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Purification of egg yolk phosvitin by anion exchange chromatography

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ABSTRACT

The objective of this study was to develop a simple method of phosvitin purification from hen egg yolk without using organic solvents. Egg yolk was diluted with equal volume of water and stirred for one hour at room temperature, followed by centrifugation to remove soluble proteins along with most of the yolk lipids in the supernatant. The granules were collected as the precipitate containing minimum amount of lipids (dry granules). The dry granules were dissolved in 0.05 M carbonate–bicarbonate buffer at pH 9.6, which yields a light yellowish solution used for anion exchange chromatography. Phosvitin fraction was collected from anion exchange chromatography as the last eluting peak with a purity of 92.6% and a yield of 35.4% of total phosvitin in the yolk or a recovery of 1.9% of total yolk dry matter, which are comparable to current methods employing organic solvents or chromatography after salt fractionation and dialysis. This method developed is simple and fast without using organic solvents.

1. Introduction

Hen egg yolk phosvitin is a phosphoglycoprotein representing 11% of egg yolk proteins and 4% of yolk dry matter [1]. It contains about 10% phosphorus, and is one of the most highly phosphorylated proteins in nature [2]. The composition of phosvitin is very unique, about 50% of its amino acids are serine, and out of which 90% are phosphorylated [3]. As a consequence of this high level of phosphorous, phosvitin has very strong metal binding capability, particularly for iron, 95% of yolk iron is bound to phosvitin [4,5]. Phosvitin has been demonstrated to have a variety of biological properties including antioxidant [6,7], anti-bacterial [8], and anti-cancer [9], along with some well known functional characteristics such as emulsion-stabilising property [10]. In comparison, a phosphoglycoprotein from bovine milk such as casein has already been commercialized as a valuable component for functional food uses [11]. However, application of phosvitin is hampered probably due to the lack of an industrial method of extraction of phosvitin from egg yolk.

Phosvitin was first extracted from hen egg yolk by a very lengthy method as reported by Mecham and Olcott [2]. Egg yolk was diluted sequentially with $MgSO_4$ solution to a final concentration of 0.09 M, in which phosvitin was precipitated and was collected by centrifugation. The precipitated phosvitin fraction was further purified by the repeated combination of ammonium sulphate dispersion, ethyl ether extraction, centrifugation, filtration, and dialysis. Phosvitin thus obtained has 9.7% phosphorus, 11.9% nitrogen (molar ratio N/P = 2.72), and 0.7% lipids. The recovery of P from egg yolk was 60–70%. Later on, Jourbert and Cook [12] studied the effect of different concentrations of $MgSO_4$ on phosvitin precipitation in more detail, and found out that phosvitin could be precipitated from egg yolk solution in 0.4 M $MgSO_4$ by adding an equal volume of water, resulting in a precipitate containing 73% phosvitin, 12% IgY, and 15% high density lipoprotein (HDL). Further purification of phosvitin was achieved by repeated precipitation and dialyzing at pH 4. Phosvitin prepared was free of lipid, containing 9.6% phosphorus, 12.6% nitrogen, and had a molecular weight of 30 kDa. The recovery was not mentioned, but probably is low because of the repeated precipitation and dialysis steps. Shortly, Sundararajan et al. [13] published a simpler method for preparing phosvitin using butanol, which precipitates lipovitellin and release phosvitin to the solution, the later could be recovered by isoelectric precipitation at pH 1.8. The precipitate had to be further treated with $MgSO_4$, ether and acetone to make the final product comparable to Mcham & Olcott's preparation. This method is used for preparing Sigma–Aldrich phosvitin standard [14].

