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Proteomic Analysis of Hen Egg White

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Hen egg white is an original biological fluid in which major proteins have been widely studied, unlike the minor components. In this study, two-dimensional electrophoresis associated with mass spectrometry enabled the separation of 69 protein spots and their matching with major proteins, which were already known, and with minor proteins. Sixteen proteins were identified, and among them, two had never been previously detected in hen egg white, i.e., Tenp, a protein with strong homology with a bacterial permeability-increasing protein family (BPI), and VMO-1, an outer layer vitelline membrane protein. Thirteen proteins present a very wide polymorphism (ovotransferrin, ovomucoid, clusterin, etc.), some of them up to nine isoforms (ovoinhibitor). Eleven functional protein families were identified (serpin, transferrin, protease inhibitors Kazal, glycosyl hydrolases, lipocalin, bactericidal permeability-increasing protein, clusterin, UPAR/CD59/Ly6/ snake neurotoxin, cysteine protease inhibitor, VMO-1, and folate receptor families). These various biological functions could be interesting for further valorizations. In addition, three spots remain unidentified, probably because these proteins are not yet indexed in the international protein databanks.

KEYWORDS: Hen egg white protein; peptide mass fingerprinting; peptide sequencing; two-dimensional gel electrophoresis; mass spectrometry; proteome

INTRODUCTION

Egg white represents a major raw material for the food industry because of its technological properties, especially foaming and gelling. For that reason, much research has been focused on the knowledge of the components implicated in these properties: The structure and functionality of major egg white proteins have been widely studied in various physicochemical conditions (*1*−*3*). A new way to increase the value of egg products could be the extraction of biologically active molecules, especially proteins. Unfortunately, until recently, no highly efficient methods were available to analyze simultaneously all of the hen egg white proteins. Indeed, this biological fluid is original and presents some difficulties for analysis. The proteins have very different molecular masses (12.7−8000 kDa) and pI values (4−11) (*1*). Their concentration differs highly from one protein to another, and ovalbumin represents more than 50% of total proteins, thus making minor protein detection very awkward. These reasons probably explain why very little research has been devoted to the identification and characterization of the minor egg white proteins, even though they probably play essential roles with respect to the original biological action of egg white, i.e., embryo protection and development. So, hen egg white is surprisingly uncharacterized. Only 13 proteins are usually referenced in egg white, some of which are not fully characterized (*1*). Prior to any valorization objective, it seemed necessary to engage in research to identify hen egg white proteins, especially the minor proteins. Some of these minor components could be interesting for nonfood applications, such as health uses. In addition to well-known proteins such as hen egg white biological agents (e.g., lysozyme or ovotransferrin), we could expect the identification of new antimicrobial or antiviral proteins, transport proteins, or growth factors. Indeed, all of the biological properties of hen egg white cannot be explained only by the already known egg white components.

The identification of these minor proteins necessitates highly resolutive methods for protein separation. For this, and despite its known limitations (*4*, *5*), two-dimensional (2D) electrophoresis proved to be particularly efficient. This technique highlighted that many unidentified minor proteins were present in hen egg white (*6*). Moreover, the 1200 Mb genome of *Gallus gallus* has been recently sequenced (*7*). The different tools useful for an efficient proteomic study of hen egg white are thus now available.

The aim of the present work was to identify the expressed proteins in hen egg white and especially to identify some new minor proteins. Using different pH gradients, this study established three 2D reference maps (pH 3−10, 4−7, and 5.5−6.7) for hen egg white proteins containing 69 entries corresponding to 16 different and identified proteins and three still unidentified proteins. One major conclusion of this work is the extraordinary

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extent of polymorphism observed for many proteins. Moreover, some new protein families were identified, which led us to think that unknown biological activities could be identified in hen egg white.

MATERIALS AND METHODS

Chemicals and Materials. Acrylamide/bis-acrylamide 37.5:1 (2.6% C) premixed stock solution, ammonium persulfate, glycine, N,N,N′,N′-tetramethyl-ethylenediamine, and Coomassie brilliant blue G250 and R250 came from Biorad (Marnes-la-Coquette, France). Tris, ammonium carbonate, trifluoroacetic acid (TFA), thiourea, glycerol, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), iodoacetamide, sodium thiosulfate, aluminum sulfate, orthophosphoric acid, silver nitrate, sodium carbonate, potassium ferricyanide, and formaldehyde were purchased from Sigma (St. Quentin-Fallavier, France). High-performance liquid chromatography (HPLC) grade acetonitrile was from Merck (Darmstadt, Germany). Molecular mass markers and immobilized pH gradient (IPG) electrophoresis buffers and strips were from Amersham Pharmacia Biotech (Upsalla, Sweden). Trypsin (sequencing grade) was purchased from Boehringer Ingelheim (Reims, France).

Egg White Preparation. Unfertilized eggs, less than 8 days after being laid, were used. The white was manually separated from the yolk and gently homogenized with a magnetic stirrer.

Two-Dimensional Electrophoresis. Proteins were subjected to high-resolution 2D polyacrylamide gel electrophoresis (2D PAGE) according to the method described by O'Farrel (*8*) and modified by Görg et al. (*9*) on nonlinear (3−10) and linear (4−7, 5.5−6.7) pH gradients. Protein samples were solubilized in a focusing solution containing 7 M urea, 2 M thiourea, 0.3% DTT, 2% CHAPS, and 2% IPG buffer corresponding to the pH gradient used. The surfactant CHAPS and chaotropic thiourea were used throughout the isoelectric focalization to improve protein solubility and transfer to the second dimension (*10*). Isoelectric focusing (IEF) was conducted with a Multiphor II unit (Amersham Pharmacia Biotech) on 13 and 18 cm IPG strips using a gradient mode yielding 60000 Vh.

