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Tandem Ion Exchange Fractionation of Chicken Egg White Reveals the Presence of Proliferative Bioactivity

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Supporting Information

ABSTRACT: Chicken eggs are recognized for their versatility as a food product and as a model for research in biology and medicine. This study investigated the egg white as a source of bioactive compounds. Egg white was fractionated using tandem ion exchange chromatography (SAX and SCX), and seven fractions were assessed for any associated bioactivity. Four fractions at various protein concentrations were shown to contain proliferative bioactivity that exceeded the FBS control. The most potent fraction (6) was used in an in vitro wound closure assay to demonstrate a positive influence on cell migration and restored scratch wounds more rapidly than the control. LC-MS/MS identified 33 proteins in fraction 6 of egg white, most of which play important roles in cell growth and development, signaling, motility, and proliferation. These candidate bioactives suggest that the egg white contains essential compounds that contribute to the growth of an embryo prior to fertilization.

KEYWORDS: *egg white, bioactivity, tangential flow filtration (TFF), proteomics, wound closure, cell proliferation, tandem ion exchange chromatography*

INTRODUCTION

The chicken egg white is primarily composed of water (88% w/w) and protein (10% w/w). It is responsible for a number of functions including antimicrobial[1] and mechanical protection of the yolk and amniotic sac and as a nutritional source for the embryo contributing up to 15% (w/w) of the nutritional requirements for gut development and maturation.[2−5] In addition to their biological functions, egg whites are renowned for their versatility as a food product and for their properties that are exploited by industrial food producers. They provide a low-calorie source for a number of quality proteins and valuable nutrients including lipids, carbohydrates, folic acid, riboflavin, iron, and vitamins A, B, D, E, and K. The protein component of the egg white comprises a small number of highly abundant proteins, notably ovalbumin, ovotransferrin, and lysozyme, making up the majority of the protein component of egg white mass, whereas carbohydrates in their free and protein-bound form make up only 1% (w/w).[6]

Some major egg white proteins have been demonstrated to have useful applications in biology and medicine. For example, ovalbumin when digested by pepsin in the stomach releases an ovokinin peptide (FRADHPFL), corresponding to residues 358−365 of ovalbumin. This specific ovokinin peptide is a bradykinin B1 agonist that significantly lowers systolic blood pressure.[7] Additionally, ovotransferrin is structurally and functionally similar to lactoferrin such that it is responsible for the transport of iron in a soluble form and contains antimicrobial activity against a variety of microorganisms (such as *Escherichia coli* and *P.aeruginosa*).[8]

Despite the well-chracterized functions of some of these egg white proteins, studying the egg white is technically challenging due to the presence of many abundant proteins that greatly affect separation strategies.[9] Previous work to fractionate the chicken egg white using gel filtration, reverse phase, and ion exchange chromatography identified only the majority of the abundant egg white proteins such as ovalbumin and ovotransferrin.[10,11] More recently, proteomic studies have identified large collections of proteins in the egg white using methods such as 1D SDS-PAGE,[9] 2DGE,[12] combinatorial peptide ligand libraries,[9,13] and shotgun proteomic approaches.[9,14] For example, Mann and Mann[14] identified 158 egg white proteins from different protein families (such as serpins, protease inhibitors, chaperones, hydrolases, membrane receptors, and transporters), suggesting that the egg white may play additional biological roles in sustaining the growth of an embryo.[15]

Recent studies on bioactive compounds have shown that multiple biological functions have been attributed to novel basic proteins isolated from duck egg white [Duck Basic Protein Small 1 (dBPS1) and 2 (dBPS2)], conferring RNA binding activity and inhibitory pancreatic lipase activity,[16] similar to other egg white proteins such as protamine, avidin, and lysozyme.[17−19] Peptic hydrolysates of dBPS(1) and dBPS(2) showed potent angiotensin I-converting enzyme (ACE) inhibition[16] in a manner similar to peptic peptides from chicken egg white hydrolysates.[20] Moreover, chicken egg membranes have been evaluated as a new biological dressing in split-thickness skin graft donor sites in burn patients.[21] Egg membranes possessed properties of wound protection and promotion of healing and pain relief at a faster rate than commercially available products Biobrane, B.G.C, and Surgilon, with no occurrence of infection or rejection.[21] These findings provided a rationale to study the chicken egg white to

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investigate whether proliferative bioactives were present and for its potential use in therapeutic applications.

In this study, we employed a novel tandem ion exchange chromatography approach that incorporates strong anion (SAX) coupled in-line with a strong cation exchange (SCX) column[22] to fractionate and simplify the complexity of egg white proteins that is mostly contributed by the abundance and nonspecific binding of ovalbumin to other proteins. Tandem ion exchange chromatography was utilized to (1) capture the majority of egg white proteins in their native form to preserve their maximal biological activity, (2) fractionate captured egg white proteins on each column independently by increasing salt concentration, and (3) retain the majority of egg white proteins on both SAX and SCX columns compared to a single ion exchange column, therefore minimizing protein losses. Each eluted fraction was assessed for proliferative activity, and the most potent fraction was used in wound closure migration assays to further assess bioactivity. The presence of proliferative bioactivity in the chicken egg white is of great interest, as it will provide a further understanding of the physiology of the egg white and offer a source of biologically active compounds that contain therapeutic potential.

