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Journal of Chromatography B, 791 (2003) 273–284
JOURNAL OF CHROMATOGRAPHY B
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Egg yolk phosvitin: preparation of metal-free purified protein by fast protein liquid chromatography using aqueous solvents

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Received 31 January 2003; accepted 11 March 2003

Abstract

Two chromatographic methods for hen egg yolk phosvitin purification avoiding organic solvents were evaluated. Hydrophobic interaction and ion-exchange chromatographies were applied to isolated phosvitin. Hydrophobic interaction chromatography has better capacity than ion-exchange chromatography to fractionate phosvitin in their different polypeptides, but its protein yield was lower (0.7 vs. 1.7% of egg yolk dry matter). Finally, ion-exchange chromatography was selected and allowed to fractionate phosvitin polypeptides, including the recovering of phosphoproteins with high electrophoretic mobility: phosvettes. Highly purified (>98%) and free metal protein was obtained in reduced time. Phosvitin polypeptide heterogeneity was evidenced.

Keywords: Purification; Egg yolk proteins; Phosphoprotein; Phosvitin

1. Introduction

Phosvitin is the principal phosphoprotein in egg yolk. It represents 2% of egg yolk dry matter [1–3]. Almost 50% of its amino acids are serine, out of which 90% are phosphorylated [4]. Only the C-terminal region, of approximately 15 amino acids, has hydrophobic characteristics [5].

Ninety-five percent of iron present in egg is in the yolk and almost all is bound to phosvitin [6]. This protein had shown exceptional polyvalent cation binding properties, specially for the iron [7,8]. In relation to this property, its phospholipid antioxidant activity was demonstrated in both, egg yolk emulsion and meat model systems [9,10].

The phosvitin purification methods described in the literature are generally composed by two phases, a first one of isolation and a further chromatographic fractionation. According to the author objectives, the latest phase help to accomplish the protein purification and/or to separate different phosvitin polypeptides [1–3,11,12]. These methods are laborious and time consuming, or they usually make use of non-aqueous or non-food-grade solvents to separate proteins from lipids. These solvents could produce loss of some protein fractions, possibly modifications on phosvitin structure, and the limitation of application possibilities. Depending on the isolation method

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1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(03)00230-7

used, egg yolk phosvitin contains between three and six atoms of iron per molecule [1,6]. The study of iron binding activity of purified phosvitin implies to obtain the protein metal free. This was usually achieved by the use of separation methods after isolation procedure [13,14].

Using chloroform–methanol for the isolation, and further size-exclusion and ion-exchange chromatographies, phosvitins having different serine content were fractionated [15]. The authors also demonstrated that phosvitin was composed of several main sub-components with molecular masses from 27 000 to 38 000. The presence of α- and β-phosvitin polypeptides was evidenced by native polyacrylamide gel electrophoresis [16]. The main components have molecular masses of 45 000 for β-phosvitin, and 37 500, 42 000, and 45 000 for α phosvitin.

Wallace and Morgan developed a method of isolation employing aqueous solutions [12]. For further phosvitin purification, and even though phosvitin has a little proportion of non-polar amino acids [5], these authors had successfully used hydrophobic interaction chromatography [17]. They described the presence of α- and β-phosvitin polypeptides, and of three additional phosphoproteins with high electrophoretic mobility: phosvettes. The phosvettes were not present in the more often used phosvitin preparations [1,11,18]. On the contrary, the protein yield for their isolated method was only 0.96% of dry matter egg yolk, that is considerably lesser than the total phosvitin present in the egg yolk.

Although there are some isolation and fractionation methods described for the phosvitin, yield, purity and polypeptide composition of final product are variable. So, methods to purify phosvitin could be always improved, especially if one desires to obtain the protein in specific conditions for further investigations. We were interested in the development of a relatively fast purification method avoiding the use of organic solvents, with good protein yield. At the same time, in view of our future studies, another objective was to obtain the protein free of polyvalent metals.

The new purification methods presented here are composed by an isolation phase, based on the insolubility of Mg^{2+}/phosvitin salt, and further chromatographic fractionation by FPLC. Two chromatographic methods, using always aqueous and food grade solutions, were tested: hydrophobic interaction and ion-exchange chromatographies.

2. Experimental

2.1. 1 Materials

Isabrown eggs were obtained from local wholesale distributor, they were 3 or 4 days old. All chemicals (analytical grade) and commercial phosvitin, were purchased from Sigma (Saint Quentin-Fallavier, France).

2.2. Phosvitin isolation

Fresh hen eggs were manually broken. The yolks were carefully freed of adhering white and chalazae by rolling on a filter paper (Whatman). The vitellin membrane was punctured with a lancet and the content was collected in a beaker cooled in iced water. The temperature was then maintained at 4 °C throughout the process. Granules were extracted from yolk according to the method of McBee and Cotterill [19]. Yolk was diluted with an equal mass of a 0.17 *M* NaCl solution and mixed with a magnetic stirrer. After 1 h, the solution was centrifuged at 10 000 *g* for 45 min in a Jouan centrifuge (model GR 2022, St. Herblain, France) and the pellet (granules) was collected and dissolved in a 1.74 *M* NaCl solution (10% p/v). The mixture was stirred to complete dissolution keeping the pH adjusted to 7.25. The solution was then dialysed against several changes of distilled water for 24 h and centrifuged at 10 000 *g* for 30 min. The supernatant was diluted with 0.9 *M* $MgSO_4$ solution to obtain a 0.2 *M* Mg^{2+} final concentration, where phosvitin precipitates. After centrifugation (10 000 *g* for 30 min), a precipitate was collected at the bottom of the tubes and it was freeze-dried (isolated phosvitin, Pvt_i). The corresponding supernatant was taken to pH 1.5 by gentle addition of 12 N HCl solution. After 60 min equilibration, suspension was centrifuged (10 000 *g* for 30 min), the sediment was recuperated (phosvitin at pH 1.5, $Pvt_{1.5}$) and the supernatant was discarded.

