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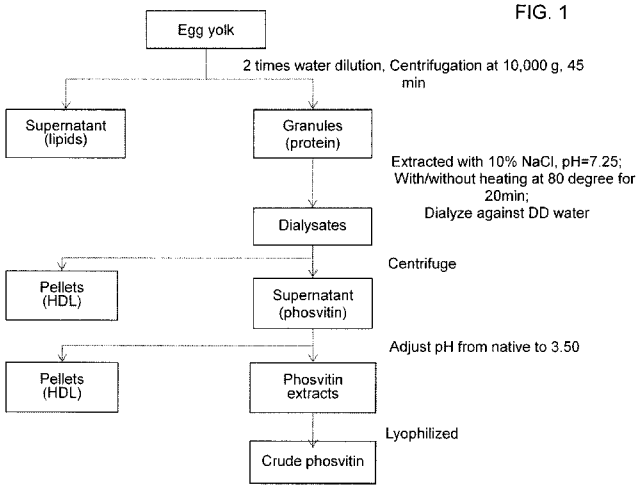
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(54) Title: METHODS OF PHOSVITIN EXTRACTION AND PHOSPHOPEPTIDE PREPARATION FROM EGG YOLK

FIG. 1



(57) Abstract: The invention is directed to methods for extracting phosvitin from egg yolk involving contacting the egg yolk or egg yolk protein granules with a solution having a salt concentration of about 10% to form a mixture; optionally, heating the mixture; adjusting the pH of the mixture to separate phosvitin from other proteins; recovering the phosvitin. The phosvitin extract may be dephosphorylated and hydrolyzed to produce phosvitin phosphopeptides.

METHODS OF PHOSVITIN EXTRACTION AND PHOSPHOPEPTIDE PREPARATION FROM EGG YOLK

Field of the Invention

[0001] The invention relates to methods for extracting phosvitin from egg yolk, and preparing phosvitin phosphopeptides.

Background of the Invention

[0002] Eggs have long been recognized as a source of high-quality protein and other important nutrients in the human diet. Phosvitin is a principal phosphoprotein present in egg yolk and represents about 11% of egg yolk proteins and 4% of yolk dry matter. Phosvitin is derived from the large multidomain vitellogenin precursors, which are synthesized in the liver of vertebrates under stimulation of estrogen and later cleaved into phosvitin, lipovitellin and other proteins (Finn, 2007). Phosvitin contains 12% of nitrogen and 10% of phosphorus, and has a molecular weight of 35 kDa (Mecham and Olcott, 1949; Powrie and Nakai, 1986). Phosvitin contains 217 amino acid residues, of which 123 are serine (Byrne *et al.*, 1984). Of the 123 serine residues, 118 are phosphorylated, making it the most highly phosphorylated protein in nature (Byrne *et al.*, 1984; Clark, 1985; Grogan *et al.*, 1990). Due to the large amount of negatively charged phosphoserine residues, phosvitin exhibits strong metal chelating ability, and is believed to provide metal ions during embryonic development (Taborsky, 1983). Phosvitin exhibits numerous other biological properties including antioxidant and anti-bacterial abilities, and excellent emulsion-stabilizing properties (Albright *et al.*, 1984; Chung and Ferrier, 1992; Nakamura *et al.*, 1998; Sattar Khan *et al.*, 2000).

[0003] Phosvitin may exhibit greater metal-chelating ability than casein phosphopeptides which are the phosphorylated fragments derived from bovine milk casein digests and are currently used as calcium supplementing agents. A subunit of casein phosphate has only one to

thirteen phosphoserine residues to stabilize amorphous calcium phosphate, whereas a molecule of phosvitin has a greater number of phosphoserine residues, implying higher calcium chelating capacity. Phosvitin may thus serve as a source for the production of bioactive peptides that may improve calcium availability *in vivo* and increase incorporation of calcium into bone (Choi *et al.*, 2005). Since calcium and phosphorus can be simultaneously supplied, phosvitin peptides may be useful in preventing osteoporosis in women and bone loss in aging population.

[0004] However, phosvitin is usually considered nutritionally negative due to its strong affinity to metal ions and resistance to proteolytic actions of proteases (Goulas *et al.*, 1996). 95% of the iron in egg yolk is bound to this protein, but only 30% is biologically available (Greegard *et al.*, 1964; Morris and Greene, 1972). Egg yolk protein and phosvitin may inhibit calcium, magnesium and iron absorptions (Ishikawa, 2007). In contrast, casein phosphopeptides enhance vitamin D independent bone calcification in rachitic children (Mellander and Isaksson, 1950; Mellander, 1950). Phosphoserine residues in casein phosphopeptides play a key role by forming soluble organophosphate salts with calcium to limit its precipitation in the distal ileum (Meisel *et al.*, 2003). The common motif, which consists of three phosphoserine residues and two glutamic acids (Ser(P)-Ser(P)-Ser(P)-Glu-Glu) is widely recognized to contribute to the metal chelating ability of casein phosphopeptides (West and Towers, 1976; Schlimme and Meisel, 1995).

[0005] Unlike casein phosphopeptides, common knowledge about phosvitin-derived phosphopeptides is limited due to insufficient hydrolysis. The strong negatively charged side chain of phosvitin hinders the enzymatic access to cleavage sites, so the core of phosvitin remains intact after hydrolysis (Goulas *et al.*, 1996; Khan *et al.*, 1998). Compared with casein, which contains no more than fifteen phosphoserine residues in total and three consecutive phosphoserines in a run (Swaisgood, 2003), phosvitin could be an ideal protein source to produce phosphopeptides, since it contains as many as 123 phosphoserine residues and many of them are in consecutive runs (Grogan *et al.*, 1990).

[0006] Phosvitin phosphopeptides exhibit antioxidant activities and calcium-absorption promoting ability *in vitro* and *in situ* (Jiang and Mine, 2000; Feng and Mine, 2006; Katayama *et*

al., 2006; Katayama *et al.*, 2007; Xu *et al.*, 2007; Young *et al.*, 2011). However, their application has been hindered by the lack of efficient and economic methods to extract phosvitin from egg yolk and to prepare phosphopeptides.

[0007] Phosvitin has been precipitated from egg yolk using magnesium sulfate, followed by ammonium sulfate and ethyl ether extraction (Mecham and Olcot, 1949). The purity of phosvitin (expressed as atomic ratio of nitrogen to phosphorus or "N/P") was 2.72 with an estimate recovery of 60-70% from egg yolk. Another method involves using butanol to remove lipovitellin, recovering phosvitin by repeated precipitation at pH 1.8, purifying with ether and ammonia extraction, and finally precipitating phosvitin using magnesium sulfate (Sundararajan *et al.*, 1960). The resultant N/P ratio was 2.65. A simplified approach involved diluting egg yolk ten-fold with water to prepare an egg yolk pellet, extracting the lipids in the pellet using hexane/ethanol (v/v, 3/1), and extracting phosvitin with 1.74 M sodium chloride, followed by lyophilization (Losso and Nakai, 1994). The purity of the phosvitin, expressed as N/P ratio, was 3.60. A larger N/P ratio may be related to protein impurities and lower purity. Extraction was improved by conducting 10% sodium chloride extraction and magnesium sulfate precipitation, resulting in a N/P ratio of 3.5 and a yield of 3.3 g per 100g of dried egg yolk (Castellani *et al.*, 2003). However, such methods are of small scale, inefficient, mediocre in purity, and rely upon organic solvents and non-food compatible chemicals such as magnesium sulfate.

[0008] Therefore, there is a need in the art for improved methods of extracting phosvitin, and preparing phosvitin phosphopeptides.

Summary of the Invention

[0009] The present invention relates to methods for extracting phosvitin from egg yolk, and preparing phosvitin phosphopeptides.

[00010] In one aspect, the invention comprises a process for extracting phosvitin from egg yolk comprising the steps of:

a) contacting the egg yolk or egg yolk protein granules with a salt solution to form a

mixture;

b) adjusting the pH of the mixture to precipitate non-phosvitin proteins and leave phosvitin substantially in solution; and

c) recovering a fraction comprising phosvitin.

In one embodiment, egg yolk is diluted and mixed with water to produce egg yolk protein granules. The mixture of egg yolk or egg yolk proteins and salt solution may be heated, prior to the pH adjustment step. The adjusted pH may be about pH 7.0 or lower.

[00011] In another aspect, the invention may comprise a method of preparing phosvitin phosphopeptides, comprising the steps of dephosphorylating phosvitin, egg yolk, or an egg yolk fraction comprising phosvitin and hydrolyzing the dephosphorylated phosvitin. In one embodiment, the phosvitin may be dephosphorylated with an alkaline solution, such as a solution of sodium hydroxide (NaOH).

[00012] In one embodiment, the extracted phosvitin, or egg yolk, or an egg yolk fraction comprising phosvitin, is hydrolyzed with proteases such as trypsin, pancreatin, pepsin, thermolysin (from *Bacillus thermoproteolyticus rokko*), α–chymotrypsin (bovine pancreas), Protex 6L (from *Bacillus licheniformis*), protex 30L (derived from *Bacillus subtilis*), or protex 7L (from *Bacillus amyloliquefaciens*). The hydrolysis may be conducted using pancreatin at an enzyme/substrate ratio of about 1:50 (w/w). The resulting phosvitin hydrolysate may have a degree of hydrolysis of greater than about 6%, or greater than about 10%. In one embodiment, the degree of hydrolysis is about 12.9%. In one embodiment, the hydrolysate has a nitrogen to phosphorus (N/P) of less than about 5, or less than about 4. In one embodiment, the phosvitin hydrolysate comprises at least one phosvitin phosphopeptide.

[00013] In another aspect, the invention comprises high purity phosvitin obtained by a process as claimed or described herein. In one embodiment, the phosvitin may have a purity in a range of about 50% to about 95%.

[00014] In another aspect, the invention may comprise a phosvitin hydrolysate, which

comprises phosvitin phosphopeptides. The hydrolysate may be produced from high-purity phosvitin, a phosvitin extract having a widely varying purity, from egg yolk itself, or an egg yolk fraction comprising phosvitin.

[00015] In yet another aspect, the invention may comprise a pharmaceutical or nutraceutical composition comprising a high-purity phosvitin, or the above phosphopeptide, in combination with one or more pharmaceutically acceptable carriers, or a foodstuff comprising a high-purity phosvitin, or the above phosphopeptide.

[00016] In yet another aspect, the invention may comprise a method of promoting health by administering to a subject the pharmaceutical or nutraceutical composition or a foodstuff described above, in an amount sufficient to effect a health benefit.

[00017] Additional aspects and advantages of the present invention will be apparent in view of the description, which follows. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

[00018] The invention will now be described by way of an exemplary embodiment with reference to the accompanying simplified, diagrammatic, not-to-scale drawings:

[00019] Figure 1 is a schematic diagram showing one embodiment of the method of the present invention.

[00020] Figure 2 shows gel filtration chromatograms of phosvitin extracts at different pHs (unheated). Absorbance was monitored at 215 nm.

[00021] Figure 3 is a photograph of a SDS-PAGE gel showing the protein profiles of phosvitin extracts prepared at different pHs without heating.

[00022] Figure 4 shows gel filtration HPLC analysis of phosvitin at different pHs after heating.

[00023] Figure 5 is a photograph of a SDS-PAGE gel showing the protein profiles of phosvitin extracts at different pHs with heating.

[00024] Figure 6 shows the elution profile of anion exchange chromatography of dissolved granule solution prepared from egg yolk.

[00025] Figure 7 is a photograph of a SDS-PAGE gel showing the protein 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 phosvitin standard (Sigma, "PVT Std").

[00026] Figure 8 shows the HPLC elution profiles of different fractions during phosvitin purification from egg yolk.

[00027] Figure 9 is a photograph of a SDS-PAGE gel showing the protein profiles of pancreatin hydrolysates of granules and extracted phosvitin (both granules and phosvitin were dephosphorylated by 0.2 M NaOH for 0.5 h before hydrolysis). 1 and 8, molecular weight markers; 2, phosvitin standard; 3 and 4, pancreatin hydrolysates of granules; 5 and 6, pancreatin hydrolysates of purified phosvitin; 7, egg yolk granules.

[00028] Figure 10 shows the HPLC elution profiles of granules, extracted phosvitin and their hydrolysates.

[00029] Figure 11 shows the elution profile of anion exchange chromatography of pancreatin hydrolysates of extracted phosvitin.

[00030] Figures 12A-C show the results of de novo sequencing of one representative peptide LEDDSSSSSSSSVLSK (SEQ ID NO: 30) with 9 phosphate groups from fraction P-4 by using MS/MS spectra. Figure 12A shows the total ion chromatogram of P-4 fraction. Figure 12B shows a mass spectrum of a selected time at 22.62 min. Figure 12C shows the MS/MS spectrum of the ion m/z 1167.7. Losses of H_3PO_4 (98 Da) were detected as can be seen in the MS/MS spectrum. Ions with a (*) represent phosphorylated form of the ions. Ions with a (^{0}) represent dephosphorylated form of the ions.

Detailed Description of Preferred Embodiments

[00031] The present invention relates to methods for extracting phosvitin from egg yolk, and preparing phosvitin phosphopeptides.

[00032] When describing the present invention, all terms not defined herein have their common art-recognized meanings. To the extent that the following description is of a specific embodiment or a particular use of the invention, it is intended to be illustrative only, and not limiting of the claimed invention. The following description is intended to cover all alternatives, modifications and equivalents that are included in the spirit and scope of the invention, as defined in the appended claims.

