PARTITIONING OF CHICKEN EGG WHITE PROTEINS USING AQUEOUS TWO-PHASE SYSTEM

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Abstract. *The egg white is a rich source of proteins with high biological and nutritional value which are largely applied in the food and pharmaceutical industries. Therefore, the development of industrially applied techniques to concentrate and separate these proteins is of commercial interest. In the present study was used aqueous two-phase systems composed by poly(ethylene glycol) (PEG) and an inorganic salt (potassium phosphate and lithium sulfate) for the separation of proteins of liquid chicken egg white. It was evaluated the influence of type of salt, molar mass of PEG and temperature on the partitioning coefficient (Kp) of the proteins of "in natura" egg white. The PEG molar masses tested were of (1500 and 4000) g.mol^{-1}. The salt and PEG concentrations ranged from (11 to 28) mass % and (8 to 16) mass %, respectively. The temperature ranged from (5 to 45) °C. The results of the present work indicate that ATPS are suitable to concentrate proteins of "in natura" egg white in one phase of the aqueous system. Usually, the proteins concentrated on the salt-rich phase. The Kp values ranged from 0.04 to 2.75. The systems containing PEG1500 and potassium phosphate presented the highest value of Kp. It was observed the Kp increase with the tie line length increase and this effect was more pronounced at lower temperatures. It was verified that several factors such as the type of salt, the polymer molar mass, polymer and salt concentrations and temperature influence the partition coefficient of the egg white proteins.*

Keywords: *aqueous two-phase system, partition, egg white, proteins.*

1. INTRODUCTION

The egg white is a natural source of proteins with recognized biological and nutritional values, presenting high technological interest. The predominant protein in egg white is ovalbumin comprising 54% of the protein content; other proteins in egg white are ovomucoid (11%), ovotransferrin (12%), ovomucin (3.5%), and lysozyme (3.5%) (Mine and Zhang 2002, Stadelman and Cotterill 1995). Due to their functional properties (solubility, gelatinization, coagulation, jellification and foam formation) and antimicrobial action, the proteins of hen egg white have been extensively utilized as ingredient in processed foods, like bakery products, meringues, biscuits and meat derivates (Machado 2007, Mine and Bergougnoux 1998, Wong *et al.* 1996).

Egg white proteins have already been purified on a laboratory scale by liquid chromatography, reduction of ionic strength and precipitation using salts or solvents (Awade *et al.* 1994, Vachier *et al.* 1995). Some disadvantages related to these techniques, are high costs, protein denaturation and difficulties in the process scale-up. Thus, it is necessary in the development of economically viable techniques for the separation and purification of proteins look for their stability maintenance and a high level of purification.

The partitioning of biological components in aqueous two-phase system (ATPS) is a simple and efficient separation technique (Albertsson 1986), which may also be applied for separating egg white proteins. Su and Chiang (2006) studied the partitioning of lysozyme from chicken egg white by PEG/salt ATPS.

Traditionally, aqueous two-phase systems are constituted of two incompatible polymers in water solution, or a polymer and an inorganic salt, that when mixed in certain ranges of composition and temperature separate into two aqueous phases with different compositions. ATPS containing a polymer and an inorganic salt, or two structurally different hydrophilic polymers, show high applicability in the separation and purification of biomaterials (animal and plant cells, chloroplasts, enzymes, mitochondria, nucleic acids, proteins and others) and metallic ions (Albertsson 1986, Da Silva *et al.* 2007, da Silva *et al.* 2006, Silva and Meirelles 2000, Zaslavsky 1995).

ATPS have been successfully used for separation and purification of proteins due to their advantages over traditional methods as their high water content in both phases, which means high biocompatibility and low interfacial tension, thus minimizing degradation of biomolecules. ATPS also provide good resolution, high yield, and high capacity. In addition, this system is easily scaled-up. ATPS composed by polymer and salt could be a good alternative as a first purification

step since such systems allow removal of several contaminants by a simple and low-cost process (Da Silva *et al.* 2007, Giraldo-Zuniga *et al.* 2006Zaslavsky 1995).

