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## Exploring the Chicken Egg White Proteome with Combinatorial Peptide Ligand Libraries

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The use of two types of peptide ligand libraries (PLL), containing hexapeptides terminating either with a primary amine or modified with a terminal carboxyl group, allowed the discovery and identification of a large number of previously unreported egg white proteins. Whereas the most comprehensive list up to date (Mann, K., *Proteomics* 2007, 7, 3558–3568) tabulated 78 unique gene products, our findings have almost doubled that value to 148 unique protein species. From the initial nontreated egg, it was possible to find 41 protein species; the difference (107 proteins) was generated as a result of the use of PLLs from which a similar number of species (112 and 109, respectively) was evidenced. Of those, 35 proteins were the specific catch of the amino-terminus PLL, while 33 were uniquely captured by the carboxy-terminus PLL. While a number of these low-abundance proteins might have a biological role in maintaining the integrity of the egg white and protecting the yolk, others might be derived from decaying epithelial cells lining the oviduct and/or represent remnants of products from the magnum and eggshell membrane components secreted by the isthmus, which might ultimately be incorporated, even if in trace amounts, into the egg white. The list of egg white components here reported is by far the most comprehensive at present and could serve as a starting point for isolation and functional characterization of proteins possibly having novel pharmaceutical and biomedical applications.

**Keywords:** egg white • peptide libraries • hexapeptide ligands • low-abundance proteome • mass spectrometry

### Introduction

Egg white has been used for decades in the food industry, generally as a gelling, emulsifying and foaming agent.<sup>1–3</sup> The avian egg is a nutritious food and also a major source of biologically active compounds that are beneficial for human health. These biologically active molecules are widely used by pharmaceutical, cosmetic and food industries.<sup>4–6</sup> However, egg white proteins could also raise medical concerns due to the presence of potential allergens, such as ovomucoid<sup>7</sup> and ovalbumin.<sup>8</sup> Egg proteins were previously studied using classical biochemical techniques such as chromatographic and electrophoretic separations, together with Edman sequence analysis. Surprisingly, up to 1989, only 13 proteins were usually referenced in egg white, some of which were not even fully characterized.<sup>9</sup> This might be due to the unfavorable composition of its proteinaceous content, by which six proteins (ovalbumin, 54%; ovotransferrin, 13%; ovomucoid, 11%;

lysozyme, 3.5%; ovomucin, 3.5%; and ovoglycoprotein, 1%) constitute 86% of its entire proteome.<sup>1,10</sup> The simplicity of egg white proteins composition is however only apparent when observing the extraordinarily large degree of glycosylation of many proteins. From this general picture, it appears clear that if one wants to elucidate the full composition and then determine the function of protein constituents of egg white, it becomes necessary to use fractionation techniques capable to evidence species that are hidden and thus difficult to detect. In principle, this problem could be circumvented by adopting prefractionation techniques, such as ion-exchange chromatography,<sup>10</sup> or preparative electrophoresis,<sup>11</sup> both of which have indeed been tried, although the recovered fractions were not submitted to proteomic analysis.

Proteomics analysis via 2-D mapping, in fact, has not borne its fruits. In one instance, Guérin-Dubiard et al.,<sup>12</sup> via 2-D electrophoresis and ESI LC-MS/MS, identified only 16 gene products, out of 69 spots eluted and analyzed from the various wide and narrow pH range maps. Apart from well-known egg white constituents, this study identified some new egg white proteins, such as the vitelline membrane protein VMO-1, Tenp (a potentially antimicrobial protein) and the chondrogenesis-associated lipocalin CAL g. Several proteins presented a very wide polymorphism, some of them generating strings of spots up to

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10 in number. Several known egg-white components were not recognized; this fact was blamed onto limitations of 2D PAGE related to extreme molecular masses or extreme pI values of some proteins, although in the 2-D map most proteins exhibited pIs in the pH 4–6 range, with the notable exception of lysozyme and avidin (pI 10.7 and 10.0, respectively). In another instance, Raikos et al.,<sup>13</sup> via 2-D PAGE and MALDI-TOF MS, identified five proteins, namely, ovalbumin, ovotransferrin, clusterin, activin receptor IIA, and the hypothetical protein FLJ10305. These results indicated that the known protein inventory of egg white was far from being complete. A major breakthrough came only recently, with the work of Mann<sup>14</sup> who, using 1D SDS-PAGE and LC-ESI FT-ICR (Fourier-transform, ion cyclotron resonance) MS and performing MS/MS and MS<sup>3</sup> experiments, was able to identify 78 chicken egg white proteins, 54 of them detected for the first time, thus, tabulating what appears to be by far the most comprehensive list available at present.

With the understanding that even this last achievement, albeit impressive, might not quite represent the full proteome of egg white, we have revisited the field, by applying the now well-ingrained combinatorial library of hexapeptides, as originally described by Thulasiraman et al.<sup>15</sup> (for reviews, see refs 16–18). Our group has already used this technology to search for the low-abundance proteome in various biological extracts, such as urine,<sup>19</sup> serum<sup>20</sup> and human platelets lysate,<sup>21</sup> thus, greatly expanding the identification of rare species. In another application, this technique has performed extremely well in bringing to the limelight host proteins and other impurities present in trace amount in biotech products.<sup>22</sup> A typical application recently achieved was the strong reduction of dominating species, while concomitantly amplifying very low abundance proteins from a red blood cell lysate.<sup>23</sup> The results here obtained are quite impressive as they have doubled the list of identified species reported by Mann.<sup>14</sup>

## Materials and Methods

**Materials.** The solid-phase combinatorial peptide Library-1 (ProteoMiner) and its carboxylated version (Library-2), as well as materials for electrophoresis such as gel plaques and reagents and ProteinChip arrays, were from Bio-Rad Laboratories (Hercules, CA). N-Ethylmaleimide, urea, thiourea, 3-[3-cholamidopropyl]dimethylammonio]-1-propansulfonate (CHAPS), isopropanol, acetonitrile, trifluoroacetic acid and sodium dodecyl sulfate were all from Sigma-Aldrich (St Louis, MO). Complete protease inhibitor cocktail tablets and yeast alcohol dehydrogenase (ADH) were from Roche Diagnostics, (Basel, Switzerland). Sequencing grade trypsin was from Promega (Madison, WI). All other chemical were also from Aldrich and were of analytical grade.

**Sample Collection and ProteoMiner Treatment.** Unfertilized chicken eggs, freshly laid, were used. The white was manually separated from the yolk and gently homogenized with a magnetic stirrer until strong reduction of viscosity was achieved. To prevent protease action, the egg white was immediately added with two tablets of complete protease inhibitor cocktail. One hundred milliliters of egg white was collected, corresponding to a total of 3.5 g of protein, and added with solid salt powder so as to equilibrate it in PBS (25 mM phosphate buffer, pH 7.2, containing 150 mM NaCl). This sample was put in contact with ProteoMiner Library-1 and shaken overnight at room temperature. During the treatment, mucin aggregates were formed as white precipitate around the beads and were eliminated. After filtration and collection of

the supernatant, Library-1 was extensively washed to remove excess soluble proteins and the captured proteins were eluted with three sequential eluants, as follows: TUC solution (2 M thiourea, 7 M urea, 2% CHAPS), UCA solution (9 M urea, citric acid up to pH 3.3) and a hydro-organic solution (HOS) composed of 6% (v/v) acetonitrile, 12% (v/v) isopropanol, 2% (v/v) ammonia and 80% (v/v) water. The supernatant from Library-1 was then mixed with Library-2 and the suspension shaken overnight. After filtration, Library-2 was extensively washed with PBS to remove excess of proteins and the captured species were eluted according to the same procedure used for Library-1. The six eluates were immediately neutralized, submitted to protein content analysis by the Bradford-Lowry standard spectrophotometric method, desalted by dialysis at 4 °C against a 10 mM ammonium carbonate solution (cutoff of dialysis membrane was 1000 Da) and lyophilized.

**One-Dimensional Electrophoresis.** Ten microliters of each sample was mixed with 10 µL of Laemmli buffer<sup>24</sup> (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris HCl, pH approximately 6.8). The mixture was heated in boiling water for 5 min and immediately loaded in the gel. The SDS-PAGE gel was composed of a stacking gel (125 mM Tris-HCl, pH 6.8, 0.1% SDS) with a large pore polyacrylamide gel (4%) cast over the resolving gel (8–18% acrylamide gradient in 375 mM Tris-HCl, pH 8.8, 0.1% SDS buffer). The cathodic and anodic compartments were filled with Tris-glycine buffer, pH 8.3, containing 0.1% SDS. Electrophoresis was at 100 V until the dye front reached the bottom of the gel. Staining and destaining were performed with Colloidal Coomassie Blue<sup>25</sup> and 7% acetic acid in water, respectively. The SDS-PAGE gels were scanned with a Versa-Doc image system (Bio-Rad). For MS analysis, 30–120 µg of total proteins was loaded per track; at the end of the run, 15 slices were excised that covered the whole gel resolving region.