[00033] "Phosvitin" is a principal phosphoprotein present in egg yolk with a molecular mass of about 35 kD and contains about 10% phosphorus. A "phosvitin extract" is a fraction extracted from egg yolk and which comprises phosvitin.

[00034] A "peptide" is a short polymer of amino acids linked by peptide bonds. A "phosphopeptide" is a peptide which incorporates at least one phosphate group. A "phosvitin phosphopeptide" is derived from phosvitin and may include phosphopeptides derived from proteins which remains associated with phosvitin during the extraction process described herein, such as lipovitellin.

[00035] In one embodiment, the present invention is directed to methods for extracting phosvitin from egg yolk, and preparing phosvitin phosphopeptides. Since the intention is to create value-added phosvitin and phosvitin-derived phosphopeptides, and pharmaceutical compositions and foodstuffs comprising same for human and/or animal consumption, the use of organic solvents and/or non-food compatible chemicals is preferably avoided. The process of the present invention is thus preferably carried out without any organic solvent and/or non-food compatible chemicals. As will be appreciated by those skilled in the art, a process which does not require the use of any organic solvent or toxic chemicals positively impacts the environment and dairy, food, pharmaceutical, and cosmetic industries. Further, the method of the present invention provides purity and recovery rates which may be beneficial in a large scale.

[00036] In one embodiment, the process for extracting phosvitin from egg yolk comprises the steps of:

a) contacting the egg yolk or egg yolk protein granules with a solution having a salt concentration of about 10% to form a mixture;

b) optionally, heating the mixture;

c) adjusting the pH of the mixture to precipitate non-phosvitin components; and

d) recovering a fraction comprising phosvitin.

[00037] As generally shown in Figure 1, the steps of the process to extract phosvitin are generally as follows. Fresh egg yolk, such as chicken egg yolk, is used as the starting material (Example 2). Egg yolk components include protein granules, lipids, and phospholipids. The egg yolk may be used directly, or may initially be diluted with deionized water in a ratio of ranging from 1:1 to 1:20, preferably about 1:2 (v/v), and then centrifuged to yield a precipitate comprising protein granules and a supernatant comprising lipids. The precipitate comprising protein granules is collected and used for phosvitin preparation.

[00038] The protein granules comprise phosvitin in the form of a high density lipoprotein (HDL)-phosvitin complex in protein granules. Since phosvitin is a salt-soluble protein, the protein granules are extracted using a solution having a high salt concentration to solubilize the HDLs and phosvitin, and to yield a supernatant. The high salt solution may comprise any

suitable salt, such as a sodium salt solution, and is used to substantially break up the HDL-phosvitin complex. A salt concentration of about 10% may be suitable. In one embodiment, the solution has a pH of about 7 and the solubilization takes place at a refrigerated temperature, about 4 °C in one example. Without being bound by any theory, it is believed that the use of a sodium salt solution such as NaCl causes the substitution of divalent calcium by monovalent sodium.

[00039] The resulting solubilized phosvitin/salt solution may be dialyzed against distilled water to reduce the salt concentration, if necessary or desired.

[00040] In one embodiment, phosvitin may be extracted without heating. The solubilized phosvitin/salt solution is collected, and the pH is adjusted to extract the phosvitin from HDLs, by precipitating non-phosvitin components such as the HDLs, and leaving the phosvitin in solution. As demonstrated in the Examples and in Table 1 (without heating), the purity of the recovered phosvitin increases as the pH for adjustment increases. In one embodiment, the pH is adjusted to about pH 5.0 or greater.

[00041] In one embodiment, the solubilized phosvitin/salt solution may be heated. In one embodiment, the supernatant from the protein granules-high salt solution is collected and heated at a temperature of about 80 °C for twenty minutes, and then dialyzed against double distilled water. The pH is then adjusted to extract the phosvitin from HDLs. As demonstrated in the Examples and in Table 2 (with heating), the purity of the recovered phosvitin is unaffected by pH; however, at pHs lower than pH 5.0, heat-treated phosvitin extracts show higher phosvitin recovery than that of unheated extracts. As further discussed below, heating appears to release more phosvitin from HDLs. In one embodiment, where the solubilized phosvitin is heated, the pH is adjusted to about pH 5.0 or lower.

[00042] In one embodiment, the pH of the solubilized phosvitin/salt solution may allow for recovery of the phosvitin without pH adjustment. As demonstrated in the Examples and in Tables 1 and 2, native pH (i.e., without pH adjustment) results in the highest recovery with reasonable purity.

[00043] After adjusting the pH in either one of the above embodiments (i.e., with or without heat treatment), the supernatant is centrifuged to yield a precipitate and supernatant comprising crude phosvitin. In one embodiment, centrifugation is conducted at 10,000 x *g* for about twenty-five minutes. In one embodiment, centrifugation is conducted at a temperature of about 4 °C. The crude phosvitin is then freeze-dried or lyophilized for use in various applications.

[00044] The purity of the phosvitin extract can be determined for example, by gel filtration HPLC using a commercial phosvitin standard (e.g., available from Sigma) to prepare a standard curve (Example 14). Recovery can be calculated, for example based on the ratio between phosvitin obtained from the method to the amount of phosvitin in the original egg yolk. As demonstrated in the Examples, the purity of the phosvitin obtained by the methods of the present invention appears to increase at decreasing pHs at the cost of recovery or yield; for example, a recovery of >100% with purity of 54.5% was obtained at native pH for unheated extract, and with greater purity (64% and 75%) for heated extracts.

[00045] In one embodiment, the protein granules may be solubilized at alkaline pHs, followed by isolation of phosvitin using anion exchange chromatography (Example 8). The phosvitin has a purity of 93% with a recovery yield of 57%.

[00046] In one embodiment, the purity of the phosvitin extract resulting from the methods of the present invention is in a range of about 50% to about 95%. In one embodiment, the purity is greater than about 75%, and preferably greater than about 80%. It is believed that the remaining non-phosvitin components primarily comprise HDLs such as lipovitellin.

[00047] In another aspect of the invention, the phosvitin extract may be used to prepare phosphopeptides. In one embodiment, the phosphopeptides of the present invention are produced from phosvitin using the methods described herein (Example 6). The method generally involves at least the steps of dephosphorylating the phosvitin and hydrolyzing the dephosphorylated phosvitin. The resultant hydrolysate comprises phosphopeptides which may

have functional properties and suitability for particular applications such as, for example, as antioxidants, antibacterial or remineralizing agents, or improving mineral absorption in food, feed, cosmetic, dental and pharmaceutical products.

[00048] Phosvitin or an egg yolk fraction comprising phosvitin, such as that produced using the methods described or claimed herein, may be used as the starting material. In the process of bioactive peptide production, the limited enzymatic susceptibility of phosvitin can be considered as a major drawback; thus, the dephosphorylation of phosvitin by alkaline treatment increases the proteolytic susceptibility of phosvitin, and yields phosphopeptides after enzymatic digestion. In one embodiment, the phosvitin is treated with an alkaline solution at a sufficient time and temperature for dephosphorylation. In one embodiment, the alkaline solution is a solution of sodium hydroxide. The mixture is stirred for about half an hour at about room temperature. The pH may then be adjusted to about 8.0 using an acidic solution. In one embodiment, the acidic solution is a solution of hydrochloric acid.

[00049] The dephosphorylated phosvitin is then hydrolyzed with an enzyme such as trypsin, pancreatin, pepsin, thermolysin (from *Bacillus thermoproteolyticus rokko*), α–chymotrypsin (bovine pancreas), Protex 6L (from *Bacillus licheniformis*), Protex 30L (derived from *Bacillus subtilis*), or Protex 7L (from *Bacillus amyloliquefaciens*). In one embodiment, the enzyme comprises a a digestive enzyme, under selected conditions of pH and temperature, to yield phosvitin phosphopeptides. In one embodiment, the enzyme comprises pancreatin. As used herein, the term "pancreatin" refers to a mixture of several digestive enzymes produced by the exocrine cells of the pancreas, and is composed of amylase, lipase and protease. In one embodiment, hydrolysis with pancreatin is conducted at an enzyme/substrate ratio of about 1:50 (w/w). In one embodiment, hydrolysis is conducted at a pH of about 8.0, and at a temperature of about 37 °C. In one embodiment, hydrolysis is conducted for at least about three hours. If needed, the pH is adjusted to about 8.0 by adding an alkaline solution, such as sodium hydroxide. The hydrolysate solutions are then treated to inactivate the enzymes, such as by heat inactivation. In one embodiment, the hydrolysate solutions are heated at about 95 °C for about fifteen minutes. The hydrolysate solutions are then centrifuged to separate the soluble hydrolysates from non-soluble substances. In one embodiment, centrifugation is conducted at 10,000 x *g* for

about thirty minutes. In one embodiment, centrifugation is conducted at a temperature of about 4 °C. The soluble hydrolysates may be freeze-dried for storage and future use.

[00050] The hydrolytic degradation of the phosvitin can be confirmed, for example, by gel filtration HPLC using a commercial phosvitin standard (e.g., available from Sigma) to prepare a standard curve (Example 14). The peptide/protein yield can be calculated, for example as the amount of protein in hydrolysate from 100 g yolk solids. The peptide/protein recovery can be calculated, for example as a percentage based on the weight ratio of amount of peptide/protein in the hydrolysate to the total protein in the egg yolk. As demonstrated in the Examples, egg yolk phosvitin was hydrolyzed by pancreatin to reach a degree of hydrolysis of 12.9% with a recovery of 6.1% based on egg yolk.

[00051] The soluble hydrolysates may be fractionated using anion exchange chromatography or ultra-filtration to yield one or more fractions. The properties of the fractions may be further analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to identify the peptides within the fractions.

[00052] In one embodiment, the soluble hydrolysates are fractionated using anion exchange chromatography to identify a fraction which the highest phosphorus content compared to any other fractions. In one embodiment, this fraction comprises at least one phosvitin or lipovitellin phosphopeptide comprising at least one phosphoserine residue.

[00053] In another aspect, the invention comprises pharmaceutical or nutraceutical compositions comprising a phosvitin extract, a phosvitin phosphopeptide or both in combination with one or more pharmaceutically acceptable carriers. Those skilled in the art are familiar with any pharmaceutically acceptable carrier that would be useful in this regard, and therefore the procedure for making pharmaceutical compositions in accordance with the invention will not be discussed in detail. Suitably, the pharmaceutical compositions may be in the form of tablets, capsules, liquids, lozenges, lotions, aerosol, and solutions suitable for various routes of administration including, but not limited to, orally, via injection or infusion, intraperitoneally, topically, nasally, ocularly, vaginally or rectally, in solid, semi-solid or liquid dosage forms as

appropriate and in unit dosage forms suitable for easy administration of fixed dosages.

[00054] In one embodiment, the invention comprises a nutraceutical or a foodstuff comprising a phosvitin extract, a phosvitin phosphopeptide or both. A nutraceutical is a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with foods. A nutraceutical generally has a physiological benefit or provides protection against disease. A foodstuff may be any compound or composition which is intended and suitable for human and/or animal consumption, including compounds and compositions which are consumed per se as well as compounds and compositions used as ingredients, components, and/or flavorings in other compositions.

[00055] It is well known to those skilled in the art that phosvitin and phosvitin phosphopeptides may provide potential health benefits, including antioxidant, anti-bacterial, metal chelating abilities, and calcium uptake into bones. In one embodiment, the invention comprises a method of providing a health benefit by administering to a subject a phosvitin extract, a phosvitin phosphopeptide or both, a pharmaceutical composition, a nutraceutical composition, a cosmetic product, a dental product or a foodstuff comprising the phosvitin extract, phosphopeptide or both, in an amount sufficient to effect any of the above health benefits.

[00056] The following is a specific example of one embodiment of the present invention. This example demonstrates one embodiment of the method of the present invention to extract phosvitin from egg yolk, and the production of phosphopeptides. This example is offered by way of illustration and is not intended to limit the invention in any manner.

[00057] High density lipoproteins (HDLs) have been reported to be soluble at alkaline pHs, insoluble at pH 7.5-7.8 for α-HDL and at pH 6.5-7.0 for β-HDL, but soluble at pH 4.5-5.0. In a report on the solubility between pH 5 to 7, the solubility of phosvitin was not affected, whereas HDLs were completely precipitated under pH 5.5 (Castellani *et al.*, 2003). The isoelectric point of phosvitin was reported to be around pH 4 (Ternes, 1989). In view of such reports, the effect of pH upon the extraction of phosvitin was initially examined in order to

determine whether phosvitin and HDLs might be separated by pH adjustments.

[00058] Phosvitin extracts prepared at various pHs without heat treatment were analyzed by gel filtration chromatography (Figure 2). Phosvitin prepared by different procedures is typically heterogeneous in gel filtration HPLC and electrophoresis (McBee and Cotterill, 1979; Abe *et al.*, 1982; Wallace and Morgan, 1986a; Wallace and Morgan, 1986b). Standard phosvitin (Sigma) was fractionated into three major components. The first peak having a larger molecular weight is generally considered to represent HDL contaminant, while the other two peaks represent β-phosvitin (190 kDa) and α-phosvitin (160 kDa) (Tsutsui and Obaba, 1984).