After focalization, the gel strips were equilibrated for 10 min in an excess of buffer containing 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% glycerol, 1% SDS, and 25 mM DTT. This step enabled the complete reduction of disulfide bridges and denaturation of polypeptides by SDS. A second 10 min equilibration step in the same solution containing 250 mM iodoacetamide instead of DTT was then performed in order to block SH groups according to Görg et al. (*11*). Proteins were then subjected to SDS−PAGE according to Laemmli (*12*). In that aim, strips were placed on top of second-dimension 12% acrylamide gels (16 cm × 16 cm × 0.1 cm or 20 cm × 20 cm × 0.1 cm slab for 13 and 18 cm length, strips, respectively). The migration buffer used was 25 mM Tris, 192 mM glycine, and 0.1% SDS. Electrophoresis was carried out at 65 V overnight and at 150 V to 200 V to finish the migration.

Protein Detection. After migration, gels were fixed and stained in 0.1% Coomassie blue R250, 40% ethanol, and 10% acetic acid. These gels were then washed with 40% ethanol and 10% acetic acid solution until the background was clear. To improve some spot staining, the Coomassie blue G250 staining method was also used as described by Kang and al. (*13*). Last, a more sensitive silver staining method was used when necessary, according to Rabilloud et al. (*14*). This latter method was also compatible with mass spectrometry analysis. Then, gels were finally stocked in 20% ethanol until spot excising.

Molecular Mass and pI Determination. Molecular masses were calibrated by migrating low molecular mass markers (97.0, 66.0, 45.0, 30.0, 20.1, and 14.4 kDa) from Amersham Pharmacia Biotech on SDS−PAGE gel. Isoelectric points were estimated using linear pH gradient strips and calculated using Image Master 2D Platinum software (Amersham Pharmacia Biotech). Theoretical molecular masses and pI have been calculated with Expasy Compute pI/Mw tool considering the primary sequence of each protein, without any post-translational modification and without signal peptide if need be.

Protein Enzymatic Digestion. Gel pieces were excised from the gel and washed with acetonitrile and NH_4HCO_3 for spots stained with Coomassie R250 or G250 or with sodium thiosulfate/potassium ferricyanide as described by Gharahdaghi et al. (*15*) for silver-stained spots; these were then dried in a Speed-vac. In-gel trypsin digestion was performed overnight at 37 °C and stopped with TFA. The resulting peptides were extracted by successive washing steps of the gel pieces with acetonitrile. The supernatants containing peptides were then dried in a Speed-vac and maintained at −20 °C until mass spectrometry analysis.

Nano-Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS). Tryptic hydrolysates were subjected to nanoscale reverse phase liquid chromatography on a modular LC Packings Ultimate HPLC system equipped with a Famos autosampler and a Switchos microcolumn switching device (LC Packings, Dionex Co., Amsterdam, The Netherlands). Samples were previously concentrated and desalted on a 5 mm × 300 μm PepMap C18 precolumn (100 Å, 5 μm, LC Packings). Peptides were eluted onto a 150 mm × 75 μm analytical PepMap C18 column (100 Å, 3 μm, LC Packings). The nanoscale LC eluent from the analytical column was sent to the nanoelectrospray ionization source of a QSTARXL global hybrid quadrupole/time-of-flight mass spectrometer (Applied Biosystem, Framingham, CA) operated in positive ion mode. Raw data were automatically analyzed on a local server harboring Mascot and updated Swissprot and NCBI-NR library databanks. Results were corroborated with ProID software (Applied Biosystem).

Peptide Mass Fingerprinting (PMF). Some tryptic digests resulting from 2D PAGE separation were analyzed by matrix-assisted laser ionization/desorption time-of-flight (MALDI-TOF) mass spectrometry on a Voyager DE STR spectrometer (Applied Biosystem) equipped with a nitrogen laser (337 nm, 20 Hz). Spectra were acquired in the reflector positive mode with a 130 ns extraction delay. An external calibration was first performed in the range of 900−3000 Da. An internal calibration was then performed by using trypsin peptides. The monoisotopic mass lists were compared to the Swiss-Prot and Trembl protein databanks available on the Expasy proteomic server (http://us.expasy.org/) using Protein prospector software (http://prospector.ucsf.edu/) for PMF data analysis. The mass accuracy was lower than 40 ppm. The carbamidomethylation of cysteins, methionine oxidation, and two missed cleavages were considered during the search. A minimum of four matching peptides and a sequence coverage above 20% were required before considering this a result of the databank search. Additional parameters were used to assume correct identification: theoretical molecular mass and isoelectric point in good agreement with experimental values and a MOWSE score value above e + 003.

RESULTS AND DISCUSSION

The literature has contained the original protein composition of hen egg white for a long time, with a very broad molecular mass range (from 12.7 for cystatine to 8000 kDa for soluble ovomucin) and pI value ranges (from 4 for ovomucoid O4 to 10.5 for lysozyme) (*1*, *16*). That is the reason that we performed the first proteomic analysis of hen egg white with broad pH gradients. The standard 2D PAGE profile of 1000 μg of hen egg white proteins loaded on a pH 3−10 IPG strip actually reveals protein spots throughout the pH and molecular mass ranges (**Figure 1A**). The Coomassie blue staining used enables the visualization of 35 distinct protein spots. Some of these protein spots are sufficiently isolated from other egg white proteins to enable their direct identification from the gel. To improve the separation of the spots in the densely populated pH 5−7 zone, we performed 2D PAGE experiments using pH 5.5−6.7 IPG strips (**Figure 1B**,**C**) and pH 4−7 IPG strips (**Figure 1D**−**F**). Moreover, because some proteins are much more abundant than others, i.e., ovalbumin, ovotransferrin, and ovomucoid, with poor resolution when large protein quantities were loaded on the gels, some experiments were performed with lower protein quantities, i.e., 500 μg (**Figure 1C**,**D**).

