for these two partition parameters were averaged and reported as mean and standard deviation.

3. Results and discussion

3.1. Segregation of WPC/HPMC mixed systems

WPC and HPMC mixtures at pH 7 are generally unstable and upon a critical biopolymer concentration they separate in a protein-rich lower phase and a polysaccharide-rich upper phase (Pérez et al., 2006). Phase separating protein–polysaccharide systems can be considered as water-in-water emulsions. A W/W emulsion is a disperse system, where droplets of one of the immiscible aqueous solutions are dispersed throughout another aqueous biopolymer solution (Tolstoguzov, 2003). These emulsions are characterized by a co-solubility of biopolymers in the co-existing phases (Turgeon, Beaulieu, Schmitt, & Sanchez, 2003). Table 1 shows the upper phase (UP) and lower phase (LP) compositions after separation, join to the initial composition of each mixed system. The UP of all systems resulted slightly more concentrated in the polysaccharide than initial solutions, but the LP strongly increased in protein, except those having initially WPC 20% wt/wt.

Nevertheless the polysaccharide-rich UP of all systems had a significant protein concentration which increased as initial WPC concentration increased, being this effect slightly lower with 2% than 0.5% wt/wt HPMC. This is due to increased exclusion volume effects in more concentrated polysaccharide solutions. The decrease of initial HPMC concentration, at constant WPC concentration, would decrease the driving force for the segregation, allowing more protein to coexist with the HPMC in the UP. As a result, the systems with an initial lower concentration of HPMC could concentrate more protein in the UP, being the mixed system WPC 20% wt/wt/HPMC 0.5% wt/wt the most efficient.

Additionally, the UP and LP volumes together with the weights of each phase are given in Table 1. These values reflect the solutions density increase as solid content increases.

3.2. Fractionation of α-lac, β-lg and BSA in the segregated phases

In order to select the two-phase system with the best fractionation capability of the desired proteins from WPC80, the proportion of these proteins in the UP and LP was calculated. Fig. 2a shows the SDS-PAGE pattern for WPC80 and commercial β-lg. A molecular weight standard (STD) is also included in the first lane. WPC80 is the starting protein material to be fractionated, while the commercial β-lg constitutes a β-lg concentrate which serves as a control. In both lanes five defined bands can be observed. Three of them were associated with the following molecular weights by comparison with the standard: 14.2 kDa, 18.3 kDa and 66 kDa, which were identified as α-lac, β-lg and BSA, respectively (Liu, Powers, Swanson, Hill, & Clark, 2005). The other two bands, denominated B I and B II, have molecular weights of approximately 36 kDa and 116 kDa, respectively. The first one could correspond to β-lg dimmers and the second one to non-specific protein aggregates (Liu et al., 2005). Probably, this aggregation could be a consequence of protein denaturation that takes place during the commercial WPC manufacturing. Fig. 2b is an example of a processed image from a scanned SDS-PAGE gel, while Fig. 2c shows the surface plot of the processed image, which is a 3-D view of bands intensity. These images were used to quantify the proportion of each protein in each sample.

For the WPC/HPMC mixed systems, the LP band profiles were similar to that obtained for WPC80, while in the UP the bands of α-lac and β-lg presented more intensity; additionally, the B I band (36 kDa) was absent and the B II band (116 kDa) was diminished in

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Table 1
Composition, volume and weight of segregated phases from WPC/HPMC mixed systems at 25 °C and pH 6.5.