2.3. Chromatographic fractionation

All chromatographies were carried out, at least in triplicate, on a FPLC system, equipped with an automatic injector, dual monitors at 220 and 280 nm containing 0.2-ml flow cells, and a fraction collector. The equipments and gel media were from Amersham–Pharmacia Biotech (Uppsala Sweden). All samples were initially suspended 1 h in the corresponding solution, centrifuged (2000 *g* for 30 min at 15 °C) and filtered through 0.22-μm pore filters. They were then injected onto the column through 0.45-μm filters. The absorbancy at both wave lengths (220 and 280 nm) was measured because of the weak Pvt_i absorption at 280 nm.

2.3.1. Hydrophobic interaction chromatography

A Resource PHE column (30×6.4-mm I.D.) and a Phenyl Sepharose High Performance column (110×26-mm I.D.) were used. For the first column procedure, 10 mg/ml of lyophilised isolated phosvitin (Pvt_i) were suspended in a sodium phosphate buffer (0.05 *M*, pH 7.0) containing 3 *M* $(NH_4)_2SO_4$. The second column was charged with a solution of isolated phosvitin (5 mg/ml). Because of solubility problems, in this case, the Pvt_i was suspended in sodium phosphate buffer (0.05 *M*, pH 7.0) containing 2 *M* of $(NH_4)_2SO_4$. Both columns were equilibrated with phosphate buffer (0.05 *M*, pH 7.0) containing 3 *M* $(NH_4)_2SO_4$ and the elution was carried out by decreasing $(NH_4)_2SO_4$ concentration as indicated in the corresponding figures of Section 3.

2.3.2. Ion-exchange chromatography

A Source 15Q column (100×4.6-mm I.D.) was used for anion exchange chromatography. Pvt_i was dissolved (5 mg/ml) at pH 5.0 (0.05 *M*, NaAc/AcH) in the presence of 10 m*M* Na_2EDTA or at pH 7.5 (100 m*M*, Tris–HCl) in the presence of 0.3 *M* NaCl. These solutions were injected into the column that was previously equilibrated with the same buffer system without Na_2EDTA nor NaCl. The elution was performed with an increasing gradient of NaCl in the corresponding buffer as indicated in the figures (Section 3).

2.4. Nitrogen and protein determinations

The nitrogen content of phosvitin samples was determined by an automated total protein nitrogen method [20]. For solubility determination, a Pvt_i solution (5 mg/ml) in 0.05 *M* NaCl at pH 7.0 was prepared. Then, the solution was fractionated into five samples, and the pH of each sample was brought to the desired value (between 1.5 and 7.0) with 2 *M* HCl. After 90 min, fractions were centrifuged (1800 *g* for 30 min at 15 °C) in a Beckman centrifuge (model J-6B). Five ml of each supernatant were analysed by the total protein nitrogen method. The conversion factor employed was 6.25 mg protein/mg N. All determinations were made in triplicate.

2.5. Phosphorus determination

The phosphorus content of different samples was determined by triplicate, using the colorimetric method described by Bartlett [21] using hydrazine sulphate and sodium molybdate.

2.6. Native polyacrylamide gel electrophoresis

Protein samples and eluted fractions (after desalting by dialysis against distilled-deionized water) were diluted (4:5) with the sample buffer (0.25 *M* Tris–HCl, pH 6.8, 0.04% bromophenol blue and 30% glycerol). When protein was lyophilised, solutions of 2 mg/ml of protein were directly made.

Stacking gel was constituted by 3.5% of acrylamide in 0.124 *M* Tris–HCl buffer at pH 6.8, and the running gel by 6.5% of acrylamide in 0.05 *M* Tris–HCl/0.36 *M* glycine at pH 8.8. Electrophoresis buffer was constituted by 0.05 *M* Tris–HCl, 0.42 *M* glycine at pH 8.8. About 30 μg of total protein were loaded onto the gels for the two stained methods used: Coomassie Brilliant Blue with aluminium mordant [22] or Stains all [23]. When gel was stained with Silver method [24], about 1 μg of protein was loaded. Electrophoretic migration was performed at 30 mA per gel for 1.5 h in an Amersham Biosciences system (Hoefer, Mighty Small II SE250/SE260, San Francisco, CA, USA). Gels were scanned on an imagining densitometer Biorad GS710 and both R_f and percentage ratio of each band were determined

with Quantity One 4.1 software (Biorad, Ivry-sur-Seine, France).

2.7. Amino acid analyses

Analyses were performed following the procedure of Spackman et al. [25] using an Amersham–Pharmacia Biotech analyzer Alpha Plus (Uppsala, Sweden). Two replicates were made.

2.8. Fe and Mg determinations

The Fe and Mg contents were measured by atomic absorption spectrometry with a spectrometer SpectrAA 55B (Varian, Mulgrave Victoria, Australia). For the different metal determinations, the manufacturer indications were followed, and samples were analysed in triplicate. The standard and sample solutions for Mg determination were made in 0.002 *M* HCl with 0.9 mg/l of lanthanum, while those for Fe determination were made in 0.002 *M* HCl only.

3. Results

3.1. Phosvitin isolation

The phosvitin isolation procedure from hen egg yolk is an improvement of a procedure developed in this laboratory, where phosvitin was precipitated in the presence of 0.2 *M* $MgSO_4$ [26]. All the reagents were aqueous salt solutions and the techniques employed were easy to use (centrifugation and dialysis). The yield was 3.3±0.3 g of lyophilised phosvitin/100 g of dried egg yolk, and the nitrogen to phosphorus atomic ratio (N/P), that could be considered as a criterion for phosvitin purity [2], was 3.5±0.2.