MATERIALS AND METHODS

Materials. Tris-HCl, disodium orthophosphate, sodium chloride, Bradford reagent, HEPES, PBS tablets, lyophilized ovalbumin from chicken egg white, porcine trypsin (13000−20000 BAEE units/mg protein), acetonitrile, ammonium bicarbonate, formic acid, dithiothreitol, iodoacetamide, and trypan blue were obtained from Sigma (St. Louis, MO, USA). NuPAGE 10% Bis-Tris precast gels and 3-(*N*-morpholino)propanesulfonic acid (MOPS) running buffer were obtained from Invitrogen (San Diego, CA, USA). DMEM culture medium and fetal bovine serum (FBS) were obtained from GIBCO Industries BRL (Gaithersburg, MD, USA). Penicillin and streptomycin were obtained from Invitrogen (Molecular Probes, Eugene, OR, USA). Sequencing grade trypsin was obtained from Promega (Madison, WI, USA). WST-1 reagent was obtained from Roche Applied Sciences (GmbH, Penzberg, Germany).

Egg White Preparation. Fresh unfertilized eggs from Isa Brown chickens (*Gallus gallus*) fed a standard grain diet containing canola were purchased from the local supermarket. Approximately 60 eggs were broken, and then egg white and yolk were separated manually using a kitchen egg separator. The egg white (500 mL) was diluted 10-fold with 10 mM Tris-HCl and 10 mM disodium orthophosphate, pH 7.0 (4.5 L), stirred for 30 min, and stored overnight at 4 °C. The diluted sample was then centrifuged at 10000*g* for 30 min to remove all insoluble debris, and the supernatant was collected. Filtration of the viscous supernatant was performed using a 1.2 μm glass fiber filter membrane (AP20) (Millipore, MA, USA) using a Millipore Sterifil Aseptic System (Millipore, MA). The supernatant was prepared for tandem ion exchange chromatography.

Tandem Ion Exchange Chromatography. The separation of egg white proteins was carried out using tandem ion exchange chromatography as described previously.[22] This method incorporates both HiLoad 26/10 High Performance Q and SP Sepharose Fast Flow columns [55 mL of resin per column with dynamic binding capacity of 60 mg BSA/mL (Q Sepharose) and 55 mg ribonuclease/mL (SP Sepharose), GE Healthcare, Uppsala, Sweden] for the separation of both anionic and cationic species, respectively, on an ÄKTA explorer FPLC System (GE Healthcare) (Supplementary Information). Columns were initially washed with elution buffer (10 mM Tris-HCl, 10 mM disodium orthophosphate, 1 M sodium chloride, pH 7.0) to remove any bound species and further equilibrated with at least 4 column volumes of equilibration buffer (10 mM Tris-HCl, 10 mM disodium orthophosphate, pH 7.0).

A sample of 100 mL of diluted egg white (1:10 equilibration buffer) was loaded at a flow rate of 8.0 mL/min initially onto the Q Sepharose column to bind anionic compounds. The unbound cationic components that did not bind to the Q Sepharose column were then trapped onto the SP Sepharose column. Compounds that did not bind either Q Sepharose or SP Sepharose were collected as the flow through (FT). Egg white proteins were first eluted from the Q Sepharose column with elution buffer (10 mM Tris-HCl, 10 mM disodium orthophosphate, 1 M sodium chloride, pH 7.0) with isocratic steps at 5% for 30 min, 10% for 30 min, 20% for 20 min, and 100% for 20 min. Following the elution of compounds on the Q Sepharose column, the in-line valve was switched to isolate the SP Sepharose column. Elution was then achieved with elution buffer (10 mM Tris-HCl, 10 mM disodium orthophosphate, 1 M sodium chloride, pH 7.0) on the HiLoad SP Sepharose column using isocratic steps at 5% for 20 min and 100% for 20 min. Fractions were collected for which the A_{280} was detected above 20 mAu except in FT fraction 2 (F2), for which it was necessary to collect an additional fraction to accommodate the large volume retrieved in fraction 1 (F1).

Tangential Flow Filtration (TFF) and Stirred Cell Filtration. Tandem ion exchange chromatography separated diluted egg white into nine fractions. Fractions were concentrated on two 1 kDa nominal molecular weight limit (NMWL) Pelicon Mini cartridges (regenerated cellulose = 0.1 m^2 total surface area) via TFF using a ProFlux M12 (Millipore, Bedford, MA, USA) at a transmembrane pressure of 40 psi. Ultrafiltration and buffer exchange (diafiltration) were used to ensure conductivity of the sample was approximately ≲300 μS.

Owing to the large hold-up volumes associated with TFF, an Amicon stirred cell with a 1 kDa NMWL regenerated cellulose Ultracel membrane (Millipore) was used to further concentrate protein fractions and diafilter the samples in PBS. Nitrogen gas was applied to the apparatus at a maximum pressure of 75 psi while the sample was slowly stirred at 25 rpm to avoid "gelling" and accumulation of egg white proteins on the filter membrane. The concentration of each protein fraction was quantified using the colorimetric Bradford assay.[23] The purified sample fractions were stored at −80 °C if not used immediately.

SDS-PAGE. All samples were prepared for electrophoresis according to the manufacturer's instructions (Invitrogen, San Diego, CA, USA). Briefly, approximately 10 μg of protein (as determined by Bradford assay) was reduced and denatured in 10 mM dithiothreitol and LDS buffer (Invitrogen), respectively, and boiled at 96 °C for 10 min. Each sample was run on NuPAGE 10% Bis-Tris precast gels with MOPS as running buffer. Electrophoresis conditions were set to 200 V and 125 mA for 60 min. The gels were fixed in 7% (v/v) acetic acid and 10% (v/v) methanol and stained overnight with Sypro Ruby (Invitrogen). Gels were destained in 7% (v/v) acetic acid and 10% (v/v) methanol and imaged using the Typhoon Trio Variable Mode Imager (GE Healthcare).

