DOI:10.1021/jf101808b

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Process for Coproduction of Crude Immunoglobulin Y and High-Density Lipoproteins from Hen Egg Yolk

Yao Luo,[‖] Runsong Xiong,[‖] Lefeng Hou, Jiandong Li, and Jinchun Chen*

College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, People's Republic of China. [‖] Both authors made equal contributions to this work.

High value-added products from hen egg are receiving increasing attention from both egg academic and industrial circles due to their potential applications in research and medicine as well as the benefits they bring to egg-breaking industries. This paper reports a simple method for the preparation of immunoglobulin Y (IgY) and high-density lipoproteins (HDL) from hen egg yolk. A water dilution method coupled with $(NH_4)_2SO_4$ precipitation was employed to prepare the two target proteins. SDS-PAGE under reducing or nonreducing conditions and liquid chromatography−electrospray ionization−mass spectrometry (LC-ESI-MS/MS) were used to characterize the two products. Western blotting and histobiochemical staining were utilized to qualitatively analyze IgY and HDL, respectively. The purities of prepared IgY and HDL were detected by ELISA and a direct high-density lipoprotein-cholesterol assay, respectively. Results show that dilution times 15 makes IgY and HDL separate well from each other. Western blotting proves IgY has an immunocompetence similar to that of human IgG. Histobiochemical staining shows HDL is composed of sugar, lipid, and protein. The quantitative evaluation of the products indicates that approximately 3 kg of IgY and 2 kg of HDL with purities of 72.7 and 71.9%, respectively, are available from 1 ton of shell eggs via this process.

KEYWORDS: Immunoglobulin; high-density lipoprotein; hen egg yolk; coproduction

INTRODUCTION

First, it should be pointed out that the hen egg-product industry was originally established in United States as early as 1878 and then moved to Shanghai, China, by Henningsen Foods, Inc., in 1916 (*1*). The industry has witnessed enormous development in the past 30 years since the beginning of the Chinese open policy. Now there are more than 400 plants for egg products throughout China. The industry provides not only a great number of job opportunities for urban populations but also offers sources of economic benefits to farmers. The research and development of new processes and new products, particularly value-added products from this industry, have never stopped despite a long history of more than a century. For example, the market price of golden yolk powder in China is about \$5 kg^{-1}, which means \$650 for all dried yolk powder from 1 ton of fresh hen egg breaking. Indeed, the net profit of this processing for the egg-breaking industry is quite poor. Value-added products such as immunoglobulin Y (IgY) could be extracted from the fresh yolk, which produces in dried yolk powder a very limited loss by weight. The market value of 3 kg of IgY extracted from the fresh yolk of 1 ton of shell egg might be at least \$1300. Therefore, along with deep processing or research and development of high value-added products, the clear profit could be dramatically increased compared with simple processing for the egg-breaking industry.

*Author to whom correspondence should be addressed (phone +86-10-64439673; fax +86-10-64439673; e-mail jingchunchen@hotmail.com).

We are interested in the research and development of high value-added products such as high-density lipoproteins (HDL), IgY, and lecithin from hen egg yolk, and this work was aimed at developing an efficient and suitable process for simultaneous large-scale production and separation of IgY and HDL from hen egg yolk. It is already known that IgY, like human IgG, is a water-soluble protein in egg yolk, which is being widely added to children's milk powder or food as a functional factor for children's resistance against some bacteria or viruses (*2*−*5*). Moreover, there is increasing interest in the use of IgY as a medical agent for immunotherapeutic and immunodiagnostic purposes, and thus the importance of eggs as a source of antibodies is well recognized (*6*−*10*). Data on the isolation and purification of IgY are now relatively complete and keep increasing. Mainly, there are four methods for separation of IgY from chicken egg yolk, namely, methods based on water dilution (*11*), polyethylene glycol (*12*), dextran sulfate (*13*), and natural gums (*14*). Although the IgY content is not very high, about 3.0 kg per ton of fresh eggs in practice, there are two plants in China to produce it at present. In contrast, HDL in hen egg yolk exist as liposoluble glycoproteins, and the content can reach near 10.4 kg per ton of fresh eggs, in theory. HDL in egg yolk is composed of 40% neutral lipid and 60% phospholipids. The neutral lipid in HDL includes about 4.1% free cholesterol and 0.14−0.2% of the ester. Human HDL is believed to be able to prevent the occurrence of atherosclerosis by eliminating cholesterol adhered to blood vessels. Hen HDL may not directly perform the same function as human HDL in the human body because after consumption

 Published on Web 10/15/2010

it is subject to actions of digestive enzymes. However, much evidence has indicated that intake of hen HDL could elevate the level of human serum HDL, which makes hen HDL a potential functional food for human health. Unfortunately, hen HDL has not been produced in large scale to date due to the lack of a promising method, although many preparation methods have been reported in the past including ultracentrifugation (*15*), dilution with $MgSO_4$ and centrifugation (*16*), moving-boundary electrophoresis (*17*), and various chromatography methods (*18*−*20*). To our knowledge, there is no literature on the preparation and coproduction method of IgY and HDL from hen egg yolk.

Fresh egg yolk contains insoluble structures called granules, which can be easily separated from the soluble phase called plasma by centrifugation. The plasma of egg yolk contains 85% low-density lipoprotein (LDL) and 15% livetins including IgY, whereas the granules constitute about 70% HDL, 16% phosvitin, and 12% granular LDL. The granular LDL is very similar to plasma LDL (*21*). These physicochemical properties of IgY and HDL make it possible for us to find a satisfactory method for mass coproduction of chicken egg yolk IgY and HDL. The method has two advantages: (i) more effective utilization of hen egg yolk; (ii) more profit for the egg-product industry.

MATERIALS AND METHODS

Materials and Apparatus. Fresh eggs were purchased from a local market. Histochemical dyes Methyl green and Sudan black were provided from Beijing Hengye Zhongyuan Chemical Co., Ltd. 5-Sulfosalicylic acid was supplied by the Academy of Military Medical Sciences (AMMS). $AgNO_3$ was purchased from Beijing Yi-Li Fine Chemical Co. Ltd. All other chemicals were of analytical grade.