Electrophoretic analysis still indicated the presence of phosvitin in the supernatant from the pellet of isolated phosvitin during the isolation method (data not shown). So, with the aim to recuperate more protein, Pvt_i solubility (Table 1) and electrophoretic profile (Fig. 1) were determined between 1.5 and 7.0 pH values. From 7.0 to 2.5, little reduction in solubility (98–83.5%) was observed (Table 1). For pH 5.0 and below, an important diminution of HDL was observed (Fig. 1). At pH 1.5, the solubility decreases sharply to 10%. In view of the results, the improvement of isolation process regarding phosvitin yield was attempted by leading the pH value of the residual solution to 1.5 and taking the insoluble protein. In this way, and after lyophilization, we obtained $Pvt_{1.5}$ which contained 1.5 times more protein than Pvt_i.

Table 1
Solubility of isolated phosvitin at different pH values

pH	% of solubility[a] ± SD[b]
1.5	9.3±4.2
2.5	83.5±1.7
3.6	84.5±1.5
5.0	90.6±2.5
7.0	97.7±1.4

[a] Expressed in percent of N from lyophilised isolated protein.
[b] Standard deviation.

Progression of phosvitin isolation during the procedure was followed by native electrophoresis (Fig. 2). The yolk protein bands (Y line) were identified as

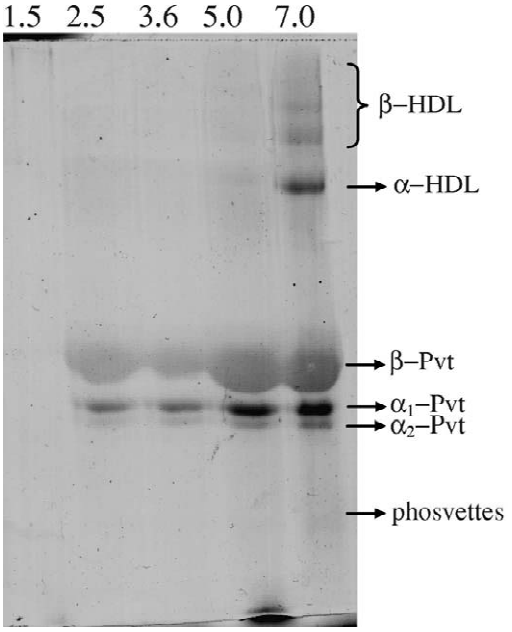

Fig. 1. Native polyacrylamide gel electrophoresis (6.5% acrylamide) of the soluble protein at different pH values (indicated on the top). Stain: Coomassie Blue with aluminium mordant.

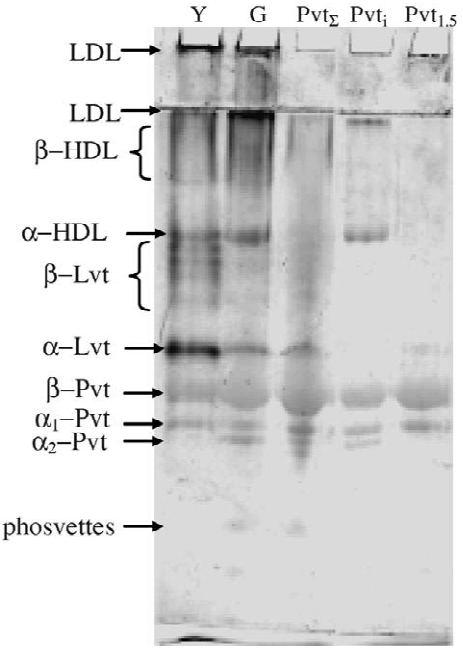

Fig. 2. Native polyacrylamide gel electrophoresis (6.5% acrylamide) of different isolation procedure fractions. Y, egg yolk; G, granules; Pvt$_\Sigma$, sigma phosvitin; Pvt$_i$, isolated phosvitin; Pvt$_{1.5}$, phosvitin precipitated at pH 1.5. Stain: Coomassie Blue with aluminium mordant.

in a previous work [27] according to their R_f values, and were formed by LDL, HDL proteins (α- and β-polypeptides), livetins (α- and β-polypeptides) and phosvitin (α_1-, α_2-, and β-polypeptides). The granules (G line) were mainly constituted by HDL and phosvitin proteins (α_1, α_2, β, and phosvettes), LDL polypeptides were also present (material on the top of the gel). The phosvitin commercialised by Sigma (Pvt$_\Sigma$ line) is composed by all phosvitin polypeptides, some HDL polypeptides, and α-livetin (α-Lvt). The two obtained phosvitins are observed in the two last lines (Pvt$_i$ and Pvt$_{1.5}$). They were similar concerning α_1- and β-phosvitin polypeptides, but Pvt$_{1.5}$ did not contain α_2-polypeptides. Moreover, Pvt$_i$ presented HDL contamination, while Pvt$_{1.5}$ was mainly contaminated by α-livetin and also some protein aggregates visualised on the top of the gel. Contrarily to Pvt$_i$, Pvt$_{1.5}$ presented some difficulties for solubilization. In view of our future studies, only Pvt$_i$ was further purified. The extreme conditions applied to obtain Pvt$_{1.5}$ could lead to undesired effects on the protein.