Initial composition (% wt/wt)		Lower phase				Upper phase			
WPC80	HPMC	Protein (% wt/wt)	HPMC (% wt/wt)	*V* (mL)	*w* (g)	Protein (% wt/wt)	HPMC (% wt/wt)	*V* (mL)	*w* (g)
8	0.5	15.0 ± 1.0	0.40 ± 0.02	1.2 ± 0.1	1.60 ± 0.08	4.5 ± 0.3	0.50 ± 0.03	6.7 ± 0.6	7.7 ± 0.3
12	0.5	17.0 ± 1.1	0.40 ± 0.02	1.70 ± 0.14	2.30 ± 0.09	7.0 ± 0.4	0.53 ± 0.04	6.2 ± 0.5	7.3 ± 0.3
15	0.5	19.0 ± 1.1	0.40 ± 0.03	2.2 ± 0.2	3.0 ± 0.1	8.0 ± 0.5	0.53 ± 0.03	5.50 ± 0.45	6.60 ± 0.23
20	0.5	20.5 ± 1.2	0.45 ± 0.03	2.50 ± 0.22	3.50 ± 0.14	13.0 ± 0.9	0.55 ± 0.03	5.0 ± 0.4	6.30 ± 0.25
8	2	18.0 ± 1.1	1.5 ± 0.1	1.00 ± 0.08	1.40 ± 0.05	4.0 ± 0.2	2.0 ± 0.3	7.2 ± 0.6	8.3 ± 0.3
12	2	19.5 ± 1.3	1.6 ± 0.1	1.50 ± 0.13	2.10 ± 0.08	6.5 ± 0.1	2.10 ± 0.13	6.3 ± 0.5	7.6 ± 0.3
15	2	21.0 ± 1.4	1.6 ± 0.1	1.70 ± 0.15	2.40 ± 0.09	8.0 ± 0.4	2.1 ± 0.1	6.0 ± 0.5	7.3 ± 0.2
20	2	23.0 ± 1.5	1.7 ± 0.1	2.0 ± 0.17	3.0 ± 0.1	11.0 ± 0.7	2.20 ± 0.15	5.00 ± 0.45	6.30 ± 0.21

Mean ± SD, $n = 3$.
V and *w* are the phase volume and weight, respectively.

its intensity (Fig. 3). The proportion of each protein in the UP and LP for all mixed systems is given in Tables 2 and 3, respectively.

When the initial concentration of HPMC was 0.5% wt/wt, the UP presented a higher proportion of α-lac than the corresponding LP. Moreover, these upper phases resulted richer in α-lac (24%) than the WPC80 (17%), independently of the initial WPC concentration used in the mixed system preparation. An inverse behaviour can be observed for the mixed systems with HPMC 2% wt/wt initial concentration. On the other hand, it can be seen that the β-lg proportion in the UP was always higher than in the corresponding LP. Moreover, the UP was always more enriched in β-lg (48–62%) than the original WPC80 (47%), independently of initial WPC80 or HPMC concentration used. However, these values did not reach the proportion of the commercial β-lg (77%). Finally, the segregated phases did not show any significant enrichment in BSA, which is expected since this protein proportion in the starting WPC80 is too low.

The proportion calculated in this work correlates with the parameter so called "purity", which is defined as a ratio between the content of a particular protein and the total protein amount in given phase (Picó, Romanini, Nerli, & Farruggia, 2006). Thus, the α-lac and β-lg enrichment obtained in these two-phase systems would resemble the purities generally expected for an ATPS, which usually are within a range of 30–60% (Boaglio, Bassani, Picó, & Nerli, 2006; Capezio, Romanini, Picó, & Nerli, 2005). In conclusion, the UP of mixed systems with HPMC 0.5% wt/wt initial concentration could be selected as the preferred ones to isolate α-lac and β-lg.

The above results indicate that a differential fractionation of whey proteins between the UP and LP took place during phase separation. The partitioning of molecules between the two phases is a complex phenomenon that depends on the type, molecular weight, and concentration of the polymers as well as pH and ionic concentration (Sarubbo et al., 2000). In the case of proteins partitioning, their molecular weight and chemical properties are the most important factors. Regarding to molecular weight, α-lac is the whey protein with the lowest molecular mass (14.2 kDa), while at pH close to 7 β-lg exists as a dimer (36 kDa) (Capezio et al., 2005). Thus, the different fractionation behaviour between these two molecules could be attributed to the easiest inclusion of the small protein in the available free volume of the HPMC-rich phase. However, it is worth to notice that in the mixed systems with an initial HPMC 2% wt/wt concentration, this behaviour was not observed mainly because of a reduction of protein diffusion to the UP due to an increase of the HPMC-rich phase viscosity. It is well known that viscosity strongly impacts on the phase separation (Schorsch, Jones, & Norton, 1999).