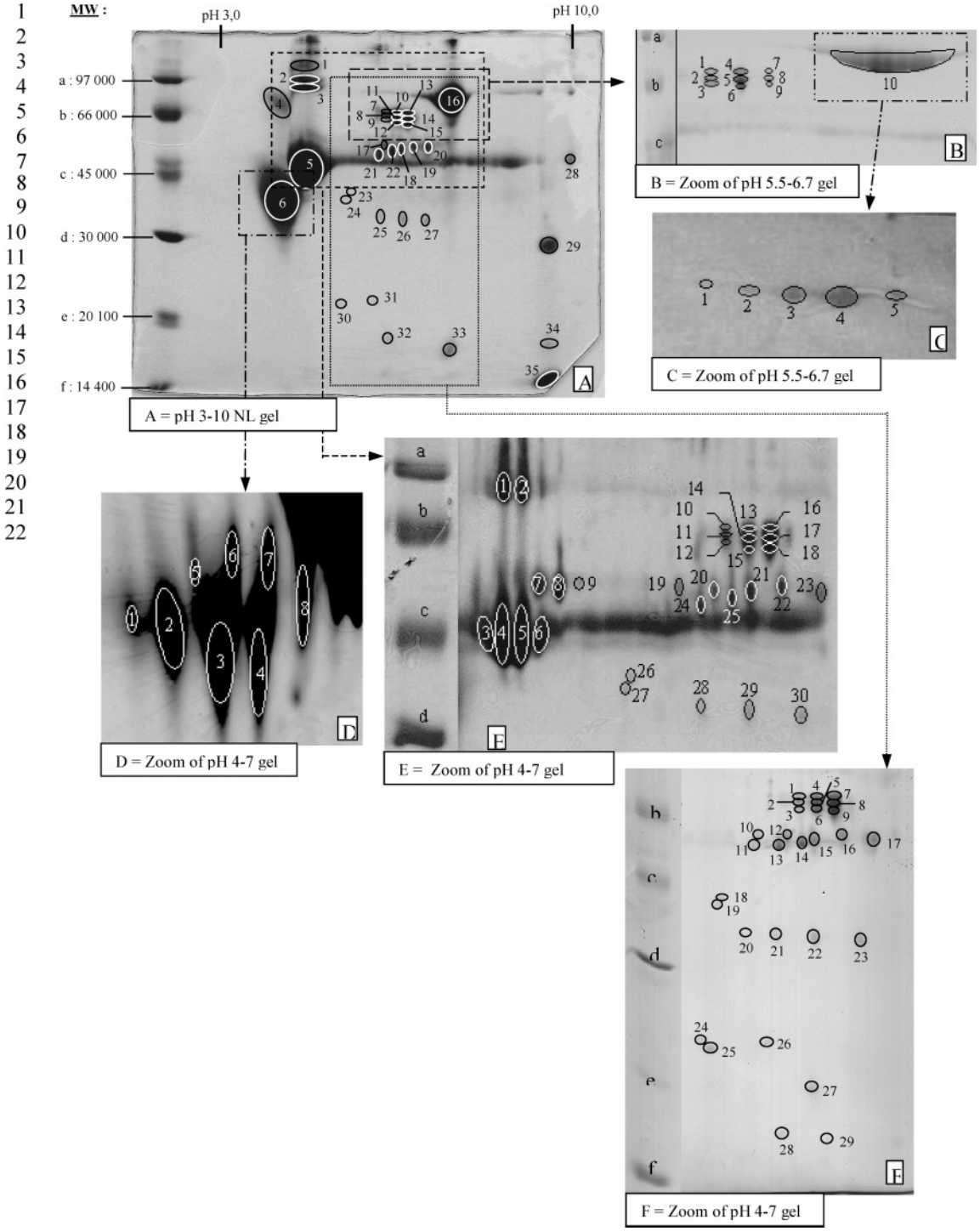

Figure 1. Two-dimensional electrophoresis of hen egg white proteins. An amount of 1000 or 500 μg of total protein was loaded on gels **A**, **B**, **E**, and **F** or gels **C** and **D**, respectively. (**A**) pH 3–10 NL IPG strip, (**B**, **C**) pH 5.5–6.7 IPG strip, and (**D**–**F**) pH 4–7 IPG strip. Gels were stained either with Coomassie blue R250 (**A**, **B**, **E**, and **F**), Coomassie blue G250 (**C**), or silver stained (**D**). Gels **B**–**F** are not entirely presented; only the interesting areas are considered, corresponding to specific areas on gel **A**.

A total of 69 protein spots were separated and analyzed on the six gels. **Table 1** summarizes protein identification on these six reference gels. Protein entries are classified according to their predicted functions and families. Eleven spot proteins were successfully identified by PMF (MALDI-TOF) alone after standard trypsinolysis. These spots represent well-known proteins such as ovalbumin, ovostransferrin, or ovoinhibitor. For 55 protein spots, internal peptide sequencing by nano-LC-MS-MS was performed to confirm the PMF MALDI-TOF analysis and/or for an unambiguous identification.

Finally, 16 proteins were identified; among them, two have never been previously detected in hen egg white: Tenp, a protein with strong homology with bacterial permeability-increasing protein (BPI), and VMO-1, an outer layer vitelline membrane. Many proteins are revealed with a very large polymorphism: Nine proteins are found to be distributed in 2−9 spots. Three protein spots have not yet been identified. On the other hand, some proteins, previously well-known proteins such as hen egg