3.2. Chromatographic fractionation of Pvt$_i$

3.2.1. Hydrophobic interaction chromatography

Two hydrophobic interaction chromatography (HIC) columns were tested applying different $(NH_4)_2SO_4$ elution gradients (Fig. 3): Resource PHE

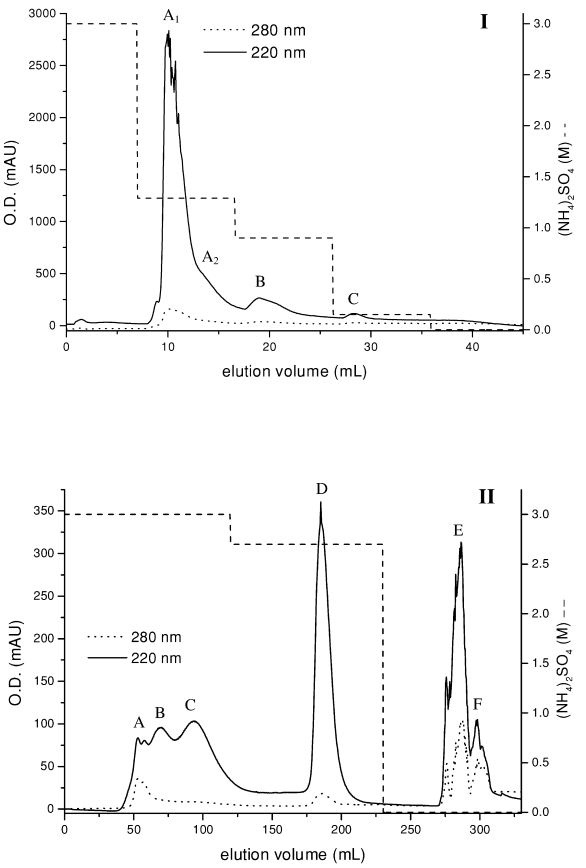

Fig. 3. Hydrophobic interaction chromatographies of Pvt$_i$ at pH 7.0 (0.05 *M* sodium phosphate buffer). (I) Resource PHE column; 1 ml of 10 mg/ml phosvitin solution in 3 *M* $(NH_4)_2SO_4$ was injected and eluted with a decreasing gradient of $(NH_4)_2SO_4$. Flow-rate was 1 ml/min, and 1.5-ml fractions were collected. (II) Phenyl Sepharose column; 8 ml of 5 mg/ml phosvitin solution in 2 *M* $(NH_4)_2SO_4$ were injected and eluted with a decreasing gradient of $(NH_4)_2SO_4$. Flow rate was 4 ml/min, and 15 ml fractions were collected. The peaks are indicated by letters as described in the text.

and Phenyl Sepharose. The chromatography profile obtained with Resource PHE column (Fig. 3I) shows a major peak at 1.2 *M* $(NH_4)_2SO_4$ (94% of 220 nm absorbancy and 80% of 280 nm absorbancy). We noticed a shoulder at the end of this peak (A_2). Peaks B and C had remarkably less intensity (at 280 nm, 13 and 4%, respectively).

When Pvt_i was fractionated by Phenyl Sepharose, the profile obtained was more complex (Fig. 3II). At 220 nm, it exhibits three non-resolved peaks eluted at 3 *M* $(NH_4)_2SO_4$, and only one of them (peak A) shows a considerable absorbancy at 280 nm. The main peak (D in the figure) was eluted at 2.7 *M* $(NH_4)_2SO_4$. Finally, material in peaks E and F was eluted with only phosphate buffer (0.05 *M*, pH 7.0), they also presented a relatively high absorbancy at 280 nm.

Phosvitin solution before chromatography (Pvt_i) and the corresponding eluted fractions were analysed by native electrophoresis (Fig. 4). For Pvt_i the profile was similar to that presented in Fig. 2, with the exception of HDL proteins which were present in less proportion. This must be the consequence of the high salt concentration in the sample preparation (2–3 *M* $(NH_4)_2SO_4$) that causes loss of protein solubility. The fractions eluted from chromatographies of Fig. 3I and II are shown in Fig. 4I and II, respectively.

Concerning the Ressource PHE column, line A_1 (Fig. 4I) shows the presence of β-phosvitin and phosvettes in peak A_1 of Fig. 3I, while the last part of this peak (line A_2) is composed by β- and α_1-phosvitin. The polypeptides of peak B (line B in Fig. 4I) are β- and α_2-phosvitin in similar proportions. Finally, there was no protein material detected by native PAGE in peak C of Fig. 3I, even when silver staining was used (data not shown).

Concerning the Phenyl Sepharose column (Fig. 3II), we have observed that the three peaks eluting at 3.0 *M* $(NH_4)_2SO_4$ (peaks A–C) have different composition (Fig. 4II). The first peak (A) was mainly composed of β-phosvitin and presented some protein material in the HDL region; α_1-phosvitin and phosvettes were present as fading bands as well. The second peak (B) exhibited all phosvitin polypeptides, and peak C was formed by β- and α_1-phosvitin. The