Based on the above research, Wallace and Morgan [14] formulated a general method for phosvitin purification from vertebrate eggs including chicken. The procedure consisted of three steps including granule isolation, which utilized salt precipitation and centrifugation; ammonium sulphate precipitation, where phosvitin was extracted by ammonium sulphate precipitation and dialysis, and finally phosvitin was purified by DEAE cellulose chromatography. The chicken phosvitin thus prepared had a N/P ratio of 2.44, but the paper did not mention the recovery. A simple

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procedure for isolation of phosvitin from egg yolk was reported by Losso and Nakai [15] who started with ten times water dilution of egg yolk, the diluted egg yolk was centrifuged to collect precipitate, the latter was delipidated by hexane:ethanol (3:1, v:v), and then was extracted with 10 times its volume of 1.74 M NaCl to disrupt the phosphocalcic bridges between HDL and phosvitin in granules and thus release phosvitin from the HDL and phosvitin complex. After dialysis and lyophilisation, the phosvitin isolated was quite pure (N/P = 3.60) with higher yield (100–113 mg/egg or 0.4% egg dry matter).

Most of the methods currently used are based on the modifications of the above mentioned procedures, which usually include step of yolk dilution with water or salt followed by centrifugation to isolate granules, which were then extracted for phosvitin by various methods including salt precipitation, organic solvent fractionation before subjected to chromatographic purification. The procedures were either lengthy because of high speed centrifugation, ammonium sulphate precipitation/dialysis or used organic solvents. Recently, Castellani et al. [16] reported a simpler aqueous method that omitted the need of ammonium sulphate precipitation compared with the method of Wallace and Morgan [14], instead, it used different concentration of $MgSO_4$ and isoelectric focusing to fractionate phosvitin before chromatographic process. Even though this process is significantly simpler and more environmentally friendly compared with previously reported methods, it is very difficult to scale up for industrial application, because of the high salt concentration used as well as the large amount of water consumed owing to the procedures of $MgSO_4$ precipitation and dialysis before column fractionation. The objective of the study was to develop a simplified method of phosvitin purification without using organic solvents.

2. Materials and methods

2.1. Reagents

Sodium carbonate, sodium bicarbonate, sodium chloride, sodium hydroxide, and hydrochloric acid were from Fisher Scientific (Nepean, ON, Canada). Glycine, Precision Plus Protein Standard, sodium dodecyl sulphate (SDS), and precast gel (10–20% Tris–HCl) were from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Phosvitin standard (P1253, from chicken egg yolk) was purchased from Sigma–Aldrich, Ltd. (Oakville, ON, Canada). The deionized distilled water (dd water) used for all the experiments was produced by a Barnstead water purification system.

2.2. Egg yolk

Eggs were purchased from a local supermarket at the day of the experiment. Egg shell was manually broken, and yolk was isolated by eliminating albumen, and then yolk was carefully rolled on a Whatman filter paper (Whatman Inc., Florham Park, New Jersey) to remove albumen and chalazas adhering to the vitelline membrane. This membrane was then perforated to collect unspoiled egg yolk in a beaker cooled in ice water.

2.3. Granule solution preparation

Yolk was fractionated into plasma and granules based on the method of McBee and Cotterill [17] with modifications. Yolk was diluted with deionized water, and mixed by magnetic stirring for one hour. The mixture was centrifuged at 10,000 × *g* for 45 min at 4 °C. The precipitate (granules) was collected and re-suspended in 0.05 M carbonate–bicarbonate buffer pH 9.6. Granules were completely dissolved after 1 to 2 h magnetic stirring.

2.4. Anion exchange chromatography

Granule solution was filtered through 0.22 μm filter, and then loaded to a HiPrep 16/10 Q FF anion exchange column (GE Healthcare Bioscience, USA). The column was equilibrated in advance with 0.05 M carbonate–bicarbonate buffer pH 9.6. The chromatographic elution was run by an AKTA-FPLC system (GE Healthcare Bioscience, USA) at a flow rate of 2 mL/min with buffer A as 0.05 M carbonate–bicarbonate buffer pH 9.6, and buffer B as 0.5 M NaCl in buffer A. The column was first run for 6 bed volumes with increasing proportion of buffer B from 0% to 45%, and then to 75% in 20 bed volumes, and the effluent was monitored at 280 nm.