Table 1. Proteins Identified by Mass Spectrometry after 2D Electrophoresis

spot no.	identification	accession no.[a]	MW exp.	MW theor.	pI exp.	pI theor.	MS equipment used	score	% cover	peptide matched
			serpin family							
A1	ovalbumin	P01012	103200	42750	5.10	5.19	nanoLC-ESI-Q-TOF	102[b] (>34)	13	4
A2	ovalbumin	P01012	97000	42750	5.10	5.19	nanoLC-ESI-Q-TOF	384[b] (>34)	36	17
A3	ovalbumin	P01012	94000	42750	5.10	5.19	nanoLC-ESI-Q-TOF	485[b] (>34)	42	13
E1	ovalbumin	P01012	84800	42750	5.10	5.19	MALDI-TOF	9.97e + 008[c]	64	22
E2	ovalbumin	P01012	84800	42750	5.20	5.19	MALDI-TOF	1.25e + 007[c]	51	15
A3 = E1 + E2										
A4	ovalbumin	P01012	74800	42750	5.00	5.19	nanoLC-ESI-Q-TOF	79[b] (>35)	4	1
E3	ovalbumin	P01012	42400	42750	5.10	5.19	MALDI-TOF	7.92e + 007[c]	52	17
E4	ovalbumin	P01012	42400	42750	5.10	5.19	nanoLC-ESI-Q-TOF	715[b] (>34)	48	64
E5	ovalbumin	P01012	42400	42750	5.20	5.19	MALDI-TOF	1.78e + 008[c]	52	17
E6	ovalbumin	P01012	42400	42750	5.30	5.19	nanoLC-ESI-Q-TOF	555[b] (>34)	52	39
A5 = E3 + E4 + E5 + E6										
E7	ovalbumin Y	P01014	53400	43772	5.30	5.20	nanoLC-ESI-Q-TOF	398[b] (>35)	29	21
E8	ovalbumin Y	P01014	53200	43772	5.40	5.20	nanoLC-ESI-Q-TOF	444[b] (>35)	32	32
E9	ovalbumin Y	P01014	54300	43772	5.50	5.20	nanoLC-ESI-Q-TOF	421[b] (>40)	24	11
E19 = F10	ovalbumin-related Y protein (ovalbumin X)	XP_418984 (NCBI-Nr)	53000	55878	6.30	6.47	nanoLC-ESI-Q-TOF	218[b] (>37)	25	8
A17 = E20 = F12	ovalbumin-related Y protein (ovalbumin X)	XP_418984 (NCBI-Nr)	53000	55878	6.00	6.47	nanoLC-ESI-Q-TOF	156[b] (>39)	21	5
A18 = E21 = F15	ovalbumin-related Y protein (ovalbumin X)	XP_418984 (NCBI-Nr)	53000	55878	6.20	6.47	nanoLC-ESI-Q-TOF	225[b] (>40)	25	8
A19 = E22 = F16	ovalbumin-related Y protein (ovalbumin X)	XP_418984 (NCBI-Nr)	53000	55878	6.50	6.47	nanoLC-ESI-Q-TOF	180[b] (>36)	16	4
A20 = E23 = F17	ovalbumin-related Y protein (ovalbumin X)	XP_418984 (NCBI-Nr)	53000	55878	6.70	6.47	nanoLC-ESI-Q-TOF	151[b] (>36)	20	5
			transferrin family							
C1	ovotransferrin	P02789	85000	75828	6.20	6.69	MALDI-TOF	2.70e + 15[c]	56	37
C2	ovotransferrin	P02789	81000	75828	6.30	6.69	MALDI-TOF	7.84e + 08[c]	36	20
C3	ovotransferrin	P02789	75000	75828	6.40	6.69	nanoLC-ESI-Q-TOF	981[b] (>34)	37	34
C4	ovotransferrin	P02789	76000	75828	6.50	6.69	nanoLC-ESI-Q-TOF	430[b] (>34)	17	11
C5	ovotransferrin	P02789	83000	75828	6.70	6.69	MALDI-TOF	8.21e + 13[c]	46	25
A16 = B10 = C1 + C2 + C3 + C4 + C5										
			protease inhibitors Kazal family							
A7 = B1 = E10 = F1	not determined		69200		6.20					
A8 = B2 = E11 = F2	ovoinhibitor	P10184	66300	49405	6.20	6.07	MALDI-TOF	3.67e + 02[c]	23	9
A9 = B3 = E12 = F3	not determined		64400		6.20					
A10 = B4 = E13 = F4	ovoinhibitor	P10184	69200	49405	6.30	6.07	nanoLC-ESI-Q-TOF	284[b] (>40)	12	8
A11 = B5 = E14 = F5	ovoinhibitor	P10184	65900	49405	6.30	6.07	nanoLC-ESI-Q-TOF	393[b] (>40)	25	11
A12 = B6 = E15 = F6	ovoinhibitor	P10184	63600	49405	6.30	6.07	nanoLC-ESI-Q-TOF	464[b] (>40)	23	11
A13 = B7 = E16 = F7	ovoinhibitor	P10184	69500	49405	6.40	6.07	nanoLC-ESI-Q-TOF	484[b] (>40)	26	12
A14 = B8 = E17 = F8	ovoinhibitor	P10184	66300	49405	6.40	6.07	nanoLC-ESI-Q-TOF	587[b] (>40)	41	15
A15 = B9 = E18 = F9	ovoinhibitor	P10184	63600	49405	6.40	6.07	nanoLC-ESI-Q-TOF	610[b] (>40)	34	14
D1	ovomucoid	P01005	40000	20098	5.00	4.82	nanoLC-ESI-Q-TOF	88[b] (>30)	8	3
D2	ovomucoid	P01005	39200	20098	5.00	4.82	nanoLC-ESI-Q-TOF	206[b] (>30)	38	6
D3	ovomucoid	P01005	37400	20098	5.20	4.82	nanoLC-ESI-Q-TOF	331[b] (>30)	37	9
D4	ovomucoid	P01005	37200	20098	5.30	4.82	nanoLC-ESI-Q-TOF	298[b] (>30)	37	7
D5	ovomucoid	P01005	42600	20098	5.10	4.82	nanoLC-ESI-Q-TOF	67[b] (>30)	8	2
D6	ovomucoid	P01005	43100	20098	5.20	4.82	nanoLC-ESI-Q-TOF	81[b] (>27)	8	3
			family 22 of glycosyl hydrolases							
A29	lysozyme C	P00698	28400	14313	ND[d]	9.32	nanoLC-ESI-Q-TOF	268[b] (>38)	37	6
A34	lysozyme C	P00698	17600	14313	ND[d]	9.32	nanoLC-ESI-Q-TOF	68[b] (>40)	10	1
A35	lysozyme C	P00698	15000	14313	ND[d]	9.32	nanoLC-ESI-Q-TOF	350[b] (>37)	48	12
			lipocalin family							
A30 = F25	ex-FABP (quiescence-specific protein, Ch21 protein)	Q6E6M8	21000	18065	5.70	5.39	nanoLC-ESI-Q-TOF	179[b] (>28)	24	4
F24	chondrogenesis-associated lipocalin (CAL γ, prostaglandin H2 D-isomerase)	Q8QFM7	22200	20843	5.60	6.30	nanoLC-ESI-Q-TOF	88[b] (>29)	14	2
A31 = F26	chondrogenesis-associated lipocalin (CAL γ, prostaglandin H2 D-isomerase)	Q8QFM7	21600	20843	6.00	6.30	nanoLC-ESI-Q-TOF	293[b] (>28)	34	6