In-Solution Trypsin Digestion. Approximately 20 μg of each egg white fraction (volumes and concentrations for each fraction are provided in the Supporting Information) was buffer exchanged with 100 mM ammonium bicarbonate, pH 7.8, using a Microcon YM-10 centrifugal filter unit (Millipore). The samples were reduced with 25 mM dithiothreitol (50 μL) for 30 min and alkylated in the dark with 55 mM iodoacetamide (50 μL) for 45 min. The samples were buffer exchanged again with 100 mM ammonium bicarbonate, pH 7.8, and concentrated. After concentration, the proteins were digested with 1:50 trypsin/protein (w/w) with sequencing grade trypsin for 18 h at 37 °C. Each fraction was desalted using C_{18} tips (PerfectPure C_{18} tips, Eppendorf, Germany), and the eluate was dried using a vacuum centrifuge followed by resuspension in 0.1% (v/v) formic acid (10 μL) in preparation for nanoRPLC-MS/MS.

RPLC-MS/MS. All tryptic peptides from collected ion exchange fractions were analyzed by nanoLC-MS/MS using an LTQ linear ion-trap mass spectrometer (ThermoFinnigan, San Francisco, CA, USA). Reversed phase columns were packed in-house to approximately 7 cm (100 μm i.d.) using 100 Å, 5 mM, Zorbax C_{18} resin (Agilent Technologies, Santa Clara, CA, USA), in a fused silica capillary with an integrated electrospray tip. A 1.8 kV electrospray voltage was applied via a liquid junction upstream of the C_{18} column. Samples were injected onto the C_{18} column using a Surveyor autosampler. Each

sample was loaded onto the C_{18} column followed by an initial wash step with buffer A [5% (v/v) acetonitrile, 0.1% (v/v) formic acid] for 10 min at 1 μL/min. Peptides were subsequently eluted from the C18 column with 0−50% buffer B [95% (v/v) acetonitrile, 0.1% (v/v) formic acid] for 58 min at 500 nL/min followed by 50−95% buffer B for 5 min at 500 nL/min. The column eluate was directed into a nanospray ionization source of the mass spectrometer. Spectra were scanned over the range of 400−1500 amu. Automated peak recognition, dynamic exclusion, and tandem MS of the top six most intense precursor ions at 40% normalization collision energy were performed using Xcalibur software (ThermoFinnigan).

Spectra files were converted to mzXML format and processed through the global proteome machine (GPM) software (Ver 2.1.1), an open source protein identification system that uses the Tandem algorithm[24] (available at http://www.thegpm.org/). Peptide identification was determined using a 0.4 Da fragment ion tolerance. Carbamidomethyl was considered as a complete modification, and partial modifications were also considered, which included oxidation of methionine and threonine and deamidation of asparagine and glutamine. MS/MS spectra were searched against the *G. gallus* database (from SwissProt, Ensemble, and NCBI released 01/06/2006), and reverse database searching was used for estimating false discovery rates.[25] Protein identifications were validated using a 1% false discovery rate assessed by reverse database searching, which was applied to nonredundant protein lists, and scores were reported as log(e) values. Reported log(e) values are expectation values for the peptides distributed as expected from random matching.[26]

Biological processes for each protein identified in fraction 6 of egg white were determined using gene ontology annotation and information derived from previous studies that have been curated in the SwissProt/UniProt databases.

Cell Proliferation Assays. Swiss 3T3 fibroblast cells from the Balb/c mouse were grown in DMEM with 110 mg/L sodium pyruvate and pyridoxine HCl, which was supplemented with 10% (v/v) FBS, 1% (v/v) penicillin, 1% (v/v) streptomycin, and 10 mM HEPES and hereafter called complete DMEM.

Approximately 5×10^3 cells were seeded into 6-well culture plates and allowed to adhere overnight. Following incubation of cell cultures, complete medium was removed from the well plates. Aliquots of fractionated egg white proteins were diluted with serum-free DMEM (containing no FBS) to obtain various protein concentrations (5 μg/mL, 50 μg/mL, and 0.5 mg/mL). Measurements for cell proliferation were noted every 24 h in batches of six wells using the WST-1 colorimetric assay to monitor cell proliferation. Cells were incubated at 37 °C with 10 μL of WST-1 reagent for 4 h. Absorbances of plates were analyzed using an ELISA plate reader (FluoroStar OPTIMA, BMG Labtechnologies) at 440 and 600 nm to quantitate the level of cell proliferation.

Wound Closure Assay. Swiss 3T3 cell monolayers subjected to narrow scratch wounds were tested for wound closure over time. Cell monolayers were grown to confluency in 6-well plates. Medium was removed from the wells, and the adhered cells were washed twice with PBS to remove unbound cells. Confluent monolayers were wounded by scraping using a pipet tip of 1.2 mm width and incubated at 37 °C. Equivalent protein concentrations (5 μg/mL, 50 μg/mL, and 0.5 mg/mL) of fraction 6 used in proliferation assays were added to serum-free DMEM and incubated with cell monolayers for 6 h. Cell images were obtained using a Leica DFM 280 light microscope.

RESULTS AND DISCUSSION

Tandem Ion Exchange Chromatography Fractionation of Egg White Proteins. With the goal of investigating bioactives in chicken egg white, we used a tandem ion exchange column system consisting of SAX coupled in-line with SCX with the ability to elute bound species from each column sequentially.[22] Here, we applied this strategy for fractionating diluted chicken egg white by capturing both anionic and cationic compounds on corresponding columns where positively charged proteins bind to the strong cation exchange column and negatively charged proteins bind to the strong anion exchange column, as well as utilizing buffers containing high sodium chloride to competitively elute bound species. This tandem ion exchange approach has been used successfully and reproducibly for separating complex human plasma antigens,[22] and, similarly to separate chicken egg white proteins,[11] using SCX followed by SAX. Guerin-Dubiard and co-workers separated unfertilized egg white proteins using a tandem SCX/SAX approach, which redistributed ovalbumin among six fractions, revealing a number of different ovalbumin gene X and Y isoforms.[11]

Egg white was diluted 10-fold, syringe filtered, and fractionated using in-line tandem ion exchange chromatography into nine separate highly reproducible fractions (Figure 1).