**Surface Enhanced Laser Desorption Ionization–Mass Spectrometry (SELDI-MS).** Protein fractions a concentration of 0.02 µg/mL, were deposited upon ProteinChip Array surfaces, using a Bioprocessor device. Two types of arrays were selected: CM10 (weak cation exchanger) and IMAC30 (immobilized metal ions affinity chromatography) loaded with copper ions. Each array contained eight distinct spots over which the adsorption of protein could be performed. After applying the samples (starting material and the first two eluates from each combinatorial peptide ligand library), chip surfaces were washed to remove non-associated proteins, dried and prepared for the analysis after application of 1 µL of energy adsorbing matrix solution composed of a saturated solution of sinapinic acid in 50% acetonitrile and 0.5% trifluoroacetic acid. All arrays were then analyzed with a PCS 4000 ProteinChip Reader. The instrument was used in a positive ion mode, with an ion acceleration potential of 20 kV and a detector gain voltage of 2 kV. The mass range investigated was from 3 to 20 kDa. Laser intensity was set between 200 and 250 units according to the sample tested. The instrument was mass calibrated using a kit of standard masses mixture “All-in-1 protein standard”.

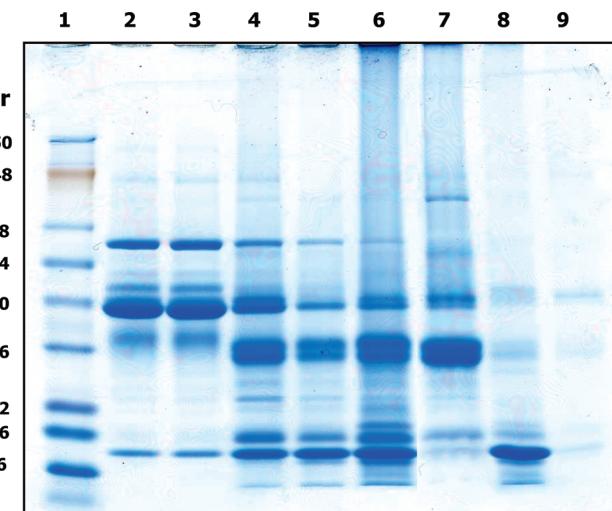
**2D-PAGE Analysis.** The desired volume of each non-treated sample and eluates was solubilized in the “2-D sample buffer” (7 M urea, 2 M thiourea, 3% CHAPS, 40 mM Tris, 5 mM TBP and 10 mM acrylamide) to a final concentration of 2 mg/mL protein and the alkylation reaction was allowed to proceed at room temperature for 60 min. To stop the alkylation reaction, 10 mM DTT (diluted from a bottle of neat DTT) was added to the solution, followed by 0.5% Ampholine (diluted directly from

the stock, 40% Ampholine solution) and a trace amount of bromophenol blue. The 18-cm long IPG strips (Bio-Rad), pH 3–10 NL, were rehydrated with 400  $\mu$ L of protein solution for 5 h. Isoelectric focusing (IEF) was carried out with a Protean IEF Cell (Bio-Rad) in a linear voltage gradient from 100 to 2000 V for 5 h, 2000 V for 4 h, followed by an exponential gradient up to 10 000 V, until each strip was electrophoresed for 25 kV/h. For the second dimension, the IPG strips were equilibrated for 25 min in a solution containing 6 M urea, 2% SDS, 20% glycerol, and 375 mM Tris-HCl (pH 8.8) under gentle shaking. The IPG strips were then laid on an 8–18% acrylamide gradient SDS-PAGE with 0.5% agarose in the cathode buffer (192 mM glycine, 0.1% SDS and 100 mM Tris-HCl to pH 8.3). The electrophoretic run was performed by setting a current of 5 mA/gel for 1 h, followed by 10 mA/gel for 1 h and 15 mA/gel until the dye front reached the bottom of the gel.<sup>26</sup> Gels were incubated in a fixing solution containing 40% methanol and 7% acetic acid for 1 h followed by silver staining. Destaining was performed in 7% acetic acid until a clear background was observed, followed by a rinse in pure water. The 2-DE gels were scanned with a Versa-Doc image system (Bio-Rad), by fixing the acquisition time at 10 s; the relative gel images were captured via the PDQuest software (Bio-Rad). After filtering the gel images for the removal of the background, spots were automatically detected, manually edited and then counted.

**Protein Digestion.** Gel slices were triturated, *in-gel* reduced, S-alkylated and digested with trypsin as previously reported.<sup>27,28</sup> Gel particles were extracted with 25 mM NH<sub>4</sub>HCO<sub>3</sub>/acetonitrile (1:1 v/v) by sonication and peptide mixtures were concentrated. Samples were directly analyzed by  $\mu$ LC-ESI-IT-MS/MS.<sup>27–29</sup>

**MS Analysis and Protein Identification.** Peptide mixtures from 1-DE bands were analyzed by  $\mu$ LC-ESI-IT-MS/MS using a LCQ Deca Xp Plus mass spectrometer (ThermoFinnigan) equipped with an electrospray source connected to a Phoenix 40 pump (ThermoFinnigan).<sup>27–29</sup> Peptide mixtures were separated on a capillary ThermoHypersil-Keystone Aquasil C18 Kappa column (100  $\times$  0.32 mm, 5  $\mu$ m) (Hemel Hempstead, U.K.) using a linear gradient from 10% to 60% of acetonitrile in 0.1% formic acid, over 60 min, at a flow rate of 5  $\mu$ L/min. Spectra were acquired in the *m/z* 200–2000 range. Three injections were analyzed for each sample. Acquisition was controlled by a data-dependent product ion scanning procedure over the three most abundant ions, enabling dynamic exclusion (repeat count 2 and exclusion duration 3 min). The mass isolation window and collision energy were set to *m/z* 3 and 35%, respectively. Data were elaborated using the BioWorks 3.1 software provided by the manufacturer.

The Sequest algorithm<sup>30</sup> was used to identify proteins from 1-DE bands by  $\mu$ LC-ESI-IT-MS/MS experiments. Proteins were identified by comparison of tryptic peptide product ion mass spectra against those generated from the nonredundant NCBI database. The multiconsensus report function was used to assign tryptic peptides to individual proteins and compile rank listings of the proteomes. Sequest parameters included selection of trypsin with up to 2 missed cleavage sites, static and dynamic mass modification associated to Cys carbamidomethylation and Met oxidation, respectively. Identified proteins were ranked in ascending order according to consensus scores and false-positive identifications minimized by filtration against 4 of the following criteria: Xcorr > 2,  $\Delta$ Cn > 0.2, Sp > 400, rsp < 5, ions > 30%.<sup>31</sup> Where appropriate, protein identifications were checked manually to provide for a false-positive rate of <1% by using Xcorr and  $\Delta$ Cn values described and validated elsewhere.<sup>32</sup> The identified pro-



**Figure 1.** SDS-PAGE analysis of egg white. Sample load for all tracks: 10  $\mu$ L. Samples: 1,  $M_r$  markers (the scale is in kDa); 2, starting egg white; 3, flow through; 4, TUC eluate; 5, UCA eluate; 6, HOS eluate; 7, TUC eluate; 8, UCA eluate; 9, HOS eluate. Samples 4–6 refer to Library-1, whereas samples 7–9 refer to Library-2. Staining with colloidal Coomassie Blue.

teins were further evaluated by comparison with experimental  $M_r$  values. Reproducibility and reliability of the results were subjected to the following criteria: (i) proteins with more than 2 peptides were considered as reliable proteins; (ii) single peptide protein identifications were considered reliable if they were found repeated in the same fraction number of other injections.<sup>33</sup>

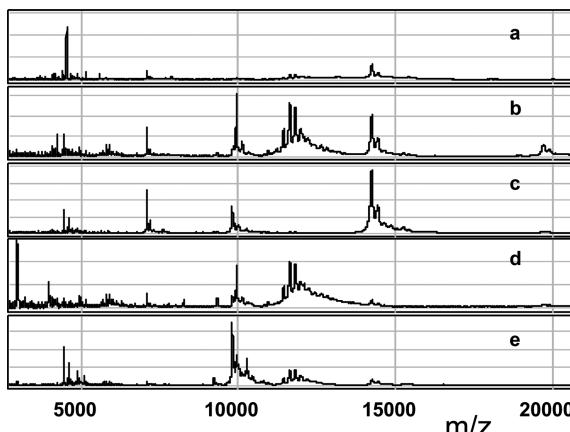
Proteins reported in NCBI database as hypothetical, putative uncharacterized, or similar were further analyzed to investigate their nature. BLAST analysis of their sequence was performed with the program provided by the NCBI against the non-redundant databases for all organisms.