Table 1 (Continued)

spot no.	identification	accession no.[a]	MW exp.	MW theor.	pI exp.	pI theor.	MS equipment used	score	% cover	peptide matched
			lipocalin family							
D1	ovoglycoprotein	Q8JIG5	40000	20317	5.00	5.03	nanoLC-ESI-Q-TOF	138[b] (>30)	15	3
D2	ovoglycoprotein	Q8JIG5	39200	20317	5.00	5.03	nanoLC-ESI-Q-TOF	209[b] (>30)	18	4
D3	ovoglycoprotein	Q8JIG5	37400	20317	5.20	5.03	nanoLC-ESI-Q-TOF	171[b] (>30)	18	4
D4	ovoglycoprotein	Q8JIG5	37200	20317	5.30	5.03	nanoLC-ESI-Q-TOF	211[b] (>30)	18	4
D5	ovoglycoprotein	Q8JIG5	42600	20317	5.10	5.03	nanoLC-ESI-Q-TOF	212[b] (>30)	18	4
D6	ovoglycoprotein	Q8JIG5	43100	20317	5.20	5.03	nanoLC-ESI-Q-TOF	223[b] (>30)	18	5
D7	ovoglycoprotein	Q8JIG5	42500	20317	5.30	5.03	nanoLC-ESI-Q-TOF	211[b] (>30)	18	5
D8	ovoglycoprotein	Q8JIG5	40500	20317	5.40	5.03	nanoLC-ESI-Q-TOF	197[b] (>30)	18	4
A6 = D1 + D2 + D3 + D4 + D5 + D6 + D7 + D8										
			BPI family							
F11	TENP	O42273	48900	47435	5.90	5.57	nanoLC-ESI-Q-TOF	182[b] (>28)	14	4
A21 = E24 = F13	TENP	O42273	48900	47435	6.10	5.57	nanoLC-ESI-Q-TOF	391[b] (>28)	25	9
A22 = E25 = F14	TENP	O42273	50200	47435	6.30	5.57	nanoLC-ESI-Q-TOF	354[b] (>27)	19	6
			clusterin family							
F20	not determined		34500		5.90					
A25 = E28 = F21	clusterin	Q9YGP0	33000	51348	6.10	5.48	nanoLC-ESI-Q-TOF	102[b] (>26)	4	2
A26 = E29 = F22	clusterin	Q9YGP0	32600	51348	6.30	5.48	nanoLC-ESI-Q-TOF	290[b] (>26)	16	7
A27 = E30 = F23	clusterin	Q9YGP0	32400	51348	6.60	5.48	nanoLC-ESI-Q-TOF	194[b] (>26)	10	4
			UPAR/CD59/Ly6/snake neurotoxin superfamily							
A32 = F27	Hep21 protein	Q8AV77	18100	10001	6.30	6.73	nanoLC-ESI-Q-TOF	57[b] (>50)	11	1
A33 = F29	Hep21 protein	Q8AV77	17100	10001	6.40	6.73	nanoLC-ESI-Q-TOF	68[b] (>51)	11	1
			cysteine protease inhibitor family							
F28	cystatin	P01038	17000	13148	6.10	6.54	nanoLC-ESI-Q-TOF	122[b] (>39)	20	2
			VMO-1 family							
A34	vitelline membrane outer layer protein 1 precursor (VMO-1)	P41366	17600	17978	ND[d]	8.85	nanoLC-ESI-Q-TOF	69[b] (>37)	8	1
			folate receptor family							
D1	RBP	P02752	40000	25355	5.00	5.05	nanoLC-ESI-Q-TOF	71[b] (>30)	6	2
D2	RBP	P02752	39200	25355	5.00	5.05	nanoLC-ESI-Q-TOF	73[b] (>30)	6	2
D3	RBP	P02752	37400	25355	5.20	5.05	nanoLC-ESI-Q-TOF	69[b] (>30)	6	2
			unidentified spots							
A23 = E26 = F18			37000		5.80					
A24 = E27 = F19			35000		5.70					
A28			48000		ND[d]					

[a] If not specified, the accession number refers to the SwissProt database. [b] Mascot total score (a score > *x* indicates the identity or extensive homology for $p < 0.05$). [c] Protein Prospector MOWSE score. [d] ND, not determined (electrophoresis gel with pH 3–10 nonlinear).

white components, were not revealed despite their physicochemical characteristics, which were compatible with the molecular mass and pH ranges used in the present study.

Serpin Family. This protein family is essentially represented by the major hen egg white protein, i.e., ovalbumin (54% of proteins), for which three phosphorylation levels have already been reported. The diphosphorylated form (pI 4.75) represents approximately 87% ovalbumin, as compared to 12% for the monophosphorylated form (pI 4.89) and 1% for the nonphosphorylated form (pI 4.94) (*17*). Ovalbumin spots are easily identified on the two gels with pH gradients 3−10 and 4−7. With a broad pH gradient, ovalbumin appears as a large spot (A5). On the other hand, on the 4−7 pH gradient gel, ovalbumin is identified as four spots (E3−E6). These spots could be attributed to ovalbumin isoforms even if four ovalbumin forms were unexpected. According to their respective intensity, spots E4−E6 could be assigned to diphosphorylated, monophosphorylated, and nonphospohorylated ovalbumin forms, respectively. However, E3 remains difficult to explain according to the known ovalbumin structure. A satisfactory agreement is observed between the experimental molecular mass and the pI with the theoretical values.

