Comparison of Different Electrophoretic Separations of Hen Egg White Proteins

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The hen egg white protein composition has not yet been fully defined. To improve the knowledge of this biological fluid, the most usual and recently developed electrophoretic methods have been used: SDS−PAGE, native−PAGE, isoelectric focusing (IEF), and 2-dimensional electrophoresis (2DE). Seven of the major known proteins were thus identified in at least one electrophoretic system. Isoforms of ovotransferrin, ovalbumin, and ovomucoid were visualized when pI was used for the separation. Two-dimensional electrophoresis allowed separation of a very large number of spots. In each of the four systems, some components were revealed but not identified, and unknown spots were particularly numerous with 2DE. With this technique, many spots corresponding to small acidic proteins were highlighted, among which was the Ch21 protein, whose presence in hen egg white was thus confirmed. This study thus constitutes, to our knowledge, the first proteomic investigation of hen egg white.

Keywords: *Hen egg white; protein; SDS−PAGE; native−PAGE; IEF; two-dimensional gel electrophoresis; immunodetection; proteomic*

INTRODUCTION

Hen egg white represents an essential ingredient for the food industry because of its useful functional properties. Egg-product processing, however, fails to generate increased revenue. To increase the value of egg products, two ways can be envisaged: better control of protein functionality and extraction of biologically active molecules, especially proteins. Unfortunately, hen egg, in particular egg white, is surprisingly uncharacterized. Less than 20 proteins have been identified thus far in the egg white, among which some are not fully characterized. Such an apparent simplicity in the protein composition of a biological fluid, which original role consists of protecting the embryo and in sustaining its development, probably indicates a large gap in the knowledge about it. Then, prior to any valorization objective, it seemed necessary to engage in research to identify the egg white proteins. For this reason, the most recent techniques for protein analysis (proteomics) have been applied to egg white.

To identify the different components of albumen, chromatographic techniques were developed (*1*, *2*). They nevertheless failed to discriminate all egg white proteins. Promising complementary separation methods include electrophoretic separations, which recently underwent considerable improvements, whereas the last studies on egg white proteins, in this field, date from the 1960s and 1970s (*3*−*5*). SDS−PAGE followed by Coomassie blue detection is the only technique described in recent work for the study of egg white proteins (*6*, *7*). This simple and easy electrophoretic method is, however, limited in terms of resolution. Some high-performance techniques were used, but these were on purified proteins (*8*−*10*). We are unaware of any work to improve global electrophoresis analysis of albumen polypeptides. The mixture of egg white proteins is original and presents some difficulties for analysis. In fact, the proteins have very different molecular weights (12700 to 240 × 10^6 Da) and pI values (4 to 11) (*11*). Their concentrations in albumen are also very different; for example, ovalbumin is the major protein (more than 50% of total proteins), and other proteins such as lysozyme or cystatin are present in very small quantities. Moreover, many of the egg white proteins are highly glycosylated, what constitutes a major limit for their detection and visualization on gels, as already reported (*12*, *13*). According to these authors, steric interferences and electric charges induced by the carbohydrate moieties would be involved.

In this work, we compared and modified existing electrophoretic techniques for egg white proteins analysis and we also applied immobilized pH gradient two-dimensional electrophoresis to this biological fluid. This technique, today accessible and user-friendly, is the most recently developed in the field of electrophoresis. It is widely used throughout the world because of its powerful resolution and its high reproductibility, which are attributable to the immobilized pH gradient (IPG) technique. Moreover, 2D gels are now easily interpreted with modern image analysis software packages. However, these recent techniques have not yet been applied to the egg white. Then, to our knowledge, this work constitutes the first proteomic investigation of hen egg white.

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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′-tetra-methyl-ethylenediamine (TEMED), nitrocellulose membranes (pore size 0.45 μm), and dye solutions (Coomassie brilliant blue R250, silver nitrate) were from Biorad (Marnes-la-Coquette, France). Tris, thiourea, glycerol, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), sodium dodecyl sulfate (SDS), dithiothreitol (DTT), 5-bromo-4-chloro indoxyl phosphate/nitroblue tetrazolium (BCIP/NBT), Tween 20, iodoacetamide, and mixed ampholytes carriers (pH linear gradient 4−7 L), poly(vinylidene difluoride) (PVDF) membranes were purchased from Sigma (St Quentin-Fallavier, France). Molecular weight markers and immobilized pH gradient (IPG) electrophoresis buffers and strips were from Amersham Pharmacia Biotech (Upsalla, Sweden).

Lysozyme (lyso) was produced by OVONOR (Annezin-lès-Bethune, France); standards of hen egg white proteins were purchased from Sigma: avidin (avid), flavoprotein (flavo), globulins, ovalbumin grade V (ovlb), ovoinhibitor type IV-O (ovinh), ovomucoïd type III-O (ovmd), ovotransferrin (ovtf); ovomucin was prepared according to Brooks and Hale (*14*), modified by Kato et al. (*15*).

Monoclonal mouse anti-ovalbumin and anti-avidin immunoglobulins were purchased from Sigma. Monoclonal mouse anti-ovotransferrin immunoglobulin was produced according to Uthoff and Böldicke (*16*) and kindly supplied by Y. Nys and J. Gautron, INRA (Nouzilly, France). Secondary anti-mouse antibody was from Sigma.

1D migrations were carried out on small slab gels (7 × 8 × 0.1 cm) using a Biorad Mini Protean II system.

The first dimension of 2D PAGE was performed using the Multiphor II electrophoresis cell (Amersham Pharmacia Biotech) and pH 4 to 7 linear gradient IPG dry strips; the second dimension was performed on 16 × 16 × 0.1 cm slab gels (Protean II, Biorad) and semi-dry blotting using a Trans-Blot Cell (OWL Scientific Inc., Woburn, MA).

