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New Processes to Extract and Purify Phosvitin by Using Aqueous Salt Solutions, Precipitation and Ultrafiltration Techniques

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Abstract: Phosvitin is the most phosphorylated naturally occurring protein and it is concentrated in the granular fraction of egg yolk. It has interesting antioxidant and cation chelating properties that can be used to preserve food and cosmetics, but its industrial application is limited as its separation relies on the use of organic solvents and chromatographic techniques, which are expensive and difficult to assimilate in a continuous procedure. In this study, we propose a new phosvitin separation process using substances legally accepted for use in the food industry (NaCl and HCl), employing egg yolk granules as raw material. In this case, the NaCl concentration and the pH of the solution of granules were screened in order to obtain a phosvitin-rich supernatant after centrifugation. Additionally, two new processes were proposed to purify this phosvitin-rich solution. The first was the precipitation of impurities during the desalting stage at optimized pH values. The second was ultrafiltration under selected pH value conditions. A low nitrogen/phosphorous (N/P) atomic ratio is considered a quality parameter, with 3.6 ± 0.2 being the value of the phosvitin-rich supernatant. The two purification processes provided highly purified phosvitin with a similar N/P value of 2.5 ± 0.1. The high level of purification of the phosvitin was confirmed using electrophoresis and ion-exchange chromatography. In particular, the purified phosvitin obtained via ultrafiltration is already desalted and membrane technology is more easily scalable than that based on chromatography, thus facilitating the industrial separation and commercialization of the phosvitin.

Keywords: extraction; granules; high density lipoproteins; phosphorylated proteins; purification; ultrafiltration; yolk

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1. Introduction

Egg yolk is a cheap source of substances of interest for the food, cosmetic and pharmaceutical industries, and is obtained without causing suffering to animals [1]. Egg yolk is composed of two phases: one, referred to as plasma, with a high water content and a high low-density lipoprotein (LDL) content, which contains agglomerations of the second phase, the granular fraction, composed mainly of aggregates of proteins and high-density lipoproteins (HDLs).

To be more specific, egg yolk granules are composed of 70% HDLs, 16% phosvitin [2] and 12% LDLs, all of which are bound to one another via phosphocalcium bridges (between both molecules of the same type and between those of the different groups) [2,3]. HDLs and LDLs have emulsifying capacities, with a lipid content ranging from 22% in HDLs to 89% for LDLs [4]. Furthermore, the emulsifying capacities of the granules allow them to be used as egg yolk substitutes in the preparation of dietary products, with a lower presence of cholesterol than when using whole egg yolk [5].

Regarding phosvitin, this protein has two major components, one large and the other small, known as the alpha and beta components, although five proteins, of 40,000, 33,000, 18,000, 15,000 and 13,000 Daltons, have also been reported [6,7]. Phosvitin is

a protein with a large number of serine residues, most of which are phosphorylated, making it one of the most phosphorylated of the naturally occurring proteins, with a 10% phosphorous content [8]. Therefore, phosvitin is more hydrophilic than HDLs and LDLs, as these proteins contain only 2% phosphorous [3,9]. The number of phosphate groups in phosvitin is thought to be related to a biological role and by accumulating 95% of the iron that appears in egg yolk, it ensures that this iron is made less available to microorganisms. Furthermore, due to its major capacity to bind divalent cations, phosvitin is an effective inhibitor of oxidation mediated by Fe^{+2} and Cu^{+2} [10]. The partial digestion of phosvitin using hydrolytic enzymes results in peptides with enhanced complexing properties compared to those of the original protein, making it more effective than other molecules such as ferritin, transferrin and EDTA [11]. Phosvitin also has emulsifying and emulsion stabilizing properties, with these qualities being superior to those of bovine whey albumin, soya protein or β-casein [12–14].

In order to take advantage of the aforementioned qualities, phosvitin was initially purified by using non-food grade chemicals or organic solvents or a mixture of the two [15–19]. However, more recent studies have focused on extracting and purifying phosvitin without using these types of chemicals. In this line, Castellani et al. [20] extracted phosvitin from egg yolk by precipitating it with magnesium sulphate and then purifying it using ion-exchange chromatography. In this case, the chromatographic separation was performed at pH 7.5 and with the addition of 0.3 M of NaCl to avoid the coelution of the HDL with the phosvitin. In a similar way, Lei and Wu [21] obtained a fraction with a high phosvitin purity by using ion-exchange chromatography, but in this case the granular fraction was solubilized at pH 9.6 and directly separated, without a previous phosvitin extraction. In another research paper, Zhang et al. [22] dissolved the egg yolk granules in a 1.74 M NaCl solution and then removed the impurities by precipitating them with PEG6000 at pH 4.0. The phosvitin in the supernatant was dialysed and subsequently purified by using anion exchange chromatography. In all these cases, it must be borne in mind that chromatographic techniques, such as ion-exchange chromatography or gel filtration, are poorly scalable. Moreover, in the case of anion-exchange chromatography, the desalting of the phosvitin solution before and after the purification process is frequently required.

Other separation methods avoid the use of chromatography but require the use of a relatively high temperature in order to purify the phosvitin. Jung et al. [23], for example, solubilized the egg yolk granules in a 12% NaCl solution and then subjected them to heat treatment at 90 °C for 60 min in order to precipitate the HDL contamination. In a similar way, Lee et al. dispersed the egg yolk granules in a 10% NaCl solution, extracting the phosvitin by lowering the pH to 4.0 and then purifying it by heating the previously concentrated phosvitin at 70 °C for 30 min. Although these temperatures may not be considered very high, some researchers suggest that phosvitin shows a certain degree of heat sensitivity at temperatures equal to or higher than 70 °C [24,25].