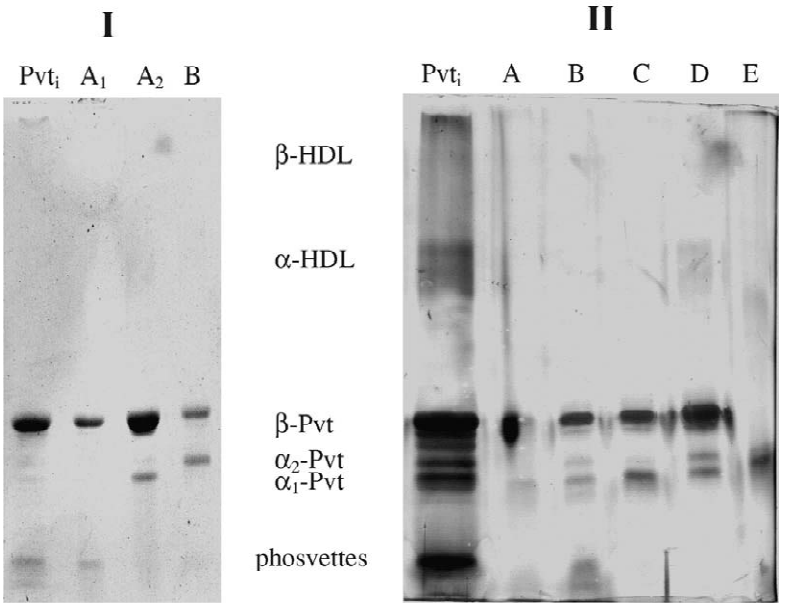

Fig. 4. Native polyacrylamide gel electrophoresis (6.5% acrylamide). (I) Samples from HIC (Resource PHE column). Pvt_i, isolated phosvitin (solution before chromatography); A_1–B, eluted samples as indicated in the corresponding chromatogram (Fig. 3I). Coomassie Blue with aluminium mordant stained gels. (II) Samples from HIC (Phenyl Sepharose column). Pvt_i, isolated phosvitin (solution before chromatography); A–E, eluted samples as indicated in the the corresponding chromatogram (Fig. 3II). Silver stained gels.

line D, corresponding to the main peak in the chromatography, exhibited the presence of β-, α_1- and α_2-phosvitin, and there is also a little α-HDL contamination. In peak E it is possible to separate α_2 and traces of phosvette polypeptides. The last peak (F) did not exhibit protein material by electrophoretic analysis (data not shown).

We have tried to scale up the volume processed with a semi-preparative column (Phenyl Sepharose), but problems of sample filtration before chromatography appeared, even when reducing the $(NH_4)_2SO_4$ sample concentration to 2 *M*. Additionally, when lowering the initial salt concentration of the sample to 1.5 *M* $(NH_4)_2SO_4$, proteins were not able to interact with the chromatographic media, even using 3 *M* $(NH_4)_2SO_4$ in the buffer for column equilibration.

3.2.2. Ionic interaction chromatography

The Pvt_i was also fractionated by anion-exchange chromatography. Two strategies at different pH values with the same media (Source 15Q) were tested (Fig. 5). At pH 7.5, and with the addition of 0.3 *M* NaCl in sample buffer to reduce HDL/Pvt_i and polyvalent metal–protein interactions, the elution profile showed two main peaks (Fig. 5I): a first one (A) corresponding to excluded fraction and another larger one (B) eluting at 0.31 *M* NaCl.

Another attempt to improve the purity and the fractionation of the isolated phosvitin was made at pH 5.0, where the solubility conditions of Pvt_i and HDL are different (see Table 1 and Fig. 1). EDTA (0.01 *M*) was added only in the sample buffer to weak metallic bridges between Pvt_i and HDL proteins. The corresponding chromatographic profile is shown in Fig. 5II. It presents four fractions, designated A–D in order of their elution from the column. In the first peak (A), optical densities at 220 and 280 nm were very similar, in opposition to the other peaks. Moreover, protein material was not detected by native PAGE (even using silver staining). Otherwise, we measured relatively high concentration of metals (16.7±1.5 mg/l of Fe and 270±7 mg/l of Mg), which suggests the presence of metal–EDTA complex compounds. The elution of peak B was at low salt concentration (0.15 *M* NaCl), peak C was eluted at 0.39 *M*, and peak D between 0.42 and 0.55 *M*. The phosvitin material distribution was 13, 6 and

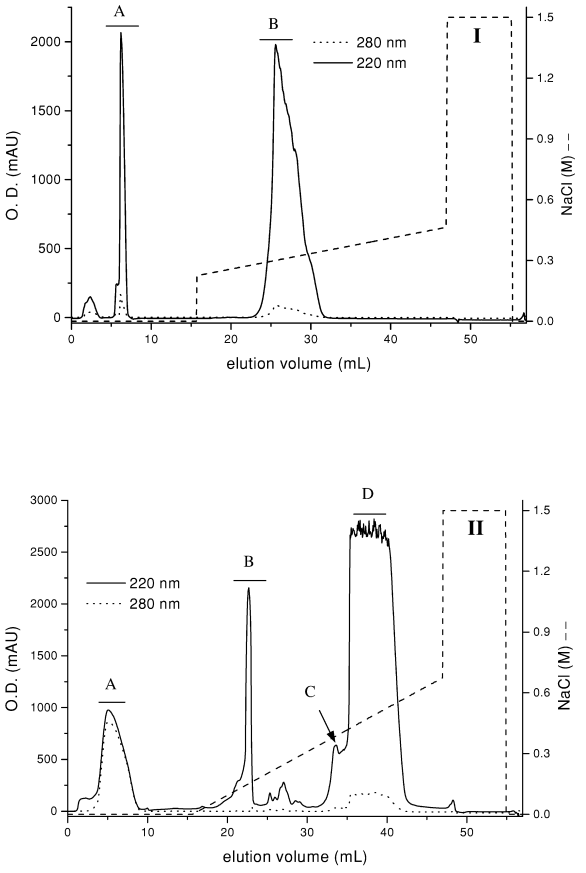

Fig. 5. Anion-exchange chromatography of Pvt_i (Source 15Q column). (I) pH 7.5, 2 ml of 5 mg/ml Pvt_i solution in 0.05 *M* Tris buffer containing 0.3 *M* NaCl were injected. (II) pH 5.0, 5 ml of 5 mg/ml Pvt_i solution in 0.05 *M* AcH/NaAc buffer containing 0.01 *M* Na_2EDTA were injected. Each elution was done with an increasing concentration of NaCl in the corresponding buffer without NaCl nor Na_2EDTA. In both chromatographies the flow-rate was 1.3 ml/min, and fractions of 2 ml were collected.

80% in peaks B, C and D, respectively, based on an integration of the areas under the 220-nm trace.