Also noteworthy that, although these proteins are slightly negative charged at pH 6.5 because they have isoelectric points between 4.9 and 5.4, the observed partitioning behaviour should not be hardly influenced by electrostatic interactions with HPMC due to the non-ionic nature of this polysaccharide.

On the other hand, a different partitioning behaviour of a protein in a mixture with respect to that of the pure protein has been previously observed (Franco, Andrews, & Asenjo, 1996). In a mixture formed by different proteins, the protein–protein interaction becomes relevant.

Finally, the presence of other substances like fat, lactose and salts in WPC/HPMC mixture, impart more complexity to the protein

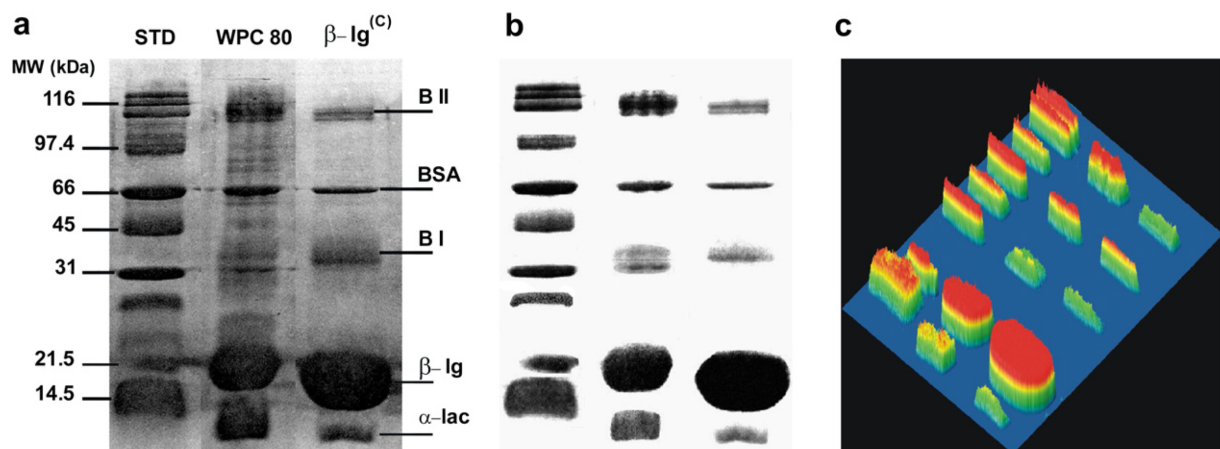

Fig. 2. SDS-PAGE pattern of commercial β-lactoglobulin (β-lg$^{(C)}$) and WPC80 (a), processed image from a scanned SDS-PAGE gel (b), and a representative 3-D view of image analysis quantification (intensity surface plot) (c). STD = standard molecular weight, α-lac = α-lactalbumin, β-lg = β-lactoglobulin, B I = band I, BSA = bovine serumalbumin, B II = band II.

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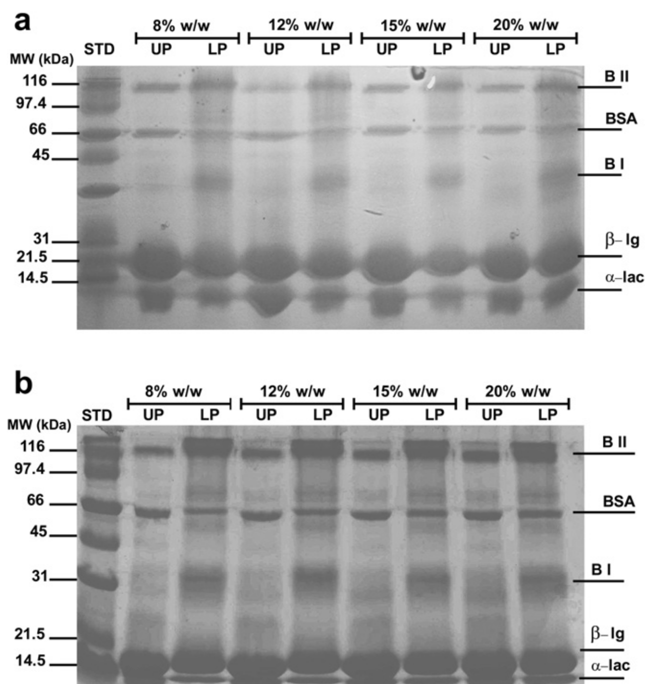