SDS−PAGE. SDS gel electrophoresis was performed according to Laemmli (*17*), using 4% acrylamide in stacking gels and separating gels consisting of 12% acrylamide, the optimal concentration for a good separation of hen egg white proteins. The SDS-containing sample buffer (2% SDS, 20% glycerol, 5% β-mercaptoethanol, and 0.5% bromophenol blue in 62 mM Tris−HCl buffer, pH 6.8) was used for protein denaturation. After dilution, samples were treated for five minutes at 100 °C. For a good visualization of major proteins, 10 μg of proteins were loaded when the gel was stained with Coomassie brilliant blue, and 2 μg of protein was loaded with silver staining. Minor proteins were visualized when 50 μg (Coomassie brilliant blue) or 20 μg (silver staining) were loaded. The migration buffer used was 25 mM Tris, 192 mM glycine, and 0.1% SDS. Electrophoresis was carried out at 75 V in the stacking gel and at 150 V in the separating gel.

Native PAGE. Electrophoresis was performed according to Ornstein (*18*) and Davis (*19*), in 0.5 M Tris−HCl buffer, pH 6.8, for stacking gel (4% acrylamide) and 1.5 M Tris−HCl buffer, pH 8.8, for separating gel (7.5% acrylamide). Samples were diluted in 62 mM Tris−HCl buffer, pH 8.8, 10% glycerol, and 0.5% bromophenol blue. Hen egg white proteins (0.31 to 10 μg) were loaded and silver stained. Migration was performed in the same conditions as SDS−PAGE in 24 mM Tris−glycine buffer, pH 8.8.

Isoelectric Focusing. Conventional isoelectric focusing (IEF) in ampholyte carrier buffers was performed using 7.5% acrylamide, 10% glycerol, and 3% ampholytes. Samples were diluted in 50% glycerol, 2% ampholytes, and 2% CHAPS. The cathode solution contained 20 mM lysine/20 mM arginine and the anode solution was 10 mM H_3PO_4. The running conditions on mini gel were 1h at 100 V, 1h at 250 V, and 30 min at 500 V.

2D Electrophoresis. Proteins were subjected to high-resolution two-dimensional electrophoresis according to the method described by O'Farrel (*20*) and modified by Görg et al. (*21*) on a pH 4 to 7 linear gradient. Protein samples were solubilized in a focusing solution containing 7 M urea, 2 M thiourea, 2 mM DTT, 4% CHAPS, 2% IPG buffer type pH 4−7L (Amersham Pharmacia Biotech). The surfactant CHAPS and chaotropic thiourea were used throughout the isoelectric focalization to improve protein solubility and transfer to the second dimension (*22*). Isoelectric focusing was conducted with a Multiphor II unit (Amersham Pharmacia Biotech) on 13-cm IPG strips (type pH 4−7L, Amersham Pharmacia Biotech) using a gradient mode yielding 30,000 Vh. After focalization, the gel strips were equilibrated 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 affords complete reduction of disulfur bridges and denaturation of polypeptides by SDS. A second 10 min equilibration step in the same solution but containing 250 mM iodoacetamide instead of DTT was then performed in order to block SH groups according to Görg et al. (*23*). Proteins were then subjected to SDS−PAGE according to Laemmli (*17*). In that aim, strips were placed on top of second-dimension 12% acrylamide gels (16 × 16 × 0.1 cm slab). Migration was then performed as described above.

Detection. *Coomassie Blue.* After migration, gels were fixed and stained in 0.05% Coomassie blue R250, 50% methanol, and 10% acetic acid.

After isofocalization, because of ampholytes and in order to improve ovomucoïd fixation, staining and destaining steps were carried out after immersion of the gel in 20% TCA diluted in 50% ethanol, as described elsewhere (*8*).

Silver Staining. Gels were silver-stained as described by Tunon and Johanssen (*24*). After IEF, staining and destaining steps were carried out after immersion of gel in 7% sulfosalicylic acid dihydrated, 20% TCA, and 30% methanol as described elsewhere (*25*).

Western Blot Analysis. Immunoblotting was performed by transferring the separated proteins from the 1D gels onto a nitrocellulose membrane and from the 2D gels onto a PVDF membrane, using a constant current of, respectively, 200 mA for 1.5 h and 400 mA for 2 h using a semi-dry blotter system. Transfer was performed using cathode and anode solutions as described by Towbin et al. (*26*). After transfer, the membranes were blocked with 0.1 M Tris−HCl, 0.05 M NaCl, 0.05% Tween 20, and 2% skimmed milk. The membranes were then incubated with primary antibodies diluted in blocking solution and with anti-mouse immunoglobulins conjugated to alkaline phosphatase. Following this incubation, the membranes were developed using BCIP/NBT as substrate.

Image Analysis. Image analysis was performed using the Melanie II software (Biorad). Molecular weights were calibrated by migrating LMW markers (94.0, 67.0, 43.0, 30.0, 20.1, and 14.4 kDa) and peptide markers (16949, 14404, 10700, 8159, 6214, and 2512 Da) on identical gels. Similarly, isoelectric points were calibrated using the broad pI kit from Amersham Pharmacia Biotech. Isoelectric points 4.55, 5.20, 5.85, 6.55, and 6.85 were resolved on the gels used in this study.

Analysis of Amino Acid Sequence. Protein spots were cut out from several identical semipreparative 2D gels that had been stained with Coomassie blue (500 μg of proteins was loaded onto each gel), pooled, and sent to the Institut Pasteur (Laboratoire de Microséquençage des Protéines, Paris, France) for sequence analysis. Spots were first digested by trypsin, and then tryptic proteolysis products were extracted and separated by RP−HPLC on a C18 column. Amino acid sequence analysis was carried out on a model 473A liquid protein sequencer (Applied Biosystems) for major peptides.