## Results

Figure 1 shows the SDS-PAGE profiling of the starting material, the flow-through and all eluates from the hexapeptide libraries. It can be immediately visualized that at least two of the most abundant proteins in the control material (ovalbumin and ovotransferrin at the vicinity of 45 and 75 kDa, respectively) were strongly reduced in concentration in all library eluates (from lane 4 to 9). Conversely, all eluates showed an increased number of new species in the 6–30 kDa region. One can also notice that, whereas the HOS eluate from library-1 (track 6) was still very rich in protein bands, the corresponding eluate from library-2 (track 9) bore very little proteins. It is also interesting to note a pretty good complementary behavior for protein category separations, especially highlighted by the two first and second eluates (lanes 4 and 7, and lanes 5 and 8, respectively).

A comparative SELDI pattern analysis (Figure 2) shows that eluates from hexapeptide ligand libraries comprised a significantly larger number of signals (from panel b to e) compared to the control (panel a), as evidenced within a mass range from 3 to 20 kDa. Although these data were obtained using a quaternary amine-coated chip, similar behavior was obtained using other types of surfaces such as carboxylic acid or IMAC-Cu<sup>2+</sup> (data not shown). The active role of the libraries is here underlined as it was already observed for a number of other biological extracts.<sup>34</sup>

Figure 3 shows the 2D maps of all PLL eluates from both columns, stained with silver. Most of the proteins captured



**Figure 2.** SELDI-MS analysis of desorbed fractions of egg white from peptide ligand library columns. The analysis was performed using a Q10 ProteinChip surface as capturing agent prior to mass spectrometry directly on the arrays. (a) Control egg white prior treatment; (b) TUC eluate from the library-1; (c) UCA eluate from library-1; (d) TUC eluate from the library-2; (e) UCA eluate from library-2. For experimental details see Materials and Methods.

seem to focus in the pH 4–7 range, with a mass distribution covering the 6–250 kDa region. However, the eluate from library-2 was also somewhat enriched in alkaline proteins, reaching pI values of 10 and above, particularly abundant in the low  $M_r$  region (10–20 kDa), thus, confirming the data from SDS-PAGE.

Figure 4 displays the 2D maps of the control, untreated egg white, as compared to a mixture of all six eluates from the two combinatorial libraries, both visualized by silver staining. Here too one can notice that the low  $M_r$  region (10–30 kDa) is particularly enriched in spots, comprising also a number of rather alkaline proteins otherwise invisible in the control sample.

To identify the species collected from the libraries as compared to the initial sample, the six eluates and the control were subjected first to SDS-PAGE by loading 30–120  $\mu$ g of total proteins per track. At the end of the run, 15 slices were excised, which covered the whole gel resolving region; these gel portions were treated with trypsin, extracted for peptides and subjected to MS analysis by running each eluted gel slice in triplicate. A total data reproducibility not inferior to 97% and the consistency criteria reported in the experimental section ensured a proper reliability in protein identification.<sup>31–33</sup> The identified gene products are listed in Table 1, and amount to a grand total of 148 unique species, about twice as many as those recently reported.<sup>14</sup> The results can be summarized as follows: from library-1, 112 gene products were found; from library-2, 109 genes products were identified. After the elimination of redundancies, 148 unique gene products were counted, whereas the starting material allowed detecting 41 gene products. Among the entries listed in Table 1, 12 corresponded to hypothetical proteins, 11 to putative uncharacterized proteins and 32 showed a similitude to other proteins within the NCBI database. In the first case, a further BLAST analysis identified 6 of 12 entries as specific gene products already assigned within the chicken genome database. In the second case, 10 of 11 entries showed an evident similarity to sequences present within the non-redundant NCBI database but from other organisms. In the third case, half of the entries were named “similar to” or not depending on the database used (NCBI or Swiss-Prot).

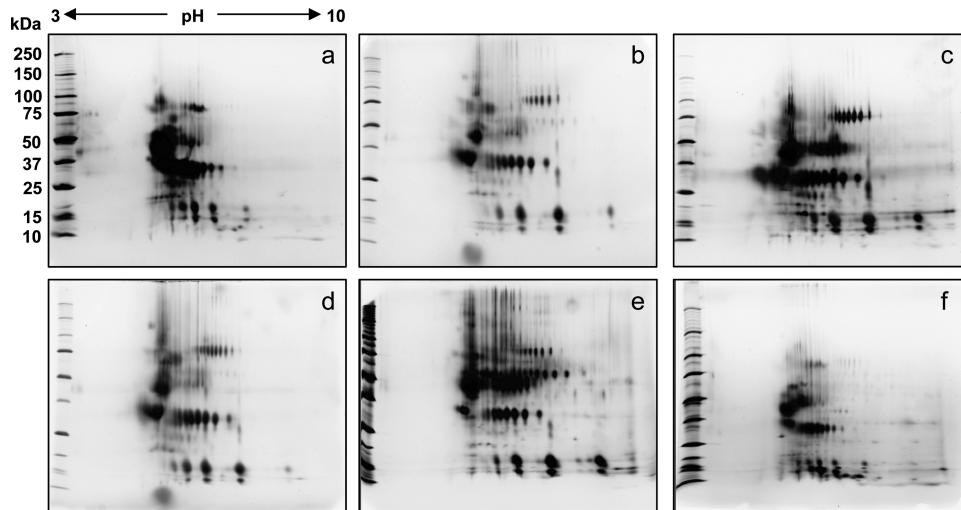
The enhancing properties of the libraries were confirmed by the number of peptides identified in eluates from library-1 and

2, compared to starting material. The overlapping Venn diagram in Figure 5A compares the unique contributions of each library; it can be seen that both libraries similarly contributed to find novel proteins (35 against 33). In addition, only 1 protein escaped the capture by the libraries together and was found only in the starting material (ovotransferrin CC type). Figure 5B shows a double comparison with recent published data,<sup>14</sup> where it appears that the initial egg white product shares about 3/4 of proteins found (31 out of 41) and shows that 10 proteins were not described earlier. The large difference in total number of proteins found in the control compared to the literature may be explainable by the use of a mass spectrometer with significant lower performances as compared to that used by other authors. Nonetheless, the number of novel proteins found as a result of the use of hexapeptide libraries is significant; out of the 147 proteins found, 86 are exclusively attributed to the combined PPLs utilized. With the present contribution, the number of identified gene products from egg white reaches 165 units.

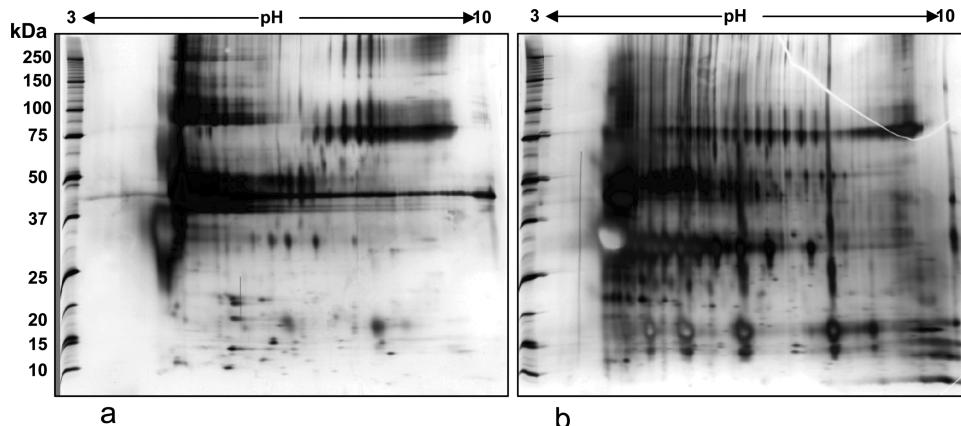
## Discussion

Although Mann's findings<sup>14</sup> were truly impressive and represented the most extensive list of egg white protein published thus far, one could argue that this list could hardly be exhaustive. Biological fluids are known to comprise hundreds, if not thousands, of unique gene products. As an example, in a recent study on human urines, with the help of our combinatorial peptide ligand library, we could detect >470 unique protein species,<sup>19</sup> versus ca. 100 in all published reports up to 2005. In human sera, again by using PLLs, we could identify 1559 or 3869 proteins, respectively, depending on how 95% confidence was estimated.<sup>20</sup> Several thousands more were reported by various authors engaged in the HUPO PPP (plasma protein project), as collected in a book edited by Omenn.<sup>35</sup> Also in cerebrospinal fluid, a recent report has described 573 peptides as well as 798 unique proteins.<sup>36</sup> Perhaps the most impressive results obtained so far with the PLL technology have been those regarding the exploration of the red blood cell (RBC) cytoplasm. The RBC cytosol was thought to comprise just a handful of proteins, beside its main component, hemoglobin, which alone constitutes 98% of its entire proteome. Most reports, even those exploiting modern proteomic technologies, had not been able to describe more than 100 unique species,<sup>37</sup> till the work of Pasini et al.,<sup>38</sup> who at once were able to detect no less than 252 cytosolic proteins, by using sophisticated MS instrumentation, such as the FT-ICR equipment. Yet, even this outstanding result faded out when we reported, again with the use of the PLL technology, no less than 1578 unique gene products in the 2% minority RBC proteome.<sup>23</sup> This result was outstanding, considering the highly adverse conditions applied to the RBC cytoplasm, in terms of relative protein composition and abundances.