A refrigerated centrifuge was supplied by Shanghai Anting Scientific Instrument Factory. A JB-2 thermostatic magnetic stirrer was purchased from Shanghai Leici Co. Ltd. The LGJ10-C freeze-dryer was bought from Beijing Sihuan Scientific Instrument Factory.

Optimization of Diluting Yolk in Distilled Water. Considering the solubility difference between IgY and HDL in water as well as product safety standards for human consumption, distilled water was utilized as dilution agent to dilute hen egg yolk. To determine the optimal separation of the lipid- and water-soluble parts of the hen egg yolk, the yolk was diluted with distilled water in the ratios 1:5, 1:10, 1:15, 1:20 (v/v), respectively, and then the mixtures were stirred thoroughly at 20 °C for 2 h followed by settlement overnight at room temperature to form a HDL-containing precipitate and a supernatant containing the water-soluble proteins, including IgY. Thereafter, samples were drawn from the precipitate and supernatant and determined by SDS-PAGE to check the dilution results (*22*).

Optimization of Separating IgY. First, $(NH_4)_2SO_4$ was added to the supernatant obtained from the diluted yolk and stirred at 4 °C for several hours. Different concentrations (20, 30, 35, 40, and 45% (w/v)) of $(NH_4)_2SO_4$ were applied in this pattern to confirm a better concentration of $(NH_4)_2SO_4$. After settling for 30 min, the mixture was centrifuged at 10000 rpm for 30 min at 4 °C. Samples were drawn from the supernatant and the pellets to be checked by SDS-PAGE under nonreducing condition to determine the deposition effect of $(NH_4)_2SO_4$ on the target protein IgY. Thereafter, the pellets were redissolved in distilled water and ultrafiltered using a hollow fiber membrane (molecular weight cutoff of 30 kDa), followed by freeze-drying in vacuum, and finally the crude IgY was available.

Quality Evaluation of IgY. *Checking of IgY Molecular Weight by PAGE.* To check IgY molecular weight, SDS-PAGE was performed under both reducing and nonreducing conditions. The experiment protocol was as in references *22* and *23*. The IgY samples were mixed with an equal volume of reducing or nonreducing loading buffer and incubated at 100 °C for 10 min prior to SDS-PAGE (the concentration of stacking gel was 3% acrylamide and that of separation gel was 5%). The composition of reducing loading buffer was 2.0 mL of 0.5 M Tris-HCl (pH 6.8), 2.4 mL of glycerol, 1.0 mL of 20% (w/v) SDS, 0.2 mL of β-mercaptoethanol, 0.4 mL of 0.1% (w/v) bromophenol blue, and 4.0 mL of ddH_2O. Nonreducing loading buffer is composed of 2.0 mL of 0.5 M Tris-HCl (pH 6.8), 2.4 mL of glycerol, 1.0 mL of 20% (w/v) SDS, 0.4 mL of 0.1% (w/v) bromophenol blue, and 4.2 mL of ddH_2O. After PAGE, the gel was stained by Coomassie blue.

Western Blotting IgY. After nonreducing SDS-PAGE, the samples on gel were transferred to a nitrocellulose membrane under semidry condition for 40 min at 10 V and then kept in blocking buffer containing bovine serum albumin for 1 h. Afterward, rabbit anti-chicken IgG conjugated previously with horseradish peroxidase was combined with IgY for 30 min, and thus the color reaction could be observed after using diaminobenzidine solution for 5 min, which facilitated observation of the electrophoresis strip.

Amino Acid Sequence Analysis by LC-ESI-MS/MS. The probable target protein band on the SDS-PAGE gel (conducted under nonreducing condition) was excised in size of about 1 mm^3 and washed in equal volumes of 100% methanol and 200 mM ammonium bicarbonate for 15 min at least twice to remove the CBB R-250. Then the gel pieces were dehydrated with 100% ACN for 5 min, vacuum-dried in an SPD1010 SpeedVac system (ThermoSavant, Milford, MA), and rehydrated with 250 ng of sequencing-grade, modified trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate (pH 8.5) at 37 °C for 16 h. The digested samples were collected, vacuum-dried, and then dissolved in 50% (v/v) ACN containing 0.1% (v/v) formic acid, and then 20 μL of the trypsin digestion sample was loaded onto a BioBasic-18 column, 100 × 0.18 mm, particle size 5 μm (Thermo Electron, Waltham, MA) and separated using a gradient of 5% A and 95% B for 15 min, 65% A and 35% B for 45 min, and 95% A and 5% B for 25 min, where A was ACN with 0.1% formic acid and B was water with 0.1% formic acid. The column was connected directly to the LCQ Deca XP plus ion trap mass spectrometer (ThermoQuest, San Jose, CA). Full-scan spectra were recorded in positive mode over the mass range of 300−1500 Da. The MS/MS data were automatically acquired on the three most intense precursor ions in each full-scan spectrum.

The MS/MS spectra were first searched against the NCBInr and EST databases using the Mascot search engine (http://www.matrixscience.com) on line. Maximally, one missed tryptic cleavage was allowed; variable methionine oxidation and cysteine carbamidomethylation modifications were considered. Peptide mass tolerance and MS/MS ion mass tolerance were set to 1 Da. A positive protein identification was defined when individual ion scores indicated identity or extensive homology ($p < 0.05$) as well as the estimated p*I* and molecular weight values.

The MS/MS spectra were searched again using the SEQUEST algorithm in the Bioworks 3.1 software package against the *chicken fasta* peptides database. Peptides identified were filtered according to Xcorr score equal to or above 1.9, 2.2, or 3.75 for singly, doubly, or triply charged precursor ions, respectively, and normalized difference in correlation score, Delta Cn > 0.1.

Quantitative Analysis of IgY. To estimate the yield of IgY, three batches (10 kg of egg/batch) were tried in this research. The crude products were dried in vacuum and then weighed.