Several studies show that the distribution of proteins is a function of various factors such as concentration and molar mass of the polymer, pH, ionic species, hydrophobic groups and temperature. Therefore, the study of these variables is necessary in the design and scale−up of separation processes (Alves *et al.* 2000, Da Silva *et al.* 2007, Faravash *et al.* 2007, Gautam and Simon 2006, Madeira *et al.* 2005, Silva and Meirelles 2001, Rosa *et al.* 2007).

In view of that, this work aims to apply the liquid-liquid extraction using aqueous two-phase system to a complex system, the "in natura" egg white, and analyze the partition behavior of the protein fraction in the systems composed by PEG and inorganic salts, as a function of the polymer molar mass, salt type, polymer and salt content and temperature. These data are necessary for the optimization of industrial processes that use liquid-liquid extraction to separate "in natura" egg white proteins.

2. MATERIALS AND METHODS

2.1. Materials

The reagents used in the experiments were mono and dibasic potassium phosphate (Vetec, Brazil), lithium sulfate (Vetec, Brazil), PEG 1500 $g.mol^{-1}$ (Sigma, USA) and PEG 4000 $g.mol^{-1}$ (Isofar, Brazil). All chemicals were of analytical grade and were readily available commercial products. Chicken eggs were purchased at the Viçosa local market. The water was deionized (Milli-Q, Millipore, USA).

2.2. Preparation of the egg white sample

The egg white "in natura" was diluted in phosphate buffer, with a pH value of 7.0, in the proportion of 1:1 (v/v). The solution was stirred at 4 °C for 2 h, and then centrifuged (Beckman J2-MC centrifuge, USA) at 18900 *g* for 45 min at 4 °C. The supernatant was collected and further used for the extraction of the proteins.

2.3. Preparation of aqueous two-phase system

ATPS were obtained following the methodology used by Carvalho *et al.* (2007), in which PEG/potassium phosphate aqueous systems were prepared from stock solutions of PEG (50 % w/w) and potassium phosphate (30 % w/w). The pH of PEG + potassium phosphate aqueous systems was corrected to 7.0, by adding mono and dibasic potassium phosphate in the ratio of 1:1.82 (w/w). PEG + lithium sulfate aqueous systems were prepared from stock solution of PEG (50 % w/w) and by adding the pure salt. Such systems presented a pH value close to 7.0 and required no adjustment.

Each system was prepared in graduated centrifuge tubes by the addition of 2 mL of the egg white solution containing approximately 65 mg/mL of proteins, and water until the final mass reached 30 g. The systems were weighed with a precision of ± 0.0001 g (Denver M-310 Analytical Balance, USA). The centrifuge tubes were stirred for 5 min and left in a thermostatic bath (Tecnal TE-184, Brazil) for 24 h to obtain clear phase separation and to reach the equilibrium. The operational temperature were of (5, 25, 35 and 45) °C for PEG/potassium phosphate system, and (5 and 25) °C for PEG/ lithium sulfate system. The resulting mixture was further centrifuged (Eppendörf AG-5804 centrifuge, Germany) at 2880 *g* for 5 min, at 25 °C. After than the top and bottom phases were carefully collected with a syringe, leaving a 5 mm thick layer above the interface. The partition experiments were carried out in duplicate.

The concentration of the total proteins (mg/mL) in each phase was determined by spectrophotometry (spectrophotometer Cary 50, Varian, Australia), applying the Biuret Method (Gornall *et al.* 1949).

The partition coefficient of the proteins was calculated using the Eq. 1:

$$K_p = C_T/C_B \tag{1}$$

where K_p is the partition coefficient of the proteins, and C_T and C_B are the equilibrium concentrations of the proteins in the PEG-(top) and salt-(bottom) enriched phases. This coefficient is used to quantify the degree of separation reached in an extraction process.

The partition of the proteins in the different systems can be also available using a numerical reference for the composition of each phase, which is tie line length (TLL). This measure is determined by the difference between the concentrations of the system constituents in each phase (Carvalho *et al.* 2007). The TLL is expressed in percentage (%), being calculated by Eq. 2:

$$TLL = [(C^T_{PEG} - C^B_{PEG})^2 + (C^T_S - C^B_S)^2]^{1/2} \tag{2}$$

where C^T_{PEG}, C^T_S, C^B_{PEG} and C^B_S, are the PEG and salt concentration (% w/w) in the top (T) and bottom (B) phases, respectively.