The findings described above might not apply to egg white. Since all the biological fluids previously described in reality bathe a number of tissues in the human organism, one would expect to find plenty of proteins, even in traces, released by the surrounding organs. Nevertheless, it had to be expected that egg white, as a life sustaining and protecting material surrounding the yolk, could not possibly comprise just the 13 proteins reported up to 1989, and for that matter not even the 78 species reported by Mann.<sup>14</sup> As a matter of fact, we think that even our present investigation, which seems to have mined the egg white proteome to a much deeper extent, might not be exhaustive. First of all, because we failed to detect some 17 chicken proteins, namely, ovosecretoglobin, similar to plasma



**Figure 3.** 2D maps of TUC, UCA and HOS eluates from library-1 (a–c) and library-2 (d–f), respectively. Spot staining with ammoniacal silver. First dimension, 18-cm long IPG strips, pH 3–10 NL (nonlinear); second dimension, 8–18% acrylamide gradient SDS-PAGE.



**Figure 4.** 2D maps of control (left gel) and treated (right panel) egg white samples. In the last case, the sample was constituted by a mixture of all six eluates from the two combinatorial libraries. Experimental conditions as reported in Figure 2. Silver staining.

protease C1 inhibitor, pancreatic secretory trypsin inhibitor II, similar to histone protein Hist2h3c1, oncoprotein-induced transcript 1, protein FAM3C, 60S ribosomal protein L27, similar to Ig  $\alpha$  chain, type II keratin, similar to ovulatory protein 2, similar to ectonucleotide pyrophosphatase, Niemann-Pick type C2 protein, similar to meteorin, fibrinogen-related FReD protein, similar to aminopeptidase A, similar to desmoplakin and similar to carboxypeptidase D, which appear to be unique to Mann's list. Second, because our MS instrumentation was not as powerful as the linear trap FT-ICR equipment utilized by Mann, known to have a sensitivity at least 1 order of magnitude higher than that of our instrument, which was probably essential for identification of the 17 proteins mentioned above. Thus, it is quite possible that additional proteins will be detected in egg white by combining PLL technology with sophisticated MS instrumentations.

Among proteins listed in Table 1, only three (scavenger receptor class A member 5, RNA-binding protein 27 and similar to RNA-binding protein Raly) were identified by a single peptide composed of a sequence of less than 8 amino acids (seven amino acids were counted). This may render the identification somehow questionable; however, the mass of these proteins is relatively small and the resulting coverage may be representative enough. What is also of relevance is that few proteins were identified as belonging to other species. In particular, five

are of human origin (fibrous sheath-interacting protein 1, glutathione peroxidase 3, ligatin, mitochondrial inner membrane protein and zinc-finger protein 181). Six proteins are from *Mus musculus* (angiopoietin-related protein 3, cytochrome P450 19A1, mitochondrial 28S ribosomal protein S29, Ser/Thr-protein kinase PLK5, Sh3yl1 protein and vomeronasal receptor V1RH4). Three proteins are from *Rattus norvegicus* (complement component C6, regulating synaptic membrane exocytosis protein 2 and Ser/Thr-protein kinase TNNI3K). One protein is from *Cavia porcellus* (T-cell surface glycoprotein CD1b4) and one from *Bos taurus* (hypothetical LOC509055). The presence of proteins identified as belonging to other eukaryotic organisms was certainly not due to contaminations during the egg white extraction from fresh eggs. In fact, this operation was performed in a clean laboratory space, by using mask, gloves and glasses, thus, preventing any animal or human source of contamination. Therefore, the explanation is probably due to the fact that the chicken genome has not been fully decoded yet (5–10% of the sequence has not been determined at present),<sup>39</sup> so some proteins could not be found by searching for chicken genes, but could be found in other databases, such as human, mouse and the like.

Although the biological significance of all egg white proteins is yet to be explained, some of them were found in chicken

**Table 1.** List of 148 Unique Gene Products Found in Egg White after Combinatorial Ligand Library Treatment<sup>a</sup>

sample	protein name	Swiss-Prot or NCBI accession number	species	sequence	peptides	coverage%
L1, L2	Actin*	P53478	<i>G. gallus</i>	DLYANTVLSGGTTMYPGIADR DSYVGDEAQSK	2, 2	8
L1, L2, ST	ADP-ribosylation factor 5*	P49702	<i>G. gallus</i>	DAVLLVFANK	1, 1, 1	5
L1	Aminopeptidase E*	O57579	<i>G. gallus</i>	MLSDFLTEDVFK VVTIAHELAHQWFGNVLTLR DLMVLNEYTVMATDALTTSHPLTFR	3	5
L1, L2	Angiopoietin-related protein 3	Q9R182	<i>M. musculus</i>	LELQDWK ILANGLLQLGHGLK	1, 1	4
L1, L2, ST	Angiotensin-converting enzyme*	Q10751	<i>G. gallus</i>	IAFLPFGYLIDQWR	2, 1, 1	2
L1, L2	Apolipoprotein A-I*	P08250	<i>G. gallus</i>	YHIPGNTPYIR LADNLDTLSAAAK LISFLDELQK	1, 2	9
L1, L2	Apolipoprotein D*	Q5G8Y9	<i>G. gallus</i>	VINKEMLSSGK GSCVQANYSILKENGK SPDMHPDTVEHLK GSCVQANYSILK GSCVQANYSILKENGKFK INAIEGEIMHTDVKEPAK INAIEGEIMHTDVK IDTDKMMPTDQLNCPAEM MMPTDQLNCPAEM WYEIEKLPSNEFK	10, 5	48
L1, L2, ST	Avidin*	P02701	<i>G. gallus</i>	GEFTGTYYTAVATATSNEIK ESPLHGTQNTINK VGINIFTR TQPTFGFTVNWK	1, 4, 3	35
L2, ST	Calsequestrin-1	P19204	<i>G. gallus</i>	LAKKGLVEEGSLYVFKEER	1, 1	5
L1, L2, ST	Chondrogenesis associated lipocalin*	Q8QFM7	<i>G. gallus</i>	NSLYIR	2, 4, 2	28
L1, L2, ST	Clusterin*	Q9YGP0	<i>G. gallus</i>	STGSSNMVLLYSR VVETNYDEVALVATQISK WYSIGLASNSNWFK RNSAGCLR ICHSGSGLVGR RFEDLEER IDALLDREQR FEDLEER ELHPFLQHPVHGFHR EQFEDALR EILAVDCSQTDPVQSQLR MLDGGHGAWDHLGGFESESR EILAVDCSQTDPVQSQLREQFEDALR SSPSIIVVNGERIDALLDR RYDDLLSAFQAEMLNNTSSLQLN FGLMEDGVEDIFQDSTQLYGPAPFFR TSKEHQAMLHTLEETKR EHQAMLHTLEETKR EHQAMLHTLEETK TLMDKTSKEHQAMLHTLEETKR YIDTEVENAINGVK	18, 16, 3	44
L1	Complement component C6	Q811M5	<i>R. norvegicus</i>	CSAHTSNCVCLPPQCPK	1	2
L1	Corticotropin releasing hormone	Q703P0	<i>G. gallus</i>	AAAQLQGSPEGDEGAGEAVER	1	14
L1, L2	C-SKI protein	Q9PSS4	<i>G. gallus</i>	KQEEKLSAALQAKR	1, 1	14
L1, L2, ST	Cystatin*	P01038	<i>G. gallus</i>	LLGAPVPVDENDEGLQR YTCTCFVYVSYIPWLNQIK	2, 1, 2	19
L2	Cytochrome P450 19A1	P28649	<i>M. musculus</i>	IEDIQNLK	1	2
L1, L2, ST	Dickkopf-related protein 3*	Q90839	<i>G. gallus</i>	DVECCGDQLCVWGECR ELLFPVCTPLPEEGEPCHDPSNR LLNLITWELEPDGVLER NAVQEMEAEEEGAK TGSTIFSETITSIK	5, 2, 2	24
L1, L2, ST	Extracellular fatty acid-binding protein*	P21760	<i>G. gallus</i>	SYAVIFATR	2, 2, 1	16
L2	Fibrous sheath-interacting protein 1	Q6X2C8	<i>H. sapiens</i>	ISFLGEDELEVSAYAPS PK KRLVELLK	1	1
L2	Follistatin	Q90844	<i>G. gallus</i>	LTTSWTEEDVNDNTLFK GPVCGLDGK	2	8
L1, L2	Gallinacin-11*	Q6IV20	<i>G. gallus</i>	CVGYHGYCIR CLEEQLGLCPLKR VCPKPFAAFGTCWSR	3, 2	37
L2	Glucose-regulated protein 78 kDa	Q90593	<i>G. gallus</i>	NQLTSNPENTVFDAKR	1	2
L1, L2	Glutathione peroxidase 3*	P22352	<i>H. sapiens</i>	YVRPGGGFVPNFQLFQK PGGGFVPNFQLFQK	2, 1	8
L1, L2	Glutathione S-transferase 2*	P20136	<i>G. gallus</i>	LGLDFPNLPYLDGDK AGPAPDFDPDSWTNEKEK	1, 2	15
L1	Glycine dehydrogenase [decarboxylating]	P15505	<i>G. gallus</i>	HLRPAAGGPR	1	1
L2	Golgi apparatus protein 1*	Q02391	<i>G. gallus</i>	ALNEACESVIQTACK	1	1
L1, L2, ST	Hep21 protein*	Q8AV77	<i>G. gallus</i>	YSCCETDLCNEK	4, 5, 1	52