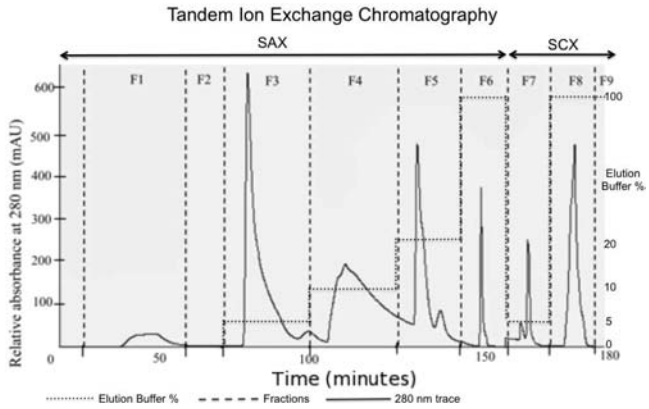

Figure 1. Tandem ion exchange chromatogram of separated diluted egg white from SAX and SCX. The separation of diluted egg white was highly reproducible for over 20 runs. Egg white (100 mL) was loaded at a flow rate of 8.0 mL/min, and nine fractions (F) were collected. F2−F6 were eluted from the SAX column, and F7−F9 were eluted from the SCX column with increasing concentration of sodium chloride.

Fractionation of diluted egg white proteins using tandem ion exchange chromatography was reproducibly carried out for 20 consecutive runs. Nine fractions were collected in total: two fractions composed of the flow through and seven fractions collected from SAX and SCX at intervals where A_{280} was detected above 20 mAu during the elution. The first fraction obtained was the flow through (F1), which contained proteins that did not bind to either the SAX or the SCX column. Increasing concentrations of 5% elution buffer containing 10 mM Tris-HCl, 10 mM disodium orthophosphate, and 1 M sodium chloride, pH 7.0, resulted in the elution of a second set of proteins from the SAX column (F2). Additional increases in the concentration of sodium chloride in three additional steps (10, 20, and 100% elution buffer) yielded the elution of fractions F3, F4, F5, and F6, respectively. Following the elution of proteins from the SAX column, the SAX column was switched offline, and subsequently a two-step increase in the concentration of elution buffer (10 mM Tris-HCl, 10 mM disodium orthophosphate, 1 M sodium chloride, pH 7.0) (i.e., 5 and 100%) was applied to the SCX column to elute three additional fractions (F7, F8, and F9). The specific conditions were determined by preliminary studies using a linear elution gradient from 0 to 100% elution buffer. The majority of egg white proteins were eluted between 5 and 20% elution buffer with very little separation and resolution (data not shown), and

therefore step elutions were employed in our tandem ion exchange methodology to obtain the best separation and narrower elution peaks for this specific sample.

The tandem fractionation approach was effective in redistributing highly abundant egg white proteins (Figure 2),

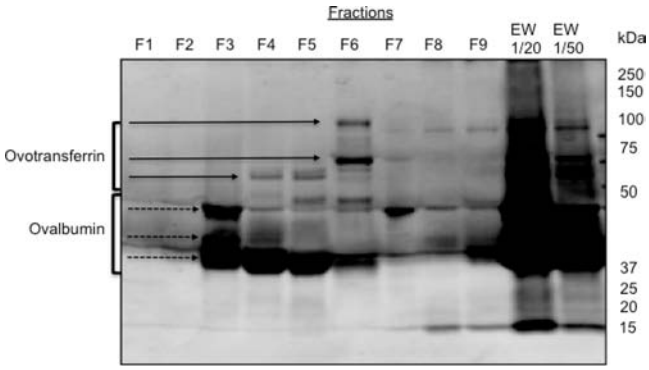

Figure 2. 1D SDS-PAGE (10% acrylamide) of egg white and egg white (EW) fractions (F1−F9). Eleven protein bands were randomly selected, excised, and identified by MALDI-TOF/TOF MS (Supporting Information) and identified mostly highly abundant egg white proteins ovalbumin and ovotransferrin.

such as ovalbumin and ovotransferrin, among all nine fractions. This was an unexpected outcome because ovalbumin, which is approximately 43 kDa in size and has an isoelectric point of 5.2, should be eluted from only the SAX column. However, due to the abundance of ovalbumin making up ∼70% of the protein content in egg white and its ability as a carrier protein, it is prone to binding nonspecifically to other proteins under native conditions. Other depletion methods that are more suited for identifying lower abundant egg white proteins, such as organic and immunoprecipitation for removing ovalbumin, were considered; however, these methods caused protein denaturation and loss of bioactivity in our initial studies, which were not amenable to the aim of our study to identify bioactive compounds in egg white. We utilized our tandem ion exchange approach for the purposes of studying bioactives.