The native electrophoresis of the fractions eluted by chromatography (Fig. 5) are shown in Fig. 6. Fig. 6I is related to the chromatography in Fig. 5I (pH 7.5). The phosvitin solution before chromatography (line Pvt_i), is similar to that presented in Fig. 2, and presents 29% of HDL contamination. Phosvitin solution was fractionated into two peaks. Peak A was constituted by β-phosvitin, α_1-phosvitin and a small proportion of both α_2-phosvitin and phosvettes. The first part of the peak B (B_1 and B_2 lines) was

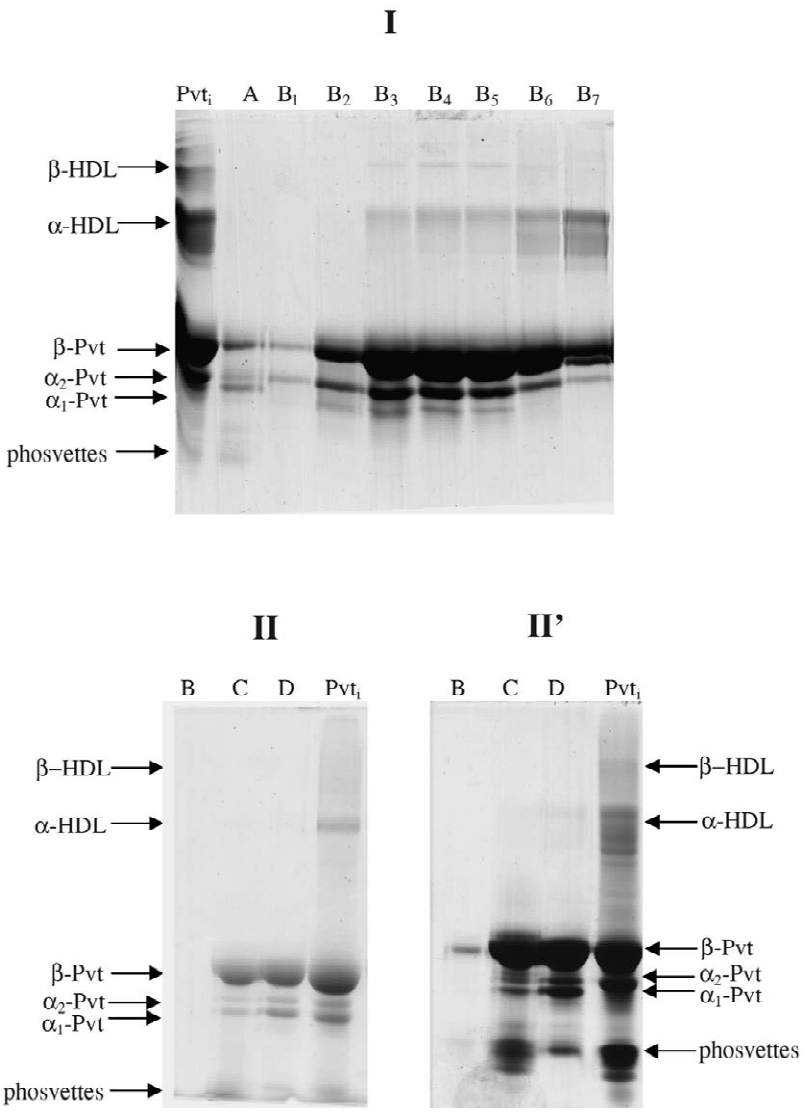

Fig. 6. Native polyacrylamide gel electrophoresis (6.5% acrylamide gel). (I) Stains all stained gels from IEC at pH 7.5. Pvt$_i$, isolated phosvitin (solution before chromatography); A–B$_7$, eluted samples like indicated in the corresponding chromatogram (Fig. 5I). (II) Coomassie Blue with aluminium mordant stained gels; and (II′) Stains all stained gels, from IEC at pH 5.0. Pvt$_i$, isolated phosvitin (solution before chromatography); B–D, eluted samples like indicated in the corresponding chromatogram (Fig. 5II).

constituted by β-phosvitin and α$_2$-phosvitin. B$_3$, B$_4$ and B$_5$ fractions were all composed of β-, α$_1$-, and α$_2$-phosvitin polypeptides with a minor HDL contamination (until 3%). The last part of peak B (fractions B$_6$ and B$_7$) presented β- and α$_2$-phosvitin polypeptides, and was more contaminated with HDL proteins (until 27% based on the absorbancy of electrophoretic profiles).

Fig. 6II and II′ show the corresponding electrophoresis of fractions issued from IEC at pH 5.0 (Fig. 5II). Two similar gels were performed, one was stained with Coomassie Blue in the presence of aluminium mordant (Fig. 6II) and the other one with Stains all (Fig. 6II′), which is a specific coloration for acidic proteins [12]. In this way, it is possible to examine all kind of contamination. Phosvitin before

chromatography (line Pvt_i) had an electrophoretic profile rather similar to that one already described in Fig. 2. Nonetheless, with Stains all (Fig. 6II′), phosvette polypeptides are better visualised. Contamination was 11 and 3% for α- and β-HDL, respectively. Protein eluted from chromatography (lines B–D) showed different proportions of phosvitin polypeptides and the absence of HDL. This was observed even when they were stained with Stains all, which is a more sensitive dyer for this type of proteins. Peak B presents β-phosvitin polypeptides (99%), peak C is enriched in β-phosvitin and phosvettes, and peak D is composed mostly of α- and β-phosvitin (Table 2). This later peak showed the same composition for all their eluted fractions. In accordance with Wallace and Morgan [12], Stains all method was highly sensitive for the smaller phosvitin components (phosvettes), but in addition the same is truth for all type of phosvitin.