Table 1. Continued

sample	protein name	Swiss-Prot or NCBI accession number	species	sequence	peptides	coverage%
L1	Heparin cofactor II	O73840	<i>G. gallus</i>	IPYVGCFHGANETTCER YSCCETDLCNEKWWDDPDT VTLYYQQGCTSALNCGR YKIPYVGCFHGANETTCER	1	7
L1, L2, ST	Histone H1*	P09987	<i>G. gallus</i>	ILKLTKGLIKEALVNPNPTLMMILNCLYFK ALAAGGYDVEK	1, 1, 1	5
L1, L2	Histone H2A3*	P35062	<i>G. gallus</i>	VTIAQGVLPNIQAVLLPK	1, 1	15
L1, L2, ST	HMG-1*	Q9PUK9	<i>G. gallus</i>	RPPS AFFLFCSEFRPK HPDASVNFSEFSK LGEMWNNTAADDK NSSVETATEVLNTTAER	3, 1, 1	19
L1	Hypothetical protein LOC509055	Q0IIJ7	<i>B. taurus</i>	PPPLTAAAPNR	1	4
L1	Hypothetical protein (UDP-glucuronate decarboxylase 1)*#	50730484	<i>G. gallus</i>	LLLGWEPVVPLEEGLNK	1	4
L2	Hypothetical protein LOC770578	118084926	<i>G. gallus</i>	DLQNISQNGGSSR	1	5
L1, L2	Hypothetical protein (acyloxyacyl hydrolase)*, #	118086191	<i>G. gallus</i>	FLWDNLHNR ALQLSNVLK NKLPFEDFDGDKFSTFPTLR MGGEWPQLIEPVDFGHPSQIAALGTSVTWQK FANFDVFYMDFPLK STVGFPNLCAFPLIADLCER LAAAKDELEK	7, 1	22
L1, L2	Hypothetical protein (leucine zipper protein 2)*#	118091049	<i>G. gallus</i>	LNEAVEKAEK GRATFSSSQEAIR	1, 2	7
L1	Hypothetical protein LOC423282	118091766	<i>G. gallus</i>	QAAPRPGGGGSGAAEGHR	1	2
L2	Hypothetical protein LOC770218	118094358	<i>G. gallus</i>	DIEKQLDILELK	1	7
L2	Hypothetical protein (MICL-like protein 2) #	118097764	<i>G. gallus</i>	NLADELKKQVREK	1	2
L1	Hypothetical protein (acetyl-Coenzyme A synthetase 1)*#	118100532	<i>G. gallus</i>	DSIPLEIR	1	2
L1	Hypothetical protein (selenium binding protein 1)*#	118102241	<i>G. gallus</i>	WGTVCDDDGWDLADAEEVCR	1	2
L1, L2	Hypothetical protein	118129730	<i>G. gallus</i>	QLGCGEAISTPGSAR	1, 1	2
L1, L2	Hypothetical protein LOC426826	118129740	<i>G. gallus</i>	LVNGPNLCTGR HGENAGVVCSTAEAAPLR VAPTTLFPPSKEEELNEATK ATLVCLINDFYPPSPVTDWVIDGSTR VITPSFVDIFISK MECGLEPVVQQDIAIR ALTQPSSVSANPGETVK	3, 3	13
L1, L2	Ig lambda chain C region*	P20763	<i>G. gallus</i>	ALTQPASVSANPGETVK	1, 2	45
L2	Ig mu chain C region*	P01875	<i>G. gallus</i>	ICPIDITLAQR	2	6
L1, L2	Immunoglobulin heavy chain variable region*	P04210	<i>G. gallus</i>	TVLSYSETLCGPGLSK	1, 1	16
L1, L2	Immunoglobulin light chain	54111245	<i>G. gallus</i>	GYSLGNWVCAAKFESNFNTQATNR	1, 1	14
L2	Ligatin	P41214	<i>H. sapiens</i>	NLCNIPCSALLSSDITASVNCACK	1	2
L1, L2, ST	Lymphocyte antigen 86*	Q90890	<i>G. gallus</i>	CELAAMK	1, 1, 1	10
L1, L2, ST	Lysozyme C*	P00698	<i>G. gallus</i>	GTDVQAWIR	14, 15, 10	80
L1, L2, ST	Melanotransferrin/EOS47	Q92062	<i>G. gallus</i>	CKGTDVQAWIR		
L2	Metalloproteinase inhibitor 2*	O42146	<i>G. gallus</i>	FESNFNTQATNR		
L1	Metalloproteinase inhibitor 3*	P26652	<i>G. gallus</i>	IVSDGNGMNAWVAWR		
L1, L2	Mitochondrial 28S ribosomal protein S29	Q9ER88	<i>M. musculus</i>	KIVSDGNGMNAWVAWR		
L1, ST	Mitochondrial inner membrane protein	Q16891	<i>H. sapiens</i>	NLCNIPCSALLSSDITASVNCACK		
L2	Mitochondrial ribosomal protein L37	125630641	<i>G. gallus</i>	NTDGSTDYGILQINSR		
L2	Muscle, skeletal receptor tyrosine protein kinase	Q8AXY6	<i>G. gallus</i>	SIVHPSYNSNTLNNDIMLIK		
L2	Neural secreted glycoprotein	O57596	<i>G. gallus</i>	WWCNDGR		
L2	Neuropathy target esterase	Q2LAG5	<i>G. gallus</i>	RHGLDNYR		
L2				TKEECMEMIQK		
L2				MHITLCDLVATWDSVSPQTQK		
L2				SDGSCAWYR		
L1				LEVNKYQYLITGR		
L1, L2				NDWHGGAIVLSQLTGSLFKSR		
L1, ST				RIDQLNR		
L2				QTASVTLQIAAQNAAVQAVNAHSNILK		
L2				MAAVEAMALR		
L2				KRESETPTLTTPSELLLDR		
L2				VTVNYPYISDAK		
L2				EQSGEYECASNDVAAPVVQR		
L2				SSSYCEYIRPPIDRFKTMDFGK		

**Table 1.** Continued

sample	protein name	Swiss-Prot or NCBI accession number	species	sequence	peptides	coverage%
L1	Nicalin	Q5ZJH2	<i>G. gallus</i>	ELEMVVASQFPEVKFMSMVHKK	1	4
L1	Novel KRAB box containing protein	Q5SVV3	<i>G. gallus</i>	EITQVFGLNLDDLYRKKFWEYR	1	8
L1, L2	Olfactomedin-Noelin-Tiarin factor 1*	Q25C36	<i>G. gallus</i>	EALRAEAEGAAAR LTDCSDTIAASVR RLALLEER FGSSAGLWTK QVYAWDDGYQIIYR LDPDSLDIEQMWDTPCPR VQCVFVDVSGTLPEEASLVYFPK	3, 7	25
L1, L2, ST	Ovalbumin*	P01012	<i>G. gallus</i>	ADHPFLFCIK AFKDDEDTQAMPFR DEDQTQAMPFR ELINSWVESQTNGIIR EVVGSAEAGVDAASVSEEFR GGLEPINFQTAADQAR HIATNAVLFFGR ILELPFASGTMMLVLLPDEVSGLEQLEIINFEK ISQAVHAAHAEINEAGR LTEWTSSNVMEER LTEWTSSNVMEERK LYAEERYPILPEYLQCVK LYAEERYPILPEYLQCVKELYR NVLQPSSVDSQTAMVLVNAIVFK NVLQPSSVDSQTAMVLVNAIVFKGLWEK PVQMMYQIGLFR TQINKVVR VHHANENIFYCPIAIMSALAMVYLGAK	12, 10, 18	65
L1, L2, ST	Ovalbumin-related protein Y*	P01014	<i>G. gallus</i>	ADHPFLFFIR EWTSTNAMAK FCFDVFNEMK FYTGVEEVNFK HSLELEEFR ILELPYASGDLSMLVLLPDEVSGLER KFYTGGVEEVNFK LYVDKTFSLPEYLSCAR QLINSWVEKETNGQIK TFSVLPEYLSCAR TINFDKLR VHHVNENILYCPLSILTALAMVYLGAR VLHFDSITGAGSTTDQCGSSEYVHNLFK YNPTNAILFFGR AGLETVNFK ELLSDTASK	8, 9, 14	48
L1, L2, ST	Ovalbumin-related protein X / Similar to ovalbumin-related protein Y*	118086485	<i>G. gallus</i>	ALHFDSIAGLGGSTQTK SRPILPIYLK SVNIHLLFK MGSISAANAEEFCDFVFNELK ILELPFASGDLMSMLVLLPDEVSGLER VQHTNENILYSPLSIIVVALAMVYMGAR EMPFHVTK ADHPFLFLIK HNPTNTIVYFGR QSTEHTGYLLAQVSSVK PDGCGPGWVPTPGGCLGFFSR	8, 11, 5	38
L1	Ovocalyxin-32	Q90YI1	<i>G. gallus</i>	1	6	
L1, L2	Ovocleidin-17	Q9PRS8	<i>G. gallus</i>	2, 2	23	
L1, L2, ST	Ovoglycoprotein*	Q8JIG5	<i>G. gallus</i>	EQLEEEFAQLR TAEVTKEQLEEFEAQLR	2, 2, 1	13
L1, L2, ST	Ovoinhibitor*	P10184	<i>G. gallus</i>	EHGANVEKEYDGECRPK EHGANVEKEYDGECR LEIGSVDCSK EEVPELDCKSYK LEIGSVDCSKYPSTVSKDGR DGTTSWVACPR QEIEPIDCDQYPTRK LEIGSVDCSKYPSTVSK NLKPVCGTDGSTYSNECGICLYNR	9, 5, 4	21
L1, L2, ST	Ovomucin alpha-subunit*	Q98UI9	<i>G. gallus</i>	DSCPCMFQGK ECVSLPDCTSNCNPEEK ETGITVNGNQIPLPFSLK EVIVDTLRSR GVCVSEGVEFKPGAVVPK ICGLCGNYDGNK IENYQHCEPSELCK ILSSAGVQIR IQCPVVK IQEATDPGAEK IVIQNNACGK LCTEDSKDCLCCYNGK LDDPCCTETVCECDIK LSSITCPPQQLK	22, 8, 19	14