Other spots with higher molecular masses (E1, E2, and A1−A4) are also identified as ovalbumin. This surprising localization could correspond to ovalbumin polymers. In that case, it is noticeable that these putative polymers still exist after urea, SDS, and DTT treatments, meaning that hydrophobic interactions and disulfide bonds would not be involved in such aggregates. The experimental molecular mass of A3 agrees with the dimer molecular mass. However, A1, A2, and A4 experimental molecular masses do not agree with simple polymeric forms, suggesting ovalbumin modifications such as glycosylation. Unfortunately, the nano-LC-MS-MS analysis did not enable the confirmation of such an assumption.

The natural overabundance of ovalbumin in egg white probably explains the protein trail observed around 45 kDa and from pH 5.3 to pH 9.0. It could explain the contamination of many spots with ovalbumin as well, unless this contamination indicates interactions between ovalbumin and many other hen egg white proteins.

Two other members of the serpin family are revealed on hen egg white 2D PAGE gels: ovalbumin gene Y and ovalbumin-related Y protein. Ovalbumin gene Y has been known as a member of the ovalbumin gene family since 1982, and its encoding gene was sequenced by Heilig et al. (*18*). It was recently identified in hen egg white, as well as ovalbumin gene X (*19*, *20*). Guérin-Dubiard et al. (*20*) already noticed a difference between the experimental molecular mass of ovalbumin gene X (45 kDa, as determined by SDS−PAGE) and the theoretical value (26.3 kDa). However, a blast between the

ovalbumin gene X sequence (P01013) and that of the ovalbumin-related Y protein (XP 418984) demonstrated a perfect homology between these two proteins (score *E* value, 457 e^{-127}), the first one being a fragment of the second one. The spots E19−E23 were then identified as ovalbumin-related Y protein. The good agreement between the experimental and the theoretical molecular masses reinforces this identification.

The 2D PAGE analysis revealed the large polymorphism of these proteins, with three and five spots containing ovalbumin gene Y and ovalbumin-related Y protein, respectively. A previous study established that the ovalbumin gene Y polymorphism could not be explained by various glycosylation or phosphorylation levels nor by genetic variations (*19*); the assumption of alternative splicing processes leading to casual exon skipping events was then put forward, such a mechanism being displayed elsewhere (*21*, *22*). However, no experimental evidence for that is currently available, and no information exists to explain the ovalbumin-related Y protein polymorphism. Another interesting question about these two minor hen egg white proteins concerns their putative biological activity: Are they or not, unlike ovalbumin, biological active members of the serpin family, that is, protease inhibitors?

Transferrin Family. Ovotransferrin seems to be the only member of this protein family in hen egg white. It is easily identified on the three gels (**Figure 1A−C**). A narrow pH gradient (pH 5.5−6.7) reveals a much wider polymorphism than previously noted for this protein. Ovotransferrin is found to be distributed in five spots, whereas previous studies exhibited only three isoforms (*6*, *23*). We could attribute these spots to ovotransferrin glycoforms as was shown for human transferrin. Even if only three pI values for aferric, monoferric, and diferric transferrin were known (*23*), nine glycoforms could be separated by capillary electrophoresis (*24*).

Protease Inhibitors Kazal Family. Two well-known hen egg white proteins are members of this protein family, i.e., ovomucoid and ovoinhibitor. Six spots contain ovomucoid isoforms, whereas only five isoforms were previously decribed (*25*). However, the resolution was very poor in this 2D gel area. It is then difficult to precisely determine the number of ovomucoid isoforms. According to what was previously mentioned (*6*), the apparent molecular mass of ovomucoid is much higher than the theoretical one. It is likely due to the high glycosylation level of this protein (up to 25% carbohydrates) (*1*).

It should be underlined that the ovomucoid spots also contain ovoglycoprotein (D1−D6) and riboflavin-binding protein (RBP) (D1−D3) (see below). This could suggest some interactions between these three proteins and could explain why ovomucoid fractions are often contaminated by ovoglycoprotein (*26*).

The second Kazal protease inhibitor is ovoinhibitor. It is identified in nine characteristic spots. Even if the first and third spots of the first row could not be identified by mass spectrometry because of the very low protein quantities available, their position led us to assume that it is ovoinhibitor. As for transferrin, different levels of glycosylation could be put forward as one reason, at least, for this polymorphism. This assumption is consistent with the observation of Davis et al. (*27*) who separated up to five fractions of ovoinhibitor with various glucidic compositions, by ion exchange chromatography on DEAE-cellulose.

Family 22 of Glycosyl Hydrolases. This protein family is represented in hen egg white by lysozyme C. Lysozyme is distinctly separated on pH 3−10 gel, in a major spot (A35). However, two other spots are also identified as lysozyme. Both of them correspond to the same apparent pI value but to different apparent molecular masses. The one named A34 could be the minor glycosylated form of lysozyme previously identified by Trudel and Asselin (*28*). This spot also contains a newly identified minor protein, i.e., VMO-1 (see below). The third spot (A29) is revealed for an apparent molecular mass around the double lysozyme molecular mass. This spot could then be attributed to the dimeric form of lysozyme. Such dimers exist naturally in small concentrations in egg white (*29*) or can be produced by storage with 0.5 M NaCl or 1% SDS or by storage in nonrefrigerated conditions (*30*).

Lipocalin Family. Lipocalins are transporters for small hydrophobic molecules, such as lipids, steroid hormones, and retinoids. This widely spread protein family is represented in hen egg white by three minor proteins; among them, two were recently identified in hen egg white and belong to the chondrogenesis-related lipocalins subfamily. Extra fatty acid binding protein (Ex-FABP), also called Ch21 protein or quiescence-specific protein, was observed for the first time in hen egg white by Larsen et al. (*31*). Initially found in chick embryo skeletal tissues, this protein is suspected to be a stress protein expressed during chondrocyte and myoblast differentiation (*32*); its relation with cartilage formation and inflammatory responses has been demonstrated (*33*). Because of the gap between apparent and theoretical molecular masses, we could suggest that this protein is glycosylated.