One thing that most of these research papers have in common is that prior to the extraction and purification of the phosvitin, it is necessary to solubilize the granular fraction by using a solution of approximately 1.70 M NaCl [26], and some of them take advantage of the differential solubility of the phosvitin and the HDLs at certain pHs at this NaCl concentration. However, granules are already 80% solubilized in a 0.3 M NaCl solution, and according to other authors, the complete solubilization of the egg yolk granules is produced at 0.58 M NaCl [3]. Therefore, the influence of the relationship between the ionic strength of the medium and the pH on the solubility of the phosvitin is an issue that has been scarcely studied, since NaCl concentrations lower than 1.72 M have not usually been considered. High phosvitin extraction at NaCl concentrations below 1.72 would imply a subsequent desalination step that would be less time- and energy-consuming.

In addition, another variable that has never been considered in order to separate and purify the different egg yolk granular proteins is their difference in size. HDLs are composed of 5 main apoproteins with molecular weights from 35 to 110 kDa [27] and the phosvitin is formed from several polypeptides of 35 and 40 kDa [8]. LDLs are made up of

six main apoproteins with molecular weights between 15 and 130 kDa. However, in native conditions, these apoproteins are assembled to form larger proteins: the native HDLs are a dimer of two subunits with a molecular weight of 400 kDa [2]; the α and β-phosvitins have a weight of 160 kDa and 190 kDa, respectively [28], and finally the LDLs are spherical particles of 35 nm size [29]. Taking into account that ultrafiltration techniques have been applied successfully to separate other egg and milk proteins [30–32], and because of the size differences among the granular proteins, the use of membranes to purify egg yolk phosvitin could be investigated.

Therefore, with the aim of broadening the potential range of applications, this paper presents a non-aggressive organic solvent-free extraction method for obtaining a phosvitin-rich fraction (PRF) by exploring the differences in solubility between the phosvitin and the HDLs at several pH values and NaCl concentrations, in order to select the mildest conditions (pH closest to neutrality and the lowest NaCl concentration). Once the PRF was obtained, further purification of the phosvitin was investigated using two different procedures. In the first, the dialysis of the PRF at various pHs was explored, with some of the pHs studied leading to the precipitation of impurities during the desalting step. In the second, PRF was ultrafiltered using a 300 kDa membrane, resulting in a highly purified phosvitin in the permeate, and then desalted using a 10 kDa membrane. In this sense, the ultrafiltration techniques are more scalable than the chromatographic ones and can operate in a continuous mode.

2. Materials and Methods

2.1. Extraction of the PRF

Medium size eggs (50–60 g) were purchased from a local market. Egg yolk granules were extracted following the procedures developed by Laca et al. [5]. Granules were diluted (1:1 w/w) with a 0.16 M NaCl solution and mixed with a magnetic stirrer. After 1 h, the solution was centrifuged for 45 min at 10,000× g and 4 °C; then, the supernatant was discarded, and the precipitate (washed granules) was subsequently collected. The washed granules were resuspended (8 g) in 400 mL of each NaCl solution assessed in this study (0.5 M, 1 M, 1.4 M and 1.72 M) for 2 h at room temperature. Then, each solution of 400 mL was divided into four aliquots of 100 mL, and the pH of each aliquot was adjusted to pH 2, or pH 2.5, or pH 3 or pH 4. These aliquots were left under gentle stirring at 4 °C for 6 h. Finally, each aliquot of 100 mL was entirely centrifuged at 10,000× g for 45 min at 4 °C, obtaining a pellet, the HDL-rich fraction, and a supernatant, which is the PRF.

2.2. Effect of Desalting pH on the Phosvitin Purity Obtained

PRF desalting was performed by using dialysis membranes (D7884-10FT, Sigma Aldrich, USA), having previously adjusted the pH of the PRF by employing 1 M HCl or 1 N NaOH. Considering that the pI of the phosvitin is 4.0, the dialysis pH values tested were 3.0 (below the pI of phosvitin), 5.0 (above the pI of phosvitin), 7.0 and 8.5, with both the latter being at the supposed pI of apo-HDLs [8,33]. Distilled water was used in each experiment and conductivity was monitored with a conductivity meter (Model HI98129, Hanna Instruments, USA) until a value lower than 1 microsiemen was obtained in the dialysis water. The dialysed samples were centrifuged at 10,000× g for 30 min and the sediment was discarded. The amounts of phosphorous and sodium in the supernatant were measured according to the method described in Section 2.4. The supernatant solution was lyophilized and stored at −20 °C for further analysis. The electrophoresis assay was carried out by dissolving the lyophilized sample at 1 mg/mL and 2 mg/mL.

2.3. Purification of Phosvitin from PRF by Ultrafiltration

In an alternative procedure to that described in Section 2.2, the PRF (500 mL) was filtered using a Pellicon 2 mini holder (Millipore, Burlington, MA, USA), equipped with a 300 kDa polyethersulfone tangential flow membrane cassette (Sartorius, Göttingen, Ger-

many). This membrane had a filtration area of 0.1 m^2. The transmembrane pressure was set to 13 $\times$ 10^4 Pa and all the experiments were performed at room temperature.

In order to study the influence of pH on the ultrafiltration process, the flux and the phosvitin transmission parameters were tested at several pH values from 3 to 9. During the experiments, the pH of the feed was controlled continuously. These parameters were tested in total recycle mode, with the retentate and permeate recirculating to the feed tank for 50 min, until a steady state was achieved. The concentration of phosvitin in the permeate, and in the retentate, was measured by calculating the percentage of organic phosphorus in each fraction:

$$\tau\ (\%) = (Cp/Cr) * 100 \quad (1)$$

where Cp and Cr are the concentration of phosphorus in the permeate and in the retentate, respectively.