Table 1. Continued

sample	protein name	Swiss-Prot or NCBI accession number	species	sequence	peptides	coverage%
L2	Ovomucin beta-subunit*	Q6L608	<i>G. gallus</i>	SSCEDCVCTDEQDAVTGTNR TATGAVEDSAAFGNSWK TSGLCGNFNNIQTDDFR VCGLCGDFDGR SLDEPDPLCK LDCIGETVLVK ITLIFESSEIR IVQPGNSFQEDK		
L1, L2, ST	Ovomucoid*	P01005	<i>G. gallus</i>	CQLDMQDCSEPQKQ FHGVCTYILMK CTMGQWNCK VEQGASVDKR FPNATDKEKG AFNPVCCTDGVTYDNECLLCAHK CNFCNAVESNGTTLTSHFGK ELAAVSVDCEYPKPDCTAEDRPLCGSDNK	3, 1, 5	4, 31
L1, L2, ST	Ovostatin*	P20740	<i>G. gallus</i>	LVTKDNDSPISNK VFTVNNEQR LISPEDDDGCVCAK TIGYLVSGYQK SSSNMVIIDVK VIQLFVNNK LLLQQTPLPQVPGK IFDPPELSLK TSHSFLVEEYVLPK SSLDQLIDDHTVMQVEYK ASVSYTIPDTITEWK ASAIVQWIR GLYYRPLTSGLGPDVYQFLR INVLLLESLDYQAK	8, 3, 14	12
L1, L2, ST	Ovotransferrin*	P02789	<i>G. gallus</i>	AQSDFGVDTK CLVEKGDVAFIQHSTVEENTGGK CVASSHEKYFGYTGALR DDNKVEDIWSFLSK DGKGDVAFK DLLFKDLTK DLLFKDSAIMLK ECNLAEVPTHAVVVRPEK EFLGDKFYTVISSLK EGTTYKEFLGDKFYTVISSLK FFSASCVPGATIEQK FMMFESQNK FMMFESQNKDLLFK FYTVISSLK GAIWEWIESGSVEQAVAK GDVAFIQHSTVEENTGGK GDVAFIQHSTVEENTGGKNK GEADAVADGLVYTAGVCGLVPVMAER GTEFTVNDLQGK HTTVNENAPDQKDEYELLCLDGSR IMKGEADAVADGLVYTAGVCGLVPVMAER IQWCAVGKDEK ISLTCVQK KDQLTPSPR KGTEFTVNDLQGK LCQLCQGSGGIPPEK LKPIAAEVYEHTEGSTTSYYAVAVVK LKPIAAEVYEHTEGSTTSYYAVAVVKK NLQMDDDFELLCTDGR SAGWNIPIGTLLHR SDFHLFGPPGK SDFHLFGPPGKK SDFHLFGPPGKKDPLK TCNPSDILOQMCSFLEGK TDERPASYFAVAR TGTCNFDEYFSEGCAPGSPPNSR TSCHTGLR WCTISSPEEK WCTISSPEEK WSVVSNGDVECTVDET WSVVSNGDVECTVDET GTEFTVNDLQGK DLLFKDSAIMLK SAGWNIPIGTLLHR LCQLCQGSGGIPPEK DDNKVEDIWSFLSK NLQMDDDFELLCTDGR GAIWEWIESGSVEQAVAK NLQMDDDFELLCTDGR TCNPSDILOQMCSFLEGK TGTCNFDEYFSEGCAPGSPPNSR	21, 5, 41	65
L1, L2	Ovotransferrin BB type	71274075	<i>G. gallus</i>		14, 1	32

**Table 1.** Continued

sample	protein name	Swiss-Prot or NCBI accession number	species	sequence	peptides	coverage%
L1, L2, ST	Ovotransferrin BC type*	71274079	<i>G. gallus</i>	AIANNEADAISLDGGQVFEAGLAPYK ISLTCVQK WSVVSNGDVECTVVDETKDCIIK HTTVNENAPDQKDEYELLCLDGSR GTEFTVNLDLQGK LCQLCQGSGGGIPPEK NLQMDDFELLCTDGR GAIEWEGIESGSVEQAVAK TGTCNFDEYFSEGCAPGSPPNSR AIANNEADAISLDGGQVFEAGLAPYK IALTCVQK LKPIAAEVYEHTEGTTSYYAVAVVK TGTCNFNEYFSEGCAPGSPPNSR TVENFVALATGEK	8, 6, 1	20
ST L1	Ovotransferrin CC type Peptidyl-prolyl <i>cis-trans</i> isomerase B*	71274077 P24367	<i>G. gallus</i> <i>G. gallus</i>	VGEEDAGRVVIGLFGK SSEELPYLR	1 2	3 14
L1, L2	6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase, liver isozyme	Q91348	<i>G. gallus</i>		1, 1	2
L1, L2	Pleiotrophin	P32760	<i>G. gallus</i>	YQFQAWGECDLN TALK KSDCGEWQWSVCVPTNGDCGLGTR	2, 2	29
L1, L2, ST	Poly(ADP-ribose)polymerase 1*	P26446	<i>G. gallus</i>	FYTLPIDFGMK	1, 1, 1	1
L1, L2	Polymeric immunoglobulin receptor*	153792243	<i>G. gallus</i>	SCLTVVSTSGYR	1, 2	4
L2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1*	P24802	<i>G. gallus</i>	IIDNTGFLPGPYEGR EYLAMKVIGPDEVENAEAR	1	2
L2	Pro-neuregulin-1, membrane-bound isoform	Q05199	<i>G. gallus</i>	ATLADAGEYACR	1	1
L1	Protein CEPU-1	Q90773	<i>G. gallus</i>	VTVNYPYISDAK AVGFISEDEYLEITGIR	2	9
L1, L2, ST	Putative uncharacterized protein (similar to SH3 domain-binding glutamic acid-rich-like)* <sup>○</sup>	Q5F3C9	<i>G. gallus</i>	YLGDYEAFFEAR	1, 1, 1	11
L1	Putative uncharacterized protein (similar to protein disulfide-isomerase A6) <sup>○</sup>	Q5F472	<i>G. gallus</i>	TSEAIVDAALSALR	1	6
L2	Putative uncharacterized protein (similar to serine beta-lactamase-like protein) <sup>○</sup>	Q5ZK12	<i>G. gallus</i>	WAGGGFLSSVGDLKK	1	3
L1	Putative uncharacterized protein (similar to syntaxin 16) <sup>○</sup>	Q5ZK87	<i>G. gallus</i>	TCTEQEAVRLR	1	3
L1, L2	Putative uncharacterized protein (similar to metallopeptidase) <sup>○</sup>	Q5ZL18	<i>G. gallus</i>	ETSIGILATAVSR	1, 1	3
L1, L2, ST	Putative uncharacterized protein (similar to GDP-fucose protein O-fucosyltransferase 1) <sup>○</sup>	Q5ZLZ9	<i>G. gallus</i>	GEAYIHSLLVRPVYGIHLR	1, 1, 1	7
L1, L2, ST	Putative uncharacterized protein (similar to WD repeat-containing protein) <sup>○</sup>	Q5ZMN8	<i>G. gallus</i>	EGEVVASVKDVNLQVYFFK	1, 1, 1	5
L2	Putative uncharacterized protein (similar to fumarate hydratase) <sup>○</sup>	Q5ZLD1	<i>G. gallus</i>	PMMIKNVLNSAR	1	2
L1	Putative uncharacterized protein	Q5ZMF2	<i>G. gallus</i>	DIVTLLSR	3	11
L1	Putative uncharacterized protein (similar to nucleobindin 2) <sup>○</sup>	Q5ZHR1	<i>G. gallus</i>	DASQILVDTLQK LFQDNSILSSLPLNSLSR KLQQANPPAGPAGEKL	3	12
L1	Putative uncharacterized protein (similar to zinc-finger and BTB domain-containing protein 1) <sup>○</sup>	Q5ZKW3	<i>G. gallus</i>	AATSDLENYDKTRHEEFK FLDEQELEALFTK YYGAEKIDFNEK	1	4
L1, L2	Regulating synaptic membrane exocytosis protein 2	Q9JIS1	<i>R. norvegicus</i>	NSGAMDIEER	1, 1	1
L1	Renin/prerenin receptor	Q1XIH7	<i>G. gallus</i>	GVDKLTLPAK DASQILVDTLQK LFQDNSILSSLPLNSLSR KIECFYR VSNSYWNR	3	11
L1, L2	Riboflavin-binding protein*	P02752	<i>G. gallus</i>	VSESSCLCLQMNKK VSESSCLCLQMNKK FEALQQEEGEE LLKFEALQQEEGEE KQEMLEK	6, 1	18
L1	RNA-binding motif protein 27	118097624	<i>G. gallus</i>	KQEMLEK	1	1