To determine the purity of the prepared IgY, a modified ELISA was used (see the Supporting Information). A standard IgY (AMMS, Beijing, China) was diluted in 0.01 M phosphate-buffered saline (PBS; pH 7.2) to a series of concentrations (0.05, 0.1, 0.25, 0.5, 1, 2, 4, 8, and 10 μg/mL), and then the standard IgY and the prepared IgY (diluted to 1 μg/mL) were added to a 96-well microtiter plate (Costar, Lowell, MA). Thereafter, unbound sites were blocked with 200 μL/well of 3 mg of bovine serum albumin (BSA)/mL in 0.01 M PBS (pH 7.4). This was followed by the addition of HRP-conjugated goat anti-chicken IgY (AMMS, Beijing, China) diluted 1:1000 in PBS-T containing 0.05% Tween 20. Each sample was added in duplicates. Finally, the wells were incubated with TMB substrate (Zymed) for 15 min. The reaction was stopped with the addition of 2 M H_2SO_4 (50 μL/well). From all solutions 100 μL was added per well if nothing else was noted. Between each step, the plate was washed three times with 300 μL of PBS-T/well. The absorbance was read at 450 nm in a microplate reader (Thermo Scientific Multiskan Spectrum, Thermo Fisher Scientific, Waltham, MA).

Separating HDL. Most HDL of yolk lies in the water-insoluble granules in morphology. The preparation protocol differences from that of preparing IgY were as follows.

Briefly, the precipitate part of yolk diluted with water was washed with a low concentration of NaCl (≤0.4 M), followed by centrifugation and discarding of the supernatant. The pellets (granules) were redissolved by

adding NaCl to certain concentrations and then adding certain amounts of $(NH_4)_2SO_4$, stirring for 10 min, and settling for 30 min to deposit the target protein. Thereafter, the mixture was centrifuged at 10000 rpm for 30 min. After the pellets had been washed with distilled water, the crude HDL was available. All of the manipulations were performed at 4 °C to avoid the denaturation occurrence of the target protein, and series of concentrations of NaCl (0.1, 0.2, 0.3, 0.4, 0.6, 0.7, 0.8, 0.9, and 1.0 M) and $(NH_4)_2SO_4$ (5, 10, 15, and 20%, w/v) were used in this process to identify their optimization, which were subsequently checked by SDS-PAGE.

Quality Evaluation of HDL. *Histobiochemical Staining*. Because the HDL molecule is classified as a glycolipoprotein, having properties of sugar, fat, and protein residues, the sample can be determined by histobiochemical staining methods, which include electrophoretic painting of lipoprotein, phospholipid, and glycoprotein. The sample was electrophoresed to obtain four identical gel strips, which were dyed using Coomassie brilliant blue, Sudan black B, Methyl green, and $AgNO_3$, respectively.

(*a*) *Coomassie Brilliant Blue Staining (24)*. After electrophoresis, the gel was fixed for 30 min, following dying with Coomassie brilliant blue solution (0.1% (w/v) Coomassie brilliant blue R-250, 45% (v/v) methanol, and 10% (v/v) acetic acid) for 30 min. After washing with distilled water, the gel was destained with a solution containing 10% (v/v) methanol and acetic acid, respectively, until the strips on the gel were clear.

(*b*) *Analysis of Lipoprotein (25)*. After washing with distilled water, the gel band was incubated in a 1% Sudan black B staining solution (prepared by dissolving 2 g of Sudan black B in 20 mL of acetone, adding 15 mL of acetic acid and 165 mL of ddH_2O into the mixture, stirring for 30 min, and filtration) for 12 h. Then the strip was put in destaining solution (composed of methanol, acetic acid, and distilled water at the ratio of 5:5:1 (v/v)) until the strip was clear.

(*c*) *Analysis of Phospholipid (26, 27)*. The strip was first fixed in 10% (w/v) sulfosalicylic acid (SSA) for 2 h and washed with distilled water. After immobilization in 0.5 M NaOH at 60 °C for 30 min, the band was washed with 1% $(NH_4)_2MoO_4$ twice and incubated with 1% $(NH_4)_2SO_4$ for 30 min. Thereafter, the band was stained with 0.5% Methyl green solution (prepared with 7% (v/v) acetic acid solution) for 30 min and destained using 10% SSA until the background was shallow while the phospholipid strip was clear.

(*d*) *Analysis of Glycoprotein*. After fixing for 30 min in the solution containing 10% acetic acid and 2.5% isopropyl alcohol (v/v), washing with distilled water, and placing in 7.5% acetic acid solution for 30 min, the band was fixed in 1% periodic acid for 1 h at 4 °C and washed with distilled water six times (5 min every time), followed by dipping in 0.2% $AgNO_3$ (freshly prepared) to react for 30 min. When time was up, the band had to be immediately washed with ddH_2O for < 5 s. Then the color reaction could be observed once the strip was put in the staining solution containing 2.3% Na_2CO_3, 0.01% formaldehyde, and 0.001% $Na_2S_2O_3$ (w/v) (the color reaction could be terminated by filling in 2.5% acetic acid).

Amino Acid Sequence Analysis by LC-ESI-MS/MS. The amino acid sequence analysis of the HDL sample was performed in the same way as mentioned in the characterization of IgY.

Quantitative Analysis of HDL. The content of free cholesterol and the ester in HDL (HDL-C) averages 4.27% (*28*). Thus, the amount of HDL in the sample can be determined by measurement of the content of HDL-C. This work was conducted by a direct high-density lipoprotein-cholesterol (HDL-C) assay. A commercial test kit (Zhongshengbeikong, Beijing, China) was used in this process. First, the free and combined cholesterol in chylomicron, low-density lipoprotein (LDL), and very low density lipoprotein (VLDL) in the sample were digested by solution R1, which contains cholesterol esterase (CEH), cholesterol oxidase (CHOD), and catalase. The reaction solution remained colorless in this step. Then, solution R2 was added to the reaction mixture. The surfactant in solution R2 made the HDL-C release from HDL, which was digested by CEH, CHOD, and peroxidase (POD). Color developed in this step, which was directly proportional to the amount of HDL-C. The optical density was read at 546 nm. The purity of the prepared HDL was calculated by

$$C_{HDL} = \frac{A_{sample}}{A_{control}} \times \frac{C_{control}}{4.27\%}$$

where $C_{control}$ is the concentration of control HDL supplied by the kit, which is 5.2 mg/mL.

