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Identification and Characterization of Ovalbumin Gene Y in Hen Egg White

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Ovalbumin gene Y has been known as a member of the ovalbumin gene family since 1982, when its encoding gene was sequenced. In the present study, ovalbumin gene Y has been demonstrated as a new minor protein of hen egg white. This protein has been isolated by isoelectrofocalization and two-dimensional polyacrylamide gel electrophoresis and has been characterized using peptide mass fingerprinting. The concentration ratio of ovalbumin gene Y:ovalbumin is about 13:100. Unlike ovalbumin, ovalbumin gene Y is not phosphorylated, but like ovalbumin, this protein is glycosylated. Ovalbumin gene Y exists as a mixture of three molecular species, which differ in their isoelectric points. The polymorphism of this protein cannot be explained by various glycosylation levels.

KEYWORDS: Hen egg white; ovalbumin gene Y; isoelectrofocalization; two-dimensional electrophoresis; mass spectrometry

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, i.e., the major proteins. Their structures and the modifications according to the physicochemical conditions are well-known (*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 the hen egg white proteins. Indeed, this biological fluid is original and presents some difficulties for analysis. The proteins have very different molecular masses (12.7−240 000 kDa) and pI values (4−11) (*1*). Their concentrations in albumin are also very different, and one of them, i.e., ovalbumin, represents more than 50% of total proteins, which makes it very awkward for minor protein detection. All of 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, the hen 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. Prior to any valorization objective, it seemed necessary to engage in research to identify the egg white proteins and especially the minor proteins. Some of these minor components could be interesting for nonfood applications, such as health uses. In addition to well-known biological agents such as hen egg whites and biological agents such as lysozyme or ovotransferrin, we could expect the identification of new antimicrobial or antiviral proteins, transport proteins, or growth factors. Indeed, the whole hen egg white biological properties cannot be explained only by the already known egg white components.

The identification of these minor proteins necessitates highly efficient methods for protein separation. For this, isoelectric focusing (IEF) and two-dimensional (2D) electrophoresis proved to be particularly suitable and highlighted that many unidentified minor proteins were present in hen egg white (*4*). Some of them have already been identified and characterized (*5*, *6*).

In the present study, we focused our attention on three components, which were clearly separated by IEF and quantitatively significant. Because their identifications using pI and molecular mass estimations as compared to literature data were not satisfactory, we decided to apply the powerful peptide mass fingerprinting analysis, using mass spectrometry. This method enabled us to identify, for the first time in hen egg white, ovalbumin gene Y, a protein whose gene was previously sequenced (*7*). Moreover, evidence of polymorphism for this protein was provided, since three isoforms with varying pI values have been observed. This polymorphism cannot be completely explained by different phosphorylation or glycosylation levels nor by genetic variations.

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, and Coomassie brilliant blue R250 came from Biorad (Marnes-la-Coquette, France). Tris, ammonium carbonate,

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trifluoroacetic acid, 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, Triton X 100, iodoacetamide, and mixed ampholyte carriers (pH linear gradient 4−7 and 3−7 L) and poly(vinylidene difluoride) (PVDF) membranes 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). Immobilon P^{SQ} membranes were from Millipore Corporation (Bedford, MA).

Polyclonal rabbit anti-phosphoserine, anti-phosphothreonine, and anti-phosphotyrosine antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA). Secondary anti-rabbit antibody conjugated to alkaline phosphatase was from Sigma. IEF migrations were carried out on small slab gels (7 cm × 8 cm × 0.1 cm) using a Biorad Mini Protean II system.

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

Acid phosphatase from potato was obtained from Sigma (80 U/mg). N-glycopeptidase F was obtained from Sigma (5000 U/mL), and sequencing grade trypsin was purchased from Promega (Charbonnières, France).