**Table 1.** Continued

sample	protein name	Swiss-Prot or NCBI accession number	species	sequence	peptides	coverage%
L1	Scavenger receptor class A member 5	118089138	<i>G. gallus</i>	VEVFHDR	1	1
L2	Serine/threonine-protein kinase PLK5	Q4FZD7	<i>M. musculus</i>	HRNIVAFHAHFADR	1	2
L1, L2	Serine/threonine-protein kinase TNNI3K	Q7TQP6	<i>R. norvegicus</i>	LEDNLQIK FLLDQNNAVNINHRGR	1, 1	3
L2	Serum albumin*	P19121	<i>G. gallus</i>	EVGQMK KMTTIGTK LCSAPAEER	3	7
L1	Sh3yl1 protein	Q6P7V3	<i>M. musculus</i>	GGNLTLGGNFTVAVGPLGR	1	8
L1	Similar to ASB-4 protein	118085773	<i>G. gallus</i>	KNFLEALKSNDYETLEELLNQK	1	5
L1, L2, ST	Similar to axoneme central apparatus protein	118085695	<i>G. gallus</i>	CQVLSALSQIAKHSVDLAELVVEAEIFPVVLTCMK	1, 1, 1	7
L1, L2	Similar to bactericidal permeability-increasing protein	118082796	<i>G. gallus</i>	LNEENICLNK LMATSPVVSLQSDTCR	2, 3	10
L1, L2	Similar to bactericidal/ permeability-increasing protein-like 2*,\$	118082798	<i>G. gallus</i>	VLSQIDAFAQIDYSLVSSPAVFK LTATEIPLVSLQQDSFTLEIR	1, 1	8
L2	Similar to beta 1,3-N-acetylglucosaminyltransferase 5	50757167	<i>G. gallus</i>	SLATNSCPNIR FKQFNLDL	1	2
L1, L2	Similar to complement component 8, gamma polypeptide*,\$	118099118	<i>G. gallus</i>	VVTEGNLSLGELVGR	3, 2	23
L1, L2	Similar to decoy receptor 3\$	118100703	<i>G. gallus</i>	SSQLSNAIVDKFEQR LEATAMTVAVPDGQSLAISTFR RLQQILER LQQILER KQENSAVITELLAALR LGSPSENTQCR GSFSSSSSTEPCR VTCQQCPCGTVAQHCTK LVNGPSHAGRVFVHFDR	1, 6	23
L1, L2	Similar to deleted in malignant brain tumors 1*	118129746	<i>G. gallus</i>	QLGCGKAISTPGSAR HGEDAGVVCSGTAEEAPLR QLGCGAALSAPGSAR FPGWGSNCK QWGTVCDDNWDAEANVCR VEVFYGHQWGTVCDDNWDISDAEVCQQLGCGR TFDRNNNDNCISVVEWVEGLSVFLRGTLER	3, 7	19
L1	Similar to EF hand calcium binding domain 1 isoform 2\$	118085755	<i>G. gallus</i>	HLNENLLEELEKSK	1	14
L1	Similar to Efocab4a protein	118091334	<i>G. gallus</i>	SDLRRHNVNSGIVNMDIK	1	3
L1	Similar to feline leukemia virus subgroup C receptor FLVCR\$	118087898	<i>G. gallus</i>	QVEYLDLDDSGKSTPPRK	1	4
L2, ST	Similar to GRB2-associated binding protein 1 isoform 2\$	118089858	<i>G. gallus</i>	YYCLQGTQFYR MYLIQGSQVSIVYSGR	1, 1	3
L1, L2, ST	Similar to hemopexin*,\$	Q90WR3	<i>G. gallus</i>	YPLSGGSALITFEK	2, 1, 1	10
L2	Similar to Interferon-induced protein 35\$	50760791	<i>G. gallus</i>	ALSILSYVRGPLPK	1	3
L2	Similar to KIAA0182	118096558	<i>G. gallus</i>	DALQRQLDRLERLVPAK	1	2
L1	Similar to KIAA1372 protein	118085250	<i>G. gallus</i>	VFHSSCCGNANNFR	1, 2	8
L1, L2	Similar to Kunitz-like protease inhibitor	118100813	<i>G. gallus</i>	TYCYACIPALR SLGLSEEQLKEQLR	1	3
L1	Similar to leucine zipper-EF-hand containing transmembrane protein 1-like protein\$	118101373	<i>G. gallus</i>	MYCCLPASWK TQIWATSHGCK LLVQEMLR FEVIFEAQPK YNIPFPQVSK TFLWSVTAER DVGQLFHTAMK TQEELLDILDEK LIITGQGVNIEEK LLLVRPEGVLIEK TWLWDLYSVPNGSK QTVTVTPNTITGWK IQVTMEEFPQFQLK FWVAPPADGTEEIATVR ANSGTFIQLMDKPIYKPGQTVK KGSPAEEVSLLPPTVEGSVR SIQAAFNWLEFHQLPNGCFR	1, 2	32
L1, L2, ST	Similar to MGC82112 protein*	118083274	<i>G. gallus</i>	YNIPFPQVSK KWANTCEMQISPVQHR LVDGSTHCSGR	15, 3, 5	14
L2	Similar to Mug1 protein	118104912	<i>G. gallus</i>	1	14	
L2	Similar to ophanin	118089211	<i>G. gallus</i>	1	6	
L1, L2	Similar to PIT 54*	118129750	<i>G. gallus</i>	8, 7	33	