Selective fractionation was achieved with each eluted fraction containing complex mixtures of different proteins with diverse concentrations and molecular weights as observed by 1D SDS-PAGE (Figure 2). Although an A_{280} signal was detected in F1, the Bradford assay could not quantify the amount of protein in this fraction, and subsequent SDS-PAGE demonstrated that this was not due to unbound proteins. Despite the clear fractionation of different egg white proteins among the nine fractions, multiple protein bands were chosen randomly and excised at various molecular weights, in-gel tryptically digested, and subjected to MALDI-TOF/TOF MS identification. We attempted to identify various protein bands that were observable in fractionated egg white and not visibly resolved in the lane containing diluted egg white. MALDI-TOF/TOF MS identified mostly ovalbumin (Supporting Information) among 11 protein bands selected from the gel irrespective of the clear 1D SDS-PAGE separation of different protein species observed. Due to the unexpected identification of predominately ovalbumin by 1D SDS-PAGE and MALDI-TOF/TOF MS, we decided to identify the protein fractions 3−9 by in-solution trypsin digestion and LC-MS/MS.

All nine fractions were subsequently filtered using TFF and stirred cell filtration to concentrate and diafilter egg white fractions with PBS to ensure that the conductivity was approximately 300 μS. The low conductivity was to ensure that salts, free sugars, and small peptides were not retained in samples and had minimal effects on mouse fibroblast cells, their ability to adhere to the plate surface,[27] and subsequent bioactivity assays.

Identification of Egg White Proteins. The use of LC-MS/MS to identify fractionated egg white proteins has been shown to be more effective than traditional 2DGE methods.[15] Many proteins in low copy numbers cannot be identified using 2DGE due to the presence of high-abundance egg white proteins, which occupy a large percentage of the protein space allowed on a gel. The identification of low-abundance proteins using 2DGE may be restricted, due to the limitations of stains in detecting low protein quantities. In-solution trypsin digestion and LC-MS/MS of the most proliferative fraction (6) were carried out to identify egg white proteins in this subset. Protein identifications of fractions 3−9 are provided as Supporting Information.

LC-MS/MS identified 33 proteins in fraction 6 of tandem ion exchange separated egg white (Table 1). Of the 33 protein identifications, 18 have been characterized to play some role(s) in cell reproduction, development, immunity, and protein synthesis. Specifically, nine proteins have been characterized as essential components of cell growth and development, such as GP340 and TENP (Figure 3); five are responsible for biological processes that involve regulating the immune system and defense responses, such as lysozyme C and lymphocyte antigen 86; and four are involved in protein synthesis, such as sulfhydryl oxidase 1 and clusterin. The biological processes and molecular functions of the proteins identified in F6 of the egg white were determined by gene ontology annotations and information curated in the SwissProt/UniProt databases (Table 1).

The proteins found in this study along with previous proteomics studies to elucidate the chicken egg white proteome[9,15] provide further evidence that molecules contained within the egg white may have a more significant purpose including bacterial protection and cellular development for the next generation upon fertilization. We identified with high confidence a subset of egg white proteins (33) in F6 as being potentially responsible for the immense proliferative and migratory bioactivity including vitellogenin,[28] TENP,[29] Dickkopf 3, Wnk 2,[30,31] GP340,[32] and gelsolin,[33] some of which have been characterized to be involved in cell development and differentiation and wound healing (Figure 3). For example, TENP has been identified to play a role during cell proliferation and differentiation during neurogenesis in the developing retina and brain, but not in the heart, kidney, or liver.[29] Moreover, glycoprotein 340 (GP340) is a multifunctional protein that is linked to a number of biological processes including promoting epithelial cell differentiation,[32] wound healing, and induction of the innate immune system.[34]

Interestingly, ρ guanine nucleotide exchange factor 11, serine/threonine-protein kinase WNK 2, and GP340 were identified for the first time in chicken egg white. We could not establish the specific protein component(s) responsible for the proliferative bioactivity, and further evaluation is required. This can be achieved by assaying the individual protein components identified in our mass spectrometry screen and/or additional fractionation of F6 followed by subsequent bioactivity assays to identify the compounds that promote and inhibit proliferative activity.

Table 1. Identification of 33 Proteins in Fraction 6 by LC-MS/MS[26a]