[00059] The profile of the extract at native pH consisted of five components other than phosvitin. Fractions A and B may represent low density lipoprotein (LDL) and HDL respectively, accounting for 27.3% of the total protein. Fractions C, D and E may represent phosvettes or protein fractions from HDL or LDL based on the molecular weight, accounting for 15.4%. A shoulder peak in α–phosvitin probably suggests another contaminant in phosvitin. The phosvitin and contaminants tended to decrease at decreasing pHs. Fractions A and B decreased from 27.3% of total proteins to almost 0% at pH 3.5. Phosvitin purity first increased to 63.7% at pH 5.5, and then decreased to 39.6% at pH 3.5. Fractions C, D and E were not affected until reaching a pH lower than 5, where fraction C was eliminated. The proportion of these three fractions increased from 15.4% at native pH to 47.7% at pH 3.5.

[00060] The recovery, yield and purity of the phosvitin extract were compared against a commercial phosvitin standard (Sigma). As shown in Table 1, the purity of phosvitin extracts ranged from 39.6% to 63.7%. The best purity obtained was 63.7% at pH 5.5, with a recovery of 40.1%. The yield and recovery also decreased at decreasing pHs. The highest recovery was obtained at native pH, which was 109.60%, but was greatly decreased to 4.0% at pH 3.5. The fact that phosvitin's pI value is around 4.0 (Ternes, 1989) may explain the low recovery of phosvitin at low pH.

Table 1. Recovery and purity of phosvitin from egg yolk (without heating)

pH	Purity (area %)	Phosvitin yield (g/100g yolk solids)	Phosvitin recovery (4g phosvitin in 100g yolk solids)
Native	54.5±1.2	4.4±0.1	109.6±3.1
7.0	57.8±3.1	3.8±0.1	94.4±2.1
6.5	61.2±1.3	2.4±0.2	60.2±3.8
6.0	63.5±0.2	1.9±0.0	46.5±1.0
5.5	63.7±0.5	1.6±0.0	40.1±1.0
5.0	60.0±0.3	1.2±0.0	30.8±0.9
4.5	56.6±2.4	0.8±0.0	20.9±1.0
4.0	51.9±4.6	0.5±0.1	11.9±1.4
3.5	39.6±6.4	0.16±0.0	4.0±0.5

[00061] SDS-PAGE was used to analyze the composition of phosvitin extracts. The phosvitin standard has a molecular weight ranging from 30 kDa to 45 kDa as shown by SDS-PAGE (Figure 3). The phosvitin extract at native pH has a major band within this range, as well as nine other bands having molecular weights of 240, 130, 80, 70, 35, 30, 25 and 20 kDa. These contaminating bands might arise from the large molecular weight fractions (A, B) and small molecular weight fractions (C, D, E) in HPLC. A significant decrease of the intensity of the large molecular weight bands was observed. The band at 240 kDa was absent at pH 6.0, which was inconsistent with the results of gel filtration chromatography. At pH 4.5, proteins bands with molecular weights of 130, 80, and 70 kDa were absent. Phosvitin was overlapped by other two protein bands having molecular weights of about 35 kDa. Without being bound by theory, these results suggest that high recovery (>100%) of phosvitin can be obtained at native pH with a purity of 54.5%. A decrease in pH can reduce large molecular weight contaminants, but also leads to loss in yield and recovery.

[00062] Phosvitin is reportedly heat resistant since heating at 110 °C for twenty minutes does not cause aggregation or insolubility (Alberight *et al.*, 1984). Anton *et al.* (2000) reported a great loss of HDLs' solubility upon heating granules at 79 °C in 0.5 M sodium chloride solution for about three minutes. The effect of heat treatment upon phosvitin purity and recovery was thus determined. After egg yolk protein granules were totally dissolved in 10% sodium chloride, the extraction solution was heated at 80 °C for twenty minutes. There appears to be less contaminant present in heated extracts compared to unheated extracts (Figure 4). At the native

pH, fractions A and B accounted for about 7.5%, while fractions D and E accounted for 10.5% of total protein. As the pH was lowered to 5.5, fractions A and B were no longer present. Fractions D and E accounted for 12.7% of total protein of the extract. Further lowering the pH to 3.5 did not eliminate fractions D and E. Without being bound by theory, these results suggest that heating reduced the solubility of HDL, thereby improving the separation of HDL from phosvitin.

[00063] The purity of heat-treated phosvitin extracts ranged from 75%-80%, which was significantly higher than the purity of unheated extracts (Table 2). The purity was unaffected by pH. The phosvitin yield and recovery were lower than those for unheated extracts at pH greater than 5.0. At pH lower than 5.0, heat-treated phosvitin extracts showed significantly higher phosvitin recovery than those of unheated extracts. Without being bound by theory, heating together with 10% sodium chloride extraction may more effectively denature HDL and break the phosphocalcic bridge to release more phosvitin from HDL. HDL precipitated when the pH was lowered, but phosvitin remained in the extracts. With heat treatment, recovery and purity of the phosvitin extracts improved.

[00064] **Table 2. Recovery and purity of phosvitin from egg yolk (with heating)**

pH	Purity (area %)	Phosvitin yield (g/100g yolk solids)	Phosvitin recovery (4g phosvitin in 100g yolk solids)
Native	75.7±1.0	2.6±0.1	64.2±2.4
7.0	76.2±0.5	2.2±0.1	54.5±2.2
6.5	76.7±0.3	1.8±0.1	45.3±2.0
6.0	78.6±0.1	1.6±0.1	40.3±1.7
5.5	79.7±0.7	1.6±0.0	39.0±0.1
5.0	80.1±1.0	1.6±0.1	38.9±2.0
4.5	79.5±1.2	1.4±0.0	33.9±0.2
4.0	79.1±1.3	1.2±0.1	30.7±1.3
3.5	79.3±1.3	1.1±0.1	28.6±2.3

[00065] Figure 5 shows the SDS-PAGE profile of phosvitin extracts at different pHs (with heating). The phosvitin extract at native pH was resolved into nine proteins subunits, with molecular weights of 130, 100, 80, 70, 45, 40, 35, 30 and 10 kDa. The standard phosvitin (Sigma) was resolved into two bands, with molecular weights of 35 and 30 kDa, respectively. In

HPLC, the large molecular weight fractions A and B were absent at around pH 6.0. In SDS-PAGE, a significant reduction of protein bands with molecular weights of 130, 80 and 10 kDa at this pH was observed. From pH 5 and below, there were only two minor contaminants at molecular weights of 130 and 100 kDa. These results concur with the results of HPLC analysis. Based upon these results, heating at 10% sodium chloride extraction may improve the phosvitin purity from 63.7% to 80.1% (optimum purity of both experiments), whereas recovery decreased from 109.6% to 64.2%.

[00066] The production of phosvitin phosphopeptides comprises the steps of dephosphorylation and hydrolysis. As shown in Figure 9, no phosvitin or large molecular weight proteins were detected in phosvitin hydrolysate. It has been reported that without dephosphorylation, hydrolysis of phosvitin by pepsin, trypsin and α-chymotrypsin produces only two to four small fractions from the C- and N-termini, leaving one major fraction of 28-29 kDa as phosvitin core (Goulas *et al.*, 1996; Khan *et al.*, 1998; Jiang and Mine, 2000; Wallace and Morgan, 1986). Only two bands were detected at 6.5 kDa and 3.5 kDa (Figure 9, lanes 5 and 6).

[00067] As shown in Figure 10, gel filtration chromatography indicated that phosvitin can be degraded after dephosphorylation. The two phosvitin peaks, α- and β-phosvitin, were degraded into smaller fractions.

[00068] In one embodiment, the phosvitin is hydrolyzed to a degree of hydrolysis greater than about 6%, and preferably greater than about 10%. In one embodiment, the degree of hydrolysis (DH), recovery and yield are summarized in Table 3. The DH of phosvitin using pancreatin after dephosphorylation was 12.9%, which exceeds a reported DH of 5.2% (Chay Pak Ting *et al.*, 2011).

[00069] In one embodiment, the hydrolysate has a nitrogen to phosphorus (N/P) of less than about 5, and preferably less than about 4. It is preferable to have a low N/P ratio for phosphopeptides. In one embodiment, the N/P ratio of phosvitin hydrolysate was 3.9, which is lower than both purified casein phosphopeptides with the N/P ratio ranging from 6 to 8 (Peterson *et al.*, 1958; Bennich *et al.*, 1959) and the phosvitin hydrolysate with the N/P ratio of 11.8 (Chay

Pak Ting *et al.*, 2011).

Table 3. Degree of hydrolysis, protein yield, protein recovery and N/P value of pancreatin-digested phosvitin

Pancreatin hydrolysis	DH %	Protein yield mg/g wet yolk	Protein recovery from egg yolk %	N/P atomic ratio
phosvitin	12.9 ± 3.1	10.4 ± 0.3	6.1 ± 0.2	3.9 ± 0.1

[00070] Phosvitin hydrolysate was fractionated by an anion exchange column, resulting in four fractions representing 0.3%, 24.2%, 2.4% and 73.0% of the total phosphorus content (Figure 11). The last fraction P-4 exhibiting the highest phosphorus content was either dephosphorylated with phosphatase or used directly for structural characterization (Figures 12A-C).

[00071] Thirty-two peptides were identified from fraction P-4 derived mainly from three phosvitin domains: AEFGTEPDAKTSSSSSSASSTATSSSSSSASSPN (PV 1-34; SEQ ID NO: 1), KPMDEEENDQV (PV 37-47; SEQ ID NO: 9) and SGHLEDDSSSSSSSSVLSKIWG (PV 190-211; SEQ ID NO: 14); and six peptides were identified from one lipovitellin domain: IITEVNPESEEEDESSPYEDI (VTG 1056-1076; SEQ ID NO: 36) (Table 4). Peptides with 6-10 phosphate groups were identified from domain PV 190-211 (SEQ ID NO: 14). Although no phosphorylation was identified in the peptides derived from domain PV 1-34 (SEQ ID NO: 1), it was possible that phosphopeptides may have been generated but could not be retained by the dC-18 column prior to MS/MS analysis. Young *et al.* (2011) reported phosvitin peptides from three domains: PV 4-40, PV 155-197 and PV 244-257. PV 4-40 and PV 244-257 partially overlapped with the domains detected herein. The last domain PV 244-257 (EDDSSSSSSSSVLSKIWGRHEIYQ; SEQ ID NO: 38) overlapped with the domain PV 190-211 detected herein. Phosvitin contains 217 total amino acids (Byrne *et al.*, 1984; Goulas *et al.*, 1996). Peptides from the domain PV 155-197 were not detected. Without being bound by theory, this result may be due to the different enzymes which were used (i.e., pancreatin versus trypsin). Young *et al.* (2011) reported only two phosphorylated peptides out of thirteen peptides with one and three phosphate groups, although the same group previously reported that the peptides prepared contained 18.9% phosphorus (Katayama *et al.*, 2006).

[00072] As summarized in Table 4, five phosphorylated peptides were identified containing six to ten phosphate groups (SEQ ID NOS: 17, 24, 27, 30 and 33). Peptides with the same amino acid sequences might contain different numbers of phosphate groups. Peptide sequences identified from native and dephosphorylated peptides were combined together. Sequences including bold letters were found in both native and dephosphorylated peptides. Sequences including italic letters were found in both phosvitin hydrolysate and granules hydrolysate. M/Z values were obtained by mass spectrometry. Mr (expt) is the determined molecular mass of the peptides, and Mr (calc) is the theoretical mass of the closest matching peptides. Phosphorylation was identified from native peptides without dephosphorylation. In some cases, one peptide contains a different number of phosphate groups. The smallest peptides from each domain comprised 8, 9 or 10 consecutive amino acid residues.

Table 4. Peptides identified in anion exchange chromatography fraction P-4 (pancreatin hydrolysate of extracted phosvitin)

	Position	Sequence	M/Z	Z	Mr (Expt)	Mr (Calc)
Domain I	1112-1145	AEFGTEPDAKTSSSSSSASSTATSSSSSSASSPN (PV 1-34; SEQ ID NO: 1)				
1	1115-1133	GTEPDAKTSSSSSSASSTA (SEQ ID NO: 2)	879.4	2	1756.8	1756.8
2	1112-1120	**AEFGT(P)EPDA** (SEQ ID NO: 3)	936.4	1	935.4	935.4
3	1112-1131	AEFGTEPDAKTSSSSSSASS (SEQ ID NO: 4)	966.9	2	1931.9	1931.8
4	1112-1119	*AEFGTEPD* (SEQ ID NO: 5)	865.4	1	864.4	864.4
5	1120-1145	*AKTSSSSSSASSTATSSSSSSASSPN* (SEQ ID NO: 6)	1169.5	2	2337.1	2337.0
6	1115-1145	GTEPDAKTSSSSSSASSTATSSSSSSASSPN (SEQ ID NO: 7)	1419.1	2	2836.2	2836.2
7	1122-1145	*TSSSSSSASSTATSSSSSSASSPN* (SEQ ID NO: 8)	1070.0	2	2137.9	2137.9
Domain II	1148-1158	KPMDEEENDQV (PV 37-47; SEQ ID NO: 9)				
8	1148-1158	**KPMDEEENDQV** (SEQ ID NO: 10)	667.3	2	1332.6	1332.6
9	1151-1158	***DEEENDQV*** (SEQ ID NO: 11)	489.2	2	976.4	976.4
10	1148 -	KPMDEEENDQ (SEQ ID NO: 12)	667.3	2	1332.	1332.