RESULTS AND DISCUSSION

SDS−PAGE Analysis. Hen egg white proteins were separated according to their size, placed on 12% acrylamide gels, and detection was performed using silver staining. To improve polypeptide detection in such a sample where only 3 molecules (ovalbumin, ovotransferrin, and ovomucoid) represent 78% of the protein content (54, 13, and 11%, respectively), different amounts

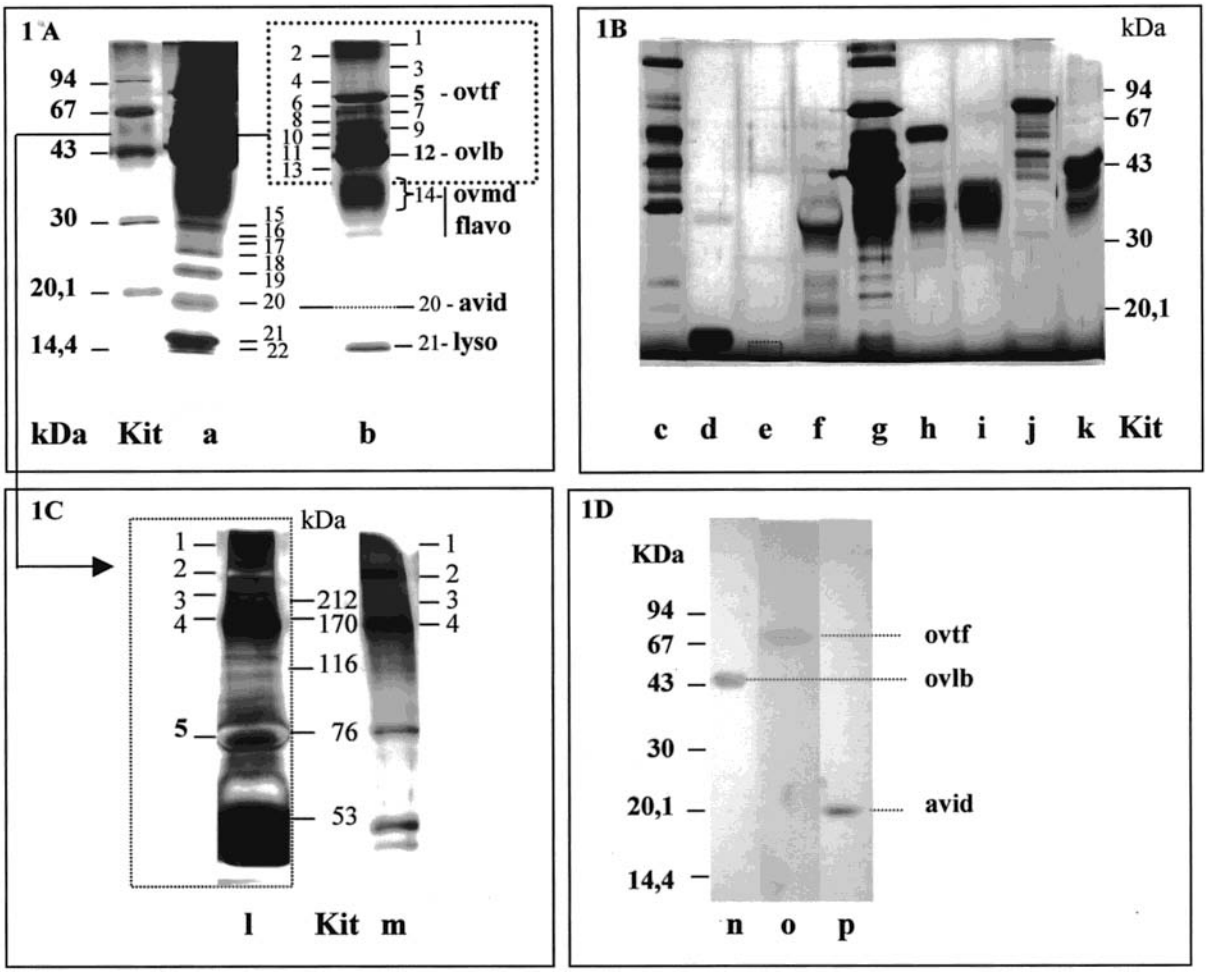

Figure 1. SDS−PAGE analysis of hen egg white proteins. (A) Whole hen egg white proteins on 12% acrylamide gel (20 μg, lane **a**; 2 μg, lane **b**), silver stained. (B) Standard proteins (globulins, lane **c**; avidin, lane **d**; lysozyme, lane **e**; flavoprotein, lane **f**; ovoinhibitor, lane **h**; ovomucoid, lane **i**; ovotransferrin, lane **j**; ovalbumin, lane **k**) and hen egg white proteins (10 μg, lane **g**) on 12% acrylamide gel, silver stained. (C) Whole hen egg white proteins (20 μg, lane **l**) and purified ovomucin (lane **m**) on 7.5% acrylamide gel, silver stained. (D) Whole hen egg white proteins on 12% acrylamide gel transferred prior to immunodetection using anti-ovalbumin (lane **n**), anti-ovotransferrin (lane **o**), or anti-avidin (lane **p**) antibodies.

were analyzed. As an example, 2 and 20 μg whole egg white proteins were loaded on the same gel (Figure 1A): 22 bands were detected in this way.

Five protein bands were unambiguously identified by comparison with isolated standard proteins or by western blotting using monospecific antibodies. These were bands **5**, **12**, **14**, **20**, and **21**. Interpretations are summarized in Table 1. Identification of other polypeptides was, however, only hypothetical (**1**, **2**, **3**, **4**, **6**, **7**, **8**, **9**, **10**, **11**, and **19**). The identities of the other bands are not yet known (bands **13**, **15**, **16**, **17**, **18**, and **22**).

Immuno-detection in western blotting experiments thus allowed the localization of ovotransferrin (band **5**), ovalbumin (band **12**), and avidin (band **20**). Molecular weights which were determined in this work for ovotransferrin (74 kDa) and ovalbumin (50 kDa) were comparable with those reported in the literature. In fact, migration of ovotransferrin in albumen (band **5**), standard ovotransferrin (lane **j**), and its western blot (lane **o**) were, respectively, 74, 77, and 75 kDa. In the same way, in the case of ovalbumin, its molecular weight in albumen (band **12**), that of the standard (lane **k**), and that with western blot (lane **n**) gave the same results: 50, 51, and 51 kDa, respectively.