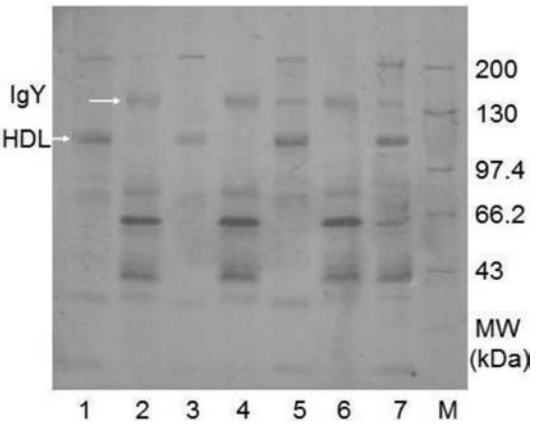

Figure 1. Separation effect of dilution times on egg yolk. Lanes: (1) precipitate of egg yolk diluted 1:20; (2) supernatant of egg yolk diluted 1:20; (3) precipitate of egg yolk diluted 1:15; (4) supernatant of egg yolk diluted 1:15; (5) precipitate of egg yolk diluted 1:10; (6) supernatant of egg yolk diluted 1:10; (7) mixture of egg yolk diluted 1:5; (M) protein standard.

The estimation of the yield of HDL was performed in the same way as mentioned for IgY.

RESULTS

Optimization Result of Diluting Yolk in Water. After dilution treatment of hen egg yolk for about 12 h, the mixture was divided into two phases: supernatant and pellet. The former was the water-soluble part containing IgY, and the latter was the lipid-soluble part including HDL. As shown in **Figure 1**, both IgY and HDL could separate very well when the yolk was diluted 1:15 in distilled water. Less than 15 times dilution could not offer a good enough result.

Optimization of IgY Separation with $(NH_4)_2SO_4$ Precipitation. According to data, IgY content in fresh hen yolk is about 2.5% of total yolk proteins if the hen is not immunized by specific antigens (*28*). Hence, salt precipitation has been employed to obtain the crude IgY from the diluted yolk mixture. As shown in **Figure 2A**, the precipitation effect of 20% $(NH_4)_2SO_4$ was not promising for too a low content of IgY in the precipitate. Thirty percent $(NH_4)_2SO_4$ supplied a much higher purity of IgY, but we could find from lane 2 that there is still some IgY remaining in the supernatant; 40 and 45% $(NH_4)_2SO_4$ made nearly all of the IgY deposit (lanes 4 and 5), but it also brought more contaminants to the precipitate, especially the latter. Thus, 35% $(NH_4)_2SO_4$ was the best among the five different concentrations via optimization, which could precipitate IgY effectively and meanwhile avoid too much contaminant in the precipitate.

Quality Evaluation of IgY. *Molecular Weight Determination by PAGE*. IgY is a big biological molecule with a molecular weight of 180 kDa and consists of two large subunits and two smaller subunits, having molecular weights of about 67 and 22 kDa, respectively. Under reducing condition, the disulfide bond usually could be broken down, which resulted in both larger subunits and smaller subunits separating. The results shown in **Figure 3A** indicate the sample behavior on SDS-PAGE was identical to the above description. In **Figure 3A**, lane 1, there was a clear band located between 130 and 220 kDa, which resulted from the sample loaded with nonreducing buffer. We inferred it was the IgY molecule. Meanwhile, in lane 2 this band did not occur again but there were two small bands of about 67 and 22 kDa, respectively, which resulted from the sample loaded with the reducing buffer. We believed that the 67 kDa band was the IgY heavy chain location and the 22 kDa band was the light chain location.

Table 1. Identification of IgY by LC-ESI-MS/MS

reference[a]	score[b]	accession[c]	peptides (hits)[d]
S00390 Ig γ chain (clone 36) chicken (fragment)	160.2	86318.0	2
thioredoxin reductase 3 (*Gallus gallus*) (MAS)	90.3	170671712.0	2
LAC_CHICK RecName: full=Ig λ chain C region	60.2	125945.0	1
unnamed protein product (*Gallus gallus*)	30.2	929597.0	1
immunoglobulin heavy chain variable region (*Gallus gallus*)	30.2	1536812.0	1

[a] Protein recommended by the protein database (NCBI). [b] Reflects the reliability of the protein. [c] Serial number of the protein in the database. [d] Number of positive peptide fragments detected by LC-ESI-MS/MS, which matched the recommended protein.