2.5. Nitrogen and protein determination

Crude protein was determined (N × 6.25) by duplicate using the Leco-N nitrogen determinator (Model FP-428, Leco Corporations, MI, USA).

2.6. Lipid determination

Lipid was extracted and determined by Goldfisch method. Freeze-dried samples were weighed into extraction thimbles and covered with glass wool. The thimbles were placed in sleeves and clamped into the lipid extractor (Model: 3500; Goldfisch, Labconco Corporation, Kansas City, MO). To each oil extraction beaker, 40 mL of petroleum ether was added, and the beaker was attached to the lipid extractor. The extraction was carried out for 6 to 8 h. After extraction, the beakers were placed in a fume hood for half hour and then in an oven at 100 to 110 °C for 0.5–1 h to evaporate the petroleum ether before being weighed. The percentage of lipid was calculated from the mass of lipid collected. Duplicate lipid analysis was performed for each sample.

2.7. Phosphorus determination

The phosphorus contents of yolk and granule samples were determined by using AOAC colorimetric method 995.11 and phosphorus contents of chromatographic fractions were determined based on Bartlett [18].

2.8. Native polyacrylamide gel electrophoresis

The SDS–PAGE was carried out using continuous system (10–20%) gel in Mini-Protean Tetra Cell (Bio-Rad, Hercules, CA, USA). The following running buffer was used: pH 8.8 Tris–HCl with 0.1% SDS. All samples were dissolved in the running buffer at a concentration of 2 mg/mL, and incubated at 95 °C for 5 min, followed by centrifugation at 15,000 × *g* for 5 min by using a benchtop minicentrifuge. A 20 μL sample from each supernatant was loaded for analysis. Electrophoresis was conducted at a constant voltage of 200 V for about 35 min. After electrophoresis, the gels were stained with 0.05% Coomassie brilliant blue R-250 in a solution of 0.1 M aluminum nitrate/25% isopropanol/10% acetic acid/1.0% Triton X-100, prepared according to the method of Hegenauer et al. [5]. Destaining was carried out in 7% acetic acid solution. Bio-Rad Precision Plus Protein Standards ranging from 10 to 250 kDa were used as molecular standards.

2.9. Gel filtration HPLC

Gel filtration was carried out by HPLC (Waters, Milford, Massachusetts, USA) in a TSK-Gel G3000SW_{XL} stainless column (0.78 cm × 30 cm, Tosoh, Tokyo). Samples were prepared in elution buffer of 0.1 M sodium phosphate (pH 7.0) at the concentrations from 0.5 to 6.0 mg/mL depending on sample characteristics. The

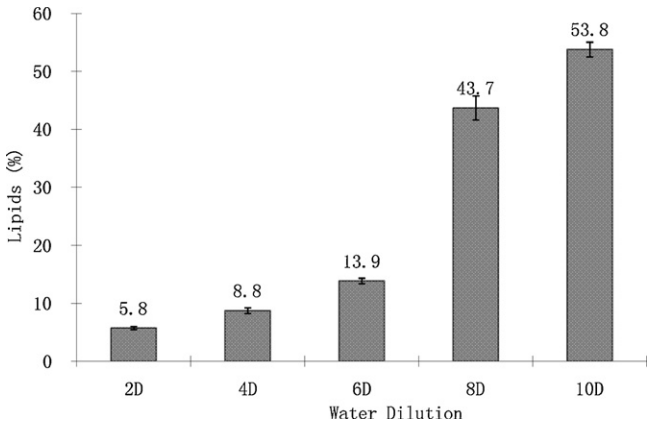

Fig. 1. Effect of water dilution on the total lipid content in granules. Lipids (%) was calculated by total lipids in granules divided by corresponding total lipids in yolk. 2DG, 4DG indicate two times water dilution, four time water dilution, etc.

load volume was 50 μL and the flow rate was 0.5 mL/min. The chromatograph was depicted by monitoring the effluent at 220 nm. The experiment control and result analysis were performed by using Empower II Software (Waters, Milford, Massachusetts, USA).