On the other hand, the migration of standard avidin (lane **d**) and the anti-avidin western blot (lane **p**) allowed us to assume that band **20** (lane **a**) observed in egg white pattern is avidin. Estimated molecular weights were, respectively, 17, 20, and 19 kDa, whereas the theoretical molecular weight of avidin is 68.3 kDa (*28*). However, this result is in agreement with that of Korpela (*29*) who highlighted that, in reducing conditions, avidin was fragmented into four monomers of 15.6 to 15.9 kDa.

Other bands were identified by comparison with mobilities of standards. The diffuse band **14** could be attributed to ovomucoid and flavoprotein. These two proteins have similar molecular weights: 28 and 29.2 kDa, respectively (*28*). Mobilities of standards have been estimated to about 40 kDa for ovomucoid (lane **i**) and 38 kDa for flavoprotein (lane **f**). In albumen, a large diffuse band was observed between 35 and 42 kDa (band **14**). The gap observed between theoretical and experimental values could be explained by the high glycosylation degree of these proteins, estimated at 25% for ovomucoid and 15% for flavoprotein (*11*). Indeed, it has been highlighted that glycoproteins migrate too slowly in SDS−electrophoresis because the sugar moieties do not bind SDS, thus lowering the SDS:protein ratio (*35*).

By comparison with standard mobility, band **21** of the albumen pattern was confidently attributed to lysozyme. The molecular weight, estimated at 14 kDa, agrees with

Table 1. Hen Egg White Proteins: Theoretical Characteristics and Experimental Values Obtained with Three Electrophoresis Systems[a]

	% of total proteins (*11*)	physical properties (ref no.)		SDS−PAGE		2D-PAGE (linear gradient pH 4−7)	
		MW (kDa)	pI	MW (kDa)	IEF pI	MW (kDa)	pI
ovotransferrin	12−13	77.7 (*27*)		74		81	7.0
apo-ovtf			7.2 (*31*))		6.8		
fe-ovtf			6.6 (*31*)		6.6		
fe2-ovtf			6.2 (*31*)		6.4		
ovalbumin	54	45 (*28*)		50		44	
A3			4.94 (*6*)		5.0		5.4
A2			4.89 (*6*)		4.9		5.3
A1			4.75 (*6*)		4.8		5.2
ovomucoïd	11	28 (*28*)		38		36	
O1			4.41 (*32*)		4.5		4.9
O2			4.28 (*32*)		4.4		4.7
O3			4.17 (*32*)		4.3		4.6
O4			4.01 (*32*)		4.1		4.5
O5			3.83 (*32*)		4.0		4.4
avidin	0.05	68.3 (*29*)	10 (*33*)	19	nd	nd	nd
lysozyme	3.5	14.3 (*28*)	10.5 (*11*)	14	nd	nd	nd
ovoglobulin		49 (*11*)		nd	6.1−5.3	nd	nd
G3	4 (?)		5.8 (*11*)				
G2	4 (?)		5.5 (*11*)				
ovoinhibitor	0.1−1.5	49 (*30*)	5.1 (*11*)	nd	7.0	nd	nd
flavoprotein	0.8	29.2 (*28*)	4.2 (*11*)	38	nd	nd	nd
ch21 protein	nd	18 (*34*)	5.19 (34)	23	nd	23	5.6

[a] nd: not determined.

the literature. Bands **1** to **4** have very high estimated molecular weights: above 100 kDa. In accordance with Larsen et al. (*36*), this could correspond to ovomucin and/or ovostatin. To confirm this hypothesis, hen egg white and ovomucin were analyzed by SDS−PAGE 7.5%. As can be seen on the electrophoretic pattern (Figure 1C), intense bands at very high molecular weights (170 to 290 kDa) could correspond to α and β subunits of ovomucin after reducing treatment (*37*, *38*).

However, Nagase et al. (*39*) also studied ovostatin in denaturing conditions. They have shown that this protein is composed of four monomers of 195 kDa, but weights were estimated at 165 kDa by SDS−PAGE. So, bands **1** to **4** could also be ovostatin. This hypothesis cannot be verified because there is no commercial standard for ovostatin.

Interpretation of the bands **6** to **11** was more difficult, because ovoinhibitor and globulins G2 and G3 have very close molecular weights and the commercial standards of globulins are not pure enough. We think that ovoinhibitor and globulins were among these bands. Band **19** (molecular weight estimate 22.9 kDa) could be a protein recently found in albumen: the Ch21 protein (*36*).

In conclusion, we were able to localize, with certainty, the following proteins: ovotransferrin, ovalbumin, ovomucoid, flavoprotein, avidin, and lysozyme in the electrophoretic pattern of albumen. We have some hypotheses for ovomucin, ovostatin, ovoinhibitor, globulins G2 and G3, and Ch21 protein. On the other hand, bands **13**, **15**, **16**, **17**, **18**, and **22** remain to be identified.

Native−PAGE and IEF Analyses. Although less commonly used than SDS−PAGE in the case of the analysis of egg white proteins, it appeared interesting to test the two usual techniques of electrophoretic separation according to the charge properties of proteins, namely native−PAGE and conventional isoelectric focusing in ampholyte carrier buffers (named IEF below). Indeed, many proteins of the egg white have relatively close molecular weights. Moreover, in the case of native−PAGE, the proteins do not undergo any denaturation. The highlighting of protein complexes, naturally present in the egg white or subsequent to certain technological processing, should thus be made possible.

Taking into account the pI values of various known egg white proteins, pH 8.8 for native-PAGE and a linear gradient of pH from 3 to 7 were chosen for IEF. Indeed, under these conditions, only avidin and lysozyme, whose pIs are respectively 10 and 10.7 (*11*) should not be detected. However, other methods exist to visualize, even to quantify, these proteins.