3. RESULTS AND DISCUSSION

3.1. Influence of the tie line length on the partitioning of the proteins

Tables 1-4 show the compositions of PEG/salt systems and the proteins partition coefficients. As can be seen in Table 1, the increase of the TLL led to an increase of K_p for the systems formed by PEG 1500/potassium phosphate at 5 °C. The same behavior was also exhibited for the system PEG 4000/potassium phosphate (Table 2). However, at 25 °C, as the TLL increases, the K_p values decreases. Although, after a certain value of TLL, K_p increases again, being the same behavior observed for the other temperatures in the systems PEG 1500/potassium phosphate. For the systems PEG 4000/potassium phosphate, a similar behavior was found, except at the temperature of 45 °C, in which firstly an increment in K_p occurs with the increase of the TLL, and subsequently a drop of K_p was verified. However, with the continuous increase of the TLL, the values of K_p start increasing again. This behavior may be explained by the salting–in effect, in which a small increase in the salt concentration promotes the hydration of the proteins, making them more soluble in the salt phase. Nevertheless, the continuous increase of the salt content leads to the salting–out effect, where the proteins become less soluble in the salt phase, transferring to the polymeric phase. The increase of the salt concentration favors the dehydration of the proteins, exposing their hydrophobic groups, favoring the bonding with the PEG (Fennema 1993, Rojas *et al.* 2006), and then increasing their solubility in the polymeric phase.

Table 1. composition of the peg 1500/potassium phosphate system and the protein partition coefficient.

PEG (% w/w)	Salt (% w/w)	TLL (% w/w)	K_p
	5 °C		
12.720	13.008	26.893	0.249 ± 0.012
14.470	13.931	35.192	0.509 ± 0.035
16.690	14.004	37.249	0.635 ± 0.057
17.860	15.926	44.196	2.745 ± 0.894
	25 °C		
11.351	12.009	19.668	0.291 ± 0.008
13.267	13.006	31.555	0.155 ± 0.003
13.481	14.004	34.860	0.168 ± 0.001
17.061	14.126	44.408	0.272 ± 0.015
	35 °C		
14.50	10.998	24.551	0.120 ± 0.005
12.72	12.003	25.511	0.124 ± 0.006
14.28	13.000	33.143	0.090 ± 0.001
15.52	14.080	38.295	0.109 ± 0.012
	45 °C		
15.991	12.007	35.411	0.052 ± 0.003
17.095	13.018	40.131	0.047 ± 0.012
17.977	14.002	44.090	0.060 ± 0.003
18.538	16.067	46.094	0.083 ± 0.007

Table 2. Composition of the peg 4000/potassium phosphate system at different temperatures and the partition coefficient.

PEG (% w/w)	Salt (% w/w)	TLL (% w/w)	K_p
	5 °C		
13.908	12.007	32.466	0.020 ± 0.001
15.934	13.017	37.092	0.030 ± 0.001
17.805	15.049	43.775	0.183 ± 0.008
19.472	16.014	48.118	0.619 ± 0.016
	25 °C		
15.139	11.116	33.613	0.019 ± 0.006
18.458	12.005	42.025	0.005 ± 0.002
19.964	13.011	46.584	0.027 ± 0.003
19.708	15.036	49.446	0.046 ± 0.001
	35 °C		
15.959	10.033	35.037	0.015 ± 0.003

17.322	11.009	39.118	0.010 ± 0.001
19.662	12.006	44,103	0.009 ± 0.001
20.464	13.992	48,056	0.130 ± 0.001
	45 °C		
16.350	9.317	32,416	0.001 ± 0.001
19.153	10.039	38,930	0.009 ± 0.006
20.813	11.083	43,840	0.005 ± 0.002
21.725	13.031	47,907	0.007 ± 0.003

Table 3. Composition of the peg 1500/ lithium sulfate system at different temperatures and the partition coefficient.

