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Partitioning of whey proteins, bovine serum albumin and porcine insulin in aqueous two-phase systems

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Abstract

Partitioning of the proteins from cheese whey, bovine serum albumin and porcine insulin were analysed using aqueous two-phase systems (ATPS) prepared with PEG–phosphate, PEG–citrate and PEG–maltodextrin (MD). Proteins were quantified through one of the following methods: FPLC, Bradford and spectrophotometry at 280 nm. Results showed that whey proteins partitioned unevenly on the phases of the systems used, with α-lactoalbumin (α-La) concentrated in the upper phase and β-lactoglobulin (β-Lg) in the lower. Albumin in PEG–MD systems concentrated in the MD-rich lower phase. Porcine insulin showed great affinity with the PEG-rich phase, its partition coefficient was always over 10 and increases with PEG molecular mass.

Keywords: Partitioning; Aqueous two-phase systems; Proteins; Bovine serum albumin; Insulin

1. Introduction

Recent advances in genetic engineering, DNA recombinant technology, cellular fusion technology, and biotechnology in general, made possible the commercial production of new active products as pharmaceuticals, vaccines and hormones. However, the purification technology for these products has developed slowly when compared to the production technology. Purification is troublesome because of system complexity and the need to retain biological activity. Usually, biological materials have been purified through precipitation with salts or organic solvents and using various chromatographic techniques, all of them having enormous difficulties in large-scale applications. According to Diamond and Hsu [1], 50–90% of production costs for biological products are determined by the purification strategy.

Liquid–liquid extraction in aqueous two-phase systems (ATPS) has proved to be a promising separation strategy for many biological products. ATPS are established spontaneously, as far as specific concentrations of two hydrophilic polymers or one hydrophilic polymer and one salt are used, as reported by many authors [2–7]. Each one of the system's components is concentrated in one of the phases, therefore favoring the partitioning of biomolecules such as proteins, cells, cell particles or nucleic acids. It is important to remember that the higher water content in both phases avoids protein denaturation.

ATPS such as polyethylene (PEG)–citrate, PEG–

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phosphate and PEG–sulfate are adequate for continuous large-scale purification of biological origin materials and allow the use of traditional liquid–liquid extraction equipment [8–10]. Development of this kind of extraction equipment needs experimental data on the partitioning of pure substances, using the knowledge of the partitioning phenomenon in order to produce a model for describing the phase equilibrium. Having a thermodynamic model, it is possible to predict a partition coefficient for the molecules of interest, which is of paramount importance in the development of a purification process using ATPS.

An example of an application of ATPS to a real case was the separation of proteins from cheese whey [8,9]. Cheese whey has a high protein content, mainly α-lactalbumin (α-La) and β-lactoglobulin (β-Lg). β-Lg is considered to be the most allergenic element in food preparations for children, based on whey proteins.

2. Experimental

2.1. Materials

The reagents used in this work were: mono and dibasic potassium phosphate (99%) from Sigma (Steinheim, Germany), sodium citrate (99%) from Merck (Darmstadt, Germany), α-lactalbumin (α-La) and β-lactoglobulin (β-Lg) from bovine milk (electrophoresis grade) from Sigma (Steinheim, Germany), bovine serum albumin (BSA) (96–99%) from Sigma (St. Louis, USA), polyethylene glycol (PEG) with molecular masses of 600, 1450, 3350, 8000 and 10 000 from Sigma (St. Louis, USA) and 1500 from Aldrich (Milwaukee, USA). Maltodextrins (MD) with molecular masses 2000 and 4000 were kindly supplied by Companhia Lorenz (Blumenau, SC, Brazil). Whey protein isolate (WPI) from cheese whey, commercially denominated as BiPRO, was supplied by Davisco Foods International (Le Sueur, MN, USA) and porcine insulin crystal (98.8%) was kindly supplied by Biobrás (Montes Claros, MG, Brazil).