In native conditions, electrophoresis makes it possible to distinguish 6 protein bands after Coomassie brillant blue staining (data not shown), and 12 bands after silver staining (Figure 2), against, respectively, 13 (Figure 3b) and 18 bands (Figure 3a) in IEF. This last method thus confirms its greater resolving power in the case of egg white. In addition, the analysis of the migration of the markers used, pI ranging from 3.6 to 6.6, confirms the linearity of the gradient formed in IEF (data not shown), which enabled us to calculate in this case the apparent pIs of the various egg white components (Table 1).

Either in native−PAGE or IEF, immunodetection after Western-blot and/or standard proteins enabled the certain identification of some bands of the egg white pattern. Thus, ovalbumin appears as 3 bands: bands **9**, **10**, and **11** in native−PAGE (Figure 2) and bands **10**, **11**, and **12** in IEF (Figure 3). This probably reflects the coexistence of the three ovalbumin isoforms, with different phosphorylation degrees. The diphosphorylated form (A1), whose pI is 4.75, represents approximately 87% ovalbumin, compared to 12% for the monophosphorylated form (A2, pI 4.89), and 1% for the unphosphorylated form (A3, pI 4.94) (*6*). The increasing intensity of the bands observed in egg white, while going toward the decreasing pI, is thus consistent with this assumption. In addition, the pIs calculated in IEF, respectively 5.0, 4.9, and 4.8, agree with those announced by these authors.

Ovotransferrin was also unambiguously localized. But while it appears as only one band in native−PAGE

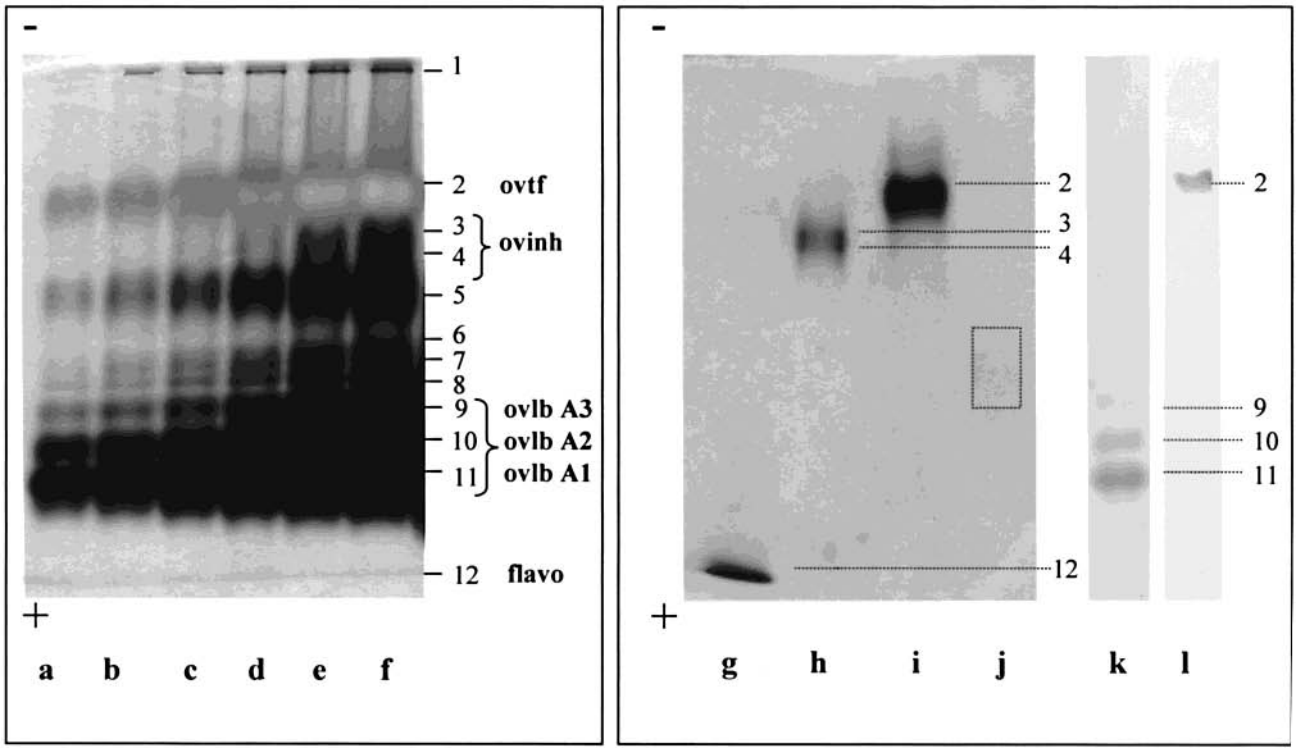

Figure 2. Native−PAGE analysis of hen egg white proteins. Loaded samples were whole hen egg white proteins (0.31 μg, lane **a**; 0.62 μg, lane **b**; 1.25 μg, lane **c**; 2.5 μg, lane **d**; 5 μg, lane **e**; 10 μg, lane **f**) or 10 μg of purified proteins (flavoprotein, lane **g**; ovoinhibitor, lane **h**; ovotransferrin, lane **i**; ovomucoid, lane **j**). Gels were either silver stained (lanes **a** to **f**), Coomassie blue stained (lanes **g** to **j**), or transferred prior to immunodetection using anti-ovalbumin (lane **k**) or anti-ovotransferrin (lane **l**) antibodies.