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

Preparation of Ovalbumin Gene Y Enriched Fraction. Egg white was first diluted with 3 volumes of water, and the mixture was adjusted to pH 6.0 with 2 N HCl. The solution was kept under stirring at 2 °C for 3 h enabling ovomucin precipitation. The gelatinous ovomucin precipitate obtained was removed by 5 min centrifugation at 3000*g*. The supernatant solution was then applied on a cation exchange chromatography S Ceramic Hyper DF resin for successive lysozyme and ovotransferrin separations, as elsewhere described (*8*). First, lysozyme separation was performed at pH 8.0, and then, ovotransferrin separation was performed at pH 5.2. The nonretained fraction, containing ovalbumin and other acidic proteins, was then used for the preparation of ovalbumin gene Y enriched fraction by anion exchange (AE) chromatography. AE-HPLC was performed on a Q-Hyper D 10 (10 cm × 0.46 cm i.d.) column (Biosepra, Villeneuve la Garenne, France). A linear gradient of NaCl concentration in a 50 mM Tris-HCl pH 8.0 buffer, from 0 to 0.18 M in 24 min and from 0.18 to 0.5 M in 5 min, was used for elution. Separation was carried out at a flow rate of 1 mL/min, and proteins were detected by absorption at 280 nm. The ovalbumin gene Y enriched fraction was manually collected according to the chromatographic profile, after previous trials for ovalbumin gene Y elution time determination.

IEF. Conventional 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 1 h at 100 V, 1 h at 250 V, and 30 min at 500 V.

Two-Dimensional Electrophoresis. Proteins were subjected to high-resolution 2D PAGE according to the method described by O'Farrel (*9*) and modified by Görg et al. (*10*) on a pH 4−7 linear gradient. The experimental protocol has been previously detailed (*4*).

Protein Detection. *Coomassie Blue.* After migration, gels were fixed and stained in 0.05% Coomassie blue R250, 50% ethanol, and 10% acetic acid. After isofocalization, due to ampholyte carriers, staining and destaining steps were carried out after immersion of the gel in 20% TCA diluted in 50% ethanol.

Glycoprotein Staining. A PAS staining method was adapted from Kapitany and Zebrowski (*11*). After migration, gels were fixed in 12.5% TCA for one night to eliminate ampholyte carriers. Glycoprotein oxidation was achieved with 1% periodic acid for 2 h. After two rinsing steps with 15% acetic acid, the gel was left in a third bath of acetic acid for one night. Finally, the gel was placed for staining in a Schiff reagent bath for 2 h, in the dark, at 4 °C. Afterward, destaining could be achieved with successive 7% acetic acid baths, before potential Coomassie blue staining.

Immunoblotting. Immunoblotting was performed by transferring the separated proteins from the IEF gels onto a PVDF membrane, using a constant current of 200 mA for 1 h and 30 min using a semidry blotter system. Transfer was performed using cathode and anode solutions as described by Towbin et al. (*12*). After transfer, membranes were blocked with 0.1 M Tris-HCl, 0.05 M NaCl, 0.05% Tween 20, and 3% BSA. Then, they were incubated for 1 h and 30 min at 30 °C with primary antibodies diluted in blocking solution. After three rinsing steps with TBS/Tween solution, they were incubated for 1 h and 30 min at 30 °C with secondary anti-rabbit antibodies conjugated to alkaline phosphatase. Then they were developed using BCIP/NBT as substrate.

Image Analysis. *Molecular Mass and pI Determination.* For 2D PAGE gels, 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, and molecular masses were calibrated by migrating low molecular mass markers (94.0, 67.0, 43.0, 30.0, 20.1, and 14.4 kDa) from Amersham Pharmacia Biotech and using Melanie II software.

Quantitative Analysis. Quantitative image analysis was performed for Coomassie blue stained gels with Image scanner II (Amersham Biosciences) using ImageQuant TL v2003.01 software.