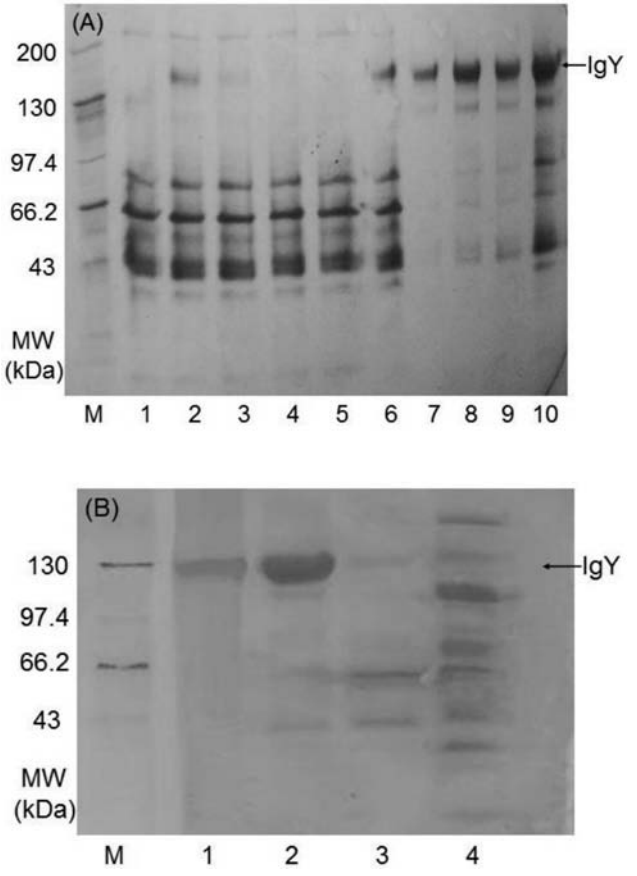

Figure 2. Optimization of IgY separation with $(NH_4)_2SO_4$. (**A**) Precipitation result with $(NH_4)_2SO_4$. Lanes: (1−5) supernatant samples of the yolk mixture treated with 20, 30, 35, 40, and 45% (w/v) $(NH_4)_2SO_4$, respectively; (6−10) precipitate samples of the yolk mixture treated with 20, 30, 35, 40, and 45% (w/v) $(NH_4)_2SO_4$, respectively. (**B**) Further purification of target protein via ultrafiltration. Lanes: (M) protein standard; (1) final product of IgY after ultrafiltration; (2) precipitate of sample 3 (lane 3) after treatment with 35% (w/v) $(NH_4)_2SO_4$; (3) supernatant of the egg yolk diluted 1:15; (4) egg yolk mixture diluted 1:15.

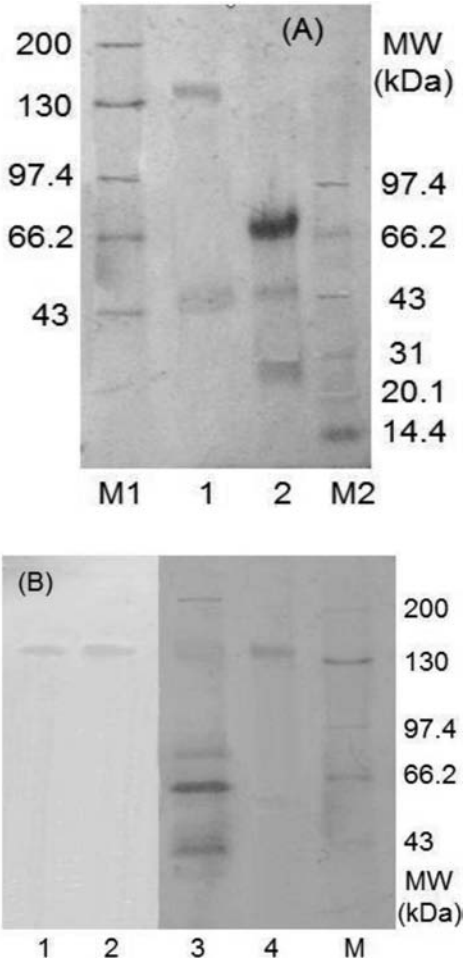

Figure 3. Quality evaluation of IgY. (**A**) Molecular weight assay after Coomassie blue staining. Lanes (M1 and M2) protein standards; (1) IgY loaded with nonreducing loading buffer; (2) IgY loaded with reducing loading buffer. (**B**) Identification of IgY by Western blot. Lanes: (1 and 2) target strips in lanes 3 and 4, respectively, after Western blot; (3) supernatant of egg yolk after diluted 1:15; (4) crude IgY sample (same with lane 1 of **A**); (M) protein standard.

Western Blot with Anti-IgY Rabbit Serum. The result of the sample Western blotting is shown in **Figure 3B**. We can infer from **Figure 3** that compared with bands of Coomassie brilliant blue staining, brown precipitation showed through 3,3′-diaminobenzidine (DAB) staining in the corresponding position of the nitrocellulose membrane of target protein after the bands were incubated with rabbit anti-chicken IgG conjugated with horseradish peroxidase, which means there is specific combination of rabbit anti-chicken IgG conjugated with horseradish peroxidase and again proves that this protein is IgY.

LC-ESI-MS/MS. The LC-ESI-MS/MS result of IgY product is shown in **Table 1**. Basically, a polypeptide can be identified when there are at least two peptide fragments that can be detected successively by MS/MS. The more fragments that match well the known protein in the protein database (from NCBI) can be detected, the more reliable the result is. A positive peptide fragment (peptide (hits)) was defined as the fragment having more than four ions that can be detected by MS/MS with high Xcorr (> 2.2) and Delta Cn (> 0.1). Meanwhile, a positive peptide should also have continuous b-type and y-type ion flows detected by MS/MS, especially when fewer than four peptides can be identified. The proteins listed in **Table 1** are recommended by the database, which matched the analyzed protein best. The score of the analyzed protein and the number of peptide (hits) reflect the matching rate between the analyzed protein and the recommended one. Also, it is noteworthy when the protein of interest