Additionally, the purification of the phosvitin was performed in diafiltration mode, recycling the retentate and measuring the amount of permeate obtained using an electronic balance to calculate the flux values every 30 s in this mode. The volume of permeate lost during the ultrafiltration process was replaced by adding a solution of 1 M NaCl to the feed tank. The concentration of phosvitin in the permeate was calculated every 30 s using a UV–vis spectrophotometer (Helios γ, Thermo Scientific, USA) at a wavelength of 280 nm. A previous calibration curve using commercial phosvitin (PsV, P1253, Sigma Aldrich, USA) was carried out. After 30 min of diafiltration, all the permeate was collected and concentrated to $\frac{1}{4}$ of the original volume, employing a 10 KDa MWCO membrane (Model PXB010A50, Millipore, USA). Finally, the desalination of the concentrate was carried out according to Ting et al. through the addition of 10 diavolumes of distilled water [34]. The desalted solution was lyophilized and stored at -20 °C for further analysis.

2.4. Chemical Analysis

The sample pH was measured at room temperature. The amount of nitrogen in the samples was estimated using a CNHS/O Elementar Vario EL analyser (Elementar, Langenselbold, Germany). The percentage of organic phosphorus was determined using a colorimetric method, using 1-amino-2naphthol-4sulfonic acid and ammonium molybdate as reagents [35].

2.5. Anion Exchange Chromatography

Chromatographic analyses were carried out on an FPLC ÄKTA system (Amersham Biosciences, Amersham, UK). The chosen column was a Ceramic HyperD® Q (Life Biosciences, Boston, MA, USA) anionic resin, the column volume was 5 mL and the elution flow rate employed was 1 mL/min. Previously desalted and lyophilized samples were dissolved to a final concentration of 3 mg/mL in a TRIS (Tris(hidroximetil)-aminometano hydrochloride) buffer solution pH 8.0, 0.1 M (Trizma®, ref. T2694, Sigma Aldrich, St. Louis, MI, USA) and then filtered using 0.45 µm pore size filters. The volume of sample injected was 0.1 mL.

Before injection, the column was equilibrated with 5 column volumes of a 0.1 M Trizma® hydrochloride pH 8.0 solution (buffer A). The elution buffer (buffer B) was composed of 1 M NaCl in 0.1 M Trizma® hydrochloride pH 8.0. The gradient elution was performed from 0% to 60% of B for 60 min. The peaks obtained were monitored at 280 nm.

2.6. SDS-PAGE Analysis

Sediments and supernatants obtained after the phosvitin extraction experiments, carried out according to the methodology described in Section 2.1, were tested using electrophoresis. Supernatants were directly assessed, whilst the sediments were dispersed in the original volume with distilled water prior to electrophoretic analysis. Additionally, previously desalted and lyophilized samples obtained, according to the methodology shown in Sections 2.2 and 2.3, were dissolved to a final concentration of 1 mg/mL and 2 mg/mL in distilled water prior to the analysis.

SDS-PAGE was performed as described by Laemmli [36] and by using a 12% *w/v* acrylamide separation gel in a Tris-Glycine buffer. Gels were stained with two different solutions in order to reveal phosphorylated proteins [24]. The first was normally used to specifically stain phosphoproteins (Coomassie blue 0.05%, acetic acid 10%, triton 1%, ethanol 25%, aluminium nitrate 0.1 M). The second was a standard Coomassie stain, which stains total protein (0.1% Coomassie blue, 50% methanol, 10% acetic acid and 40% water). The same PvS mentioned in Section 2.3 was used as a standard to identify the phosvitin bands in the samples tested.

2.7. Statistical Analysis

All measurements were carried out in triplicate, calculating average values and standard deviations. Analysis of variance was performed using Statgraphics Centurion XVI® software (StatPoint Technologies, Inc., Washington, DC, USA) with a level of significance of $p < 0.05$.

3. Results and Discussion

3.1. Preparation of a Phosvitin-Rich Fraction (PRF)

The protein compositions of the sediments and supernatants obtained were studied using electrophoresis (Figure 1).

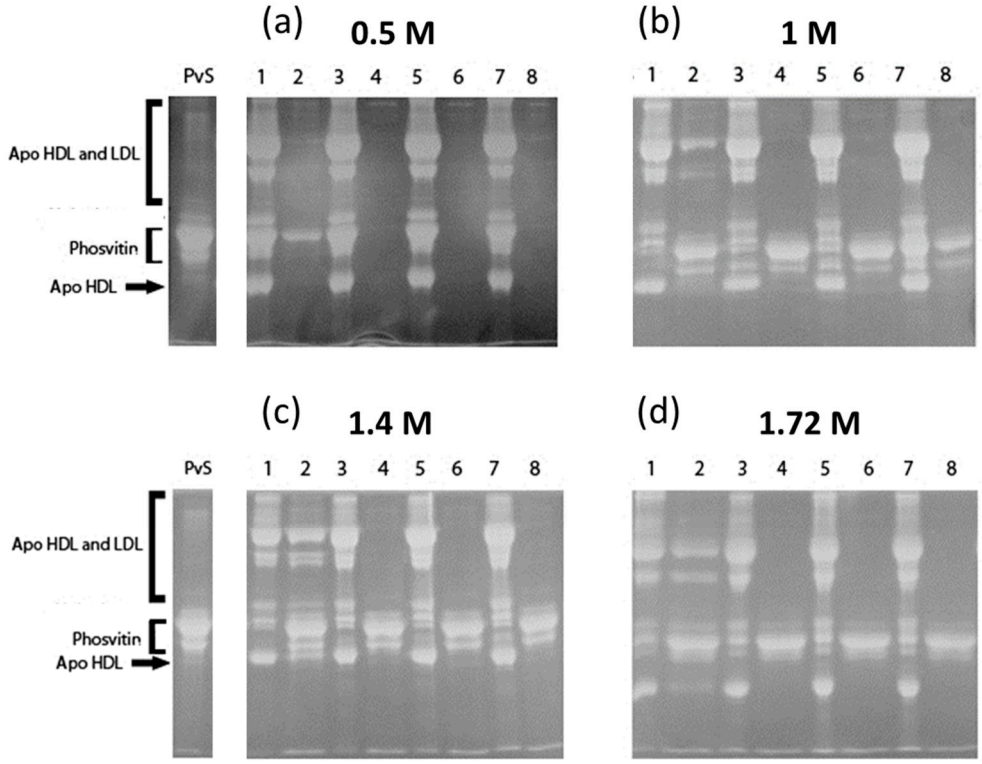

Figure 1. Granules dissolved in NaCl at different pHs and later centrifuged. (**a**) 0.5 M NaCl; (**b**) 1 M NaCl; (**c**) 1.4 M NaCl; and (**d**) 1.72 M NaCl. PvS: Phosvitin Standard, 1 mg/mL (Sigma-Aldrich). Lane 1: pH 4 sediment; Lane 2: pH 4 supernatant; Lane 3: pH 3 sediment; Lane 4: pH 3 supernatant; Lane 5: pH 2.5 sediment; Lane 6: pH 2.5 supernatant; Lane 7: pH 2 sediment; Lane 8: pH 2 supernatant.