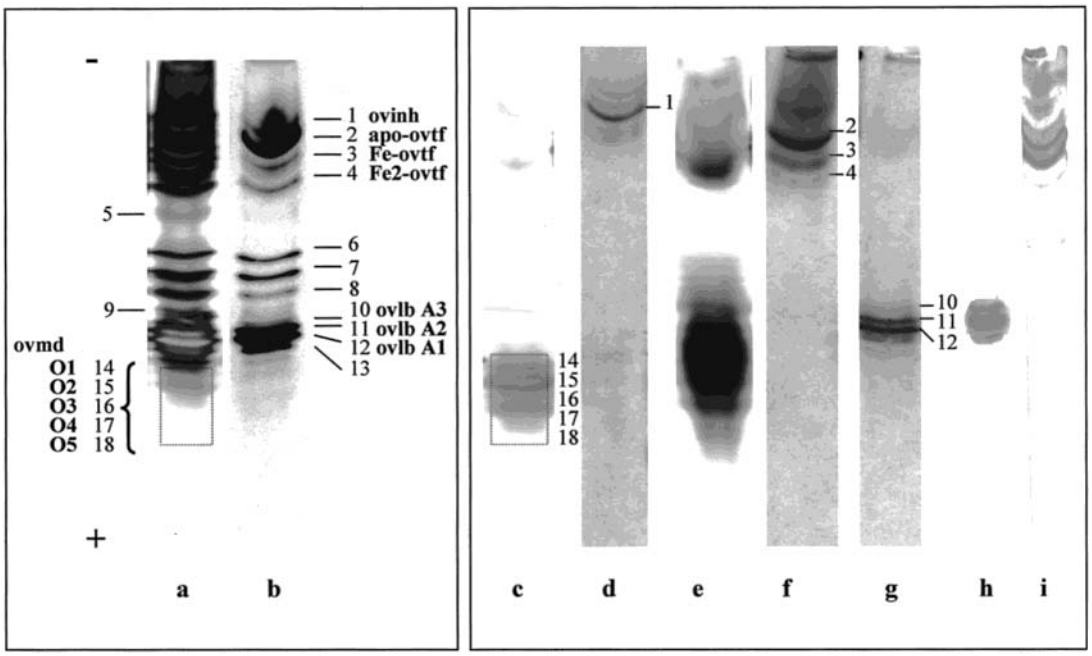

Figure 3. IEF analysis of hen egg white proteins. Isoelectrofocalization was performed on 7.5% acrylamide gel with linear gradient pH 3−7. The loaded samples were whole hen egg white proteins (20 μg, lane **a**; 50 μg, lane **b**) or purified proteins (ovomucoid, lane **c**; ovoinhibitor, lane **d**; flavoprotein, lane **e**; ovotransferrin, lane **f**; ovalbumin; lane **g**). Gels were either silver stained (lanes **a**, **c**, and **e**), Coomassie blue stained (lanes **b**, **d**, **f**, and **g**), or transferred prior to immunodetection using anti-ovalbumin (lane **h**) or anti-ovotransferrin (lane **i**) antibodies.

(band **2**, Figure 2), it is split into three isoforms in IEF (bands **2**, **3**, and **4**, Figure 3). This observation agreed with that carried out by Huang and Richards (*31*) using a capillary isoelectric focusing technique (CIEF). This polymorphism would express the existence of isoforms carrying 0, 1, or 2 iron atoms per protein molecule. The pIs calculated for these three bands (apo-ovtf, fe-ovtf, and fe_2-ovtf), respectively, 6.8, 6.6, and 6.4, are close to those announced by these authors (respectively, 7.2, 6.6, and 6.2). The native−PAGE technique does not enable the calculation of the pI molecules because separation is also carried out according to the molecular weight.

However, ovotransferrin migration in the upper part of the gel is coherent, as this protein is the most alkaline of egg white proteins, except lysozyme and avidin.

The flavoprotein also could be identified by comparison with the standard migration corresponding to band **12** obtained in native−PAGE (Figure 2). This protein, among the most acidic of the egg white proteins (pI 4.2) (*11*), but also with low molecular weight, thus constitutes the last visible band in native electrophoresis. On the other hand, in IEF, it appears as a very diffuse form not allowing the calculation of its pI nor association of it to one of the bands observed on the electrophoretic egg white pattern. Moreover, its migration on a level equivalent to that of ovalbumin, i.e., in a pH range much higher than its theoretical pI (from 5.0 to 4.4, against 4.0) is problematic.

Finally, the ovoinhibitor could be identified in the two electrophoretic systems by comparison with the protein standard migration. It corresponds to band **1** in IEF. However, the pI calculated for this component is then 7.0, that is to say a value much higher than the pI announced in the literature for this protein (pI 5.1) (*11*). This relatively incoherent positioning could be explained by the cathodic drift usually observed with this electrophoretic technique. In native−PAGE, the ovoinhibitor standard also appears in the upper part of the gel, therefore in accordance with its pI, but as two very close bands. Thus, bands **3** and **4** of the egg white pattern (Figure 2) could be identified as ovoinhibitor. The literature does not mention, however, the splitting off of this protein in electrophoretic separation. However, this observation could be brought closer to that of ion exchange chromatography on DEAE-cellulose, where up to 5 fractions of ovoinhibitor, with various glucidic compositions, could be separated (*40*).

For ovomucoid, the difficulties for staining, already announced in SDS−PAGE, were also observed in native−PAGE, as well as the diffuse aspect of this protein. It is thus not possible to visualize ovomucoid in the electrophoretic egg white pattern. However, the behavior of this protein remains inconsistent with the pI announced in the literature (pI from 4.41 to 3.83) (*32*) taking into account its position compared to the three ovalbumin isoforms. The high and variable proportion of carbohydrates fixed on ovomucoid (up to 25%) (*11*), as well as a surface electric charge different from its whole charge, could explain the abnormal behavior of this protein. These different rates of glycosylation would be, in addition, the cause of the five bands obtained for the ovomucoid in IEF, in accordance with what had been described previously (*41*). Bands **14**, **15**, **16**, **17**, and **18** of the egg white pattern thus could be identified by comparison with the ovomucoid standard. Moreover, the pIs calculated for these 5 components are close to those reported in the literature (*32*) (Table 1).