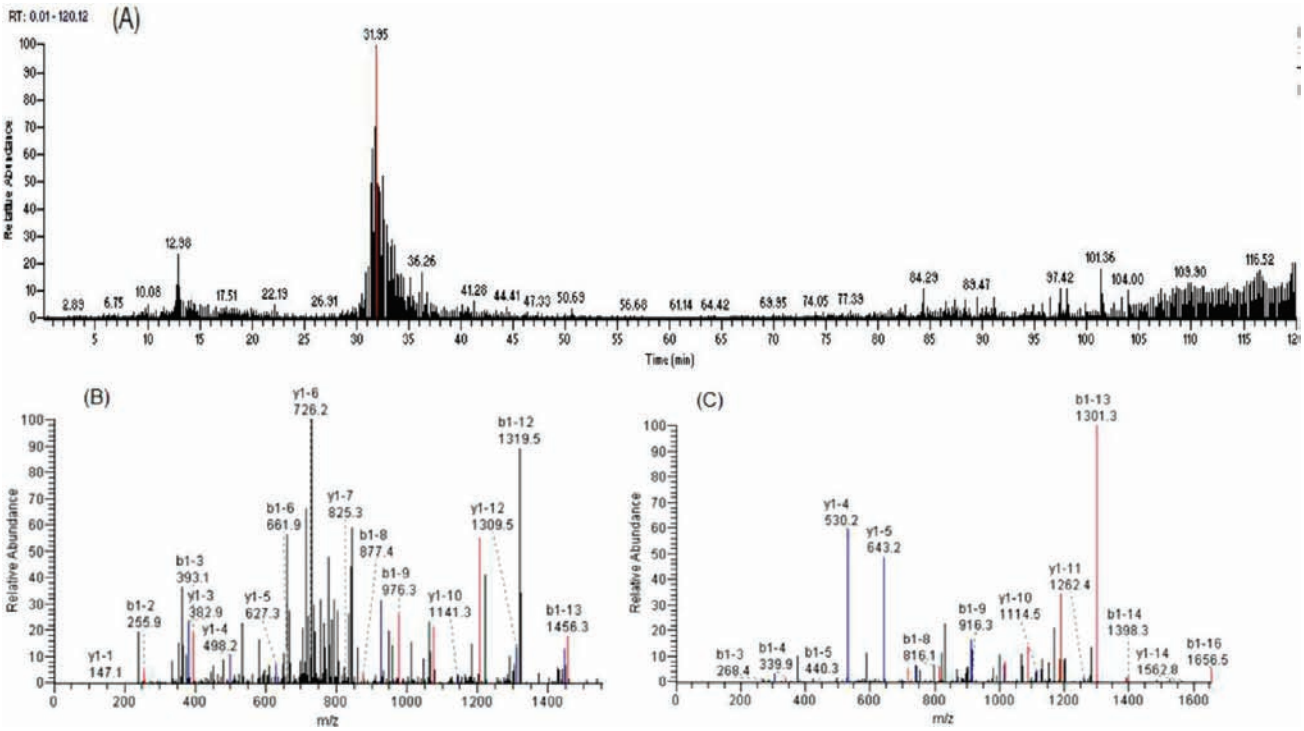

Figure 4. LC-ESI-MS/MS chromatograms of the prepared IgY: (**A**) prepared IgY sample; (**B**, **C**) peptides 1 and 2 in **Table 2**, respectively.

can achieve a score of ≥30. Considering this, **Table 1** lists the proteins that could meet this standard.

We can find from **Table 1** that the product prepared in this study can earn a score of 160.2 when compared with Ig γ chain (clone 36) chicken (fragment). **Figure 4A** exhibits the mass spectrometric profile of the obtained protein. Meanwhile, we can also find from **Figure 4B**,**C** that the detected protein has two different peptide fragments that are the same with the recommended protein, Ig γ chain (clone 36) chicken (fragment). Details of these two peptide fragments are shown in **Table 2**. These results confirmed repeatedly that the product is IgY, although there were also another four proteins with scores above 30.

Optimization Result of Separating HDL. Several concentrations of NaCl were tried in this study, and we noted that the solution with a lower concentration of NaCl (< 0.4 M) could remove some unnecessary proteins, whereas a higher concentration of NaCl (for example, 1 M NaCl) would result in all of the precipitates redissolving in the mixture (data not shown). We also found that 0.2 M NaCl could, to some extent, show a promising effect on separation of HDL before further purification approaches were conducted. Also investigated was the effect of different concentrations of $(NH_4)_2SO_4$ on the isolation of HDL from the granules, which indicated 15% (w/v) of $(NH_4)_2SO_4$ was an excellent choice. **Figure 5** exhibited a result of HDL purification using 0.2 M NaCl coupled with 15% (w/v) of $(NH_4)_2SO_4$.

Characterization of HDL. Figure 6 profiles the HDL after staining by Coomassie brilliant blue, Sudan black B, Methyl green, and $AgNO_3$, separately. Fortunately, all of the lanes (from 1 to 4) visualized the expected band with a molecular weight between 97.4 and 130 kDa. Especially, lane 2 indicated that after hydration by NaOH, the phosphate group contained in the sample reacted with $(NH_4)_2MoO_4$ to form an insoluble nitrophosphomolybdate compound, which could be stained by Methyl green. Furthermore, a modified $AgNO_3$ staining (lane 3) implied that the HDL molecule combined with some carbohydrate, whereas the specific black band obtained from Sudan black B staining (lane 4) proved the prepared product had a characteristic of lipid. This result told us that the acquired protein included phospholipid, glycoprotein, and lipoprotein simultaneously.

Table 2. Details of the Peptides Detected by LC-ESI-MS/MS from the Prepared IgY

sequence	MH^+	charge	X_{Corr}	Delta Cn	Sp	RSp	ions
1. VRHPATNTVVEDHVK	1701.90	2	3.78	0.46	775.0	1	17/28
2. AVPATEFVTTAVLPEER	1829.96	2	4.09	0.52	929.7	1	21/32

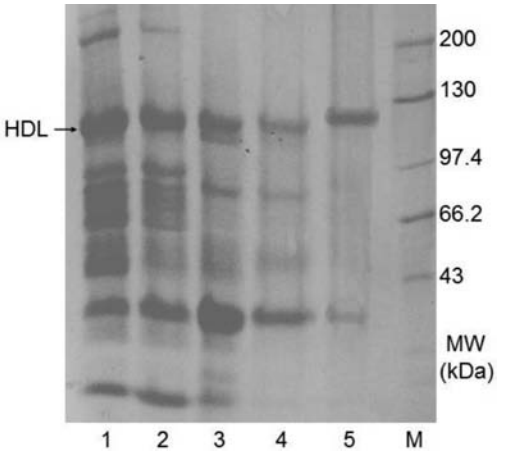

Figure 5. Ammonium sulfate precipitation classification of HDL. Lanes: (1) mixture of the egg yolk after dilution to 1:15; (2) granules of the diluted egg yolk (lane 1); (3) pellets of the granules (lane 2) after washing with 0.2 M NaCl; (4) supernatant obtained from the sample (lane 3) after washing with 5% $(NH_4)_2SO_4$; (5) crude HDL obtained from the sample (lane 4) after precipitation with 15% (w/v) $(NH_4)_2SO_4$; (M) protein standard.