Figure 1a shows how the total precipitation of the granular protein, phosvitin included, was achieved at 0.5 M NaCl and pH values of 3, 2.5 and 2. This can be seen from the absence of protein in the supernatant after pH adjustment and centrifugation (lanes 4, 6 and 8, respectively). However, at 0.5 M NaCl and pH 4, a part of the phosvitin remained in the supernatant (Figure 1a, lane 2).

The electrophoresis gel 1b shows the results for supernatants and sediments when the NaCl concentration in the experiment was adjusted to 1 M, with the same pH variation as in 1a. A reduced precipitation of HDLs into the sediment was observed at pH 4, meaning that HDL bands can be detected in the supernatant (Figure 1b, lane 2). However, mainly phosvitin bands can be appreciated in the supernatants at pH 3, 2.5 and 2 (Figure 1b, lanes 4, 6 and 8). Similar results to those of the 1 M NaCl experiment were obtained at 1.4 M (Figure 1c) and 1.72 M NaCl (Figure 1d), with no variations in the electrophoretic bands obtained. The results show that to obtain an isolate solely through acid precipitation from dissolved granules, the NaCl concentration is important. The low phosvitin solubility at 0.5 M NaCl and pH 3, 2.5 and 2 may be due to a "salting in" effect. Therefore, as has been shown in Figure 1, at an acidic pH, increasing the NaCl concentration to 1 M or higher will also lead to maintaining the phosvitin in solution.

The highest phosvitin recovery and the mildest conditions were the parameters chosen to select an optimal pH and NaCl concentration to carry out the purification process. As was mentioned, the results of electrophoresis experiments indicated no detectable differences between 1 M, 1.4 M and 1.72 M NaCl concentrations and between pH values of 3, 2.5 and 2. To verify this finding, and as phosvitin represents about 90% of the egg yolk phosphorous [37], its recovery rate was measured by analysing the phosphorus content in each sample (Figure 2).

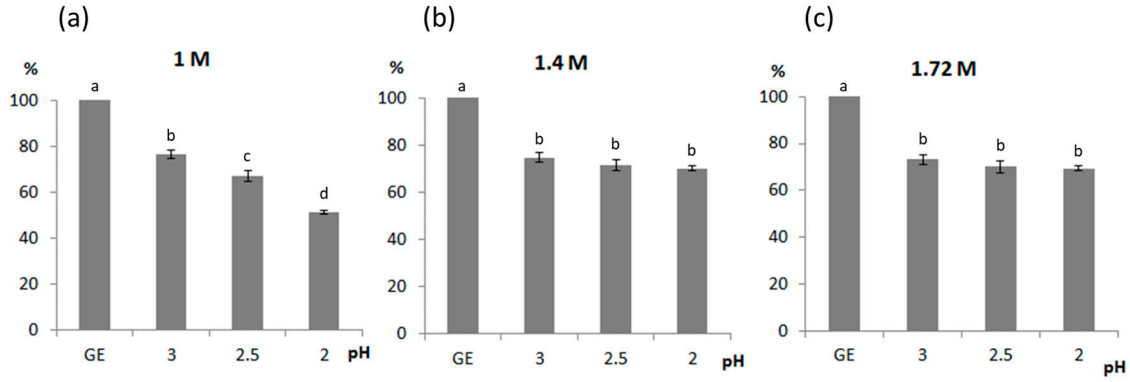

Figure 2. Percentage of phosphorus recovery in the PRF. GE: Granules from egg yolk diluted (2% *w*/*v*). (**a**) 1 M NaCl; (**b**) 1.4 M NaCl; and (**c**) 1.72 M NaCl. The different letters indicate significant differences ($p < 0.05$).

At 1 M NaCl, pH values of 2.5 or 2 lead to a decrease in the amount of phosphorous in the supernatant, with respect to the same samples at 1.4 M and 1.72 M NaCl. This means that a greater amount of NaCl was required at a lower pH to keep phosvitin in solution. According to the phosphorous content in the samples, the parameters for optimal phosvitin separation under the studied conditions correspond to 1 M NaCl and pH 3. Employing these values, a phosvitin recovery rate of 76 ± 3% was measured with an N/P atomic ratio of 3.6 ± 0.2. A low value in the N/P atomic ratio parameter is correlated with low levels of contaminants.

Yong Lee et al. [38] obtained a phosvitin-rich fraction by dissolving the egg yolk granules in a 1.72 M NaCl solution, and adjusting the pH to 4. After centrifuging the mixture, the phosvitin remained in the supernatant, but was contaminated with high density lipoproteins. These authors increased the purity of the phosvitin with a heat treatment at 70 °C for 30 min. However, as can be seen in Figure 2, most of these impurities can be removed from the phosvitin-rich fraction simply by reducing the pH to 3. Furthermore, a salt concentration of 1 M seems to be enough to extract the phosvitin efficiently.