Fig. 3. SDS-PAGE pattern of upper (UP) and lower (LP) phases segregated from WPC/E50LV mixed systems: (a) WPC 8–20% w/v/E50LV 0.5% w/v; (b) WPC 8–20% w/v/E50LV 2% w/v. STD = molecular weight standard, α-lac = α-lactalbumin, β-lg = β-lactoglobulin, B I = band I, BSA = bovine seroalbumin, B II = band II.

partitioning behaviour. Moreover, additional complexity arises from the polydispersity of HPMC. In fact molecular weight of E50LV is within 5.6 and 25 kDa (Pérez, Carrera Sánchez, Pilosof, & Rodríguez Patino, 2009). Thus, the possibility of a differential fractionation also for the polysaccharide is highly predictable, giving to the WPC/E50LV ATPS a particular protein fractionation capability.

3.3. Partitioning of α-lac and β-lg

The partition coefficients of α-lac (Kα) and β-lg (Kβ) are shown in Fig. 4. According to Johansson (1985) the partition coefficients for proteins in the most commonly employed phase segregated systems generally fall within the range 0.1–10. Thus, partition coefficients less than unity means that the target biopolymer remains in the lower phase whereas a value higher than unity indicates that biopolymer migrates to the upper phase.

Table 2
Proportion of the different whey proteins in the upper phase segregated from WPC/HPMC mixed systems, WPC80 and commercial β-lg.

Sample	P α-lac (%)	P β-lg (%)	P B I (%)	P BSA (%)	P B II (%)
WPC80	17.0 ± 0.7	47.0 ± 2.4	9.00 ± 0.45	8.0 ± 0.4	19.0 ± 1.0
Commercial β-lg	5.50 ± 0.25	77.0 ± 4.0	6.0 ± 0.3	5.0 ± 0.2	6.5 ± 0.3
$UP_{8}^{0.5}$	23.0 ± 1.2	50.0 ± 2.3	–	8.0 ± 0.4	19.0 ± 1.0
$UP_{12}^{0.5}$	24.0 ± 1.1	53.0 ± 2.6	–	8.0 ± 0.3	15.0 ± 0.6
$UP_{15}^{0.5}$	24.0 ± 1.2	57.0 ± 2.8	–	8.0 ± 0.3	11.00 ± 0.46
$UP_{20}^{0.5}$	24.0 ± 1.0	58.0 ± 4.4	–	8.0 ± 0.3	10.0 ± 0.5
UP_{8}^{2}	18.0 ± 1.0	48.0 ± 2.3	–	9.00 ± 0.45	25.0 ± 1.3
UP_{12}^{2}	17.00 ± 0.75	60.0 ± 3.0	–	8.00 ± 0.36	15.0 ± 0.7
UP_{15}^{2}	16.0 ± 0.7	62.0 ± 3.1	–	9.00 ± 0.38	13.00 ± .0.56
UP_{20}^{2}	15.0 ± 0.6	54.0 ± 2.5	–	9.0 ± 0.4	22.0 ± 1.0

Mean ± SD, $n = 18$.
P means protein proportion.
UP means upper phase; subscript and superscript indicate the initial WPC80 and HPMC concentrations, respectively, expressed as % w/w.

Table 3
Proportion of the different whey proteins in the lower phase segregated from WPC/HPMC mixed systems.