	1157				6	6
11	1150 - 1158	MDEEENDQV (SEQ ID NO: 13)	554.7	2	1107.4	1107.4
Domain III	1301-1322	SGHLEDDSSSSSSSSVLSKIWG (PV 190-211; SEQ ID NO: 14); 6-10 phosphorylation**				
12	1308-1320	SSSSSSSSVLSKI (SEQ ID NO: 15)	628.3	2	1254.6	1254.6
13	1306-1318	DDSSSSSSSSVLS (SEQ ID NO: 16)	622.8	2	1243.5	1243.5
14	1301-1319	***SGHLEDDSSSSSSSSVLSK*** (10P; SEQ ID NO: 17)	948.4	2	1894.9	1894.8
15	1307-1319	*DSSSSSSSSVLSK* (SEQ ID NO: 18)	629.3	2	1256.6	1256.6
16	1304-1317	LEDDSSSSSSSSVL (SEQ ID NO: 19)	700.3	2	1398.6	1398.6
17	1306-1319	*DDSSSSSSSSVLSK* (SEQ ID NO: 20)	1372.6	1	1371.6	1371.6
18	1307-1320	*DSSSSSSSSVLSKI* (SEQ ID NO: 21)	685.8	2	1369.7	1369.7
19	1312-1320	SSSSVLSKI (SEQ ID NO: 22)	454.3	2	906.5	906.5
20	1310-1320	SSSSSSVLSKI (SEQ ID NO: 23)	541.3	2	1080.6	1080.6
21	1301-1318	***SGHLEDDSSSSSSSSVLS*** (8P; SEQ ID NO: 24)	884.4	2	1766.8	1766.7
22	1305-1322	EDDSSSSSSSSVLSKIWG (SEQ ID NO: 25)	929.4	2	1856.8	1856.8
23	1304-1322	LEDDSSSSSSSSVLSKIWG (SEQ ID NO: 26)	986.0	2	1969.9	1969.9
24	1301-1320	**SGHLEDDSSSSSSSSVLSKI** (6-7P; SEQ ID NO: 27	1005.0	2	2007.9	2007.9
25	1306-1320	*DDSSSSSSSSVLSKI* (SEQ ID NO: 28)	1485.7	1	1484.7	1484.7
26	1304-1318	*LEDDSSSSSSSSVLS* (SEQ ID NO: 29)	743.8	2	1485.6	1485.6
27	1304-1319	***LEDDSSSSSSSSVLSK*** (7, 8, 9P; SEQ ID NO: 30)	807.9	2	1613.7	1613.7
28	1307-1322	DSSSSSSSSVLSKIWG (SEQ ID NO: 31)	807.4	2	1612.8	1612.8
29	1301-1317	SGHLEDDSSSSSSSSVL (SEQ ID NO: 32)	840.9	2	1679.7	1679.7
30	1304-1320	***LEDDSSSSSSSSVLSKI*** (7P; SEQ ID NO: 33)	864.4	2	1726.8	1726.8
31	1306-1317	DDSSSSSSSSVL (SEQ ID NO: 34)	1157.5	1	1156.5	1156.5
32	1306-	*DDSSSSSSSSVLSKIWG* (SEQ ID NO: 35)	864.9	2	1727.	1727.

	1322				8	8
Domain IV	1056-1076	IITEVNPESEEEDESSPYEDI (lipovitellin; SEQ ID NO: 36)				
33	1056-1072	***IITEVNPESEEEDESSP*** (SEQ ID NO: 37)	952.4	2	1902.8	1902.8
34	1060-1076	*VNPESEEEDESSPYEDI* (SEQ ID NO: 38)	984.4	2	1966.8	1966.8
35	1059-1076	***EVNPESEEEDESSPYEDI*** (SEQ ID NO: 39)	1048.9	2	2095.8	2095.8
36	1068-1076	DESSPYEDI (SEQ ID NO: 40)	1054.4	1	1053.4	1053.4
37	1059-1072	***EVNPESEEEDESSP*** (SEQ ID NO: 41)	788.8	2	1575.6	1575.6
38	1060-1073	*VNPESEEEDESSPY* (SEQ ID NO: 42)	805.8	2	1609.6	1609.6

[00073] Exemplary embodiments of the present invention are described in the following Examples, which are set forth to aid in the understanding of the invention, and should not be construed to limit in any way the scope of the invention as defined in the claims which follow thereafter.

Example 1 – Reagents

[00074] Sodium chloride, sodium hydroxide, and hydrochloric acid were purchased from Fisher Scientific (Nepean, ON, Canada). Glycine, Precision Plus™ Protein Standard, sodium dodecyl sulfate (SDS), and precast gel (10-20% Tris-HCl) were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Phosvitin standard (P1253, from chicken egg yolk) from chicken egg yolk was purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada). The deionized distilled water used for all the experiments and was produced by a Barnstead™ water purification system (Thermo Fisher Scientific, Waltham, MA, USA). Trypsin (T4799-10G, from porcine pancreas), pancreatin (p7545-25G, from porcine pancreas), pepsin (p7000-25G, from porcine gastric mucosa), thermolysin (p1512-25MG, from *Bacillus thermoproteolyticus rokko*) and α-chymotrypsin (c4129-1G, from bovine pancreas) were purchased from Sigma-Aldrich, Ltd. (Oakville, ON, Canada). Protex 6L (4880883444, from *Bacillus licheniformis*), Protex 30L (4880873541, derived from *Bacillus subtilis*) and Protex 7L (4860881508, derived from *Bacillus amyloliquefaciens*) were purchased from Danisco US Inc. (Rochester, NY, USA). All the other chemicals were purchased from Sigma-Aldrich, Ltd. (Oakville, ON, Canada) or Fisher Scientific

(Nepean, ON, Canada) unless otherwise specified.

Example 2 - Preparation of egg yolk

[00075] Eggs were purchased from a local supermarket on the day of the experiment. For each egg, the shell was manually broken, and the yolk was separated from the egg white and rolled on Whatman™ filter paper (Whatman Inc., Florham Park, New Jersey) to remove albumen and chalazes adhering to the vitellin membrane. This membrane was then perforated to collect unspoiled egg yolk in a beaker cooled in iced water.

Example 3 - Preparation of protein granules

[00076] Protein granules were obtained as described by McBee and Cotterill (1979) with slight modifications. Yolk was twice diluted with deionized water, and mixed by magnetic stirring for one hour at 4 °C. The mixture was centrifuged at 10,000 g for forty-five minutes at 4 °C. The precipitate (i.e., protein granules) was collected for phosvitin preparation.

Example 4 - Phosvitin preparation

[00077] The protein granules were re-suspended in 10% sodium chloride, maintaining a pH of 7.25 at 4°C. The solution was collected and divided into several beakers for pH experiments. The pH of the aliquots was adjusted to 7, 6.5, 6.0, 5.5, 5.0, 4.5, 4.0 and 3.5, and the supernatant was stirred with magnetic stirring for one hour, followed by centrifugation at 10,000 x *g* at 4 °C for twenty-five minutes. The precipitate was removed and the supernatant containing phosvitin was filtered through a 0.22 μm membrane for HPLC analysis.

Example 5 - Phosvitin preparation with heat treatment during NaCl extraction

[00078] The protein granules were re-suspended in 10% sodium chloride, maintaining the pH at 7.25. The solution was first heated at 80 °C for twenty minutes, and then dialyzed against double distilled water. The steps which follow are the same as described in Example 4 for phosvitin preparation. Samples at different pHs were analyzed by gel filtration HPLC.

Example 6 - Anion exchange chromatography

[00079] The precipitate (i.e., the protein granules) was collected and re-suspended in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Granules were completely dissolved after one to two

hours of magnetic stirring. The granule solution was filtered through a 0.22 μm filter. Samples were loaded to a HiPrep™ Q FF 16/10 anion exchange column (GE Healthcare Life Sciences, 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 Biosciences, Pittsburgh, PA, USA) at a flow rate of 2 mL/min, with buffer A being 0.05 M carbonate-bicarbonate buffer (pH 9.6), and buffer B being 0.5 M sodium chloride in buffer A. The column was first run for six bed volumes, with increasing buffer B from 0% to 45%, and then to 75% in twenty bed volumes. The effluent was monitored at 280 nm.

[00080] Prepared granules were dissolved in 0.05 M carbonate-bicarbonate buffer (pH 9.6), and loaded onto an anion exchange column (Figure 6). Three well resolved peaks eluted from the column at 315, 442, and 542 ml, which accounted for 20, 32, and 11% (w/w) of the loaded amount, apart from 36% (w/w) of loaded amount which passed through the column as unbound.

[00081] The eluted peaks were analyzed by native SDS-PAGE against phosvitin standard (Figure 7). The gel was first stained using Coomasssie brilliant blue, which shows no phosvitin bands (upper gel). The gel was then again stained using Coomassie brilliant blue and aluminum nitrate (lower gel). Phosvitin bands were observed in yolk, granules and peak 3 at position around 37 kDa (arrows). Standard phosvitin (Sigma) has a molecular weight of 34 kDa. These results indicated that phosvitin was successfully isolated from granules by an anion exchange column. Peak 2 was mainly HDL as compared with the granules band (data not shown). Anion exchange chromatography was very effective in fractionating phosvitin from the granule solution.

[00082] Gel filtration HPLC analysis further showed peak 3 has a calculated purity of 93% against standard phosvitin, at a recovery of 57.7% (Figure 8).

Example 7 – Dephosphorylation and Hydrolysis of phosvitin

[00083] Lyophilized phosvitin extract was dephosphorylated in 0.2 M NaOH at room

temperature (25 °C) for 0.5 hours. After adjusting the pH of slurry to 8.0 with 3 M HCl, pancreatin was added at a ratio of 1/50 (enzyme/phosvitin, w/w). Incubation was carried out in a 100 mL jacket beaker at constant temperature of 37 °C for 3 hours, and the pH was maintained constant during the incubation by adding of 0.2 M NaOH if necessary. To terminate the enzymatic reactions, the temperature was raised to 95 °C and held for 15 minutes. Hydrolysates were centrifuged at 10,000 x *g* for 30 minutes at 4 °C and lyophilized.

Example 8 - Molecular weight (MW) distribution of hydrolysates

[00084] A Superdex™ peptide 10/300 GL column (GE Healthcare, Piscataway, NJ, USA) was used to determine the molecular weight distribution of hydrolysates on an AKTA™ Explorer 10S system (GE Healthcare, Piscataway, NJ, USA). Samples prepared at 0.5 mg/ml in 30% aqueous acetonitrile containing 0.1% trifloroacetic acid were injected into the column at a volume of 100 µL and eluted with the same buffer. Elution was monitored at 215 nm. Molecular weights were calculated according to a calibration curve created by running molecular weight markers under identical conditions.

[00085] Pancreatin hydrolysate from granules and phosvitin were prepared at 15 mg/mL with buffer A (0.05M Tris-HCl at pH 8.0) and filtered through a 0.45 µm membrane (Millipore, Billerica, MA, USA) before loading to the HiPrep 16/10 Q FF anion exchange column. The elution was monitored at 215 nm and operated by the AKTA Explorer 10S system (GE Healthcare, Piscataway, NJ, USA) from 100% buffer A to 100% buffer B (1.0 M NaCl in buffer A). Fractions were collected and lyophilized for analysis.

Example 9 - Dephosphorylation of fraction G-4 and P-4 prior to LC-MS/MS

[00086] Fraction G-4 and P-4 from anion exchange chromatography were treated with phosphatase (E/S=1/50, w/w) in a buffer containing 10 mM Tris-HCl, 5 mM $MgSO_4$, and 1 mM $ZnCl_2$ at pH 9.0 according to the manufacturer's specification. The incubation was carried out at 37 °C for 3 h and terminated by increasing temperature to 80 °C for 10 min. Free phosphates and salts were removed by ZipTip™ pipette tip (reverse phase, 10 µL, Millipore, Billerica, MA, USA) and peptide samples were concentrated by Savant™ SpeedVac Concentrators (Fisher Scientific ltd., Nepean, On, Canada) for mass spectrometry analysis.

Example 10 - Liquid Chromatography-Mass Spectrometry-Mass Spectrometry (LC-MS/MS)

[00087] Identification of peptides in both native and dephosphorylated G-4 fraction was carried out by a Waters ACQUITY UPLC system connected to a Waters Micro Mass Q-TOF Premier Instrument (Milford, MA, USA). Samples were first separated by a Waters Atlantis dC18 UPLC column (150 mm×75 μm, 3 μm; Milford, MA, USA) before MS/MS analysis. Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in aceonitrile. Sample (5-10 μL) was injected to a 5 μm trapping column and trapped for 2 min at a flow rate of 10 μL/min by running 99% solvent A. The flow rate was decreased to 0.35 μL/min when the gradient was decreased from 99% A to 90% A in 5 min, to 70% A in 30 min, to 60% in 3 min, and to 5% A in 1 min. The flow rate was increased to 0.50 μL/min and held for another 2 min, and then the gradient was increased from 5% A to 98% A in 1 min and held for another 27 min. Then the flow rate was decreased to 0.35 μL/min in 1 min. The elute was ionized trough a nanoLockspray ionization source in a positive ion mode (capillary voltage of 3.80 kV and source temperature of 100 °C) before being loaded into the mass spectrometer. A Q-TOF analyzer was used to determine the mass of peptides and the mass/charge (m/z) range was set as 400-1600 in MS mode and 50-1990 in MS/MS mode. Instrument was controlled and data was collected by using MassLynx™ software (Micromass U.K. Ltd., Wythenshawe, Manchester, U.K.). Peaks Viewer™ 5.2 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used for peptide sequencing.

Example 11 - Nitrogen and protein determination

[00088] Crude protein and nitrogen were determined (N x 6.25) in duplicate using a LECO™ FP-428 nitrogen determinator (LECO Corporation, St. Joseph, MI, USA), and crude protein was calculated as N x 6.25.