PEG (% w/w)	Salt (% w/w)	TLL (% w/w)	K_p
	5 °C		
22.144	8.599	31.101	0.063 ± 0.009
24.672	9.456	35.509	0.081 ± 0.002
26.497	9.890	44.563	0.080 ± 0.003
27.817	10.994	48.295	0.076 ± 0.005
	25 °C		
22.985	8.595	29.354	0.054 ± 0.001
24.168	9.012	37.575	0.041 ± 0.001
26.517	9.454	42.794	0.049 ± 0.003
27.462	10.741	48.424	0.051 ± 0.001

Table 4. Composition of the peg 4000/ lithium sulfate system at different temperatures and the partition coefficient.

PEG (% w/w)	Salt (% w/w)	TLL (% w/w)	K_p
	5 °C		
21.169	8.004	31.705	0.057 ± 0.023
24.082	8.499	35.035	0.037 ± 0.007
26.798	9.499	44.957	0.044 ± 0.002
25.574	10.996	45.040	0.042 ± 0.002
	25 °C		
23.556	7.737	35.086	0.039 ± 0.001
24.851	8.598	41.061	0.032 ± 0.001
25.421	9.456	45.542	0.036 ± 0.001
27.931	9.998	46.964	0.010 ± 0.001

Therefore, in most of the systems the egg white proteins concentrated on the salt-rich phase with values $K_p < 1$. However at 5 °C, for the system containing PEG 1500 at higher values of TLL, the proteins presented a lower affinity by PEG-rich phase ($K_p > 1$).

In the Table 3, for the systems formed by PEG 1500/lithium sulfate, it was observed at 5 °C an increase of K_p on the TLL increase, and subsequently a K_p decreases with the TLL increases. This behavior was the opposite of the systems PEG 4000/lithium sulfate at the same temperature (Table 4). However, at 25 °C, for the systems containing PEG 1500, a lowering of K_p was observed with the increase of the TLL, and thereafter K_p increases with the raise of the TLL. In the systems PEG 4000/lithium sulfate, the value of K_p reduces with the increment of the TLL.

In the systems analyzed, not only the TLL influences the values of K_p. Other factors, like the type and concentration of the salt utilized, and the temperature also affected the proteins partition, since a difference could be observed in the results. Each salt exhibited a type of interaction with the polymer that may facilitate or make more difficult the migration of the proteins to the polymeric rich phase. The temperature has influence on protein partitioning, however studies of the temperature effects are still limited to allow some generalization. Forciniti *et al.* (1991) studied the influence of temperature on protein partitioning and ended that the influence of the temperature seems to be highly dependent on the kind of protein which is partitioned. It is also a fact that the egg white constitutes a highly complex system with several proteins, which exhibit a different behavior for each system, what hinders the analysis of the result obtained.

3.2. Influence of PEG molar mass on the partitioning coefficient of the proteins

Tables 1-4 show the larger values of K_p for the systems containing PEG 1500 when compared to those composed by PEG 4000, for both salts.

From a molecular point of view, a polymer with smaller molar mass might interact more strongly with the proteins, when compared to the polymer with larger molar mass, which has a higher capacity to form molecular bindings (Picó *et al.* 2006). In general, an increase in the polymer molar mass, for a certain phase composition, reduces the partition of biological material to the polymer phase rich. A polymer with higher molar mass reduces the volume of the solvent available to the molecules interactions, which implies in a lowering of the proteins solubility in the polymeric rich phase (Guan 1994), and consequently in a decrease of the K_p value.

4. CONCLUSIONS

The results of the present work indicate that ATPS are suitable to concentrate proteins of "in natura" egg white in one phase of the aqueous system. The egg white proteins presented a higher affinity for the salt-rich phase. Concerning to the type of salt, the systems composed of PEG and potassium phosphate promoted larger partition coefficients of proteins, when compared to those containing lithium sulfate. It was verified the influence of several factors such as the type of salt, the polymer molar mass, polymer and salt concentrations and temperature on the partition coefficient of the egg white proteins.

5. ACKNOWLEDGEMENTS

The authors wish to knowledge the National Counsel of Technological and Scientific Development (CNPq) and the Foundation to Research Support of the Minas Gerais State (FAPEMIG) for the financial support.

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