In view of chromatographic and electrophoretic results, the IEC procedure at pH 7.5 was discarded because of HDL contamination. Between the other chromatographic procedures, which presented a high degree of purity (>98%), the yield of IEC at pH 5.0 was more than 2-fold with respect to those of HIC. Like was mentioned previously, the major problems with HIC were sample solubility and filtration. In 3 *M* $(NH_4)_2SO_4$, and even in 2 *M* $(NH_4)_2SO_4$, the solubility was poor and filter occlusion occurred. Consequently, the eluted protein from IEC at pH 5.0 was the best product and further analyses were made on this fraction. Even if the composition of the different chromatographic fractions were not exactly the same, our objective was to have a purified phosvitin with a good yield more than to obtain its different polypeptides separately. So, fractions from peaks C and D (Fig. 5 II) were mixed, dialysed, lyophilised and stocked (purified phosvitine, Pvt_p). The yield was 1.7±0.2 g of lyophilised Pvt_p/100 g of dried egg yolk.

Table 3
Amino acid composition (mol%) of purified phosvitin (Pvtp) and of phosvitin sequences from literature

Amino acid	Protein		
	Pvtp	Pvt (seq. I)[a]	Pvt (seq. II)[b]
Asx	7.0	6.4	6.0
Glx	7.2	7.6	5.0
Ser	47.1	48.8	57.0
Gly	3.9	3.2	2.3
His	5.5	4.0	6.0
Arg	5.5	6.3	5.1
Thr	2.3	4.0	1.8
Ala	4.1	3.2	3.2
Pro	1.8	1.6	1.4
Tyr	0.6	1.6	0.5
Val	1.9	1.6	1.4
Met	0.6	0.4	0.5
Cys	0.4	0	0
Ile	1.1	0.4	0.9
Leu	1.6	0.8	1.4
Phe	1.0	2.0	0.5
Lys	8.7	7.1	6.9
Trp	0	1.2	0.5

[a] Phosvitin sequence [33].
[b] Phosvitin sequence [5].

3.3. Chemical characterisation of Pvt_p

The amino acid composition of Pvt_p is shown in Table 3, together with the amino acid composition of two hen phosvitin polypeptides sequenced from two vitellogenin genes. The more abundant amino acid was Ser (≅50% mol/mol), and then, in a lower proportion, Lys, Arg and His. Another characteristic is the negligible quantities of Tyr, Cys and Trp.

Table 2
Phosvitin polypeptide proportions from different samples of IEC process at pH 5 (percentages are based on the peak areas of optical density profiles from Fig. 6II′)

Sample	α-Phosvitin (%)		β-Phosvitin (%)	Phosvettes (%)	Contaminants (%)
	α_1	α_2			
Peak B	–	–	99	1	–
Peak C	6	7	59	28	–
Peak D	21	10	61	6	2
Pvt_i	23[a]		36	27	14

[a] α_1 and α_2 together.

These features are in accordance with the two described phosvitin sequences [5,33] and with amino acid composition of hen phosvitin shown by other authors [2,4]. This singular amino acid composition also agreed with the low absorptivity of the phosvitin at 280 nm.

Metallic composition of phosvitin was determined before and after the IEC purification. Pvt_i contents were 1.2 atoms of Fe and 37 atoms of Mg per molecule of protein, while Pvt_p contains only 0.03 and 0.16 atoms of Fe and Mg per molecule of protein, respectively. These results are in accordance with the high metal quantity eluted in the peak A of chromatography in Fig. 5II (IEC at pH 5.0).

Atomic ratio (N/P) was 2.63±0.07 for Pvt_p, similar to those described by different authors: 2.72 for Mecham and Olcott [1] and 2.90 for Joubert and Cook [11].

The absorbancy at 280 nm of Pvt_p aqueous solutions (range of protein concentration by total protein nitrogen method: 0.5–8.5 g/l) was measured. The linear relationship obtained (r=0.9995) was characterised by an absorptivity at 0.1% (1 g/l) of 0.32±0.02 l g^{-1} cm^{-1}. In the same way, OD_{220}/OD_{280} ratio was 26.4±3.5 for Pvt_p, and 10.2±1.7 for Pvt_i. This ratio, in view of the weak absorbancy of phosvitin at 280 nm, could help to evaluate the degree of purity of phosvitin.

4. Discussion

4.1. Phosvitin isolation

The purified state of phosvitin preparations has represented a problem to many authors [16,27,28]. Moreover, it could be interesting to avoid the presence of any organic solvent in the phosvitin preparation to widen its potential in future use. Between the most usual isolation methods, only one presents this feature [11]. The procedure described in our study separates granules from plasma, and then we isolate phosvitin from the granules by protein precipitation in the presence of Mg^{2+}, as was observed by Mecham and Olcott [1]. In this way, we have avoided the presence of any organic solvent in the isolation method. Separately from phosvitin, it is possible to obtain independent protein fractions enriched in low-density lipoproteins (LDL) or high-density lipoproteins (HDL). Additionally, it is possible to obtain more quantity of phosvitin by decreasing the pH value of the last supernatant to 1.5 at the end of the isolation procedure. It could be interesting in the production of the protein regardless of its structure or previous treatment conditions. Even though, in our study, we have only used Pvt_p because the applied strong chemical conditions (pH 1.5), could present some problems like the modification of phosvitin secondary structure [29,30].

4.2. Optimisation of phosvitin purification

HIC resolved the Pvt_i fraction better than IEC. This result was unexpected in view of both the low percentage of hydrophobic amino acid of phosvitin and its predominant unordered secondary structure at pH 7.5. However, the low proportion of structure present in this condition [28] and the fact that multiple conformational states may be induced by high concentrations of $(NH_4)_2SO_4$ [31] could be sufficient to promote a partial separation of polypeptides. The low yield obtained, a consequence of a low solubility of phosvitin at high $(NH_4)_2SO_4$ concentration, limits the use of this technique to analytical studies.