2.2. Partitioning experiments

The ATPS were prepared from stock solutions of PEG, potassium phosphate, sodium citrate and MD as shown in Table 1. For PEG–phosphate systems pH was corrected to 7 by addition of mono and dibasic potassium phosphates in a ratio of 1.087 (w/w). Phases were mixed with a magnetic agitator within an equilibrium cell (50 ml) having external temperature (25±0.1°C) control from a thermostatic bath, according to [4]. Whey protein isolate was added to this system in order to achieve a concentration of 6 mg WPI g^{-1} of total mass. According to the supplier, the Whey Protein Isolate contains 99.4% of protein in a dry basis, and its two main components are α-La and β-Lg, approximately 82.5% of the total protein content. The following additional information about WPI is also provided by the supplier: 5.1% moisture, 1.7% ashes, 0.4% fat, and pH=7.3 (10% solution at 20°C). After mixing, the system was left to equilibrate. Samples were submitted to dialysis in PD-10 columns (Pharmacia Biotech, Uppsala, Sweden) before protein determination using FPLC (ÄKTA purifier, Pharmacia Biotech, Sweden) [8]. An ion-exchange chromatographic column Mono Q HR 5/5, 5×50 mm (Pharmacia Biotech, Uppsala, Sweden) was used. The operational conditions for FPLC were: 1.5–2.0 MPa, 23°C, 1.0 ml min^{-1} flow-rate, and 1 ml sample volume. Absorbance at 280 nm was used for peak detection and quantification.

Table 1
Systems used in protein partition

System	Protein	Method of analysis
PEG (1500)–potassium phosphate	α-La and β-Lg (WPI)[a]	FPLC [8]
PEG (600, 1450, 3350)–sodium citrate	Porcine insulin	Spectrophotometry at 280 nm [11]
PEG[c] (1450, 8000, 10000)–MD[c] (2000, 4000)	α-La, β-Lg, BSA[b]	Bradford [12]

[a] Proteins from Whey Protein Isolate (WPI).
[b] Pure proteins from Sigma.
[c] PEG, polyethylene glycol; MD, maltodextrin; the numbers in parentheses are the polymers' molecular masses.

For the PEG–sodium citrate system the saline phase pH was adjusted to 4.5, 7.0 and 9.5, by the addition of solutions of citric acid or sodium hydroxide. Porcine insulin was added to the system until a final concentration of 1 mg ml^{-1}. Experiments were conducted within equilibrium cells following the same procedure described previously. Phase samples were analyzed by spectrophotometry (DR/4000 U, Hach, Loveland, CO, USA) at 280 nm, according to [11]. As described before, the saline phase passed through dialysis before spectrophotometry and the PEG-rich phase was centrifuged at 2.900 *g* for 10 min.

For the PEG–MD system, experiments were conducted in 15-ml centrifuge tubes. The total mass was 12 g, having a protein concentration (BSA, α-La or β-Lg) of 5 mg ml^{-1}. After mixing, the tubes were centrifuged (BR 4i, Jouan, France) for 60 min at 2900 *g* and 25°C. Then they were set in a thermostatic bath (Viscotherm, VT2, Physica, Germany) at 25±0.1°C until equilibrium. Samples of both phases were analyzed using the Bradford method [12]. All analyses were conducted in duplicates at least. Partition coefficients (K) for the proteins were calculated using the following equation:

$$K = \frac{C_{\text{i,upper}}}{C_{\text{i,lower}}}$$

where $C_{\text{i,upper}}$ and $C_{\text{i, lower}}$ are, respectively, the protein concentration in the upper and lower phases. For all analyzed systems the upper phase is the one rich in PEG.