Example 12 - Phosphorus determination

[00089] The phosphorus content was determined by using a malachite green phosphate assay kit (BioAssay Systems, Hayward, CA, USA).

Example 13 - Sodium dodecyl sulfate polyacrylamide gel electrophoresis

[00090] SDS-PAGE was conducted using a continuous system (10-20% gel) (Mini-PROTEAN Tetra Cell, Bio-Rad, Hercules, California, USA) (Shapiro *et al.*, 1967). The following running buffer was used: Tris-HCl (pH 8.8) with 0.1% SDS. All samples were incubated at 95 °C for five minutes, and centrifuged at 15,000 x *g* for five minutes. 20 µl of each supernatant was loaded onto the gel. Electrophoresis was conducted at a constant voltage of 200 V for about forty minutes. The molecular weight markers (Bio-Rad Precision Plus™ Protein Standards) included 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa bands. Staining buffer was prepared as described by Hegenauer *et al.* (1977): 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. 7% acetic acid solution was used for destaining. Images of gels were captured by an Alphachem™ SP machine. Molecular weight (MW) was analyzed by AlphaEase™ FC software (version 6.0.0, Alpha Innotech Corporation, Santa Clara, CA, USA).

Example 14 - Gel Filtration HPLC

[00091] Gel filtration was carried out by HPLC (Waters, Milford, Massachusetts, USA) in a TSK-Gel™ G3000SWXL stainless column (0.78 x 30 cm, Tosoh, Tokyo, JP). Phosvitin and its hydrolysate were prepared at 2 mg/mL with 0.1 M sodium phosphate buffer (containing 0.2 NaCl, pH 7.0) and filtered through a 0.45 µm PVDF filter (13 mm x 0.20 µm, MANDEL, Guelph, ON, Canada) before loading to a TSK-Gel™ G3000SWxL stainless column (0.78 x 30 cm, Tosoh Bioscience, Inc., South San Francisco, CA, USA). Samples (30 µL) were eluted by 0.1 M sodium phosphate buffer (containing 0.2 M NaCl, pH 7.0) at a flow rate of 0.5 mL/min on a Waters HPLC system (Waters, Milford, MA, USA), and the elution was detected at 215 nm. System control and data analysis were performed using Empower™ II Software (Waters, Milford, MA, USA).

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[00092] The following references are incorporated herein by reference (where permitted) as if reproduced in their entirety. All references are indicative of the level of skill of those skilled in the art to which this invention pertains.

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WHAT IS CLAIMED IS:

1. A process for extracting phosvitin from egg yolk comprising the steps of:

a) contacting the egg yolk or egg yolk protein granules with a salt solution to form a mixture;

b) adjusting the pH of the mixture to precipitate non-phosvitin proteins and leave phosvitin substantially in solution; and

c) recovering a fraction comprising phosvitin.

2. The process of claim 1, wherein the egg yolk is diluted and mixed with water to produce egg yolk protein granules.

3. The process of claim 1 or 2, wherein pH of the mixture is about 7.

4. The process of claim 1, wherein the egg yolk or egg yolk protein granules are mixed with the salt solution at a temperature of about 4 °C.

5. The process of claim 1, wherein the egg yolk is mixed with the salt solution and heated.

6. The process of claim 5 wherein the mixture is heated to about 80 °C.

7. The process of claim 1, wherein the salt solution is a 10% sodium chloride solution.

8. The process of claim 1, wherein the pH is adjusted to about pH 7.0 or lower.

9. The process of claim 8, wherein the pH is adjusted to about pH 5.0 to about pH 7.0.

10. The process of claim 8, wherein the pH is adjusted to a pH of about 5.0 or lower.

11. The process of claim 1, wherein after step (b), the mixture is centrifuged to remove precipitated components.

12. A process of preparing phosvitin phosphopeptides, comprising the steps of extracting phosvitin from egg yolk using the method of claim 1, dephosphorylating the phosvitin and hydrolyzing the dephosphorylated phosvitin.

13. The process of claim 12 wherein the phosvitin is dephosphorylated with an alkaline solution.

14. The process of claim 13 wherein the alkaline solution comprises NaOH.

15. The process of claim 12, wherein the phosvitin is hydrolyzed with a protease.

16. The process of claim 15 wherein the protease comprises pancreatin.

17. The process of claim 16, wherein hydrolysis is conducted using pancreatin at an enzyme/substrate ratio of about 1:50 (w/w).

18. The process of claim 16 wherein the phosvitin is hydrolyzed to a degree of hydrolysis of greater than about 6%, or greater than about 10%.

19. The process of claim 18 wherein the degree of hydrolysis is about 12.9%.

20. The process of claim 16 wherein the hydrolysate has a nitrogen to phosphorus (N/P) of less than about 5, or less than about 4.

21. The process of claim 1 or claim 12, which does not use an organic solvent or a non-food compatible chemical.

22. Phosvitin extract obtained by the process of claim 1, having a purity in a range of about 50% to about 95%.

23. The phosvitin extract of claim 22 having a purity of greater than about 75%, or greater than about 80%.

24. A phosphopeptide derived from the phosvitin extract of claim 22.

24. A pharmaceutical or nutraceutical composition, or a cosmetic or dental product, or a foodstuff, comprising phosvitin of claim 22, or a phosphopeptide of claim 24, or both.

25. A method of promoting health by administering to a subject the phosvitin of claim 22 or the phosphopeptide of claim 24, in an amount sufficient to effect a health benefit.

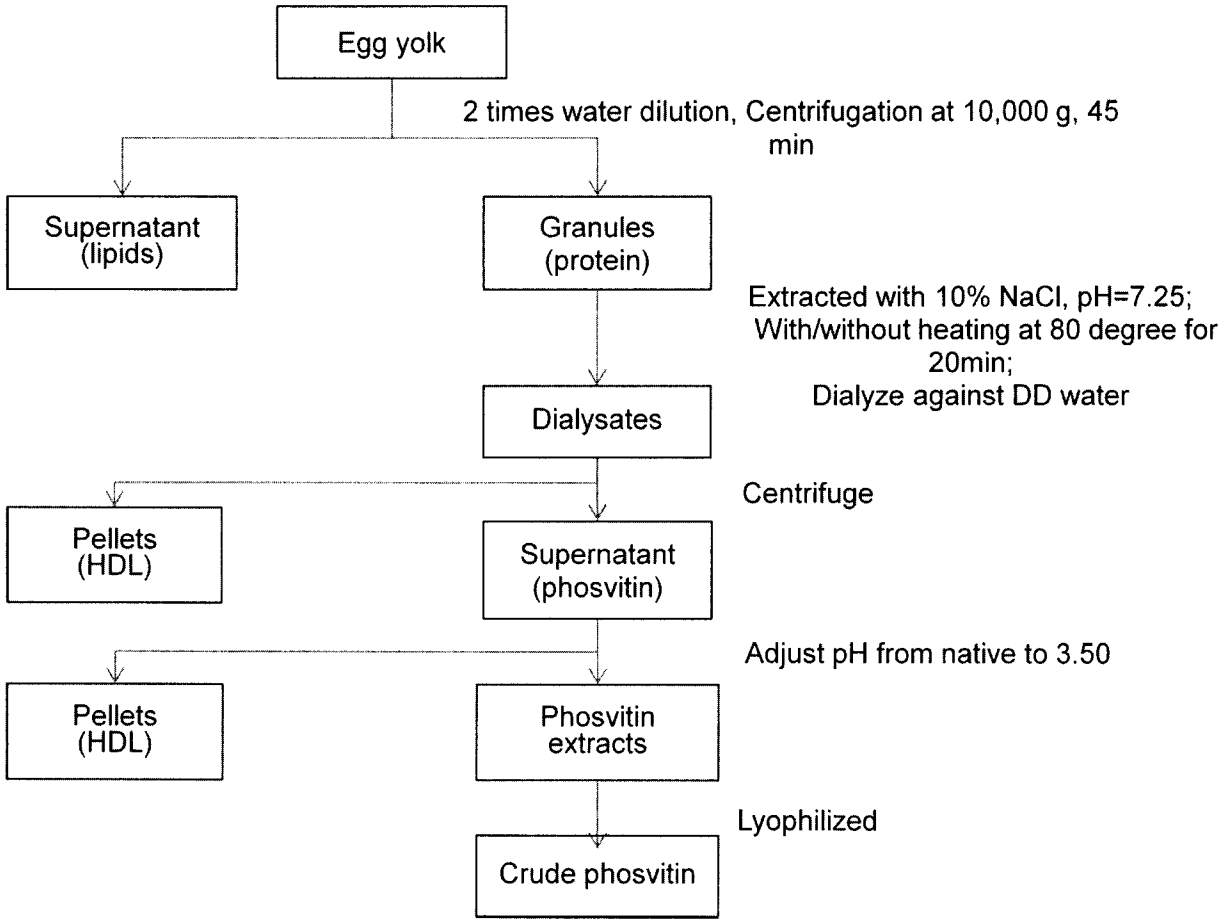

Egg yolk
2 times water dilution, Centrifugation at 10,000 g, 45 min
Supernatant (lipids)
Granules (protein)
Extracted with 10% NaCl, pH=7.25; With/without heating at 80 degree for 20min; Dialyze against DD water
Dialysates
Centrifuge
Pellets (HDL)
Supernatant (phosvitin)
Adjust pH from native to 3.50
Pellets (HDL)
Phosvitin extracts
Lyophilized
Crude phosvitin

FIG. 1

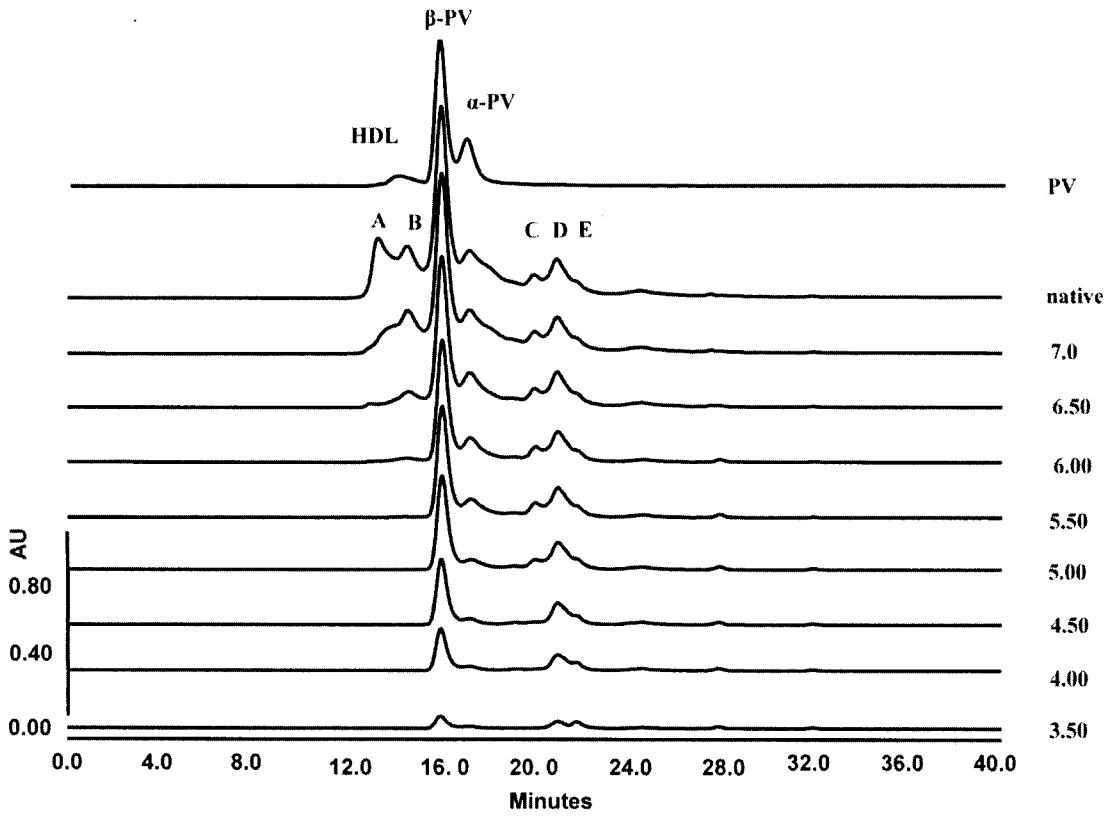

β-PV
α-PV
HDL
PV
A B
C D E
native
7.0
6.50
6.00
5.50
5.00
4.50
4.00
3.50
AU
0.80
0.40
0.00
0.0 4.0 8.0 12.0 16.0 20.0 24.0 28.0 32.0 36.0 40.0
Minutes