By IEC, using Source 15Q at pH 7.5 with the presence of 0.3 *M* NaCl in the sample buffer, it was possible to separate a low proportion of phosvitin polypeptides, and the most part of phosvitin remaining was contaminated. So, 0.3 *M* NaCl is not enough to dissociate the interactions between phosvitin and HDL. Causeret et al. [32] found that the dissociation of granules and consequently of their components (phosvitin and HDL), occurred at 0.58 *M* of NaCl independently of protein concentration. Despite that, at this salt concentration we have observed that the proteins are already eluted, and the separation by IEC is not possible.

At pH 5.0, the contamination of HDL is eliminated, in part because of solubility differences between the two protein fractions. Under this conditions, we have obtained a fraction which contains apparently only one type of β-phosvitin polypeptide, which must be less negatively charged than the other polypeptides (peak B, Fig. 5II). Moreover, one fraction (C) is enriched in phosvettes and depleted in

α-phosvitin polypeptides, whereas the other fraction (D) is relatively rich in α-phosvitin and poor in phosvettes. However, β-phosvitin is always the main polypeptide (see Table 2). Heterogeneity of phosvitin polypeptides was detected, especially for the β ones. It must be a consequence of the high degree of post-translational modification of this protein. This property could generate polypeptides with different phosphorylation degrees [16], and consequently, by IEC, they are eluted in different peaks with the increasing in salt concentration. By this method, we were able to obtain phosphoproteins with high electrophoretic mobility (phosvettes). In the past, these proteins were only obtained by the isolation method of Wallace and Morgan [12], which has a lower yield (0.96% of egg yolk dry matter) than the method presented here. In accordance with these authors we have confirmed that, by IEC, the phosvettes are eluted before the principal peak (fraction C in Fig. 5). On the other hand, by HIC, they are eluted in the earlier samples (peaks A and B in Fig. 3), and the same was observed in a TSK Phenyl-5-PW Bio-Rad column [17]. These apparently opposed characteristics must be related to the small size of the phosvettes (around 16 kDa [12]), which leads to a poor adsorption on chromatographic medium independently of the type of interaction.

Pvt_i was partially fractionated by IEC with Source 15Q. This is in accordance with IEC results from Tsutsui and Obara [15] and Abe et al. [16], who used DEAE-Sepharose and DEAE-Cellulose, respectively. In summary, to improve the Pvt_i purity including metal separation, IEC at pH 5.0 was the best method.

4.3. Characteristics of Pvt_p

For the Pvt_p, the N/P ratio (2.6) indicates a high level of purity, and it is in close agreement with those reported by many authors [1,3,11]. Losso and Nakai [2] reported a ratio of 3.6 for a relative sample phosvitin isolation, that is strikingly coincident with the value obtained for Pvt_i (3.5), which has a lesser degree of purity. Wallace and Morgan [12] reported a ratio of 3.34 for a purified phosvitin and they related the difference to the presence of phosvette polypeptides with a lesser degree of phosphorylation. Using our methodology, and even though we were also able to obtain phosvette polypeptides in the phosvitin fraction, the N/P ratio was significantly less than that obtained by Wallace and Morgan [12]. This could be due to a higher proportion of phosvettes in their preparation (27%) in comparison with the 10% in Pvt_p (weighted average of peaks C and D, Fig. 6II′), but we cannot discarded a higher degree of phosphorylation in the phosvettes from our preparation. The amino acid composition, and specially the low proportion of Trp, Tyr and Cys, is also indicative of an appropriate phosvitin preparation.

Pvt_p was essentially free of divalent metal counter ions such as iron and magnesium, without need of further chromatography or time-consuming dialysis process [13,29,30]. This is important for our further studies about the properties of phosvitin like metal chelator [8,13]. To better characterize the obtained protein, even if it is not a purified polypeptide, we have determined the absorbancy coefficient at 280 nm (1 g/l) of Pvt_p (0.32 ± 0.02 l g^{-1} cm^{-1}). The value is in agreement with the respective one (0.316) described in Swiss Prot for one phosvitin sequence [5], but is less than that calculated in the same database for another phosvitin sequence [33]: 0.840. In agreement, the levels in Trp and Tyr are more coincident with that of Byrne et al. [5]. Even though, the mixture of phosvitin polypeptides has a lower proportion of serine amino acids than this sequenced polypeptide. Consequently, its amino acid composition could not be taken as the average composition of purified phosvitin. This parameter is convenient to quantify phosvitin, because of the impossibility to use traditional methods [34,35] as a consequence of the singular amino acid composition of phosvitin. The optical density ratio (OD_{220}/OD_{280}) links the concentration of peptide bonds to the concentration of amino acids absorbing at 280 nm, so it is a sensible index of phosvitin protein contamination. Moreover, it is a parameter that could be easily evaluated during the chromatographic process, and less dependent on post-translational protein modifications than N/P ratio.

5. Conclusion

We conclude that the purification process, using a rapid anionic-exchange chromatography at pH 5.0, allows us to obtain purified phosvitin with a rela-

tively high yield (1.7% of egg yolk dried matter, that is to say 85% of total egg yolk phosvitin), using aqueous solvent solutions throughout the process. The obtained yield is comparable to those described by other authors who used organic solvents in their extraction procedures. Moreover, a high degree of purity (at less 98%) and a protein free of polyvalent metals were obtained.

Acknowledgements

We want to thank the Pole Agronomique Ouest and the Region Pays de la Loire for their financial support. We also want to thank Michele Dalgalarrondo and Michel Piot for their technical assistance.

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