2.10. Statistical analysis

Each sample was prepared in duplicate and values presented are the mean of the duplicate samples. Analysis of variance (ANOVA) was used to analyze the changes, and Dunnett range post hoc comparisons were used to determine the source of significant differences where appropriate. A $p < 0.05$ was considered statistically significant. All data were using Statistical Analysis System Software, SAS version 9.0 (SAS Institute, Cary, NC).

3. Results and discussion

3.1. Water dilution experiment to get granules containing minimum amount of lipids

The objective of water dilution experiment was to find out the best condition that retained most of the lipids in the plasma fraction, whereas produced "dry" granules containing minimum amount of lipids. Egg yolk was diluted with different volumes of dd water, followed by centrifugal separation ($10{,}000 \times g \times 45\,\text{min} \times 4\,^{\circ}\text{C}$) after one hour mixing magnetically at room temperature. Granules as precipitates were collected and analyzed for lipid contents, as shown in Fig. 1.

Lipid contents in granules increased with the increasing volume of water used in diluting egg yolk before centrifugation (Fig. 1), which explains why the increasing amount of precipitate occurred with increasing water dilutions. The granules prepared by 2 times dilution contain about 6% of total yolk lipids, while over 50% of yolk lipids are in the granules prepared at 10 times dilution. Previous research reported similar trend in lipid content in granules with different water dilutions [19], such as a 3% of total yolk lipids in granules with 2 times dilution and 93% of total yolk lipids in granules with 10 times dilution (subtracted from lipids in plasma). Lipids in yolk present in the format of lipoproteins such as LDL and HDL. The density of LDL is less than that of water and therefore LDL can be separated from yolk based on density difference. HDL in yolk is associated with phosvitin and therefore HDL present in granules after water dilution separation. The relation between yolk dilution and granules lipid content was also reported previously but the mechanism is not known. It was indicated that the lipoproteins (LP) are inherently unstable in the absence of water [20] that could precipitate out in granules at increasing water dilution, leading to increasing lipid content in granules. There is a difference between these two studies, in which the previous study [19] used overnight standing after water dilution before centrifugation, while as the current study used one-hour stirring before centrifugation to get precipitated granules. One-hour stirring is more cost-effective than overnight standing in terms of scaling up and industrial application.

Two times water dilution (2D) was selected for preparing granules as it contains the minimum amount of lipids comparing with higher dilutions (Fig. 1). Two times water dilution was also compared with the 2D containing 1% NaCl (0.17 M NaCl), and it was found out that there was no significant difference between the two methods. Therefore, salt was not used in the 2D experiment. When preparing diluted yolk mixture, it was noticed that the pH-adjusted water had no significant effect on the composition of granules, because the final pH of the yolk diluted with water at different pH (2–12) always fell between pH 6.0 and 6.5 based on our experiment (*data not shown*); therefore, dd water without pH-adjustment was used directly for further experiments.

3.2. pH and granule solubilization

The phosphocalcic bridges between HDL and phosvitin in the granules prepared from two times water dilution (2D) of egg yolk have to be disrupted in order to separate phosvitin from HDL. The most common method used is to disrupt the granules by 10% (1.7 M) NaCl or by 0.4 M $MgSO_4$ followed by water dilution to precipitate phosvitin, which has to be dialyzed to remove salt [2,17]. Granules prepared by these methods have low yield and purity, and have to be further processed by organic solvent extraction, ammonium sulphate precipitation, and/or repeated dialysis before subjecting to various chromatographic processes [2,12–14,16,17,21]. These treatments are necessary because granules are not only insoluble in water but also contain significant amount of lipids. The phosvitin separation process would be much simpler if the granules could be solubilized and disrupted to release phosvitin, which then could be fractionated directly by column chromatography. The granules prepared by 2D contain minimum amount of lipids, and thus it is feasible to load them to the chromatographic column directly given that these "dry granules" could be solubilized. A study on granule solubilization using pH ranging from 5 to 12 was conducted based on this consideration. It was found that granules can be dissolved in solution at pH 9 or higher (Fig. 2). The granules were completely solubilized into a yellowish clear solution after being re-constituted in 0.05 M carbonate–bicarbonate buffer at pH 9.6 followed by one to two hours of constant magnetic stirring. The solubilization of granules at alkaline pHs may be due to de-acidification of carboxylate groups, leading to the repulsion of negative charges (COO—) and thus solubilization of the granule [25].