FIG. 2

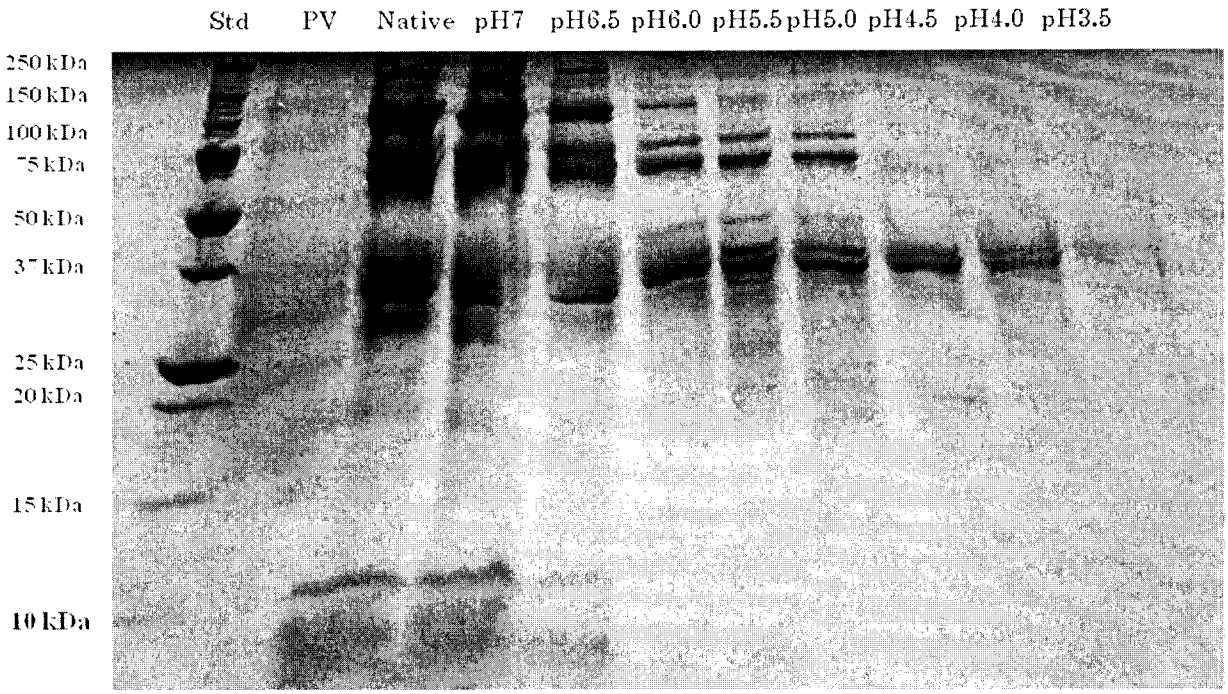

Std
PV
Native
pH7
pH6.5
pH6.0
pH5.5
pH5.0
pH4.5
pH4.0
pH3.5
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa

FIG. 3

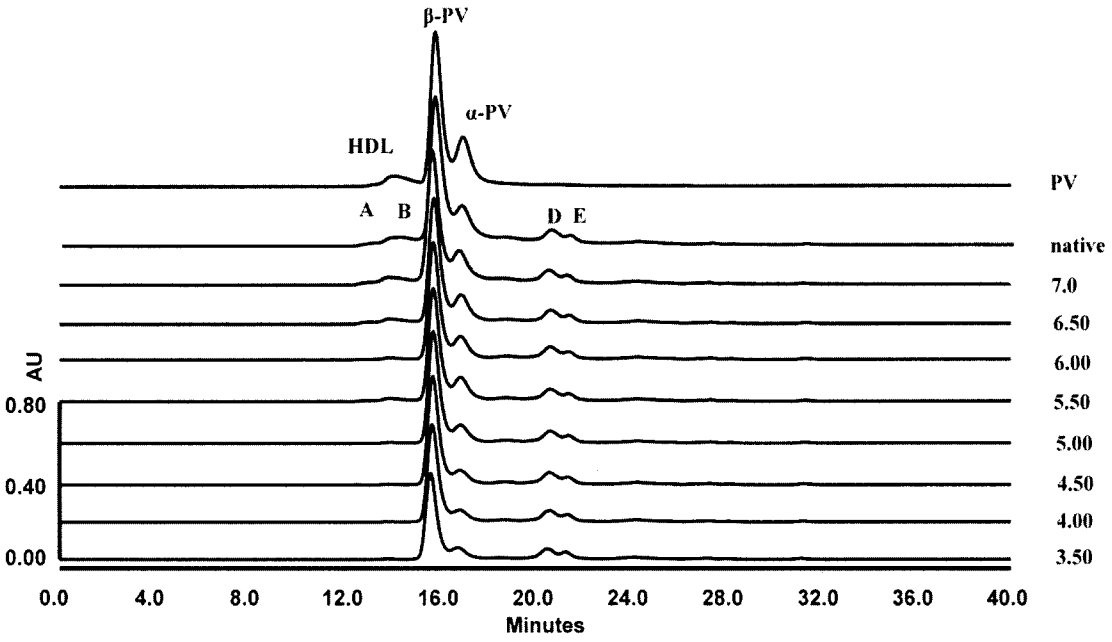

β-PV
α-PV
HDL
A
B
D
E
PV
native
7.0
6.50
6.00
5.50
5.00
4.50
4.00
3.50
AU
0.80
0.40
0.00
0.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
32.0
36.0
40.0
Minutes

FIG. 4

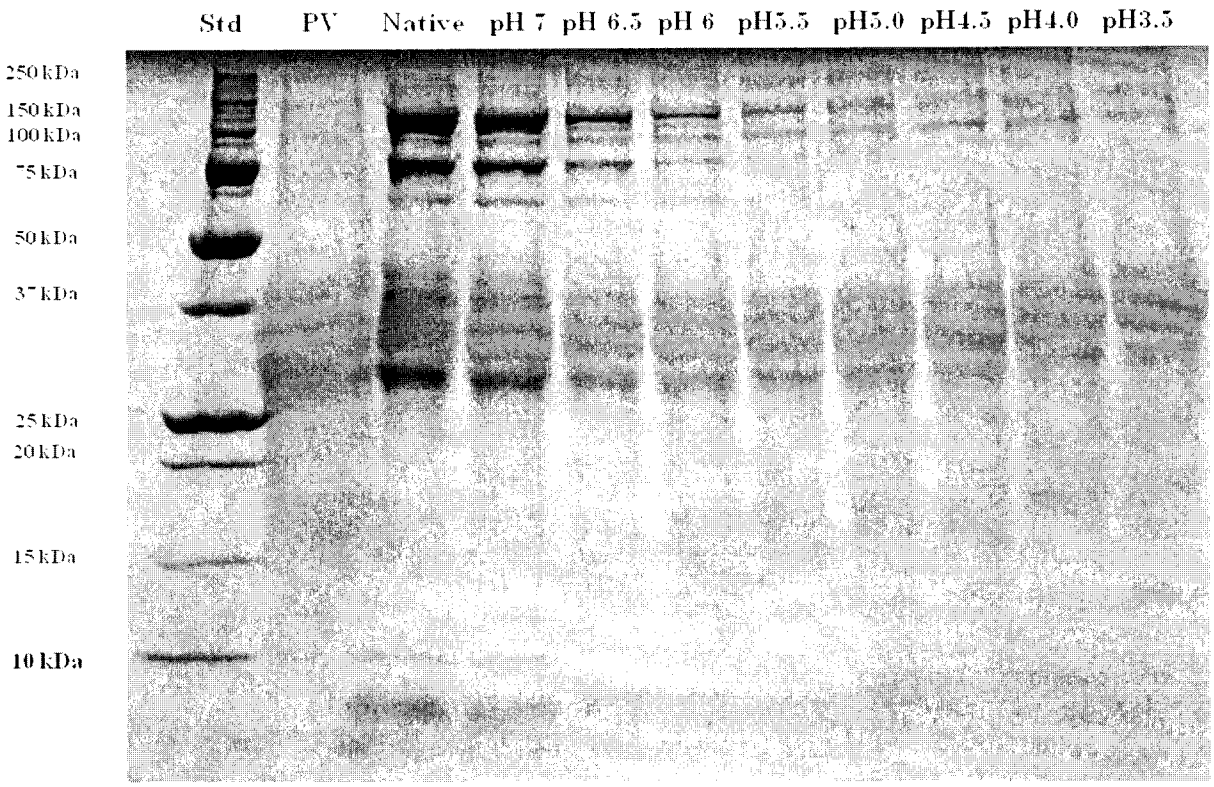

Std
PV
Native
pH 7
pH 6.5
pH 6
pH5.5
pH5.0
pH4.5
pH4.0
pH3.5
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa

FIG. 5

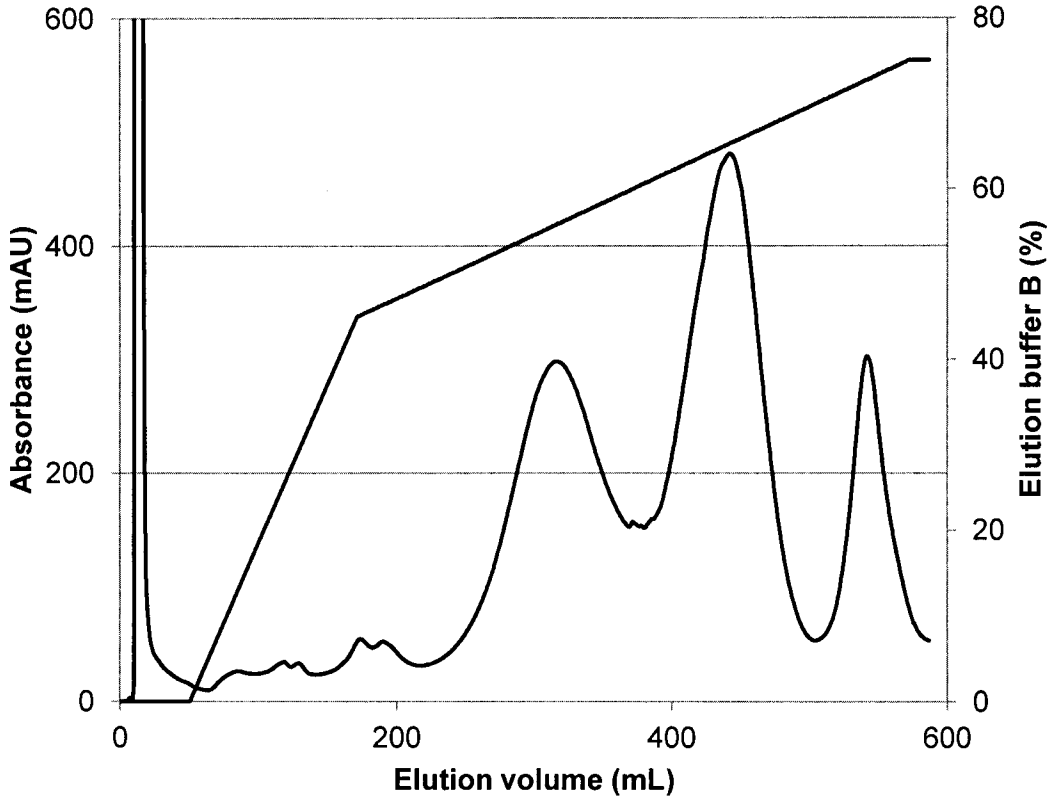

600
400
200
0
Absorbance (mAU)
80
60
40
20
0
Elution buffer B (%)
0
200
400
600
Elution volume (mL)

FIG. 6

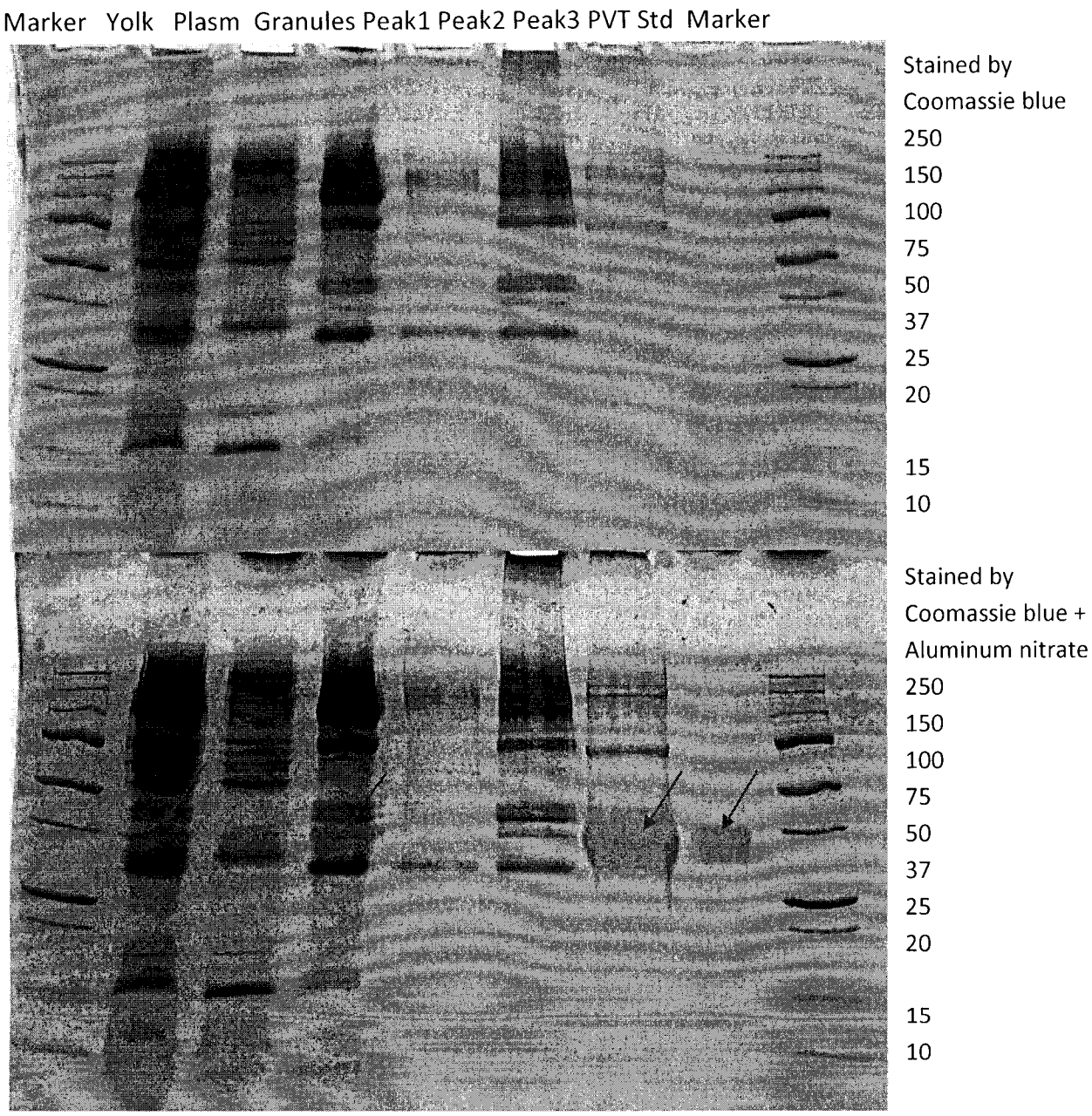

Marker Yolk Plasm Granules Peak1 Peak2 Peak3 PVT Std Marker
Stained by Coomassie blue
250
150
100
75
50
37
25
20
15
10
Stained by Coomassie blue + Aluminum nitrate
250
150
100
75
50
37
25
20
15
10