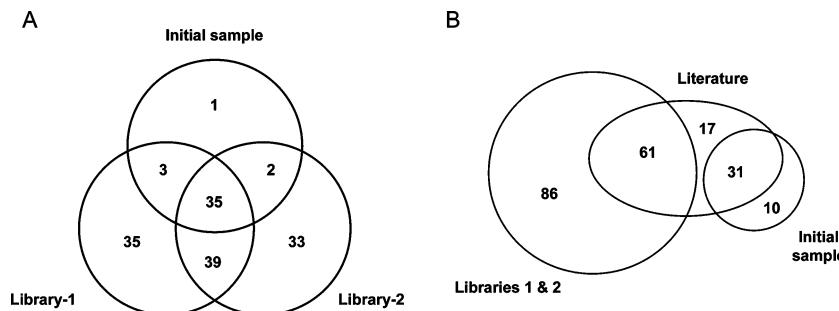
**Table 1.** Continued

sample	protein name	Swiss-Prot or NCBI accession number	species	sequence	peptides	coverage%
L1	Similar to RNA-binding protein Raly <sup>§</sup>	118100477	<i>G. gallus</i>	QLGCGEATSATGSAR GTEALSECTAKPWGK ALSAPIHGAHFQGQSDPIWLDDVSTCGTEAGLSTCK QQWGTVCDDSWDIDATVVCR VEVFHEQQWGTVCDDSWDLTDAQVVCR GSDPIWLDDIMCK VEVLYGQQWGTVCDDSWDLIDAEVVCR LFYRGR	1	3
L1, L2	Similar to serine peptidase inhibitor, Kunitz type 1 <sup>§</sup>	118091591	<i>G. gallus</i>	VGFLNFLKR	1, 1	2
L2	Similar to seven transmembrane helix receptor	126328096	<i>G. gallus</i>	RRGYKEER	1	2
L2	Similar to SLIT2 isoform 2 <sup>§</sup>	118090650	<i>G. gallus</i>	IDLSNNQISEAAPDAFQGLR ITDIEEGAFDAGSGVNELLTSNR ITDIEEGAFDAGSGVNELLTSNRLETVR LNNEFNSVLEATGIFK NNLQLLSELLFLGTPK GLFEGFLFSLQLLLLNAHK VLQLMENK LSGDCFADLACPEK FTCQGPVDVNILAK LYRLDLSNENQIQAIPR WLADYLHTNPETSGAR NLQLDYNQISCIEDGAFR GLTEIPTNLPETITEIR DVTELTYLDGNQFTLVPK VDAFDQLHNLNLSSLYDNKLQTIAK ILKVILKIQINQQSR	15	16
L1, ST	Similar to solute carrier family 39 (metal ion transporter), member 8 <sup>o</sup>	118090416	<i>G. gallus</i>	GYAVACAQEGEKK DVLDYFHRSR	1, 1	3
L2	Similar to SRp129	118082345	<i>G. gallus</i>	SNVQFLFR	1	1
L1, L2	Similar to tazarotene-induced gene 2 <sup>*§</sup>	50732085	<i>G. gallus</i>	VDSSGTFVQLHLNLAQTACR MSDVLYLPGMFAFSK GEFPWQVSLR	2, 4	32
L1, L2	Similar to transmembrane protease, serine 9 <sup>*§</sup>	118103074	<i>G. gallus</i>	LLDQALCSSLYSHALTDR IIHPHSYNTDTADYDVAVLELK VIRHSGYNSNTLNNNDIMLIK	3, 2	4
L2	Similar to trypsinogen	118083374	<i>G. gallus</i>	LAGGDTEDPQFPK	1	8
L1, ST	Sulfhydryl oxidase 1, quiescin <sup>*</sup>	Q8JGM4	<i>G. gallus</i>	RICDTVEILLTK	1, 1	2
L1	T-cell surface glycoprotein CD1b4	Q9QZY9	<i>Cavia porcellus</i>	LVPGVVDYNVNIIISVK ESAPATINAGTDLDNPK	1	4
L1, L2	Tenascin*	P10039	<i>G. gallus</i>	DYVLPVCK	1, 2	1
L1, L2, ST	Tenp*	O42273	<i>G. gallus</i>	VVDVDKLCCLDVSK ADLHDVMGPDGNLQLLTSACRPTVQAQSTR SILDKVVVDVKLCCLDVSK ITQVGSLYHEDLPITLSAALR APDCGGILTPLGLSYLAEVSKPHAEVVR VADLWLSVYPEAGLR MMISTAVIEDAELSLAASNVGLVR AALLEELFLAPVCQQPAWMDDVLR TITLEVEPSDTIENVK TLSDYNIQK	9, 9, 7	39
L1, L2, ST	Ubiquitin*	P62973	<i>G. gallus</i>	IYIMSMVNKTTK	2, 1, 1	33
L2	V(D)J recombination-activating protein 2	P25022	<i>G. gallus</i>	SEKSQGGGDDTAANNIQFR	1	2
L1, L2, ST	Vitelline membrane outer layer protein 1 <sup>*</sup>	P41366	<i>G. gallus</i>	DDTALNGIR SQGGGDDTAANNIQFR VESPGQLRDDTALNNVR CLLFICSTRKK	2, 4, 2	25
L1, L2, ST	Vomeronasal receptor V1RH4	Q8R280	<i>M. musculus</i>	ELAGRDWNEKLLQDACR	1, 1, 1	3
L1	Xanthine dehydrogenase/oxidase	P47990	<i>G. gallus</i>	NEKVNGGKK	1	1
L1	Zinc-finger protein 181	Q2M3W8	<i>H. sapiens</i>	ERKGAGTAAAPQPGAGGSGAALGPCAAR	1	2
L2	Zinc-finger transcription factor KROX20	Q98T82	<i>G. gallus</i>		1	6

<sup>a</sup> Data of the three eluates from library-1 as well as of the three eluates from library-2 were pooled together. Asterisk indicates proteins already identified by Mann.<sup>14</sup> Proteins reported as hypothetical in NCBI database but already identified as specific gene products within the chicken genome database<sup>39</sup> are indicated with (#) sign; their name is reported in parenthesis. Chicken proteins reported as putative uncharacterized in NCBI database but showing an evident similarity to entries present within the non-redundant NCBI database from other organisms are indicated with degree sign (°). Proteins reported as "similar to" or not, depending on the database used, are indicated with section mark (§). Library-1, 2 and starting material are indicated with L1, L2 and ST, respectively.

plasma<sup>40,41</sup> and chicken cerebrospinal fluid,<sup>42</sup> thus, suggesting their general occurrence in body fluids, including oviduct fluid. However, since some egg white proteins found in the present study are also of intracellular origin, they might be derived from decaying epithelial cells lining the oviduct. Moreover, quite a

number of the proteins here reported were also found in the eggshell matrix.<sup>43,44</sup> Their presence could be explained by the fact that, when the egg reaches the eggshell gland, it will be surrounded by a fluid containing remnants of products from the magnum and eggshell membrane components secreted by



**Figure 5.** Overlapping Venn diagrams of all proteins found in the control and the two ligand libraries. (A) Comparison between initial egg white extract and library eluates (data from the three eluates from library-1 as well as the three eluates from library-2 were pooled together). In the middle of the graphic are common proteins. (B) Comparison of data from the present report with those described in literature.<sup>14</sup>

the isthmus, which might ultimately be incorporated, even if in trace amounts, into the egg white.

With the discovery of these novel proteins, this study opens at least the door to numerous questions that are both of academic and practical interest. Egg white proteins are largely glycosylated with numerous isoforms; this polymorphism with a probable biological significance needs to be understood in its composition and structure first. As underlined by early 2-DE analytical studies,<sup>12,13</sup> the same proteins shows a series of protein spots that differ by one or more sugars. Although after treatment with peptide ligand libraries, the abundance of proteins changed (e.g., large reduction of ovalbumin and ovotransferrin), the amplified low-abundance protein also shows a large polymorphism. In Figures 3 and 4, new series of spots of the same protein that were not detectable from the native egg white protein material became visible. Identifying the nature of these proteins and the composition of their glycans is a next step in this investigation. As a first approach, protein collected from various eluates would have to be fractionated with immobilized lectins, already used for differential blot staining of egg white proteins.<sup>45</sup> Then, the elucidation of glycan sequences using mass spectrometry would have to follow.<sup>46</sup> Investigations in this domain are in progress.

From the discovery of novel proteins from egg white, some practical aspects are also expected. Eggs are used extensively for a large number of applications beyond just food. Pharmaceutical applications are also of extreme importance, especially for the production of human vaccines, among them, the influenza vaccine and measles-mumps-rubella vaccine. Viruses are propagated in embryonated eggs and, at the end of the production process, egg proteins are to be removed. In reality, influenza vaccines contain residual egg proteins, among which ovalbumin is probably the most representative (being of the highest abundance in egg white). This could lead to the induction of allergic reactions to patients particularly sensitive to foreign proteins. A number of cases of allergy are well-known at the point that, after a vaccination, it is recommended not to eat eggs for several days. Moreover, influenza infections with accompanying morbidity represent a major concern for high-risk individual including asthmatic patients.<sup>47</sup> These adverse reactions are attributed to the presence of ovalbumin, which is largely variable from producer to producer and probably from lot to lot.<sup>48</sup> Generally, ovalbumin is the assayed protein because it is the most abundant and hence quite easy to assay by ELISA-based quantitative tests. However, other proteins should be presumably present as well. As a consequence, the produced allergy may well be related also to other proteins of larger

immunologic reactivity. In this context, the knowledge of the protein composition of egg white and also the amplification of the concentration of low-abundance proteins may contribute to a better understanding of the origin of the allergic reactions. These findings could also allow designing better purification processes and thus rendering the influenza vaccines suitable to a larger population that today is excluded.<sup>49</sup> In this respect, some of us already described the method of amplifying the presence of protein contaminants from recombinant proteins in order to bring them to the level of detection.<sup>50</sup> Specific investigations in this direction are also in progress in our laboratories.

## Conclusions

The present report, by exploiting a PLL technology, has doubled the number of proteins (to 148) found in egg white, as compared to the most recent and comprehensive list reported by Mann.<sup>14</sup> It is not known, at present, what would be the biological significance and role of these new species in the egg white fluid. They might simply be remnants of proteins present in the oviduct lumen at the time of egg white assembly, or they could have a precise biological role in maintaining the integrity of the egg and protecting the yolk. Clearly, more work will be necessary in order to trace the origin of these novel egg white components and to determine their possible function. Additionally, some of the newly reported proteins might be potential allergens, as yet not reported, and that too will have to be assessed.

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