We revealed the presence of CAL-γ protein in hen egg white in a previous study (*34*). Its encoding gene has been previously sequenced by Pagano et al. (*35*). As for Ex-FABP, the apparent molecular mass determined for CAL-γ is consistent with the theoretical value. However, CAL-γ is here found to be distributed in two spots.

Because of their analogy and peculiar developmental patterns of expression in chicken embryos, Pagano et al. (*35*) suggested a synergistic action of CAL-γ and Ex-FABP in the process of endochondral bone formation. However, the present study indicates that these two proteins also exist in unfertilized hen egg white. It is noticeable that these authors isolated and characterized another gene (CAL-β) that may form, with Ex-FABP and CAL-γ, a genomic cluster where the three genes could be coordinately regulated (*36*). However, the present 2D PAGE analysis did not enable us to reveal this third component of the chondrogenesis-related lipocalin family.

Last, ovoglycoprotein, a third protein of this family, was first detected in egg white many years ago (*37*, *38*) and was also identified on 2D PAGE. Very little information is available about this protein, possibly due to the absence of any distinctive biochemical, functional, or biological properties. Currently, ovoglycoprotein is mainly used as a chiral selector to separate drug enantiomer by HPLC or by capillary electrophoresis (*26*). In the present study, eight poorly resolved spots are identified as being ovoglycoprotein. This could result either from a very great polymorphism or from possible interactions between ovomucoid and ovoglycoprotein, since these two proteins are also detected in some of these spots. The apparent molecular mass is much higher than the theoretical one, consistent with a sugar content of 30%, as reported by Li-Chan and Nakai (*1*).

Bactericidal Permeability-Increasing Protein (BPI) Family. The present study revealed for the first time the presence of Tenp in unfertilized hen egg white. This protein is found distributed in three spots. Tenp gene is transiently expressed in developing neural tissues, including the brain and retina (*39*, *40*). A search of domain signature patterns, realized with Pfam, PROSITE, and SMART databanks, clearly highlighted a very strong homology between the end of the Tenp protein sequence

and the BPI2 domain, among the BPI/lipopolysaccharide-binding protein (LBP)/cholesteryl ester transfer protein (CETP) C-terminal domain. The biological activity assumed for such a BPI protein is the binding to the Lipid A component of lipopolysaccharide from the outer envelope of Gram-negative bacteria (*41*). The toxic action of BPI against Gram-negative bacteria occurs in two stages: The binding of BPI causes immediate bacterial growth arrest linked to alterations in the outer membrane, followed later by bactericidal events coincident with damage to the inner membrane (*42*). Tenp could then participate in the antibacterial activity of hen egg white. This assumption has to be confirmed experimentally. If it is confirmed, it is noticeable that Tenp represents a significant quantity of hen egg white proteins (≈0.1−0.5%), as compared to the staining intensity of the corresponding spots and those of ovoinhibitor, which represents 0.1−1.5% of total protein (*43*).

Clusterin Family. Clusterin is an ubiquitous and highly conserved secreted glycoprotein. It has been found in numerous biological fluids including semen, urine, and human plasma (*44*). The present study confirms the presence of clusterin in hen egg white, as already immunodetected in several chicken tissues such as magnum, egg shell, and egg white by Mann et al. (*45*). Clusterin is a member of the chaperone proteins, which interact and stabilize unfolded or partly folded proteins, preventing their aggregation or precipitation. According to Poon et al. (*46*), clusterin interacts with and stabilizes slowly aggregating proteins, states that are often associated with Alzheimer's, Creutzfeldt−Jakob, and Parkinson's deseases. The 2D PAGE analysis reveals three clusterin isoforms. A fourth spot (F20) could not be identified precisely, because of the lack of protein material, but its position led us to assume that it could also be another clusterin isoform. Apparent molecular mass is significantly lower than the theoretical value calculated from the deduced amino acid sequence of cDNA determined by Mahon et al. (*44*). On the other hand, clusterin has been characterized as an α/β heterodimer, which was detected at 35 kDa under reducing conditions in electrophoresis by Mann et al. (*45*). These authors determined the cleavage site and the corresponding to theoretical molecular masses: 24.5 and 24.8 kDa for α- and β-clusterin, respectively. The three (or four) protein spots determined in the present study could then correspond to these two clusterin monomers, potentially more or less glycosylated.

UPAR/CD59/Ly6/Snake Neurotoxin Superfamily. A new member of the uPAR/Ly6 protein superfamily was recently identified in hen egg white: HEP21 (*47*). The 2D PAGE analysis revealed this protein as two isoforms with apparent molecular masses significantly different from each other and higher than the theoretical value, suggesting the glycosylation of this protein, as for most of the listed members of uPAR/Ly6 family. The apparent pI values are consistent with the theoretical values. The biological activity of HEP21 is still impossible to predict, because of the very wide spectrum of activities of the multifunctional uPAR/CD59/Ly6/snake toxin family. However, HEP21 appeared as an original member of this protein superfamily because it is predominantly expressed in tissue, i.e., the oviduct, and especially the magnum where the egg white components are secreted (*47*), unlike most of the other ubiquitous members of this protein family (*48*).

Cysteine Protease Inhibitor Family. Chicken cystatin, a small protein type inhibitor of cystein proteinases, represents a well-studied member of the cystatin superfamily. It was shown to be a reversible tight-binding inhibitor of papain and papain-like enzymes (*49*). According to Bode et al. (*50*) and Machleidt et al. (*51*), the enzyme−inhibitor complex is mainly formed through hydrophobic interactions between the chicken cystatin and the complementary active site cleft of papain. These inhibitors might protect the cells from inappropriate endogenous or external proteolysis and/or could be involved in a control mechanism responsible for intracellular or extracellular protein breakdown. The main characteristic of the inhibitors of the cystatin family is the presence of two disulfide bonds located toward the carboxyl terminus. They are composed of about 115 amino acid residues with a molecular mass of about 13 kDa (*52*). Chicken cystatin was isolated as a mixture of two major isoelectric forms of identical amino acid sequence, a nonphosphorylated form with a pI of 6.5 and a phosphorylated form with a pI of 5.6 (*53*−*55*). In the present study, only one spot is attributed to cystatin, with an apparent molecular mass quite consistent with the theoretical one. However, the experimental pI does not enable the identification of this protein as the phosphorylated or nonphosphorylated isoform.