3.3. Anion exchange chromatography of solubilized granule solution

The above prepared dissolved granules solution in 0.05 M carbonate–bicarbonate buffer at pH 9.6 was loaded to anion exchange column, and the elution profile was shown in Fig. 3. There were three well resolved peaks eluted from the column at 315, 442, and 542 mL, respectively, which accounted for 20, 32, and 11% (w/w) of the loaded amount in addition to the 36% (w/w) of loaded amount passing through the column as unbound fraction. The eluted peaks were analyzed by native SDS–PAGE against phosvitin standard as shown in Fig. 4. The presence of phosvitin was characterized using SDS–PAGE against Sigma phosvitin standard, and using phosphoprotein specific staining of Coomassie brilliant blue with aluminum nitrate. The gel was first stained by Coomasssie brilliant blue (upper one), which shows no phosvitin bands; then

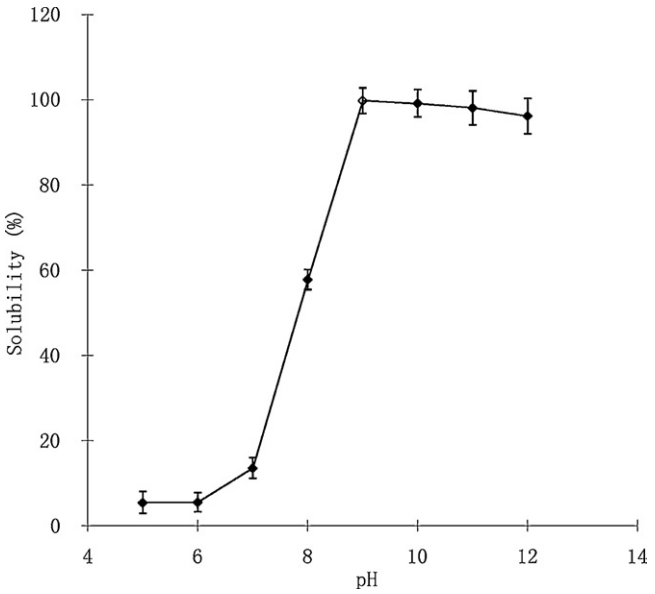

Fig. 2. The granule solubility at different pHs in water. The solubility (%) was calculated based on protein (♦) in supernatant fluid (mg/mL) divided by protein in granule solution before centrifugation. The protein concentration of granule solution was 2%, data was the results of duplicates.

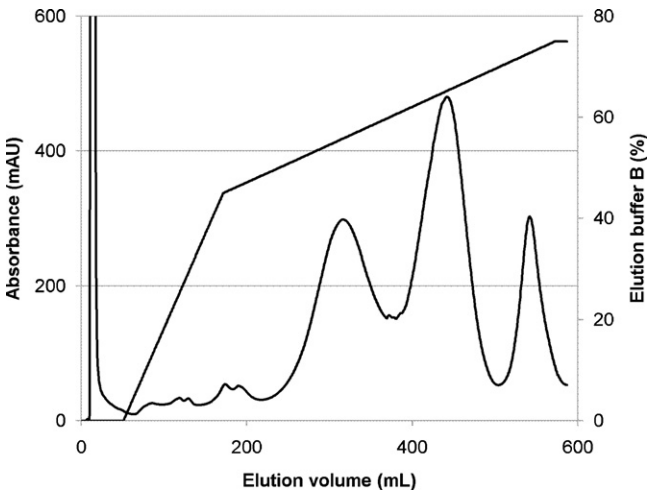

Fig. 3. The elution profile of anion exchange chromatography of dissolved granule solution from egg yolk.