FIG. 7

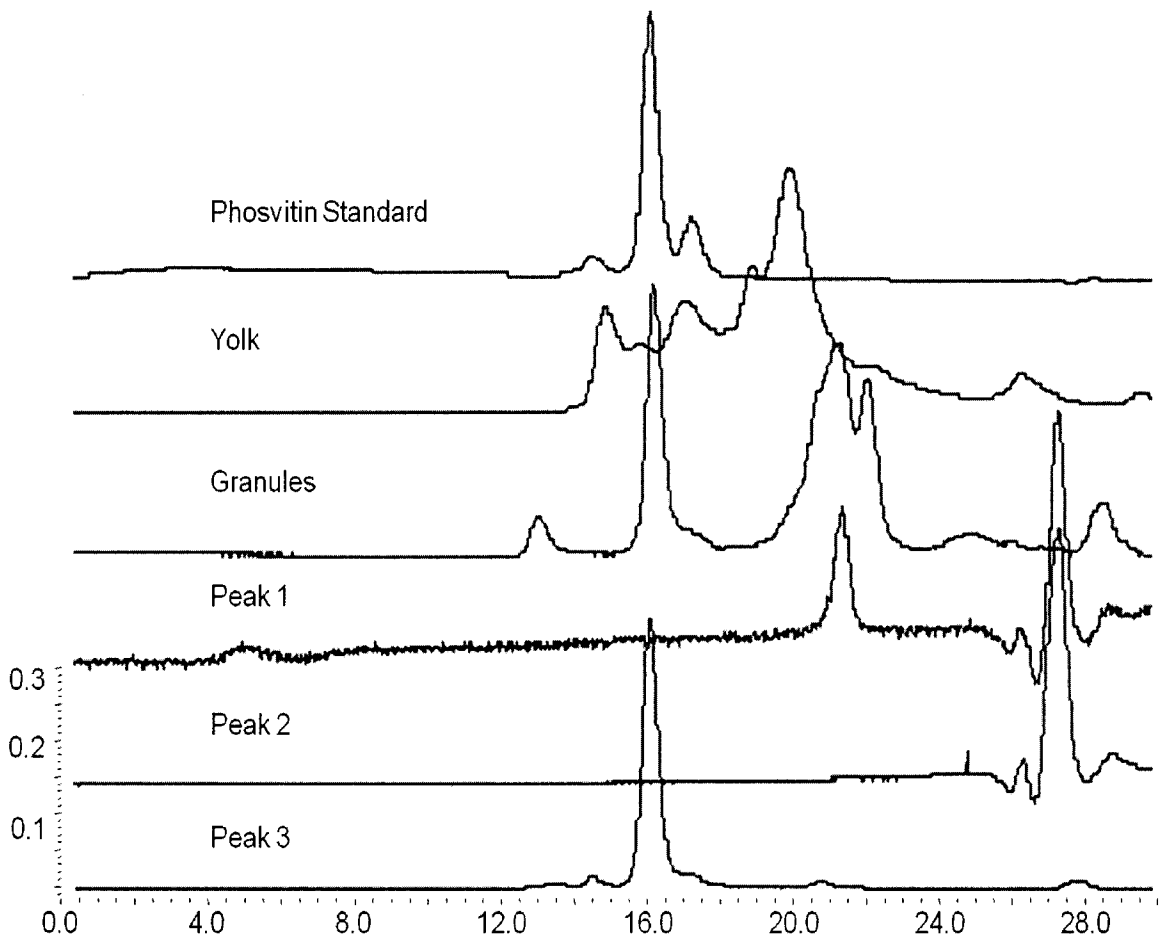

Phosvitin Standard
Yolk
Granules
Peak 1
Peak 2
Peak 3
0.3
0.2
0.1
0.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0

FIG. 8

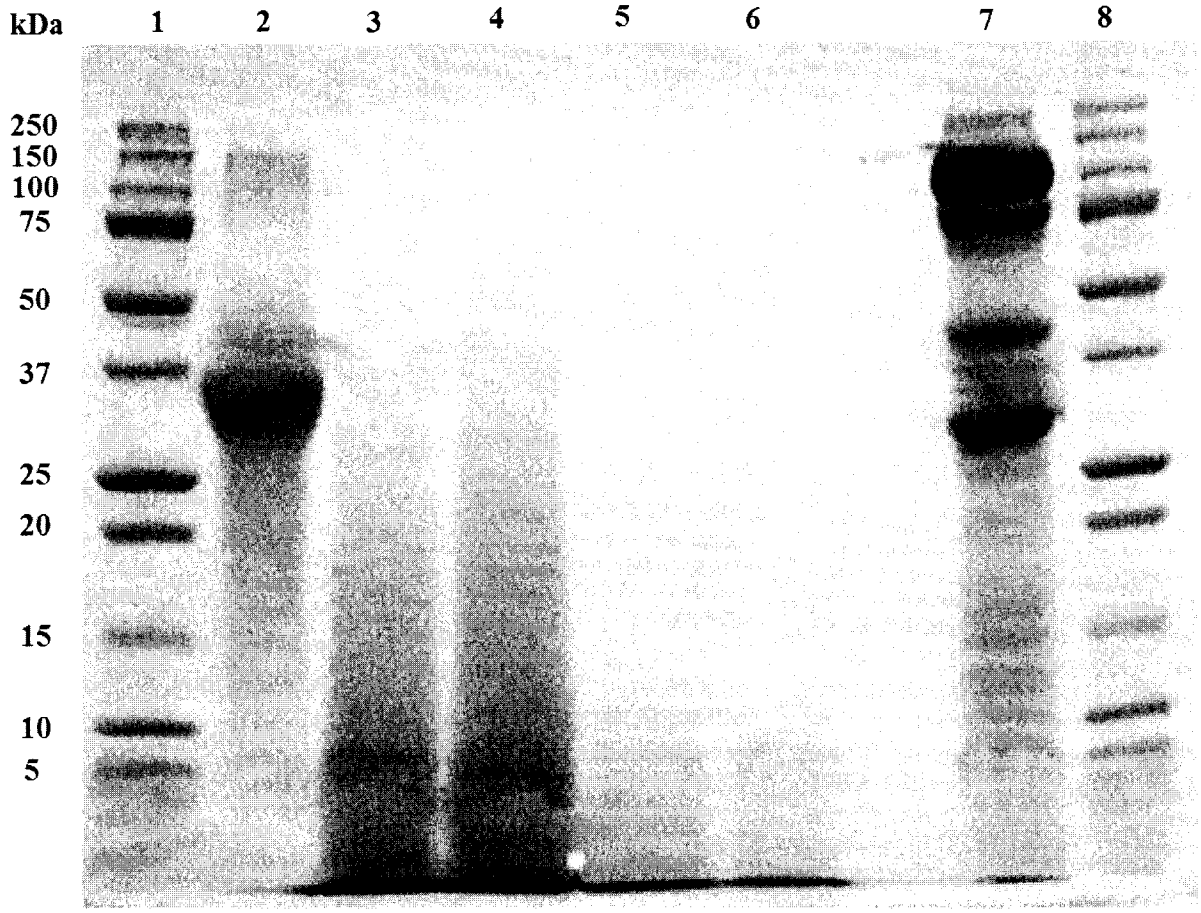


FIG. 9

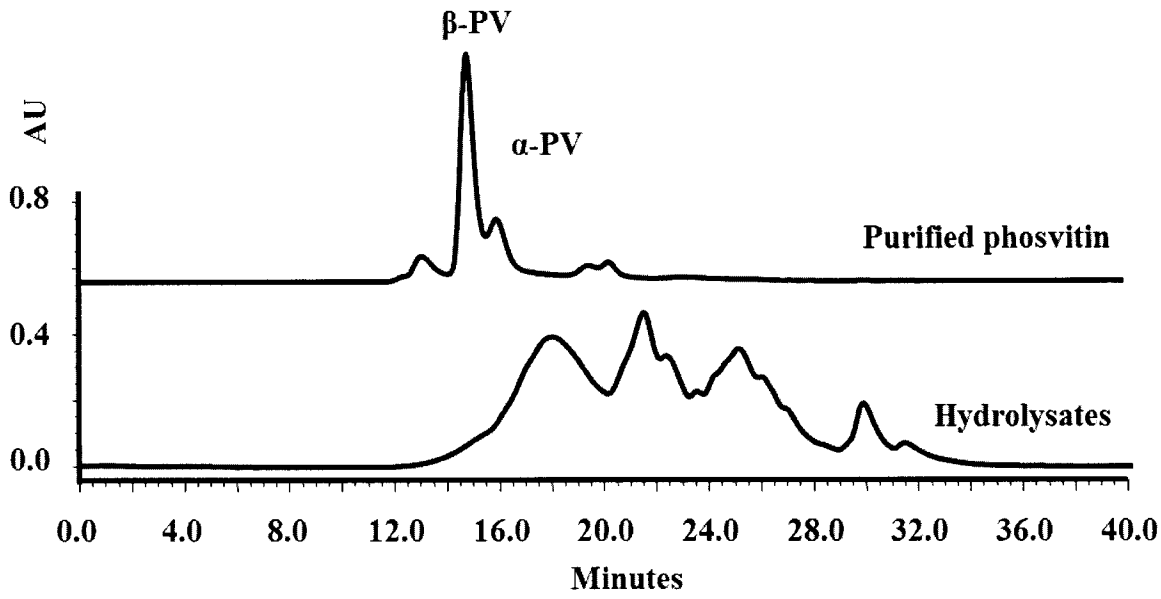

β-PV
α-PV
AU
0.8
0.4
0.0
Purified phosvitin
Hydrolysates
0.0
4.0
8.0
12.0
16.0
20.0
24.0
28.0
32.0
36.0
40.0
Minutes

FIG. 10

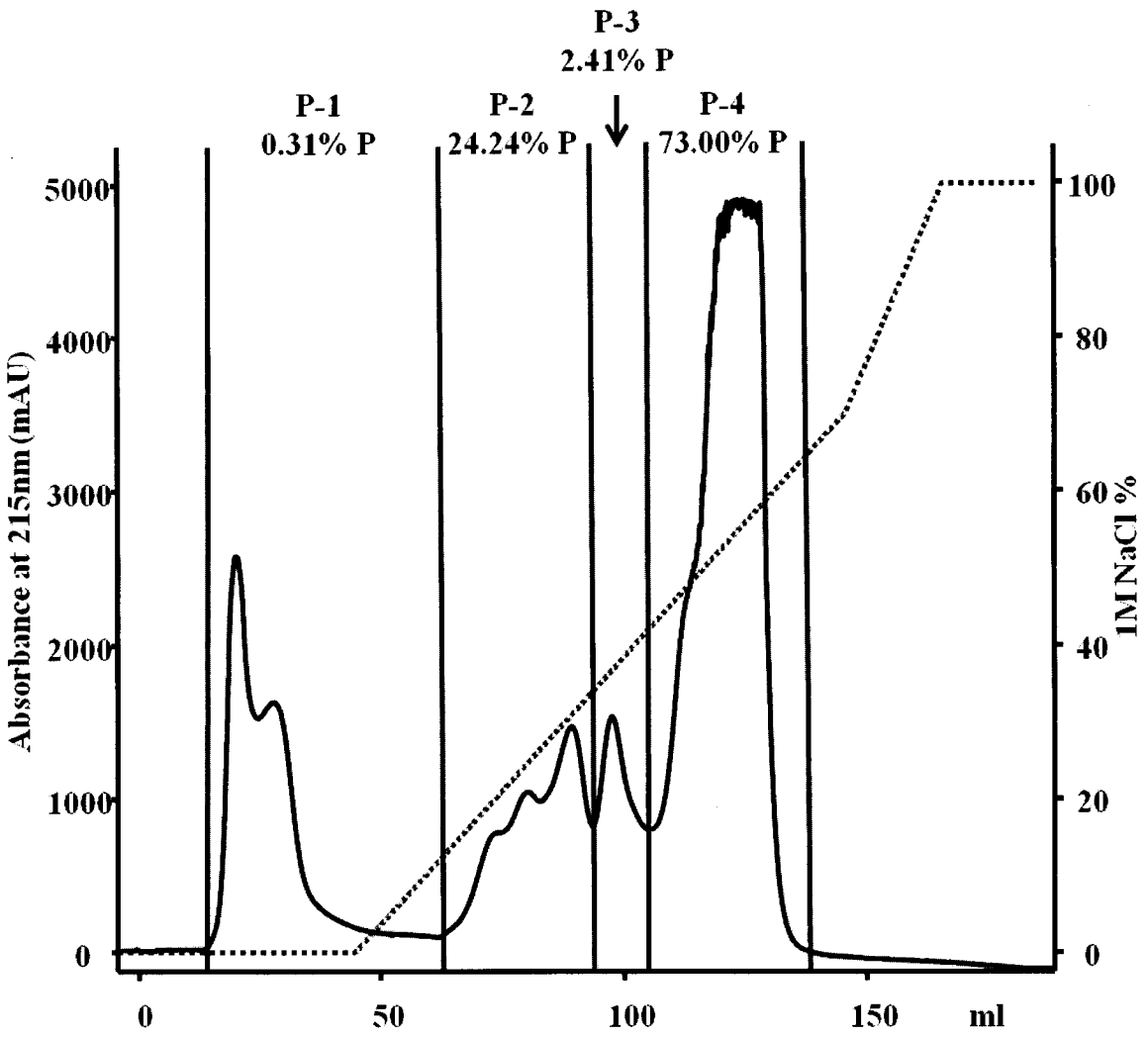

P-3
2.41% P
P-1
0.31% P
P-2
24.24% P
P-4
73.00% P
Absorbance at 215nm (mAU)
5000
4000
3000
2000
1000
0
100
80
60
40
20
0
1M NaCl %
0
50
100
150
ml