Sample	P α-lac (%)	P β-lg (%)	P B I (%)	P BSA (%)	P B II (%)
$LP_{8}^{0.5}$	12.0 ± 0.6	34.0 ± 1.7	23.0 ± 1.1	9.00 ± 0.45	22.0 ± 1.1
$LP_{12}^{0.5}$	11.0 ± 0.5	36.0 ± 1.5	22.0 ± 0.9	8.00 ± 0.32	23.0 ± 1.0
$LP_{15}^{0.5}$	14.0 ± 0.6	35.0 ± 1.6	18.0 ± 0.8	8.00 ± 0.36	25.00 ± 1.25
$LP_{20}^{0.5}$	11.00 ± 0.55	34.0 ± 1.4	24.0 ± 1.2	9.0 ± 0.4	22.0 ± 0.9
LP_{8}^{2}	18.0 ± 0.9	40.0 ± 2.0	14.0 ± 0.7	8.0 ± 0.4	20.0 ± 1.0
LP_{12}^{2}	20.00 ± 0.92	37.0 ± 1.7	15.0 ± 0.6	8.00 ± 0.34	20.0 ± 0.8
LP_{15}^{2}	21.0 ± 1.0	38.0 ± 1.5	12.0 ± 0.5	8.00 ± 0.37	21.00 ± 0.95
LP_{20}^{2}	23.00 ± 9.4	34.0 ± 1.7	14.00 ± 0.55	9.0 ± 0.5	20.0 ± 0.9

Mean ± SD, $n = 18$.
P means protein proportion.
LP means lower phase; subscript and superscript indicate the initial WPC80 and HPMC concentrations, respectively, expressed as % w/w.

Analyzing *K* values for all mixed systems, it can be seen that α-lac and β-lg remain predominantly in the LP. However, there are remarkable differences between *K* values according to the initial HPMC concentration (Fig. 4). At HPMC 2% wt/wt, *K* values never exceed 0.6, while at HPMC 0.5% wt/wt *K* values increased up to 1.4 and 1.1 for Kα-lac and Kβ-lg, respectively. Besides, in all mixed systems an increase of initial WPC concentration produced an increase of *K* value. This could be attributed to the fact that when increasing WPC concentration also increase fat, salt and lactose concentrations, leading to ATPSs with different protein fractionation capability, and therefore, the protein *K* values also change.

Particularly, the WPC 20% wt/wt/HPMC 0.5% wt/wt mixed system presented the highest Kα-lac and Kβ-lg values (1.4 and 1.1, respectively). This indicates that these proteins were distributed evenly in both phases. However, these *K* values are within ranges previously reported for whey protein partitioning in other ATPSs. For polyethylene glycol/maltodextrin ATPS, da Silva and Meirelles (2000) obtained *K* values between 0.38–4.93 for α-lac and 0.13–1.28 for β-lg. Moreover, de Oliveira, de Abreu Filho, and de Alcântara Pessôa Filho (2007) obtained Kα-lac and Kβ-lg values of 2.5 and 1.1, respectively, working with an ATPS containing ammonium carbamate and block copolymers.

The protein recovery is a measurement of the amount of the desired protein that is recovered in the UP with respect to the total initial protein in the system. The protein recovery values for α-lac and β-lg are given in Table 4. First of all, it can be observed that the protein recovery for both proteins is between 35 and 73%. In

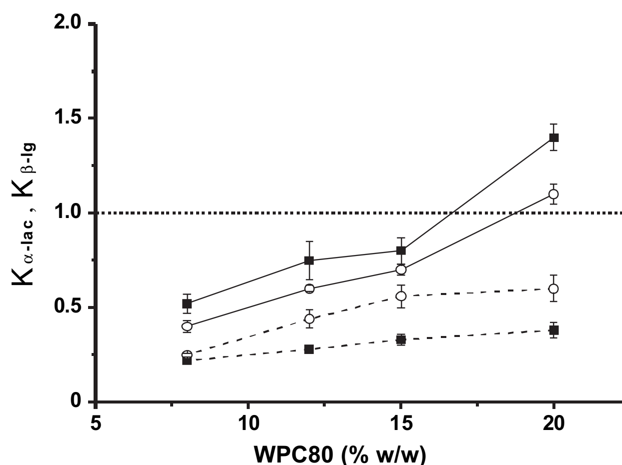

Fig. 4. Partition coefficients (*K*) for whey proteins in aqueous two-phase system with initial HPMC concentration of 0.5% w/w (solid line) or 2% w/w (dash line) and increasing initial WPC80 concentration; Kβ-lg: (○), Kα-lac: (■).

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Table 4
α-lac and β-lg protein recovery in the upper phase and the protein/HPMC concentration ratios for both phases.