Bands **5** to **8** obtained in native−PAGE (Figure 2) and IEF (Figure 3) could not be surely identified because they did not correspond to any standard protein. However, taking into account their intensity compared to the other identified components on one hand, and their migration distance on the other hand, they seem to be the same proteins detected in both electrophoretic systems. By comparison with previous electrophoretical investigations on egg white proteins, they could be globulins G3 and G2 (*5*). Moreover, the estimated pIs for these four bands, respectively 6.1, 5.7, 5.5, and 5.3, are quite in agreement with those indicated in the literature for ovoglobulins (pI from 5.8 to 5.5) (*11*).

Finally, one of the bands in native−PAGE, band **1**, remains unidentified. It corresponds, in fact, to proteins unable to penetrate into the 7.5% acrylamide gel in nondenaturing conditions. So, these could be either high-molecular-weight proteins such as ovostatin (780 kDa), or polymeric glycoproteins such as ovomucin (220 to 240000 kDa), or protein aggregates.

With IEF, 2 bands (**9** and **13**) are still unidentified, and they are not comparable with any of the bands obtained in native−PAGE. According to their estimated pI values, respectively 5.1 and 4.6, these bands could correspond to proteins such as cystatin (pI 6.5 and 5.6) (*42*), ovomacroglobulin (pI 4.9) (*39*), cobalamin binding protein (pI 4.3) (*43*), or glutamylaminopeptidase (pI 4.2) (*44*). But all these proteins are minor compounds of the egg white, i.e., present at concentrations lower than 1% of total proteins, whereas these bands appear surprisingly well stained and are thus difficult to explain.

To conclude on these two techniques, the great resolving power of the IEF technique, which is able to separate the ovalbumin, ovotransferrin, and ovomucoid isoforms, is indicated for egg white analysis. Moreover, it appears that further investigation (using proteomic analysis) is needed to clearly identify all the polypeptides evidenced in these electrophoregrams.

2D Electrophoresis Analysis (2DE). Preliminary studies using pH 3 to 10 2D gels have shown that the majority of egg white proteins were resolved in the pH range of 4 to 7. Routine analysis was thus performed on linear 4 to 7 IPG gels. As for monodimensional electrophoresis, various amounts of egg white proteins were analyzed in order to clearly visualize either major (especially ovalbumin and ovomucoid) or minor proteins (Figure 4a, b, and c).

When loading 2 μg of proteins, we highlighted five major spots in the migration area of ovalbumin by silver staining. This protein was clearly identified by immunodetection (Figure 4e). Among these five spots, considering their relative intensity and the natural proportion of isoforms A1, A2, and A3 ovalbumin in egg white, we deduced the spot identification indicated in Figure 4a. The estimated pIs for these three compounds, 5.2, 5.3, and 5.4, respectively (Table 1), are, however, higher than those announced (*6*). On the other hand, their apparent molecular weight (44 kDa) agrees with the literature (*28*). Spots ***1*** and ***2***, corresponding to an apparent molecular weight of 45.5 kDa and apparent pI values of 5.0 and 5.1, respectively, do not fit with any known egg white protein.

With 20 μg of loaded proteins, the individualization of the three ovalbumin isoforms is no longer possible (Figure 4b). On the other hand, four more or less diffuse spots appear in the molecular-weight area of 36 kDa and with apparent pI values of 4.9, 4.7, 4.6, 4.5, and 4.4. These spots could represent the ovomucoid isoforms highlighted by IEF. Indeed, their pI values are close to those published (*32*). Moreover, their molecular weight, while markedly higher than the theoretical value, agrees with that obtained by SDS−PAGE.

Spots ***3***, ***4***, and ***5***, which were relatively intense, could not be identified. Their migration on the pH scale (pI between 5.4 and 5.6) with regard to that of ovalbumin led us to assume that they correspond to bands ***6*** to ***8*** observed by IEF, which were previously identified as globulins.