VMO-1 Family. The VMO-1 protein is one of the proteins identified in the outer layer of egg vitelline membranes and characterized by Back et al. (*56*). Three others proteins also composed this outer layer: ovomucin, lysozyme, and a second vitelline membrane outer protein, VMO-2; this last protein was highlighted by Kido et al. (*57*). VMO-1, VMO-2, and lysozyme bind tightly to ovomucin and participate in the vitelline membrane structure. Schäfer et al. (*58*) showed that egg storage in non-refrigerated conditions led to disintegration of proteins VMO-1 and VMO-2 from the vitelline membrane, leading to membrane deterioration. VMO-1 was revealed here for the first time as a hen egg white protein. It is noticeable that VMO-1 is identified in a spot also containing lysozyme. However, there is no experimental evidence for interactions between these two proteins in egg white. The apparent molecular mass of VMO-1 is consistent with the theoretical value and with the apparent molecular mass (17 k Da) determined in SDS−PAGE by Schäfer et al. (*58*). Its pI could not be calculated because VMO-1 was only detected on the nonlinear pH gradient gel, but it is clearly an alkaline protein. The VMO-2 could not be detected in our experimental conditions, probably because of its low molecular mass. Schäfer et al. (*58*) identified VMO-2 as two distinct bands in SDS−PAGE with molecular masses of 5 and 8 kDa, respectively.

Folate Receptor Family. RBP also referred to as flavoprotein or ovoflavoprotein is a member of the folate receptor family. This family includes the folate receptor, which binds to folate and reduced folic acid, acid derivatives, and mediates of 5-methyltetrahydrofolate to the interior of cells. RBP is a phosphoglycoprotein found in egg white as well as in egg yolk and serum of laying hens as reported by Li-Chan and Nakai (*1*). This protein binds riboflavin (vitamin B2), an essential vitamin for embryo development. RBP is revealed as three badly resolved spots, in association with ovomucoid and ovoglycoprotein. The apparent molecular mass is higher than the theoretical value. Like ovomucoid, RBP is highly glycosylated (15% carbohydrates), which explains that its apparent molecular mass is overvalued as already described by Desert et al. (*6*). These authors also described RBP migration in monodimensional electrophoresis as very diffuse bands that are difficult to stain whatever staining method is used. A high rate of glycosylation could explain this behavior, and the nine RBP glycoforms characterized by Amoresano et al. (*59*) are certainly located in this area where spots are not well-resolved. In the present study, RBP was only detected in three spots whose pI values are consistent with theoretical values.

Unidentified Spots. Three spots are still not identified [A23

(= E26 = F18), A24 (= E27 = F19), and A28]. These three spots are strongly stained with Coomassie Blue, suggesting that their quantities are compatible with their characterization by mass spectrometry (LC/MS/MS). Indeed, tryptic hydrolysates contained satisfactory peptide quantities, as indicated by nano-LC/MS/MS spectra (data not shown). However, no identification was possible, probably because these proteins are not yet indexed in the international protein databanks. This underlines one limit of the proteomic analysis, which implies that all of the protein sequences have been previously indexed, thanks to complete genome sequencing. We were faced with the same situation when the new minor protein Hep21 was isolated in hen egg white for the first time, with an unknown internal peptide sequence (*47*).

Known Egg White Proteins Not Detected on 2D Gels. Some egg white proteins, which are already known, have not been detected by 2D PAGE analysis. It is likely that their absence on the 2D PAGE gels is due to their extreme molecular masses (higher than 100 or lower than 14 kDa) or their extreme apparent pI values (higher than 10). These assumptions are reasonable for ovomucin (molecular mass higher than 8000 kDa) (*1*), ovostatin, also named ovomacroglobulin (780 kDa) (*1*), and avidin (pI 10, *60*).

On the other hand, ovoglobulins G2 and G3 (49 kDa, pI 5.5 and 5.8, respectively), reported by Li Chan and Nakai (*1*), are not revealed despite their physicochemical characteristics, which are compatible with the molecular mass and pH ranges used in the present study. It is noticeable that these proteins are not indexed in the international protein databanks. Because of their characteristics, roughly corresponding to eight protein spots around 53 kDa (**Figure 1E**) and identified as ovalbumin gene Y and ovalbumin gene X or ovalbumin-related Y protein, we assumed that ovoglobulins G2 and G3 represent two isoforms of these ovalbumin gene-related proteins.

Finally, it was impossible to reveal in the present study the thiamin-binding protein purified from egg white by Munniyappa and Adiga (*61*), since this protein is not indexed in the protein databanks.

Finally, 2D PAGE analysis associated with mass fingerprinting has been largely used to analyze the protein composition of many microorganisms and biological tissues or secretions such as milk. However, until now, this powerful technique had never been applied to improve our knowledge of hen egg white proteins. Despite technical problems due to the overabundance of three proteins, i.e., ovalbumin, ovotransferrin, and ovomucoid, and to the very wide spectrum of molecular masses and pI values, the present study demonstrated the efficiency of this strategy to identify a large number of proteins, among which two had never been identified before in hen egg white. Moreover, three spots are still unidentified.

One remarkable result is the very wide polymorphism of the hen egg white proteins: 13 proteins (among 16 identified) are concerned, some of them with up to nine isoforms. Another interesting result concerns the large number of functional protein families for a biological liquid usually described as a biochemical protection for embryo against external aggression. Then, various biological functions could be considered, among which some could be potentially interesting for further valorizations. Antimicrobial activities being specifically in demand, the new minor protein Tenp could be an interesting candidate in that way.

However, to progress again in the identification of very minor proteins, whose biological role could be essential, the question arises of the selective elimination of major proteins. When this aim is achieved, putative minor proteins currently masked under the major protein spots may be revealed, and higher quantities of proteins could be loaded, improving the possible detection of very minor proteins. Immunological affinity could be considered in that aim.

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