Table 3 lists the SEQUEST result of the obtained HDL after LC-ESI-MS/MS, which told us that the prepared protein matched vitellogenin 2 precursor (*Gallus*) very well, with a score of up to 1020.3 and as many as 16 peptide fragments detected by MS/MS. Details of these 16 peptide fragments are shown in

Table 4. We can find from **Table 4** that all of these peptides have excellent Xcorr and Delta Cn values and that far more than four ion signals can be detected by MS/MS. Furthermore, **Figure 7** presents the chromatograms of the obtained protein (**Figure 7A**) and two typical peptide fragments detected (**Figure 7B**,**C**). These results confirmed that all of these detected peptide fragments could be identified as positive peptides from vitellogenin, and the considerable differences between the prepared protein and the other two proteins listed in **Table 3** made it reasonable that the obtained protein should be HDL.

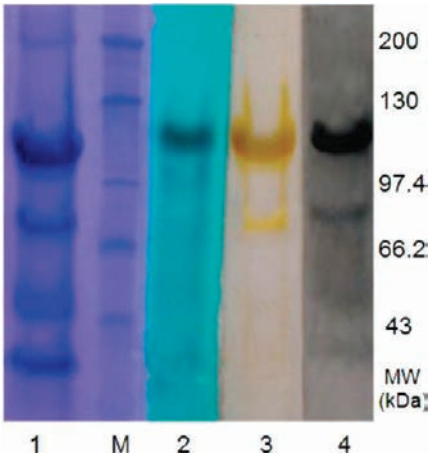

Figure 6. Histobiochemical staining of HDL. Lanes: (1) stained by Coomassie brilliant blue; (M) protein standard; (2) stained by Methyl green; (3) stained by $AgNO_3$; (4) stained by Sudan black B.

Quantitative Evaluation of the Products. Results from three batches (shown in **Table 5**) indicated that 30.2 g of IgY and 20.6 g of HDL on average could be obtained from 10 kg of shell eggs. The modified ELISA showed that the purities of prepared IgY from three batches ranged from 72.4 to 73.1%. The average purity of the prepared IgY was 72.7%. Results from the direct HDL-C assay showed that the average purity of the prepared HDLs was 71.9% (shown in **Table 5**).

DISCUSSION

The total content of IgY in egg yolk is approximately 5.5 kg/ton of shell eggs (*28*). According to Akita and Nakai (*29*), the water dilution method gave the highest yield for separating IgY from hen egg yolk. Yields of 9.8 mg of IgY/mL of yolk with a purity of up to 94% were routinely obtained, which represents about 42.65% of total IgY in egg yolk. With respect to a better separation of IgY and HDL from each other, we diluted egg yolk 1:15 in distilled water rather than 1:10. Results from our study show that about 30 g of IgY with a purity of 72.7% is available from 10 kg of shell eggs, which means that 39.65% of total IgY in egg yolk has been isolated successfully.

Table 3. Identification of HDL by LC-ESI-MS/MS

reference	score	accession	peptides (hits)
vitellogenin 2 precursor (*Gallus gallus*)	1020.3	71896765.0	16
tetra-peptide repeat homeobox-like (*Gallus gallus*)	70.3	52138705.0	6
vitellogenin (*Gallus gallus*)	30.6	63885.0	2