3. Results and discussion

3.1. Partition coefficient of α-La and β-Lg in PEG–phosphate systems

Fig. 1 presents the partition coefficients for α-La and β-Lg proteins (WPI), as a function of PEG concentration in ATPS. The mean estimated error for the partition coefficients of β-Lg was ±0.001. For the partition coefficients of α-La it was ±0.008, except for the partition coefficient measured in the system containing 14% (w/w) PEG and 18% (w/w) salt. In this last case the α-La concentration in the salt phase is actually too small, so the partition coefficient value (8.0) and its estimated error (±1.0) were higher. For β-Lg the partition coefficient shows a very weak dependency with respect to system concentration of PEG and is within the range 0.005–0.056, with an average value of 0.031. For α-La, the coefficient is within the range 0.29–8.0 and shows an increase with increasing polymer and salt concentrations. For the system with 14% (w/w) PEG and 18% (w/w) salt, the highest α-La coefficient was obtained. Selectivity for this system was also the highest attained: $K_{\alpha\text{-La}}/K_{\beta\text{-Lg}} = 318$, thus showing the feasibility of protein separation from Whey Protein Isolate in ATPS.

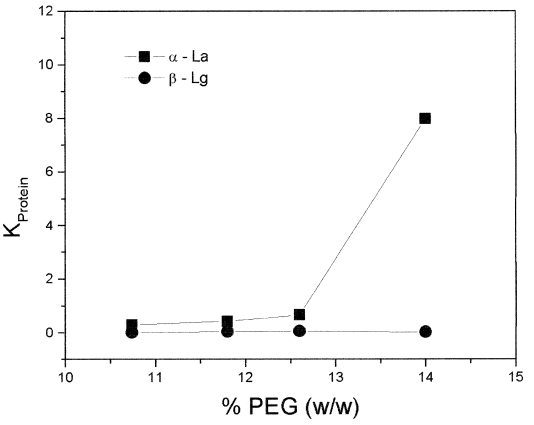

Fig. 1. Partition coefficients for whey protein isolate in PEG–phosphate systems.

3.2. Partition coefficients of α-La, β-Lg and BSA in PEG–MD systems

Fig. 2 shows the partitioning results for α-La and β-Lg (Sigma) determined in PEG–MD systems. In this case the mean estimated error for the partition coefficients was ±0.001. As is shown in Fig. 2, only the partition coefficient for α-La in the system PEG 8000–MD 2000 exhibits good sensitivity to the polymers' concentration, and it goes down as the concentration goes up. The partition coefficient of β-Lg was much less than 1 for the system PEG 8000–MD 2000, increasing to close to 1 as the MD molecular mass increased and the PEG molecular mass decreased (PEG 1450–MD 4000 system). Although these results indicate the feasibility of

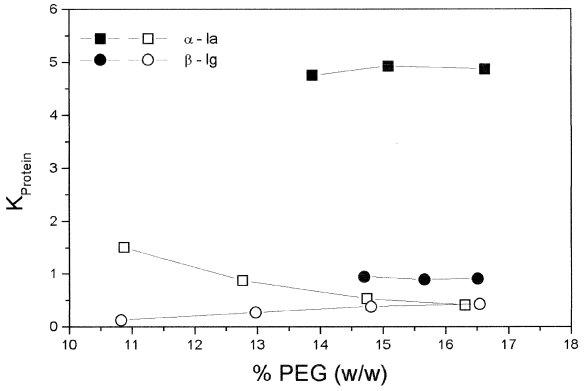

Fig. 2. Partition coefficients for α-La and β-Lg in PEG 1450–MD 4000 (■, ●) and PEG 8000–MD 2000 (□, ○).

separating these proteins in ATPS made up with PEG and MD, the observed selectivity in the system PEG–phosphate was much better, thus indicating a clear advantage for the latter system.

Fig. 3 shows partition coefficient data for BSA in the PEG–MD 2000 systems. As indicated, these coefficients show a tendency to increase with an increase in polymer concentrations. Similar results are observed for the partitioning of this protein in systems with MD 4000. Our results also show a stronger influence of PEG molecular mass on the partitioning as compared to MD molecular mass. For example, the highest partition coefficient (close to 0.9) was obtained for the system with PEG 1450. The same kind of result is reported in [13] for other proteins in PEG–MD systems. Anyway, for all PEG–MD systems the BSA had a preference to concentrate in the polysaccharide-rich phase.