Initial composition (% w/v)		PR[a] (%)		Protein/HPMC[b]	
WPC80	HPMC	α-lac	β-lg	LP	UP
8	0.5	70.0 ± 3.5	55.0 ± 2.7	37.0 ± 2.6	9.0 ± 0.6
12	0.5	70.0 ± 3.1	56.0 ± 2.2	43.0 ± 2.8	13.0 ± 0.8
15	0.5	61.0 ± 3.0	50.0 ± 2.1	47.0 ± 3.0	15.0 ± 1.0
20	0.5	73.0 ± 3.7	60.0 ± 3.0	46.0 ± 2.7	25.0 ± 1.6
8	2	52.0 ± 2.6	50.0 ± 2.5	12.0 ± 0.8	2.0 ± 0.1
12	2	50.0 ± 2.2	62.0 ± 3.1	12.0 ± 0.7	3.0 ± 0.2
15	2	43.0 ± 2.1	60.0 ± 2.5	13.00 ± 0.85	4.0 ± 0.3
20	2	35.0 ± 1.7	46.0 ± 2.3	14.0 ± 1.0	5.0 ± 0.35

PR means protein recovery.
UP and LP indicate upper or lower phase, respectively.
[a] Mean ± SD, $n = 18$.
[b] Mean ± SD, $n = 3$.

general terms, the protein recovery falls within a range of 60–95% for most commonly used ATPSs (Boaglio et al., 2006; Bolognese, Nerli, & Picó, 2005). Moreover, Alomirah and Alli (2004) obtained an average protein recovery value of 45% for α-lac and β-lg using a multiply-step purifying process to fractionate WPC. Taking into account the results of these authors, the protein recovery values obtained in the present work resulted quite acceptable.

It is noteworthy that in systems containing HPMC 0.5% wt/wt initial concentration, α-lac and β-lg recoveries reached values up to 73 and 60%, respectively. On the other hand, protein recoveries resulted between 35 and 62% when starting from a mixed system with a concentration of HPMC 2% wt/wt. Again, this different behaviour could be due to a reduction of the proteins diffusion to the UP as a result of increased HPMC-rich phase viscosity. Besides, in all mixed systems a change of initial WPC concentration produced a change in the protein recovery value. This fact has the same explanation exposed above for the partitioning coefficients, i.e. a change of WPC concentration leads to an ATPS with different fat, salt and lactose concentrations, changing its protein fractionation capability.

Finally, another interesting subject to analyze is the amount of HPMC that remains in the segregated phases because this polysaccharide could be removed in a subsequent purification step. Table 4 shows the ratio between the protein and HPMC concentration for the UP and LP of all mixed systems. Although these ratios are higher for the LP of all mixed systems, these fractions did not result to be the most convenient to isolate the desired proteins (α-lac and β-lg) due to the low enrichment reached. However, focusing in the ratio of UPs, it was found that the WPC 20% wt/wt/HPMC 0.5% wt/wt mixed system presented the highest ratio (25.0).

From the results exposed above together with the ones exposed in this section, it can be concluded that the WPC 20% wt/wt/HPMC 0.5% wt/wt mixed system presented the best combination of α-lac and β-lg enrichment (24 and 58%, respectively) and protein/HPMC ratio (25.0), for the fractionation of α-lac and β-lg.

4. Conclusions

Data collected in the present study demonstrated the capability of these aqueous two-phase systems for whey proteins fractionation. The differential incompatibility between the HPMC and each protein in WPC can be used for separating α-lac and β-lg from WPC. The upper phase of WPC 20% wt/wt/HPMC 0.5% wt/wt mixed system constitutes a fraction enriched in these proteins, where the sum of α-lac and β-lg proportions reached a value of approximately 80%. Moreover, in this phase the recovery of the initial amounts of α-lac and β-lg was 73 and 60%, respectively.

On the other hand, the low concentration of HPMC in the upper phase (protein/HPMC ratio up to 25 fold) results of key importance for avoiding its removal in a subsequent purification step. However, this little amount of HPMC could be removed from the rich α-lac and β-lg fraction by changing the conditions such as pH, salts or ionic strength, in order to obtain a new ATPS which will allow the separation of the polysaccharide.

Acknowledgements

The authors acknowledge the support from the Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT).

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