ENSEMBL identifier	log(e)	redundant peptides	M_w (kDa)	description	UniProt/ SwissProt no.	biological process
ENSGALP00000020965	−580.9	158	42.9	ovalbumin	P01012	serine protease
ENSGALP00000016728	−321.8	193	27.8	riboflavin-binding protein precursor (RBP)	P02752	transport, carrier protein
ENSGALP00000008163	−258.3	110	22.3	α1 acid glycoprotein	A7UEB0	defense response
ENSGALP00000010852	−196.1	36	233.6	ovomucin α-subunit	Q98UI9	gelling
ENSGALP00000026723	−190.3	42	51.3	clusterin	Q6PTX2	protein synthesis and regulation
ENSGALP00000010769	−169.7	23	47.4	TENP	O42273	cell development/ function
ENSGALP00000016177	−118.4	33	16.2	lysozyme C precursor	P00698	immune system
ENSGALP00000005544	−115.2	42	22.6	ovomucoid precursor	P01005	unknown
ENSGALP00000000163	−94.2	37	11.9	Hep21 protein	Q8AV77	defense response
ENSGALP00000006256	−93.4	11	43.6	sulfhydryl oxidase 1 precursor	Q8JGM4	protein synthesis and regulation
ENSGALP00000014804	−90.1	27	509.4	GP340	Q9UGM3	cell development/ function
ENSGALP00000023009	−69.4	10	165.3	α-2-macroglobulin-like 1	F1NIV0	transport, carrier protein
ENSGALP00000014869	−64.5	20	55.6	unknown		unknown
ENSGALP00000008890	−61.3	17	39.2	Dickkopf-related protein 3 precursor	Q90839	cell development/ function
ENSGALP00000000619	−57.6	8	110.2	transmembrane protease	Q7Z410	serine protease
ENSGALP00000005535	−56.2	7	52	ovoinhibitor precursor.	P10184	serine protease
ENSGALP00000020967	−38.9	8	44.5	ovalbumin-related protein X	P01013	serine protease
ENSGALP00000020856	−37.7	5	18	lymphocyte antigen 86 precursor (MD-1 protein)	Q90890	defense response
ENSGALP00000002888	−34.2	4	205	vitellogenin-2 precursor	P02845	cell development/ function
ENSGALP00000019031	−27.9	4	69.8	serum albumin precursor	P19121	transport, carrier protein
ENSGALP00000010404	−25.2	4	99.8	ovotransferrin precursor	P02789	transport, carrier protein
ENSGALP00000013544	−23.5	3	69.9	coagulation factor II	F1NXV6	other
ENSGALP00000020549	−19.9	3	73.9	lipopolysaccharide binding precursor LBP	F1NYJ8	defense response
ENSGALP00000005221	−9.9	2	16.2	uncharacterized protein C1orf90	Q9BTA0	unknown
ENSGALP00000007520	−9.9	2	56.6	Asc-type amino acid transporter 1	Q9NS82	transport, carrier protein
ENSGALP00000022989	−9.7	2	234.8	polycystic kidney disease and receptor for egg jelly related protein precursor	Q9NTG1	reproduction
ENSGALP00000002197	−9.6	2	85.8	gelsolin precursor (actin-depolymerizing factor)	O93510	cell development/ function
ENSGALP00000021624	−9.3	2	125.1	ρ guanine nucleotide exchange factor 11	O15085	cell development/ function
ENSGALP00000014228	−9.3	2	20.9	hypothetical protein	Q5ZKE1	unknown
ENSGALP00000008354	−9.3	2	166.6	serine/threonine-protein kinase WNK2	Q9Y3S1	cell development/ function
ENSGALP00000027640	−9	2	119	centromere protein J 7	Q9HC7	cell development/ function
ENSGALP00000012100	−9	2	134.3	leucyl-tRNA synthetase, cytoplasmic	Q9P2J5	protein synthesis
ENSGALP00000018665	−8.9	2	187	tensin	Q04205	cell development/ function

[a]A 1% false discovery rate was applied by reverse database searching as determined by GPM and with a minimum of two unique peptide matches for confident hits. Log(e) values are expectation values for the peptides distributed as expected from random matching.

Egg White Proteins in Fraction 6 Promote Cell Proliferation and Wound Closure. Proliferation cell-based and wound closure assays were used to determine the presence of bioactives in each of the fractionated chicken egg white samples. Due to the lack of proteins observed by SDS-PAGE in F1 and F2, only F3−F9 were further tested for bioactivity. The cell proliferation and wound closure assays were chosen to assess bioactivity of egg white fractions on mouse fibroblast cells to recapitulate phases of wound healing and repair of skin and fibroblasts. We examined wound-healing bioactivity using egg white on the basis of previous findings that showed the ability of egg membranes to promote infection-free wound healing and re-epithelialization on split-thickness skin grafts in burn patients.[21] This was the basis to examine egg white fractions for these reparative properties. The classic model of wound healing is divided into three phases: (1) inflammation, (2) proliferation, and (3) epithelial remodeling.[35] The proliferative phase of cells studied in vitro consists of collagen deposition to re-form the extracellular matrix, granulation, and wound closure and re-epithelialization.[36] Together, the cell proliferation and wound closure assays are simple and