3.3. Partition coefficients for porcine insulin in PEG–sodium citrate systems

The experimental results for porcine insulin are shown in Fig. 4. The mean error for the partition coefficients of porcine insulin was ±1.8, except for the experiment with PEG 3350 at pH=7. For this experiment the insulin concentration in the bottom phase was extremely low, resulting in a higher partition coefficient (K=59.2) and a higher estimated error (11.4). It is seen that for pH 4.5 the coefficient is very insensitive to the PEG molecular mass, showing an average value of 22.4±1.7. For pH 7.0 and 9.5 there is an increase of K with PEG molecular mass, reaching the highest values for PEG 3350 (K=59.2 for pH 7.0, and K=56.4 for pH 9.5). This behavior is explained by the higher solubility of the insulin in the upper phase when a higher PEG molecular mass is used. For systems with PEG 600 and pH 7.0 or 9.5, a precipitate was observed at the interface, which explains the lower insulin concentration found in the upper phase.

It was also observed that insulin solubility was higher at pH 4.5, a condition where no precipitate or turbidity appeared. At this pH, insulin is below its isoelectric point, which is around 5.6 according to [14]. The insulin concentrations found in the bottom

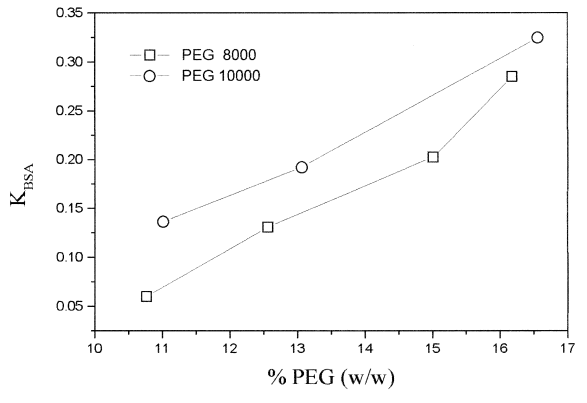

Fig. 3. Partition coefficients for BSA in PEG–MD 2000 systems.

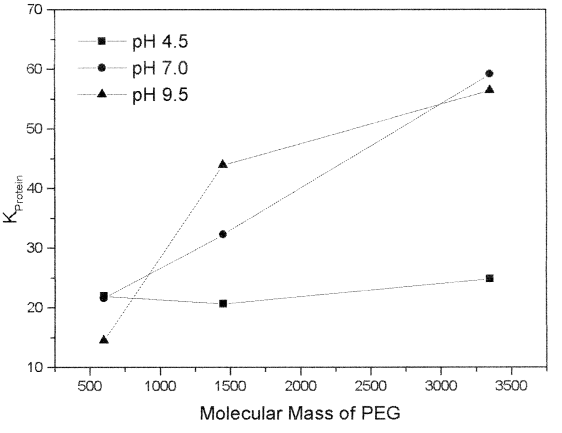

Fig. 4. Partition coefficients for porcine insulin.

phases were very low in all PEG–sodium citrate systems studied in the present work.

4. Conclusion

Partitioning of different proteins, cheese whey α-La, β-Lg, BSA and porcine insulin were experimentally analyzed in ATPS. The results showed the feasibility of α-La and β-Lg purification by using the system 14% PEG 1500–18% potassium phosphate: α-La concentrated in the PEG-rich phase and β-Lg in the saline phase.

For the PEG–MD systems the β-Lg has a tendency to concentrate in the MD-rich phase. In most PEG–MD systems α-La concentrates in the PEG-rich phase, indicating the feasibility of separating such proteins. The best results were obtained for PEG 1450–MD 4000. For BSA, the results show that it prefers the MD-rich phase over the PEG-rich phase.

Porcine insulin showed great affinity for the PEG-rich phase. For pH 7.0 and 9.5, there is a tendency for the porcine insulin partition coefficient to increase with the PEG molecular mass, attaining values above 50. For pH 4.5 the insulin partition coefficient is practically independent of the PEG molecular mass.

Acknowledgements

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