the gel was stained again by Coomassie brilliant blue added with aluminum nitrate (lower one), where phosvitin bands were showed in pink in yolk, granules and fraction 3 at position around 37 kDa (arrow labelled), comparable to the Sigma phosvitin standard. The intensity of the phosvitin bands in yolk, granules and fraction is increasing as the purity of phosvitin is increasing after granule and ion exchange chromatography. There are two minor bands, in addition to the major phosvitin band, in the fraction 3. These results indicate that phosvitin has been successfully isolated from granules by anion exchange column. Fraction 2 was mainly HDL as compared with granules band. The experiment indicates that anion exchange chromatography using HiPrep 16/10 Q Fast Flow is very effective in fractionating phosvitin from granule solution.

3.4. HPLC analysis of major phosvitin fraction

The above column fractions along with yolk and granules were analyzed against Sigma–Aldrich phosvitin standard by gel filtration HPLC, and the results are presented in Fig. 5, which shows yolk,

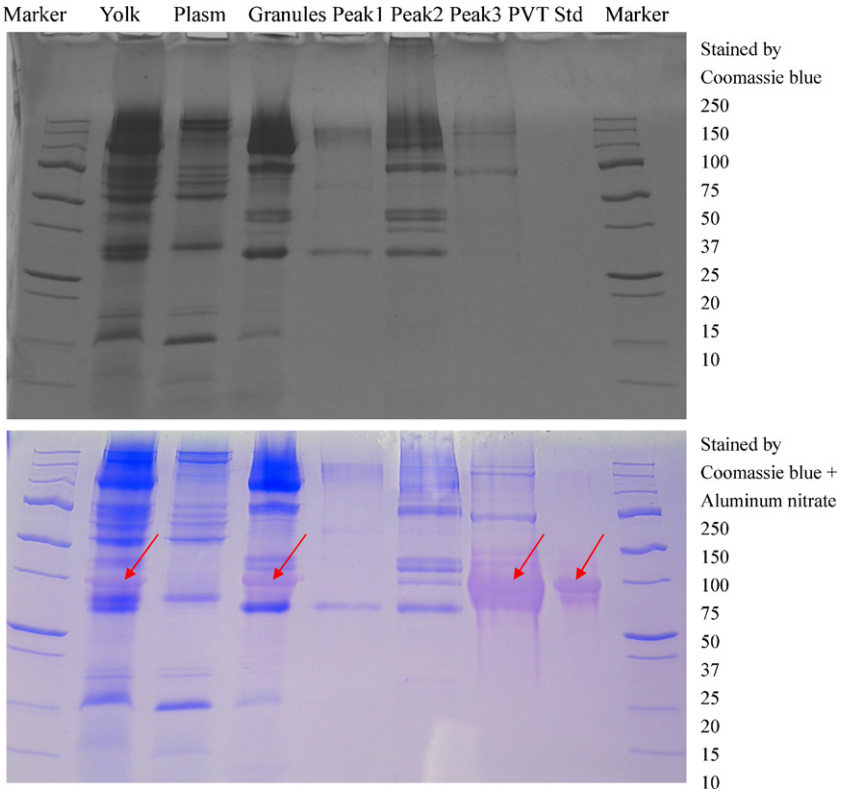

Fig. 4. The SDS–PAGE profiles of three peaks eluted from anion exchange column loaded with granule solution in 0.05 M carbonate–bicarbonate buffer at pH 9.6, comparing with egg yolk, plasma, granules and Sigma phosvitin standard (PVT Std). The arrows indicate the positions of phosvitin bands.