Ko et al. [39] treated the egg yolk granules with ethanol and after that dissolved the phosvitin in a 10% NaCl solution. They tested several pHs in this phosvitin solution and detected a loss of yield with pHs lower than 4. However, according to Figures 1 and 2, this behaviour was not found in this case. This discrepancy with Ko et al. [39] could be

produced by the use of ethanol to obtain the fat-free phosvitin. The ethanol removes most of the HDLs and could also produce conformational variations in the phosvitin; either of these changes could be the cause of the precipitation differences.

3.2. Effect of Desalting pH on the Purity of the Phosvitin Obtained

According to the results obtained in Section 3.1, the best phosvitin separation conditions were to dissolve the granules in a 1 M NaCl solution, adjust the pH to 3 and then centrifuge and discard the sediment, with the supernatant being the PRF. The pH of the PRF obtained was then adjusted before dialysis to 3, 5, 7 and 8.5. When the pH was adjusted to 3 and 5, a milky solution was obtained after dialysis due to protein aggregation. The PRF dialysed at pH 7 and 8.5 remained clear.

In order to determine the composition of the aggregates, the PRF dialysed at pH 3, 5, 7 and 8.5 was centrifuged at 10,000× *g* for 30 min. The sediments obtained at pH 3 and 5 (aggregates) and the supernatants were analysed via SDS-PAGE (Figure 3). Figure 3a shows how in the supernatants, except those obtained at pH 3, lipoproteins remained along with the phosvitin. At pH 3, the solubility of the HDLs and phosvitin decreased and they then precipitated in distilled water together. In addition, aggregates (Figure 3b) show phosvitin with other granular proteins that persist in the supernatant. In order to calculate the amounts of aggregates at pH 3 and 5, the dialysed whole PRF samples were lyophilized. Then, equal volumes of dialysed PRF at the two pH values were centrifuged at 10,000× *g* for 20 min to remove aggregates and the supernatant was lyophilized. Differences in weight corresponded to the weight of aggregates eliminated by the centrifugation process. If dialysis was carried out at pH 3, then aggregates would represent 47 ± 2% of the lyophilized weight. On the other hand, dialysis at pH 5 produced an aggregate formation of 10 ± 1% of the final lyophilized weight.

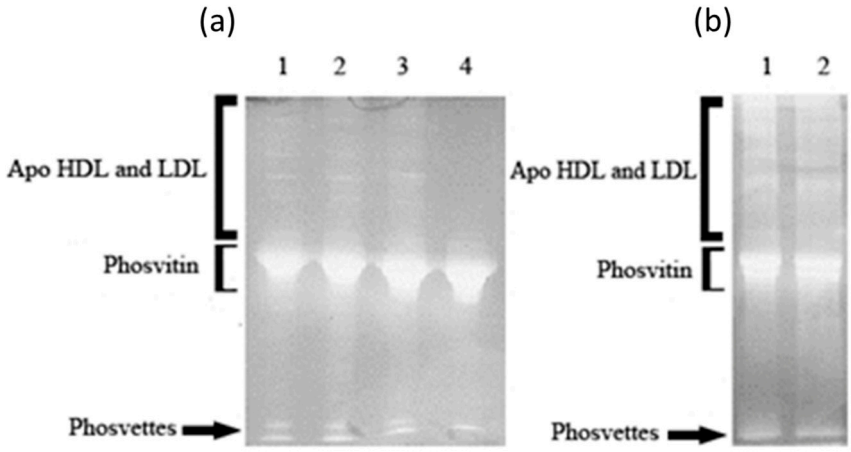

Figure 3. SDS-PAGE of the PRF dialysed at different pHs and of the aggregates formed at pH 3 and 5. (**a**) PRF adjusted to several pHs, dialysed and filtered at 0.45 microns. Lane 1: pH 8.5. Lane 2: pH 7. Lane 3: pH 5. Lane 4: pH 3; (**b**) Lane 1: Aggregates from the PRF adjusted to pH 3 and dialysed. Lane 2: Aggregates from the PRF adjusted to pH 5 and dialysed.

Changes in the secondary structure of phosvitin due to the pH of the media may modify its solubility. It has been reported that the secondary structure at pH 7 is in random coil, whereas if the medium is adjusted to a pH near to a value of 3, this changes and a beta sheet conformation is the main structure [40,41]. A similar change occurs when phosvitin is lyophilized [41]. These changes in the secondary structure of the protein lead to its aggregation [42], thus allowing its precipitation in a desalted medium. Furthermore, the charges on the phosvitin phosphoserines at pH values close to neutral keep the protein soluble. Under these conditions, it can be observed that by adjusting the PRF to pH 7 or 8.5 and then desalting by dialysis, all the protein remains soluble, and the N/P ratio is similar to that of the PRF (3.6 ± 0.2). However, by dialysing at pH 3 and centrifuging, purer

phosvitin can be obtained in the supernatant, with an N/P value of 2.5 ± 0.1 (Table 1), although this involves the loss of 47 ± 2% of the protein content of the PRF.

Table 1. N/P atomic ratio for different phosvitin preparations.

Method	N/P	Phosvitin Recovery (100 g Dry Egg Yolk)
Lei and Wu [21]	2.5 ± 0.3	2.5 g
Wallace and Morgan [19]	3.34	0.6 g
[a] Castellani et al. [20]	2.63 ± 0.07	1.7 g
PRF (our procedure)	3.6 ± 0.2	2.4 g
Pd3.0 (our procedure)	2.5 ± 0.1	1.1 g
UF (our procedure)	2.5 ± 0.1	0.9 g

[a] Value obtained in our laboratory. PRF: phosvitin-rich fraction. $P_{d3.0}$: Phosvitin purified via dialysis at pH 3.0. UF: Phosvitin purified via ultrafiltration.