Peptide Mass Fingerprinting. *Protein Enzymatic Digestion.* In-gel tryptic digestion was performed on protein bands and spots previously excised from the Coomassie blue-stained gels. Two-dimensional PAGE spots were simply washed with acetonitrile and dehydrated before enzymatic digestion. For IEF bands, reduction with DTT and S-alkylation with iodoacetamide was achieved before dehydration. In-gel trypsin digestion was performed at 37 °C overnight. The resulting peptides were extracted with successive washing steps with NH_4HCO_3, acetonitrile, and acetonitrile/HCOOH, dried with SpeedVac, and maintained at −20 °C until mass spectrometry analysis.

LC/MS Analysis. The tryptic digests resulting from IEF separation were analyzed by LC/electrospray ionization MS. The peptide molecular mass was obtained using an API-III Plus triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Canada), fitted with an atmospheric pressure ionization source. A homemade column (180 μm i.d. × 70 mm) packed with reverse phase Symmetry C18 resin (Waters, Milford, MA) was used for peptide separation, at 40 °C at a flow rate of 200 μL/min with a split to the MS ionization source that was set at a flow rate around 1 μL/min. Ion detection was performed in positive mode and mass calculation with Biomultiview 1.3.1 (Sciex software package) from scan mass to charge *m*/*z*.

Matrix-Assisted Laser Desorption/Ionization (MALDI)-Time-of-Flight (TOF) Analysis. The tryptic digests resulting from 2D PAGE separation were analyzed by MALDI-TOF mass spectrometry on a Voyager DE STR spectrometer (Applied Biosystems, Framingham, CA) equipped with a nitrogen laser (337 nm, 20 Hz). Spectra were acquired in the reflector mode (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.

Protein Databank Interrogation. The identification of proteins was performed by interrogating the Swiss-Prot and Trembl protein databanks available on Expasy proteomic server (http://us.expasy.org/) using Mascot Search software (http://www.matrixscience.com) for peptide mass fingerprinting data analysis. The average mass lists or monoisotopic mass lists were used for LC/MS and MALDI-TOF analysis, respectively. The mass accuracy was lower than 0.5 ppm. The carbamidomethylation of cysteins, methionine oxidation, and one missed cleavage 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 database search.

N-Terminal Amino Acid Sequence Determination. The bands were cut out from the Immobilon PSQ membrane resulting from the transfer of a IEF gel with an initial loading of 250 μg of egg white proteins. The N-terminal sequence was then determined by automated