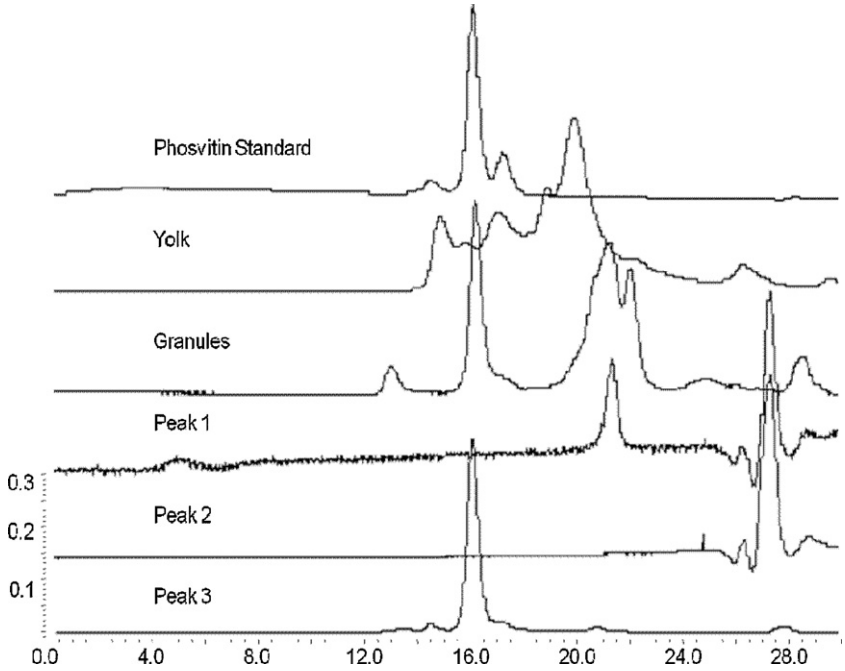

Fig. 5. The HPLC elution profile of different fractions during phosvitin purification from egg yolk.

granules and fraction 3 all had peaks overlapped with the peak position of phosvitin standard, but fractions 2 and 3 had no peaks at the position of phosvitin standard. A single major peak was eluted from fraction 3 and this peak had a purity of 92.6% calculated against a standard curve established using Sigma phosvitin standard.

3.5. Phosvitin recovery

Phosvitin contents in yolk, granules and fraction 3 (phosvitin fraction) from anion exchange chromatography were estimated by gel filtration HPLC against phosvitin standard. The results are shown in Table 1. The phosvitin content of 5.3% in egg yolk is higher than previous results of 1–4% [15,16,22], and this may be due to the different methods used. Previous data of up to 4% phosvitin in yolk were calculated based on nitrogen and phosphorus contents, but current data of 5.3% was based on HPLC method. The phosvitin content in granules was 14.7%, which was close to the reported phosvitin content in granules as 16% [22]. The phosvitin recovery from yolk in granule was 62%, while phosvitin recovered from granules was 58%, which equals to a yield of 35.4% of phosvitin from yolk; the phosvitin recovered from column chromatography constituted a 1.9% of recovery based on total dry mass of egg yolk. A recovery of 30% phosvitin was reported for the method of Losso and Nakai [15], which is lower than reported recovery here. The reported data on phosvitin recovery usually based on recovered phosvitin against total yolk dry mass but not total phosvitin in yolk, this may be because the total phosvitin content in the yolk has not been determined. According to Losso and Nakai [15], the recovery of phosvitin from their method was 100 mg/egg, which converted to about 0.4% phosvitin over dry yolk mass, and Yamamoto et al. [23] reported 0.2% phosvitin recovery against total dry yolk mass, and the data from Castellani et al. [16] were 0.7 and 1.7% of phosvitin over total dry yolk mass. All those data are lower or comparable to our current recovery of 1.9% phosvitin over yolk dry mass. A surprising high recovery of 97% was currently reported using ethanol to remove lipids and NaCl to extract phosvitin [24]. The protein content was not shown because phosvitin is a highly phosphorylated protein; therefore, calculation of protein content by a factor of 6.25 might not reflect the real protein content. Instead, we reported the nitrogen content for monitoring protein content in each step of the phosvitin preparation (Table 2). The molar ratio of N/P is often used as an index of phosvitin purity, a high value being related to higher purity. The N/P ratio of 2.5 for the phosvitin was obtained, which is comparable to the reported data as summarized in Table 2. Considering that the previous methods either used organic solvents [15,23] or other tedious methods [16], the current method as outlined in Fig. 6 is simple and environmentally friendly.