The bibliography investigation revealed studies on the effect of pH on desalted phosvitin solutions. Ren and Wu. [43] dissolved the granules in a 10% NaCl solution. This granule solution was dialysed and centrifuged, resulting in a supernatant highly contaminated with HDLs, which were precipitated upon adjusting the pH to 5.5. In these conditions, the phosvitin purity increased to 63.7% but the recovery yield also decreased significantly. This finding contrasts with that of Castellani et al. [20]. In this case, they tested several pHs, but no changes in phosvitin solubility were found until a pH of 1.5 was reached. The differences observed between these authors could be due to differences in the extraction method, since Castellani et al. had previously precipitated the phosvitin with $MgSO_4$. However, according to Figure 3 and the low N/P ratio value obtained, the adjustment of the pH to 3 before dialysis is enough to obtain a highly purified phosvitin.

3.3. Purification of Phosvitin from PRF by Ultrafiltration

As an alternative to the purification of the phosvitin from the PRF through pH adjustment and dialysis, the ultrafiltration of the PRF using a polyethersulfone membrane at several pHs was tested. In this case, HDLs have a molecular weight of 400 kDa [8], while that of phosvitin ranges between 160 and 190 kDa [28]. Therefore, a membrane with a pore size of 300 kDa was selected in order to purify the phosvitin in the permeate.

The flux values obtained during the ultrafiltration of the PRF in recycle mode at several pHs are shown in Figure 4. Furthermore, in this figure, phosvitin transmission from the retentate to the permeate at each pH tested is shown too. The lowest flux values appeared at the lowest pH tested and then the flux increased with positive increments in the pH. A plateau region was found from pH 5 to 7, and finally the best flux values were seen at pH 9. A continuous increase in the permeate flux values in a similar phosvitin-NaCl medium was found by Ting et al. [34], but in this case the plateau area was not found, probably because of differences in methodology.

In recent studies about the zeta potential of polyethersulfone membranes in a medium with NaCl, it was observed that at a low pH, the membrane tends to bind cations on its surface [44]. The isoelectric point of the phosvitin was found to be pH 4 [45], so at pH 3 the phosvitin maintains positive charges which could increase its affinity for the surface of the membrane, increasing fouling and the formation of a filter cake. With a rise in pH, there is an increase in the phosvitin charge too. At a neutral pH, the phosvitin is highly charged (−179 mV) and the phenomenon of repulsion between the protein and the membrane prevents fouling and better flux values can be obtained. According to the results shown in Figure 4, this repulsion could increase significantly from pH 7 to pH 9. Persson et al. [46], using BSA and polyethersulfone membranes, found that an increase in the ionic strength in the solution produces an increase in the transmission of the BSA at pH 3 and 7. Therefore, the electrostatic interactions between the protein and the filter cake, as well as those between the protein and the membrane, could markedly change the transmission of the proteins through the membranes.

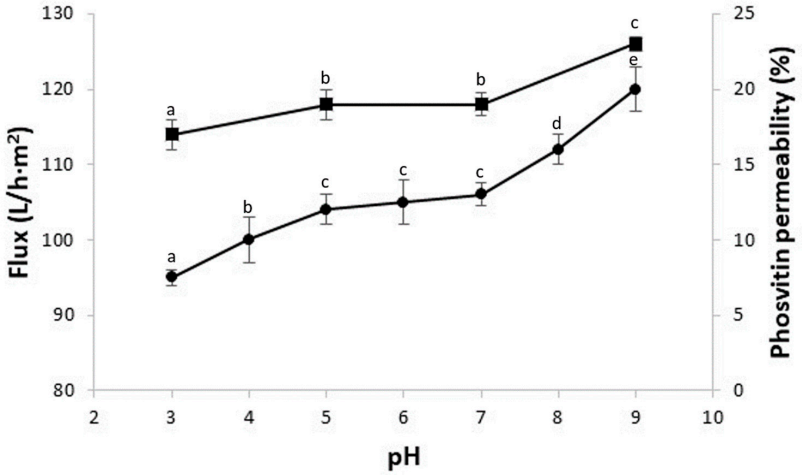

Figure 4. Ultrafiltration carried out in total recycle mode. Values of flux (squares) and phosvitin transmission (circles) at several pHs tested. The different letters indicate significant differences ($p < 0.05$).

The permeability to phosvitin follows a similar trend to the flux, and at low pHs the transmission of phosvitin was lower than at high pHs. This could be partially explained, as in the case of the flux, as being due to the positive charges on the phosvitin increasing its affinity for the membrane, and thus blocking the passage of the protein into the permeate. Since the best flux and phosvitin permeability values were found at high pHs, the experiments carried out in diafiltration mode were performed at pH 9.

In Figure 5, the variation in the flux values in the diafiltration mode as well as the phosvitin concentration in the permeate during the diafiltration process are shown. As can be seen in this figure, the flux values fell sharply from the beginning of ultrafiltration to minute 3, probably due to fouling, and then remained almost constant but with a slight tendency to decrease until the end of the experiment at 30 min. With regard to the amount of phosvitin, the concentration of this protein in the permeate decreased from an initial value of 0.6 mg/mL in the first 30 s to 0.02 mg/mL at the end of the diafiltration. This reduction in the amount of phosvitin recovered with time was expected, since the feed had been continuously refilled with a solution of 1 M NaCl and therefore the concentration of phosvitin in the feed decreased. After 30 min of ultrafiltration, all the permeate was collected, concentrated to $\frac{1}{4}$ of the original volume and desalted by using a PES membrane of 10 kDa. In this case, 35% of the phosvitin contained in the feed was recovered in the permeate.