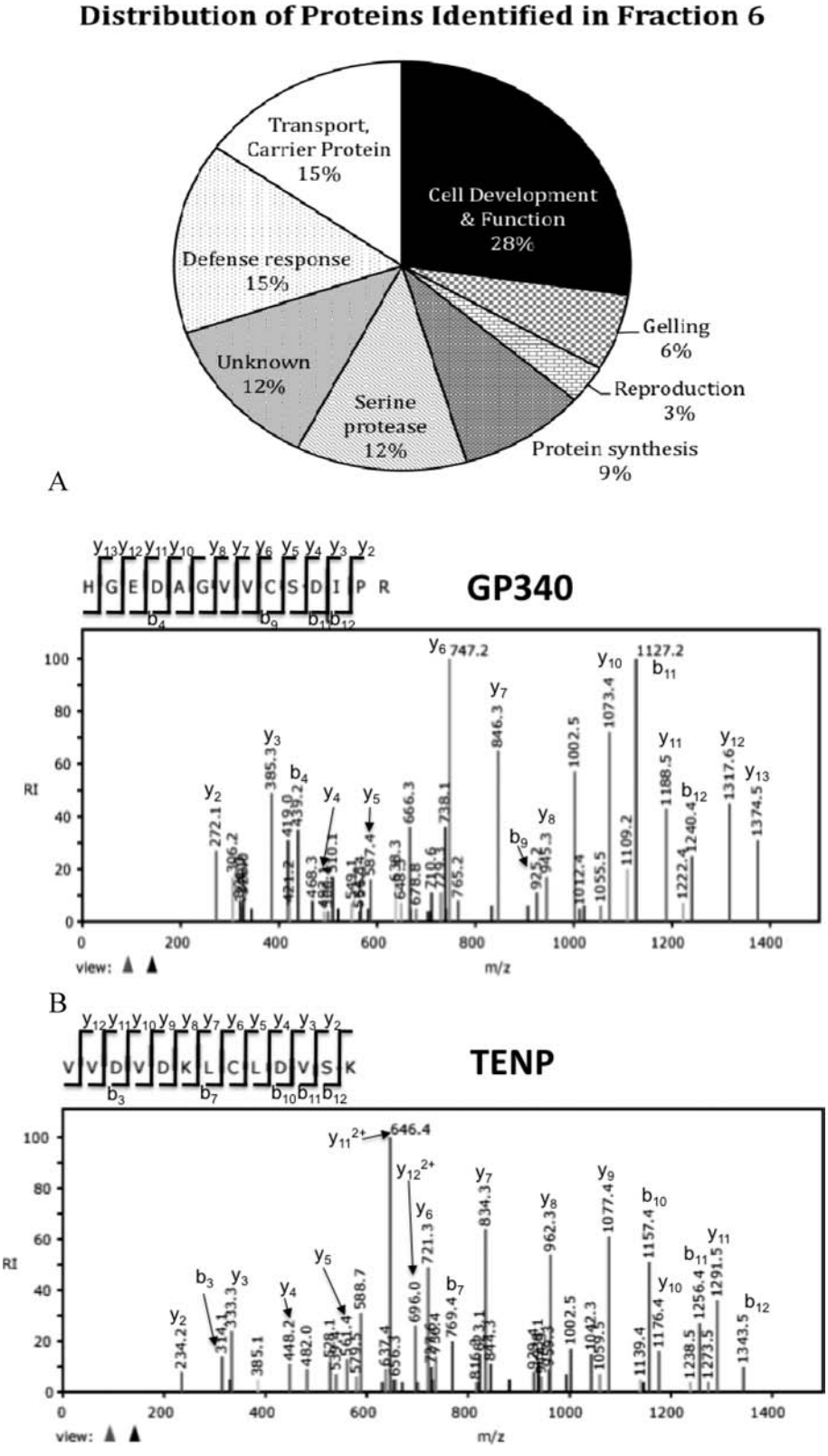

Figure 3. Distribution of proteins identified in growth-proliferative fraction 6: (A) LC-MS/MS of fraction 6 identified 33 proteins, of which 9 have been characterized to be involved in cell growth and development including GP340 and TENP; (B) MS/MS spectra of peptide HGEDAGVVCSDIPR from GP340 and peptide VVDVDKLCLDVSK from TENP.

commonly used assays to demonstrate the initial phases of wound healing in vitro.

Egg white fractions and pure egg white were diluted in serum-free DMEM containing no FBS and used to activate cell proliferation and migration of Swiss 3T3 fibroblast cells. The nine egg white fractions showed different effects on the fibroblast cells. Four of these fractions (F3, F5, F6, and F8) were shown to exhibit more effective proliferation activity than

the FBS control (Figure 4). Fractions 3, 5, 6, and 8 were used for proliferation studies over a 96 h period. The results were

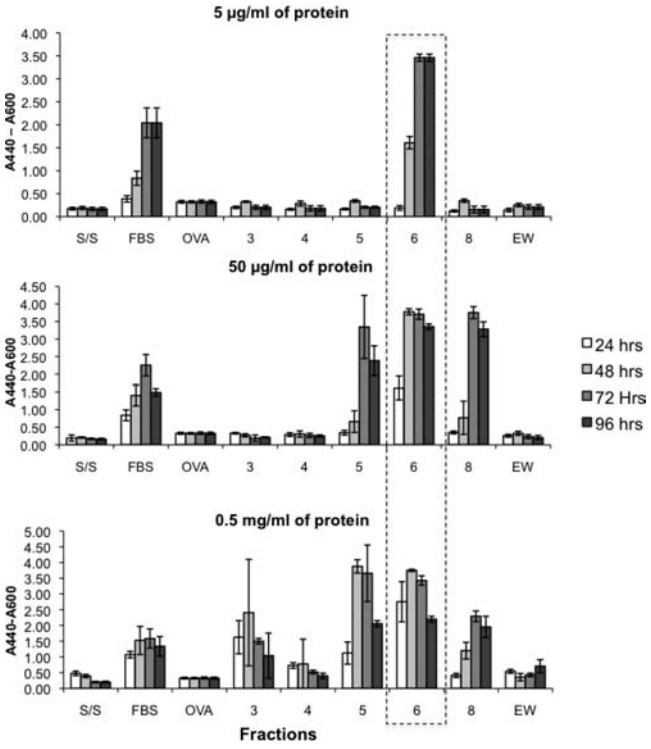

Figure 4. Viability of cells over a 96 h period supplemented with (A) 5 µg/mL, (B) 50 µg/mL, and (C) 0.5 mg/mL of protein from fractions 3, 5, 6, and 8 diluted in serum-free DMEM containing no FBS. Fraction 6 demonstrated the most potent proliferation (boxed) and was selected for wound closure assays. Cell proliferation was not observed after treatedment with no serum or with pure ovalbumin. Serum starved (S/S), fetal bovine serum (FBS), pure ovalbumin (OVA), and egg white (EW diluted 1:10) were used as controls for cell-based assays.

intriguing as they revealed for the first time the ability of fractionated egg white to stimulate proliferation of fibroblast cells in the absence of FBS. These four fractions showed significant proliferation activity when fibroblast cells were exposed to specific concentrations (5 µg/mL, 50 µg/mL, and 0.5 mg/mL) of fractionated egg white proteins (Figure 4). MALDI-TOF/TOF MS of protein bands from each eluted fraction identified a high abundance of ovalbumin (Supporting Information); hence, we used commercially available ovalbumin (5 µg/mL, 50 µg/mL, and 0.5 mg/mL) as an additional control to determine whether its presence in these fractions had an effect on cell proliferation (Figure 4). The control experiment was repeated three times and determined to have no effect on cell proliferation. This is consistent with other studies that have used different cell lines and showed no proliferation response after treatment with ovalbumin.[37−39]

Mouse fibroblast cells supplemented with serum-free media containing 5 µg/mL protein showed that F6 was the most potent for inducing proliferation, whereas significant proliferation was observed with F5, F6, and F8 at a higher concentration of 50 µg/mL protein and with F3 at 0.5 mg/mL protein. Collectively, mouse fibroblasts supplemented with serum-free medium (no FBS) containing egg white fractions (3, 5, 6, and 8) at various concentrations showed a more proliferative effect compared to the FBS control. This was an interesting observation because FBS is the most widely used growth supplement for cell cultures, primarily because of its high levels of growth stimulatory factors and low levels of growth inhibitory factors. Fraction 4 and whole egg white did not induce proliferation beyond control levels. Cells supplemented with F7 and F9 did not show any detectable proliferation within 24 h of incubation (data not shown).