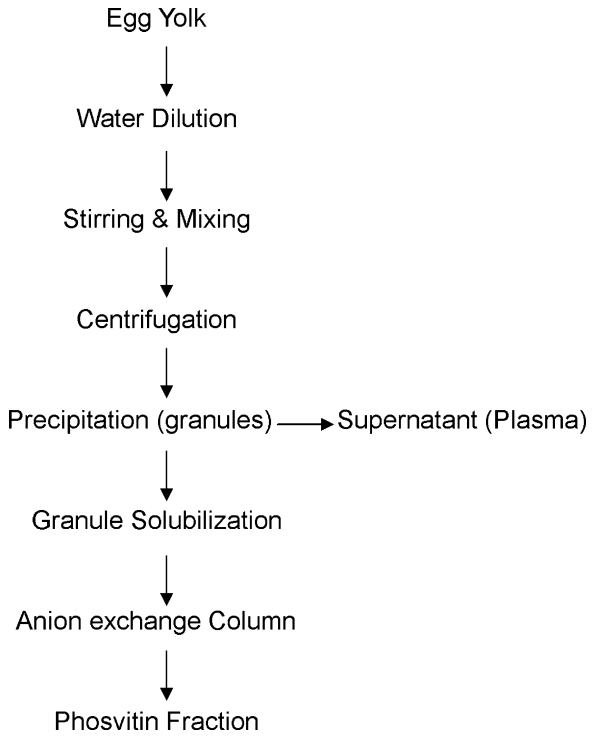

Fig. 6. The flowchart of phosvitin purification process from egg yolk.

Table 1
Phosvitin recovery in each fraction of the purification processes (all in dry matter).

Steps	Total amount (mg)	Phosvitin percentage (%)	Phosvitin quantity (mg)	Phosvitin recovery (%)
Yolk	325.4 ± 35.7	5.3 ± 0.6	17.2	100
Granule	72.0 ± 6.7	14.7 ± 1.3	10.6	61.6 (from yolk)
Peak 3	6.6 ± 0.4	92.6 ± 3.2	6.1	57.7 (from granules)
Phosvitin recovery calculated from egg yolk				35.4
Phosvitin recovery calculated from total dry egg matter				1.9

Table 2
Nitrogen and phosphorus ratios in each phosvitin prepared by different methods.

	N/P
Current method	2.5 ± 0.3
Wallace et al.	2.44
Sigma[a]	2.49
Yamamoto et al.[a]	3.70
Tsutsui & Obara[a]	2.83
Losso & Nakai[a]	3.60
Mecham & Olcott	2.5–2.9

[a] Data cited from (21).

4. Conclusions

Egg yolk diluted with equal volume of water generates the "dry" granules containing minimum amount of lipids. The dry granules thus prepared were soluble at 0.05 M carbonate–bicarbonate buffer (pH 9.6), and the dissolved granule solution was therefore able to be separated and purified by anion exchange chromatographic column directly, which produced a phosvitin fraction with a purity of 92.6% and a yield of 35.4% based on total yolk phosvitin or a recovery of 1.9% of total yolk dry mass. The whole purification process only utilized water and 0.05 M carbonate–bicarbonate buffer without any organic solvents, and avoided the necessity of various high concentration of salts employed in previous reported methods. The entire process only has three major steps (granule separation, granule solubilization, and column chromatography), which can be finished well within one working day in a lab. Using HiPrep 16/10Q FF anion exchange column, a quantity of 20–50 mg per batch can be prepared.

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