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Single-step purification of ovalbumin from egg white using aqueous biphasic systems

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ABSTRACT

The ability of aqueous biphasic systems (ABS) composed of polyethylene glycols of different molecular weights (PEG 400, 600 and 1000) and buffered aqueous solutions of potassium citrate/citric acid (pH 5.0–8.0) to selectively extract ovalbumin from egg white was here investigated. Phase diagrams, tie-lines and tie-line lengths were determined at 25 °C and the partitioning of ovalbumin in these systems was then evaluated. Aiming at optimizing the selective extraction of ovalbumin in the studied ABS, factors such as pH, PEG molecular weight and amount of the phase-forming components were initially investigated with pure commercial ovalbumin. In all ABS, it was observed a preferential partitioning of ovalbumin to the polymer-rich phase, with extraction efficiencies higher than 90%. The best ABS were then applied in the purification of ovalbumin from the real egg white matrix. In order to ascertain on the ovalbumin purity and yield, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion high performance liquid chromatography (SE-HPLC) analyses were conducted, confirming that the isolation/purification of ovalbumin from egg white was completely achieved in a single-step with a recovery yield of 65%. The results obtained show that polymer-salt-based ABS allow the selective extraction of ovalbumin from egg white with a simpler approach and better performance than previously reported. Finally, it is shown that ovalbumin can be completely recovered from the PEG-rich phase by an induced precipitation using an inexpensive and sustainable separation platform which can be easily applied on an industrial scale.

1. Introduction

Egg white is an aqueous-rich medium mainly composed of proteins, *circa* 10–12%, such as ovalbumin, ovotransferrin, lysozyme and ovomucin [1]. In addition to their nutritional importance, egg white proteins display multiple functional properties, such as foaming, emulsification and heat-setting. As a result, egg white plays a major role in the food industry [2]. Furthermore, new applications involving egg white proteins are increasingly being found due to their antimicrobial and antioxidant properties [3], which have contributed for the production of health- and pharmaceutical-related products. Ovalbumin is the main protein of egg white, being responsible for most of its functional properties, and represents about 54% of the total protein content [4]. Ovalbumin is a glycoprotein consisting of 385 amino acids (45 kDa) with an isoelectric point of 4.5 [5]. Because of the remarkable applications involving egg white proteins of high purity level [6–9], the development of novel cost-effective purification techniques is in great demand.

Ovalbumin was one of the first proteins isolated from egg white [10]. For that purpose, Hopkins [10] developed a method using high amounts of ammonium sulfate for ovalbumin precipitation. Nevertheless, the high amount of salt used can lead to the proteins irreversible unfolding. Nowadays, proteins from egg white are typically separated by electrophoresis, ion-exchange chromatography, size exclusion liquid chromatography, ultrafiltration, adsorption, among others [11–18]. However, some of these methods currently employed are multi-stage and with high equipment and operating costs. In the context of the development of alternative processes for the purification of proteins, a large interest has been devoted to aqueous biphasic systems (ABS) in the past decades [13]. ABS are a liquid-liquid extraction/purification technique which provides a highly biocompatible media since they are mainly composed of water [19]. Nevertheless, the extraction and purification of proteins from a complex matrix, such as egg white, is a difficult task

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due to some similarities between the proteins present in the raw material.

Most works in the literature have been focused on the evaluation of the extraction and recovery ability of ABS employing commercial and pure egg white proteins. For instance, Saravan et al. [20] studied the partitioning behavior of ovalbumin in poly(ethyleneglycol) (PEG)—poly(acrylic acid) ABS, achieving an extraction yield of 87.4%. Nerli et al. [21] investigated the thermodynamic forces involved in the partitioning of ovalbumin in various aqueous two-phase polymer systems (constituted by PEGs of different molecular weights and dextran). The authors [21] demonstrated that the ovalbumin transfer to the top phase is exothermic, which suggests an electrostatic interaction between the hydroxyl groups of PEG and the hydrophilic side chain of the protein. Dallora et al. [22] investigated the partition coefficients of trypsin and lysozyme, and demonstrated that they can range between 1.0 and 2.4 for trypsin and from 2.3 to 9.0 for lysozyme, depending on the polymer chain size and on the tie-line length. The analysis of the separation factor, defined as the ratio of partition coefficients of two proteins, shows that high degrees of separation could be achieved [22]. In addition to these studies employing pure proteins, Coen et al. [23] investigated the purification of egg white proteins using ammonium-sulfate precipitation and found some difficulties in the separation of lysozyme and ovalbumin. The extraction of lysozyme from egg white was also attempted using an ABS composed of PEG 600 and a sulfate-based salt at 25 °C and pH 10 [24]. The authors reported an extraction of 70% of lysozyme from egg white with no loss in the enzymatic activity. Yang and co-workers [25] demonstrated the application of PEG 4000/potassium citrate ABS to extract lysozyme from egg white. Diederich et al. [26] showed the selective separation of avidin from the remaining proteins from egg white using an ABS composed of PEG 600 + potassium phosphate + 3 wt% NaCl, with a purity level higher than 60% and recovery yields > 90% (for avidin). In an attempt to reach higher purification levels, multi-stage ABS separations, by means of liquid-liquid chromatography, have been also investigated. Shibusawa et al. [27,28] fractionated chicken egg white proteins by high-speed counter-current chromatography (HSCCC) using an ABS composed of PEG 1000 and potassium phosphate salts at different pH values. After the optimization of several operational conditions, the authors [27,28] demonstrated to be able to purify the proteins present in the crude sample solution prepared from fresh egg white using CCC. Zhi et al. [29] also employed ABS coupled to HSCCC aiming at purifying the major protein components in hen egg white, including ovaltransferrin, ovalbumin and lysozyme. Ovalbumin was successfully purified (up to 95%) from the hen egg white sample with a PEG 1000 + potassium phosphate ABS [29]. Other approaches have been described for the purification of egg white proteins, namely by the use of ion exchange columns followed by precipitations steps [30,31]. It should be however highlighted that most works reporting higher purification levels of egg white proteins comprise several stages of equilibrium and require specialized equipment, such as those using HSCCC [27–29].

