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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