FIG. 11

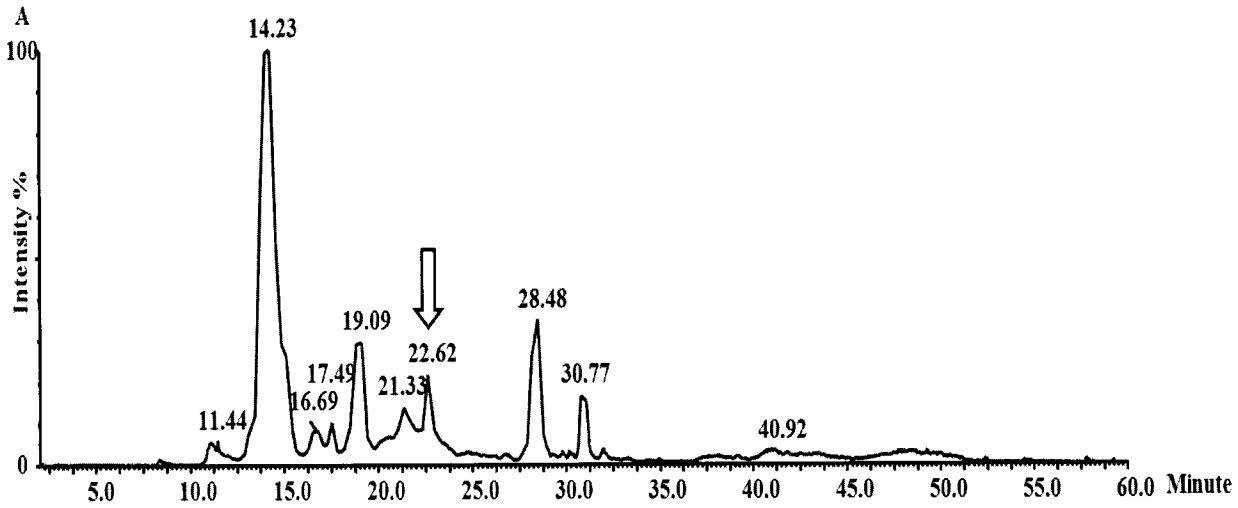


FIG. 12A

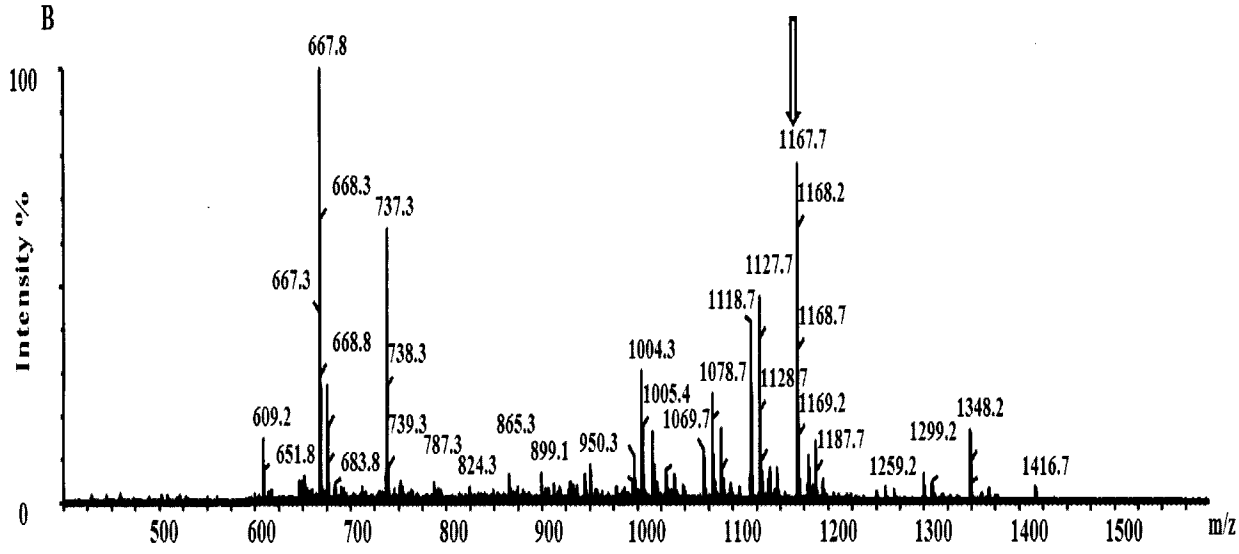


FIG. 12B

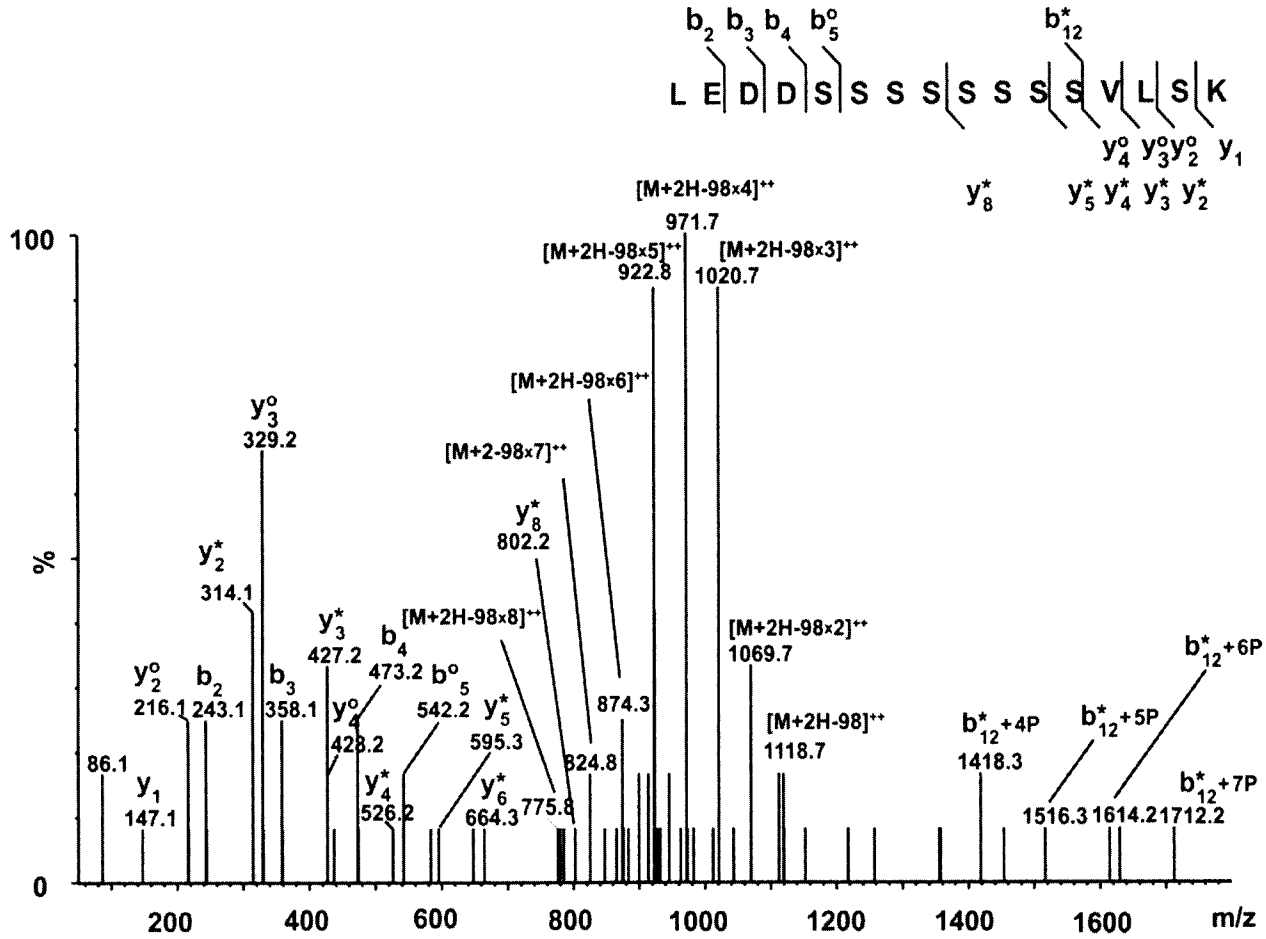

b2 b3 b4 b5o b*12
L E D D S S S S S S S V L S K
y*8 y*5 y*4 y*3 y*2
y4o y3o y2o y1
[M+2H-98x4]++
971.7
[M+2H-98x5]++
922.8
[M+2H-98x3]++
1020.7
[M+2H-98x6]++
[M+2-98x7]++
y*8
802.2
y3o
329.2
y*2
314.1
y*3
427.2
b4
473.2
[M+2H-98x8]++
b5o
542.2
y*5
595.3
y2o
216.1
b2
243.1
b3
358.1
y4o
428.2
y*4
526.2
y*6
664.3
775.8
824.8
874.3
[M+2H-98x2]++
1069.7
[M+2H-98]++
1118.7
b*12+4P
1418.3
b*12+5P
1516.3
b*12+6P
1614.2
b*12+7P
1712.2
86.1
y1
147.1
100
%
0
200
400
600
800
1000
1200
1400
1600
m/z

FIG. 12C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/000572

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***C07K 14/465*** (2006.01) , ***A23L 1/30*** (2006.01) , ***A23L 1/32*** (2006.01) , ***A61K 38/17*** (2006.01) , ***A61K 8/64*** (2006.01) , ***A61P 3/02*** (2006.01) , ***C07K 1/30*** (2006.01) , ***C12P 21/06*** (2006.01) .

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K 14/465 (2006.01) , ***A23L 1/30*** (2006.01) , ***A23L 1/32*** (2006.01) , ***A61K 38/17*** (2006.01) , ***A61K 8/64*** (2006.01) , ***A61P 3/02*** (2006.01) , ***C07K 1/30*** (2006.01) , ***C12P 21/06*** (2006.01) .

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Scopus, Pubmed, Canadian Patent Database, EPODOC, TotalPatent, Medline.
Keywords: method, extract, purify, preparation, phosvitin, egg, yolk, salt solution, pH of mixture.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CASTELLANI O. et al. "Egg yolk phosvitin: preparation of metal-free purified protein by fast protein liquid chromatography using aqueous solvents" J. Chromatogr. B., 5 July 2003, 791(1-2):273-84.	1-11 and 20-25
Y	*the whole document*	12-19
X	JIANG B. et al. "Preparation of Novel Functional Oligophosphopeptides from Hen Egg Yolk Phosvitin" J. Agric. Food Chem., April 2000, 48(4):990-4. *the whole document*	22-25
Y		12-19
A	BYRNE B.M. et al. "Amino Acid Sequence of Phosvitin Derived from the Nucleotide Sequence of Part of the Chicken Vitellogenin Gene" Biochemistry, 11 September 1984, 23(19):4275-9. *the whole document*	1-25

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 August 2012 (01-08-2012)	10 October 2012 (10-10-2012)

Name and mailing address of the ISA/CA	Authorized officer
Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Mostapha Bayaa (819) 994-6940

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2008/086431 A1 (STROHBEHN R.E. et al.) 17 July 2008 (17-07-2008) *the whole document*	1-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/000572

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [X] Claim Nos. : 25

because they relate to subject matter not required to be searched by this Authority, namely :

Claim 25 is directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search. However, this Authority has carried out a search based on the alleged effect or purpose/use of the product defined in claim 25.

2. [ ] Claim Nos. :

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [ ] Claim Nos. :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2012/000572

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO2008086431A1	17 July 2008 (17-07-2008)	US2008206436A1	28 August 2008 (28-08-2008)
		US8080272B2	20 December 2011 (20-12-2011)
		US2008166447A1	10 July 2008 (10-07-2008)
		US8133521B2	13 March 2012 (13-03-2012)
		US2012052552A1	01 March 2012 (01-03-2012)