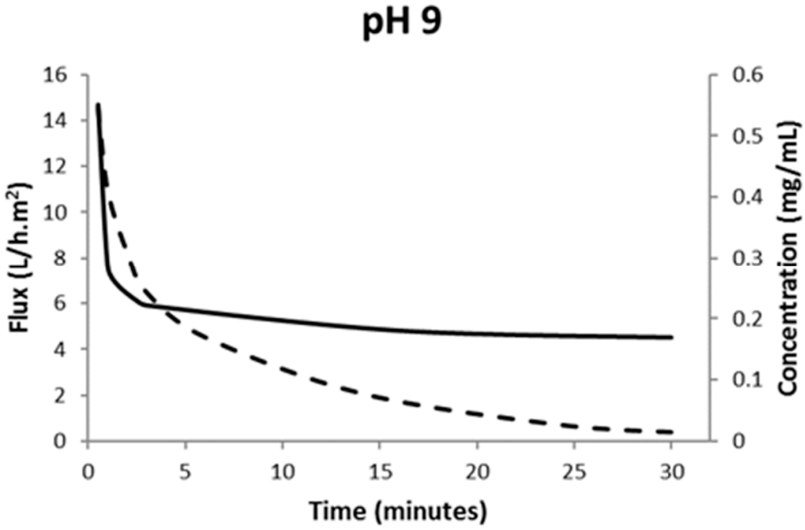

Figure 5. Ultrafiltration carried out in diafiltration mode at pH 9. Flux values (solid line) and concentration of phosvitin in the permeate (broken line) during the 30 min of operation.

3.4. Evaluation of the Purity of the Phosvitin Extracted and Purified

3.4.1. Anion Exchange Chromatography Analysis

The anion exchange chromatography of the PRF and the phosvitin obtained via ultrafiltration and by dialysis at pH 3 is shown in Figure 6. The phosvitin acquired from Sigma-Aldrich was analysed as a source of comparison.

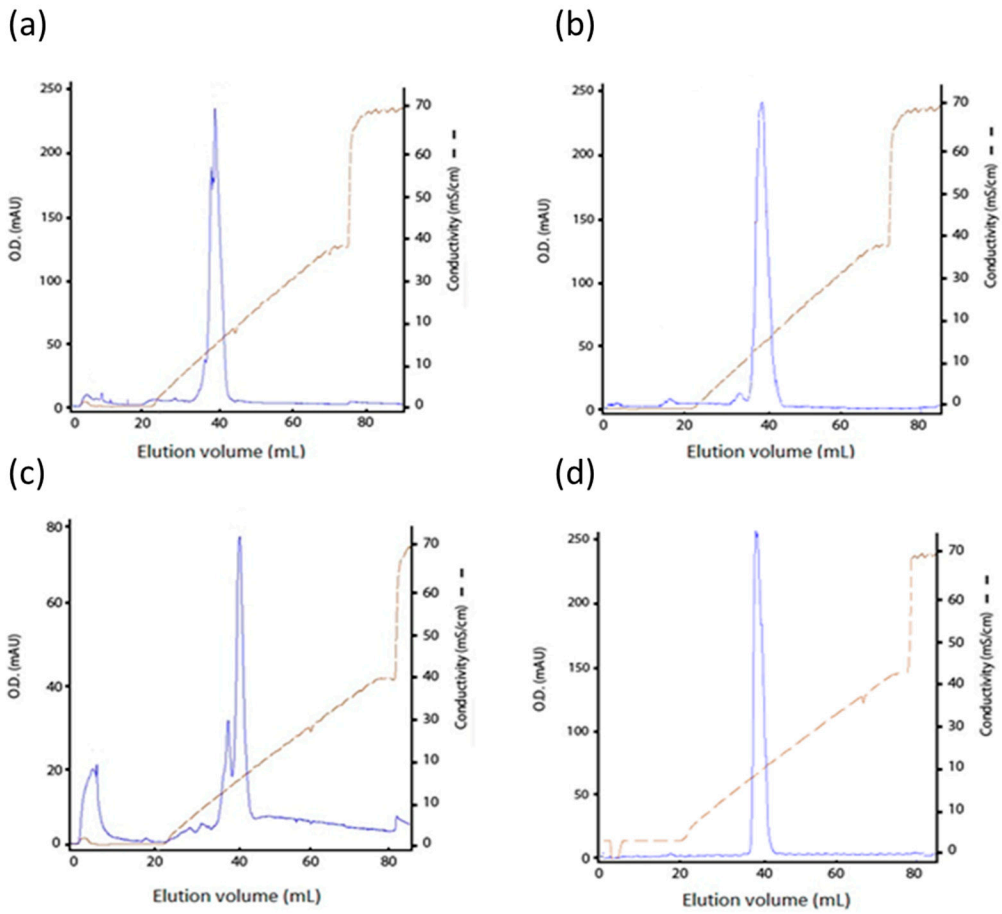

Figure 6. Chromatography of the phosvitin solutions obtained. (**a**) Purification via precipitation; (**b**) purification via ultrafiltration; (**c**) PRF dialysed at pH 7; (**d**) phosvitin standard (Sigma-Aldrich).

There are several studies looking into the evaluation and purification of phosvitin using anion-exchange chromatography. Wallace et al. [6] evaluated four different separation processes and determined the anion exchange chromatogram of each using a DEAE cellulose column. In all the cases tested, the phosvitin was eluted mainly in one peak. They concluded that the isolation of the different types of phosvitin using anion exchange is not possible, since the phosphorylation of the vitellogenin is a heterogenous process which does not give the resultant phosvitin fractions sufficiently clear differences in their binding characteristics.

According to Lei and Wu [21], the separation of the phosvitin from the egg yolk granules is possible using anion exchange chromatography, since the elution profile of the dissolved granules has three main peaks, one of them containing 57.7% of the total phosvitin present in the granular fraction, with a purity of 92.6%. The HDLs were in the other peaks. Ren and Wu [43] reported obtaining highly purified phosvitin from egg yolk granules too. These granules were previously dissolved in a 10% NaCl solution and then dialysed at pH 7.25. In this case, the phosvitin was recovered from one peak with 97.1% purity.

According to these authors, and as shown in Figure 6, only one peak was detected in the phosvitin purified using dialysis at pH 3 (6a) and using ultrafiltration (6b). This peak

also appears in the same position as for the Sigma-Aldrich phosvitin. No other peaks are observed in these cases, which can be taken as an indication of the high grade of purity of the phosvitin obtained. However, in the PRF (pre-treatment fraction), the protein not bound to the column was detected at the beginning of the anion exchange chromatogram, which suggests the presence of impurities in this fraction.