Fraction 6, the most potent for cell proliferation at 5 µg/mL, was subsequently used for an in vitro wound closure assay. We examined the ability of F6 to activate the unique directional cell spreading and motility required for wound closure in stable confluent monolayers. Fibroblast cells were monitored at 2, 4, and 6 h intervals. Fibroblast cells incubated with 5 and 50 µg/mL of F6 were the most effective in promoting cell migration (Figure 5 and Supporting Information). After 6 h of incubation, cell−cell bridge migrations became absolute, where the monolayer was restored to confluence at a faster rate than the FBS control (4-fold). The extracellular matrix remained intact and attached to the surface. The accelerated rate of cell migration at these concentrations is consistent with the rate ofwith the increased rate of proliferation of this fraction. Despite the potent proliferation of fibroblast cells with F6 with 0.5 mg/mL, reduced cell migration activity was observed at this higher concentration, and cells treated with 1.5 mg/mL of protein were detached from the plate surface (Supporting Information). We speculate that the treatment of cells with increased protein concentration in the examined wound closure assay concomitantly elevates levels of growth inhibitory factors and/or increases concentration and activity of extracellular matrix proteases causing the reduced cell migratory activity. This observation warrants further investigation.

The immense rate of proliferation and motility of F6, which exceeds FBS, supports the possibility of growth factors, hormones, or other nutrients being present in these egg white fractions. The negative control (serum starved) was observed to have very little effect on cell migration and on its morphology. After 6 h of incubation, cells begin to detach from the surface of the plate where the matrix was no longer maintained. Thus, cell migration had collapsed after 6 h. The positive control (FBS) was shown to adopt cell−cell bridge motility and, after 6 h of incubation, was exposed to promoting migration. The wound in the FBS control-treated cells had closed, and the monolayer was confluent by 24 h (Figure 5).

The wound closure assay measures the migration of cells as a net change in the area covered by cells over time when seeded.[40] In scratch wounds, where cell migration is required to close the wound, two types of wound-associated movements can occur in fibroblast cells. The first type of motility occurs when cells break contact with the monolayer, spread, and then migrate into the wound gap by forming cell−cell bridging. The second type occurs when cells within the confluent monolayer spread and migrate as whole connected sheets to fill the wound gap.[41−43] In this study, the migration of Swiss 3T3 fibroblast cells was observed to behave in both ways (Figure 5 and Supporting Information). The combination of increased proliferation and migration of fibroblast cells into the wound has been shown to trigger increased levels of extracellular matrix proteins, assisting in re-forming granulation tissue and subsequently contracting the wound.[44] Properties of normal cells such as attachment, spreading, motility, and proliferation are all involved with cell−cell and cell−matrix interactions.[45] The Swiss 3T3 cell line derived from mouse fibroblast has a low rate of spontaneous transformation, and although it does not

Figure 5. Mouse fibroblast cells were subjected to narrow scratch wounds and incubated with DMEM containing 10% fetal bovine serum (FBS, egg white fraction 6 (5 μg/mL, 50 μg/mL, and 0.5 mg/mL) and no serum (S/S, serum starved). Fibroblasts treated with 5 μg/mL of fraction 6 were shown to migrate and restore scratch wounds after 6 h of treatment, and cells treated with FBS restored wounds after 24 h of treatment.

divide in serum-free media, it is able to proliferate in bovine serum or in serum-free media containing a single added growth factor such as EGF, FGF, or PDGF.[45−47] This supports the notion that bioactive proteins, particularly these growth factors, may be present at low concentrations in the egg white fractions tested and warrants further investigation. Also, peptide growth factors have been shown to regulate many of these processes, such as proliferation and migration in vitro, and may also regulate important phases of wound healing in vivo.[48,49] Further analysis to positively identify the existence of growth factors would allow a better understanding of the properties and purpose of the white in the avian egg.

Our approach for separating chicken egg white by tandem SAX and SCX ion exchange chromatography provides a novel strategy for fractionation and preservation of proteins in their native states, simplifying complex mixtures of proteins, and examining any associated bioactivity. Fractions 3, 5, 6, and 8 of egg white contained proliferative bioactivity, with F6 being the most potent fraction in promoting cell proliferation and migration in mouse fibroblast cells. LC-MS/MS identified 33 proteins in F6 with high confidence; some of these proteins are responsible for cellular growth and development and immunity. The identification of novel egg white proteins in fractionated egg white suggests that many of these proteins are essential building blocks for the next generation upon fertilization. Additionally, this study demonstrates the diversity and complexity of such an intricate biological system and has laid the foundation for separating egg white into fractions useful for protein identification and detection of pro-growth bioactives that may contain therapeutic value.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

MALDI-TOF/TOF MS identification of excised protein bands, images of wound closure assays, and protein identifications of F3−F9. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS USED

TFF, tangential flow filtration; SAX, strong anion exchange; SCX, strong cation exchange; LC-MS/MS, reverse phase liquid chromatography mass spectrometry

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