This work is focused on the development of an one-step extraction/purification process of ovalbumin from egg white using ABS constituted by polyethylene glycol of different molecular weights (PEG 400, PEG 600 and PEG 1000) and a citrate-buffered medium (pH 5, 6, 7 and 8 according to different $K_3C_6H_5O_7$:$C_6H_8O_7$ mole fraction ratios). Initially, the phase diagrams of each ternary system, *i.e.*, the description of the binodal curves which separate the monophasic from the biphasic regimes, were determined at 25 °C. The respective tie-lines and tie-line lengths were also determined. These biphasic systems were then optimized regarding their extraction performance for commercial ovalbumin and finally employed to separate ovalbumin from egg white.

2. Experimental section

2.1. Materials

The ABS studied in this work are composed of a buffer aqueous solution constituted by potassium citrate monohydrate, $K_3C_6H_5O_7{\cdot}H_2O$, ≥ 99 wt% pure from Sigma–Aldrich, and citric acid monohydrate, $C_6H_8O_7{\cdot}H_2O$, 100 wt% pure from Fisher Scientific. Polyethylene glycol of different molecular weights, namely 400 g mol^{-1} (PEG 400), 600 g mol^{-1} (PEG 600), and 1000 g mol^{-1} (PEG 1000), and ovalbumin from hen egg white, were acquired from Fluka. All compounds were used as received. Fresh eggs were bought in a local supermarket.

2.2. Phase diagrams, tie-lines and tie-line lengths

Aqueous solutions of polyethylene glycol (PEG 400, PEG 600 and PEG 1000) at 90 wt% and aqueous solutions of the buffer $K_3C_6H_5O_7/C_6H_8O_7$ (pH 5, 6, 7 and 8 according to different $K_3C_6H_5O_7$:$C_6H_8O_7$ mole fraction ratios) at ≈50 wt% were prepared and used for the determination of the binodal curves. The phase diagrams were determined through the cloud point titration method [32,33] at 25 °C and atmospheric pressure. The system compositions were determined by the weight quantification of all components added within ±10^{-4} g.

The tie-lines (TLs) were determined by a gravimetric method originally proposed by Merchuk et al. [34]. A mixture point in the biphasic region of the phase diagram was prepared using small ampules (*ca.* 10 mL) especially designed for the purpose, vigorously stirred and allowed to reach equilibrium by the separation of the phases for at least 12 h at (25 ± 1) °C. After separation of the two phases, both the top and bottom phases were weighted. Each individual TL was determined by application of the lever-arm rule to the relationship between the top phase weight and the overall system composition. The experimental binodal curves (in percentage weight fraction) were correlated using Eq. (1) [34]:

$$[PEG] = A\exp\left(B[salt]^{0.5}\right) - \left(C[salt]^3\right) \quad (1)$$

The determination of the TLs was accomplished through the solution of the following system (Eqs. (2)–(5)) with four unknown values, namely $[PEG]_{PEG}$, $[PEG]_{salt}$, $[salt]_{PEG}$, and $[Salt]_{salt}$ [34]:

$$[PEG]_{PEG} = A\exp\left[\left(B[salt]_{PEG}^{0.5}\right) - \left(C[salt]_{PEG}^{3}\right)\right] \quad (2)$$

$$[PEG]_{salt} = A\exp\left[\left(B[salt]_{salt}^{0.5}\right) - \left(C[salt]_{salt}^{3}\right)\right] \quad (3)$$

$$[PEG]_{PEG} = \frac{[PEG]_M}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[PEG]_{salt} \quad (4)$$

$$[salt]_{PEG} = \frac{[salt]_M}{\alpha} - \left(\frac{1-\alpha}{\alpha}\right)[salt]_{salt} \quad (5)$$

where the subscripts "PEG", "salt" and "M" represent the polymer (top) and the salt (bottom) rich phases and the mixture composition, respectively. The parameter α is the ratio between the weight of the top phase and the weight of the total mixture.

The tie-line lengths (TLLs) were determined according to Eq. (6),

$$TLL = \sqrt{([salt]_{PEG} - [salt]_{salt})^2 + ([PEG]_{PEG} - [PEG]_{salt})^2} \quad (6)$$

2.3. Partitioning of commercial ovalbumin in PEG-salt ABS

Fixed mixture compositions in several ABS were selected and used to evaluate their performance for the extraction of ovalbumin. Initial optimization tests were carried out with pure commercial ovalbumin. Then, the best ABS was employed in the separation of ovalbumin from egg white. Commercial ovalbumin was diluted in water at a concentration of *circa* 0.5 g L^{-1} while egg white was