3.4.2. Electrophoresis Analysis

The SDS-PAGE electrophoresis of the PRF and of the purified phosvitin is shown in Figure 7. Some of these samples were analysed at 2 mg/mL to show the appearance of bands which represent impurities more clearly. The HDL, livetin and phosvitin bands were distributed according to several studies found in the bibliography [47–49]. In this figure, at the top of the gel in the lane of the phosvitin acquired from Sigma-Aldrich, two faint bands can be seen, which were identified as β and α HDL impurities. These bands can be more clearly observed in the phosvitin prepared according to Castellani et al., but before their anion exchange purification (lane "Pc") [20]. In the Castellani et al. preparation, the selective precipitation of the phosvitin is carried out using $MgSO_4$, although the use of Mg^{++} is subject to restrictions in the food industry. In the PRF fraction (lane "PRF"), several bands of impurities are shown, but these bands were completely removed via ultrafiltration (lane "UF") or after the dialysis at pH 3 (lane "$P_{d3.0}$"). In the case of these purification methods, even when using a high concentration of the lyophilized sample (2 mg/mL), the impurities cannot be detected.

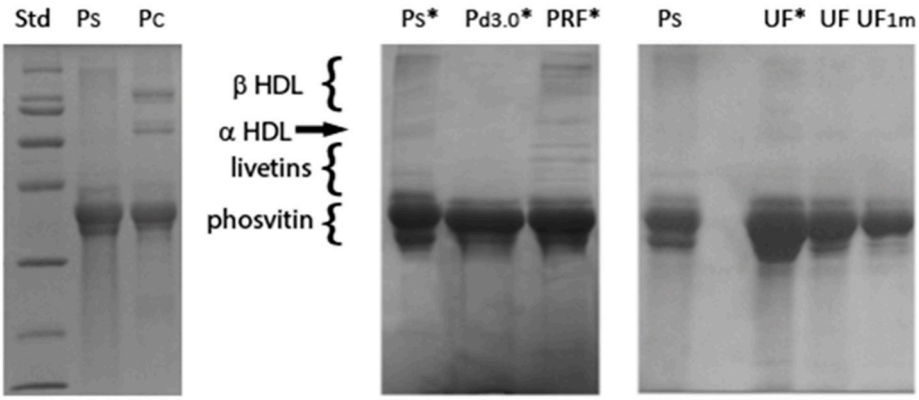

Figure 7. SDS-PAGE profiles of lyophilized samples at 1 mg/mL; *: 2 mg/mL. Std: size standard; Ps: Sigma-Aldrich phosvitin; PC: phosvitin prepared according to Castellani et al. $P_{d3.0}$: phosvitin purified via dialysis at pH 3.0; PRF: phosvitin-rich fraction; UF: phosvitin purified by ultrafiltration; UF_{1m}: permeate obtained after the first minute of ultrafiltration.

3.4.3. N/P Atomic Ratio

The N/P atomic ratio is normally assumed as an acceptable measure of purity [19,34]. The results for these ratios are shown in Table 1, comparing the samples seen in this study with some of those found in the bibliography. Regarding the phosvitin preparations presented in this study, the PRF showed an N/P value of 3.6 ± 0.2, with a recovery yield of 76 ± 3% of the egg yolk granules' phosvitin. Taking into account that the granules contain 17% phosvitin, a recovery yield of 2.4/100 g dry egg yolk was obtained. The results obtained via electrophoresis showed that the impurities were removed by dialysing the PRF at pH 3. In this case, a phosvitin solution with an N/P value of 2.5 ± 0.1 was obtained in the supernatant, with a phosvitin recovery yield of 1.1/100 g dry egg yolk. Although with this method, the recovery yield value is lower than with others found in the bibliography, this is the simplest of the methods and so the phosvitin obtained can be considered to be of high purity.

Very similar values were obtained when the PRF was treated via ultrafiltration, obtaining a permeate with an N/P value of 2.5 ± 0.1 and with a phosvitin recovery yield

of 0.9/100 g dry egg yolk. In the case of ultrafiltration, it is significant that the purified phosvitin was present in the permeate, and this permeate was concentrated and desalted by using a 10 kDa ultrafiltration membrane. Furthermore, ultrafiltration technology is more scalable than chromatography techniques.

In this sense, the most purified phosvitin obtained in this research, by adjusting the pH during the dialysis or alternatively by using ultrafiltration techniques, gave an N/P ratio value similar to the value for the phosvitin purified by Lei and Wu [21] and Castellani et al. [20] but with a lower phosvitin recovery rate (Table 1). In this regard, it must be borne in mind that the purification methods described by these researchers involve the use of chromatographic techniques and subsequent dialysis steps, and such procedures are operated discontinuously and are less scalable than ultrafiltration techniques. Furthermore, Wallace et al. [19] calculated an N/P atomic ratio of 3.34 for their phosvitin preparation, with a recovery of phosvitin of 0.6/100 g of dry egg yolk. However, this separation method involved the use of (NH4)2SO4, which was also removed by dialysis.

4. Conclusions

In this study, egg yolk granules were dissolved in 1 M NaCl and then the phosvitin was extracted using acidic treatment. The phosvitin obtained was further purified using dialysis at several different pHs. At pH 3, under dialysis conditions, the phosvitin obtained was the purest due to the precipitation of the lipoproteins, but a loss of phosvitin yield was observed. At pH 7 and 8.5, purification was not possible and the desalination of the PRF was hampered, owing to the negative charges of the phosvitin.

The phosvitin was also purified using ultrafiltration techniques. In this case, the differences in the size of the granular proteins and lipoproteins allowed the selective separation of the phosvitin in the permeate, and as in the case of the purification using dialysis at pH 3, a high purity phosvitin was obtained. In the case of purification using ultrafiltration, the yield of phosvitin was lower, but the phosvitin solution was desalinated and the process could be redesigned to allow for continuous flow operation, with this being more easily scalable than other techniques.

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