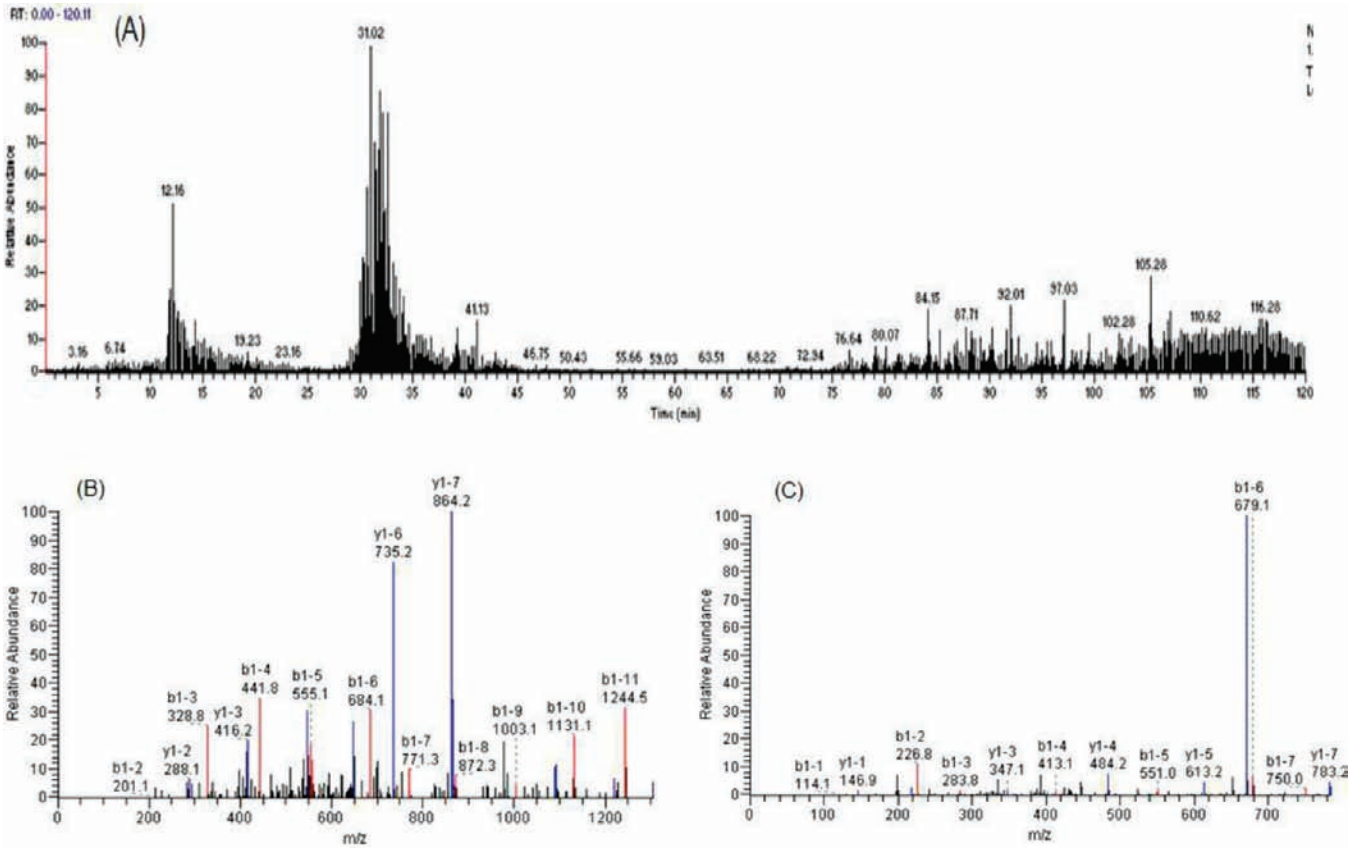

Figure 7. LC-ESI-MS/MS chromatograms of the prepared HDL: (**A**) prepared HDL sample; (**B**, **C**) peptides 2 and 16 in **Table 4**, respectively.

Table 4. Details of the Peptides Detected by LC-ESI-MS/MS from the Prepared HDL

sequence	MH+	charge	X_{Corr}	Delta Cn	Sp	RSp	ions
1. SPQVEEYNGVWPR	1560.74	2	3.19	0.58	1239.6	1	18/24
2. LSQLLESTMQIR	1418.77	2	4.24	0.46	1603.4	1	19/22
3. NSIAGQWTQPVWMGELR	1972.97	2	3.76	0.57	468.2	1	14/32
4. YVIQEDRK	1050.56	2	2.27	0.29	619.8	2	11/14
5. NIGELGVEKR	1114.62	2	2.76	0.26	747.6	1	14/18
6. ADTYFDNYR	1164.50	2	2.30	0.51	705.2	1	14/16
7. LKQSDSGTLITDVSSR	1706.89	2	5.04	0.60	2342.1	1	25/30
8. LFKFEYSSGR	1233.63	2	2.96	0.43	695.1	1	14/18
9. NIPFAEYPTYK	1342.67	2	2.50	0.42	387.0	1	14/20
10. EALQPIHDLADEAISR	1777.91	2	4.05	0.58	500.6	1	16/30
11. WLLSAVSASGTTETLK	1663.89	2	3.71	0.58	763.6	1	17/30
12. TVQGYLIQILADQSLPPEVR	2240.23	2	2.58	0.23	372.9	1	16/38
13. ILGQEVAFININKELLQQVMK	2428.36	2	4.66	0.57	1436.4	1	22/40
14. FLPISSSSAADIPVHIQIDAITALK	2607.44	2	4.12	0.61	519.2	1	22/48
15. FLEVVQLCR	1163.66	2	2.55	0.33	828.5	1	13/16
16. LIGEHEAK	896.48	2	2.29	0.19	498.9	1	13/14

Table 5. Quantitative Analysis of the Two Products

		batch[a]			
		1	2	3	av
IgY	yield/g	30.4	29.2	31.1	30.2
	purity[b]/%	72.4	73.1	72.6	72.7
HDL	yield/g	19.8	21.2	20.8	20.6
	purity[c]/%	73.0	70.7	72.0	71.9

[a] The batch scale was 10 kg of shell eggs per time. [b] Data were acquired using a modified ELISA. [c] Data were acquired using a Direct High Density Lipoprotein-Cholesterol Kit.

The content of HDL in hen egg yolk is theoretically 8%, which was confirmed again in our study (shown in lane 1, **Figure 5**), accounting for about 70% of granules in egg yolk (*28*). However, the purity of the crude HDL in this process was 71.9%, which is just slightly higher than that in granules. We think the possible interactions between the granules and other components of egg yolk (for example, LDL_1 and LDL_2 in plasma) make it difficult to isolate granules effectively from egg yolk even when the egg yolk is diluted several times with water, which subsequently results in the low concentration of HDL in the precipitates obtained from the diluted egg yolk (shown in lane 2, **Figure 5**). It also can be inferred from **Figure 5** that the purity of the crude HDL will be considerably enhanced if further purification methods, for example, ultrafiltration with hollow fiber membranes, are used. The characterization of the prepared HDL in this study has been confined to histobiochemical staining and LC-ESI-MS/MS. The results confirmed the obtained product matched HDL well. However, as a functional protein, further investigations on its biological activities are necessary.

In conclusion, it is possible to isolate IgY and HDL from hen egg yolk simultaneously. Water dilution coupled with $(NH_4)_2SO_4$ precipitation supplies a good separation of IgY and HDL from egg yolk, although the purities of target products are not very high. However, as an attempt focusing on developing a process for mass production, the yield of the target product should receive equal attention as well as the product's purity. Also important are the economy and facility of the process. Therefore, this study tried to avoid utilizing high energy consuming approaches such as ultracentrifugation to presume a higher purity while simplifying the process to gain a higher yield. We also hope this study would give a guide to thorough processing of egg products.

ABBREVIATIONS USED

ACN, acrylonitrile; CBB, Coomassie brilliant blue; HDL, high-density lipoprotein; IgY, immunoglobulin Y; LC-ESI-MS/MS, liquid chromatography−electrospray ionization−mass spectrometry; LDL, low-density lipoprotein; rpm, revolutions per minute; MW, molecular weight; NCBI, National Center of Biotechnology Information; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide electrophoresis; TMB, 3,3′,5,5′-tetramethylbenzidine.

ACKNOWLEDGMENT

We thank Sirajo Umar for the active discussion and proofreading of the paper.

Supporting Information Available: Standard curve detected in the modified ELISA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review May 12, 2010. Revised manuscript received September 21, 2010. Accepted September 27, 2010. This work was partially supported by the National Key Technology R&D Program of China (2007BAD49B05).