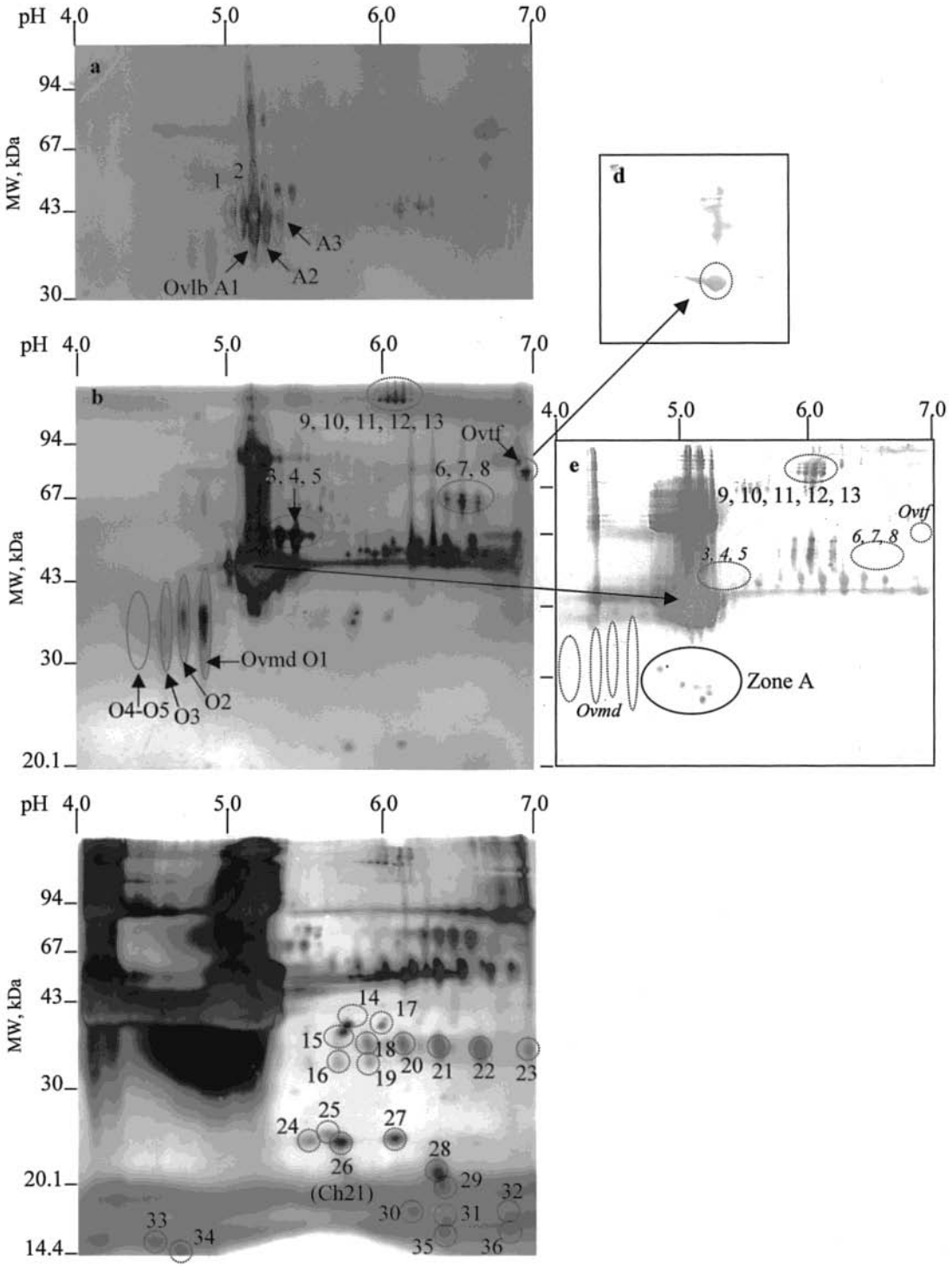

Figure 4. 2D-PAGE analysis of hen egg white proteins. Loaded samples where whole hen egg white proteins (2 μg, **a**; 20 μg, **b**; 100 μg, **c** ; 1000 μg, **d,** ; 2000 μg, **e**). Gels were either silver stained (**a** to **c**) or transferred prior to immunodetection using anti-ovotransferrin (**d**) or anti-ovalbumin (**e**) antibodies.

For the protein quantity loaded, one intense spot always appeared with an apparent molecular weight of 81 kDa and pI 7.0. These values correspond approximately to ovotransferrin. Indeed, immunodetection after transfer confirmed this hypothesis (Figure 4d).

In the pI range of 6.5 to 6.7 and molecular weight range of 69 to 63 kDa, one can see a typical picture of three spots (***6***, ***7***, and ***8***), each constituted by a chain of three subspots. We cannot identify these spots, which could correspond to different isoforms of one protein.

In the same way, in the upper part of the gel (molecular weight above 100 kDa), a very distinct chain of five spots, with apparent pI between 6.0 and 6.2 (spots ***9*** to ***13***) is visible. However, contrary to spots ***6*** to ***8***, these spots positively reacted with anti-ovalbumin antibody (Figure 4e). The intentional overexposure of the membrane to the antibody highlighted some of the silver stained spots observed between pI 5 and 7 and with molecular weights above 44 kDa reacted with anti-ovalbumin antibody. The overloading of 2D gels probably lowers the solubilization of ovalbumin, the most

abundant protein in egg white. This could explain the smears observed from the ovalbumin spot on silver stained gels (Figure 4b). When the 2D gel was overloaded to allow the visualization of minor proteins (Figure 4c), the upper part of the gel then seemed difficult to interpret. However, some of the spots revealed in the upper part, and none of the silver stained spots in the lower part of the gel (molecular weights lower than 44 kDa and pI higher than 5.5), did not react with anti-ovalbumin antibody. On the other hand, the high sensitivity of immunodetection enabled the visualization of some compounds reactive against anti-ovalbumin antibody in egg white, although their molecular weight was lower than that of ovalbumin (Figure 4e, ***A zone***). These compounds, which were in very low concentrations and undetectable with silver staining, could be products from ovalbumin hydrolysis.

With 100 μg loaded proteins, the gel area corresponding to acidic pI (from 4.0 to 5.6) and high molecular weight (above 40 kDa) seemed very saturated and, therefore, unworkable. On the other hand, the rest of the gel revealed many distinct spots, particularly in the lower part (molecular weight below 40 kDa) where 23 spots (***14*** to ***36***) were detected. Among these, spot ***26***, one of the more intense, was collected and subjected to amino acid sequencing. The sequence ISFLGED was found on one of the trypsic peptides of this protein. It corresponds to fragment 60−66 of Ch21 protein, also called quiescence specific protein. Initially found in chick embryo skeletal tissues, this protein belongs to the superfamily of lipophilic molecule carrier proteins and was recently observed for the first time in hen egg white (*36*). Its apparent molecular weight in 2DE is 23 kDa, which is slightly higher than estimated by these authors (21 kDa), and much higher than its theoretical molecular weight (18065 Da) deduced from the genomic sequence (*34*). It is thus more than likely that Ch21 undergoes a posttranslational modification responsible for its molecular weight upshift. Its apparent pI (5.6) is consistent with its theoretical one, calculated from the amino acid sequence (pI 5.2).

The 22 other spots of the lower part of the 2DE gel do not correspond to any known egg white proteins, considering their estimated pIs and molecular weights. They could thus be either minor proteins, still unidentified in egg white, or hydrolysis products from major proteins.

Finally, this comparative work highlighted the interest of electrophoretic techniques, which are less widely used than the usual SDS−PAGE with Coomassie blue staining, for egg white analysis. Thus, techniques for precise separation, such as IEF or even 2DE, enabled the visualization of protein compounds not corresponding to any known molecule of egg white. This observation led us to initiate work, now in progress, for identification and characterization of these minor compounds.

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