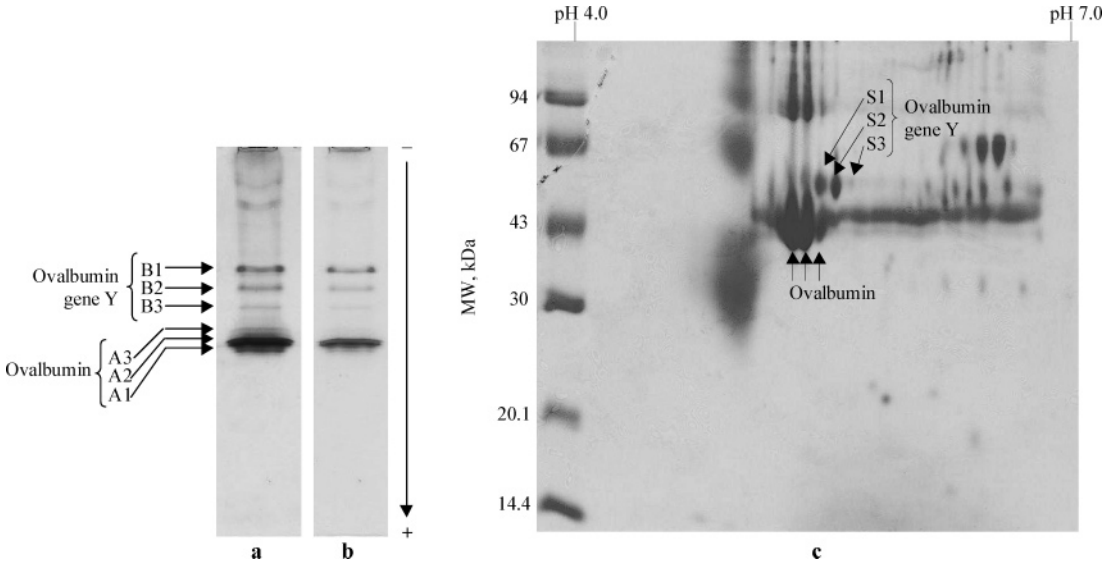

Figure 1. Electrophoresis analysis of hen egg white proteins: IEF with Coomassie blue (**a**) and Schiff reagent (**b**) staining and 2D PAGE with Coomassie blue (**c**) staining.

Edman degradation by J. D'Alayer (Institut Pasteur, Laboratoire de microséquençage des proteins, Paris, France).

Enzymatic Dephosphorylation. Enzymatic dephosphorylation was performed with the ovalbumin gene Y enriched fraction obtained as previously described. The reaction mixture contained 200 μL of the protein fraction, 760 μL of 50 mM sodium citrate buffer, pH 5.5, and 40 μL of acidic phosphatase 60 U/mL (2.4 U/mL final solution) and was incubated at 37 °C. Aliquots (100 μL) were taken after 3, 24, and 48 h; the enzymatic reaction was immediately stopped by adding 5 μL of 1 M NaOH, and the samples were maintained at −20 °C until their analysis by IEF.

Enzymatic Deglycosylation. Enzymatic deglycosylation was performed with the ovalbumin gene Y enriched fraction obtained as described above. The reaction mixture contained 10 μL of the protein fraction, 0.5 μL of Triton X 100, 38.5 μL of 50 mM Tris-HCl buffer, pH 7.5, and 1 μL of N-glycopeptidase F 5000 U/mL (100 U/mL final solution) and was incubated at 37 °C for 24 h. The sample analysis using IEF was performed as soon as the enzymatic reaction ended.

RESULTS AND DISCUSSION

Isolation and Identification of Ovalbumin Gene Y in Hen Egg White. IEF enabled the detection of minor proteins; among them, some could not be identified with certainty because they did not correspond to any standard protein (*4*). This is the case for the bands B1, B2, and B3 (**Figure 1a**) that were first supposed to be ovoglobulins because of the quite good agreement between their estimated pIs and those indicated in the literature for these proteins (*1*). To progress in the characterization of hen egg white, these minor components were submitted to peptide mass fingerprinting. The number of matching peptides (10, 10, and 9 for B1, B2, and B3, respectively) and the sequence coverages (31, 21, and 27% for B1, B2, and B3, respectively) (**Table 1**) enabled an unambiguous identification of these three bands as ovalbumin gene Y (accession number in Swiss-Prot databank: P01014). Heilig et al. (*7*) completely sequenced this protein in 1982, demonstrating an overall 72.6% homology with ovalbumin coding sequence, resulting in a 58% homology for the amino acid sequence. The same identification results could be drawn from the peptide mass fingerprinting of spots S1, S2, and S3 identified on 2D PAGE gel (**Figure 1c**). In this case, the number of matching peptides were 9, 15, and 5 for S1, S2, and S3, respectively, with sequence coverages 31, 45, and 23% for S1, S2, and S3, respectively (**Table 1**). The pI values estimated by 2D PAGE for S1, S2, and S3 (5.5, 5.4, and 5.3, respectively) were consistent with the theoretical pI calculated for the ovalbumin gene Y sequence deduced from the nucleic acid (i.e., 5.2, using Expasy Compute pI/MW software, http://us.expasy.org/). However, the molecular mass estimated by 2D PAGE for the three components (53 kDa) was significantly higher than the theoretical one (43.8 kDa, 388 amino acids). This gap between measured and theoretical molecular masses could result from posttranslational modifications, especially glycosylation. The N-terminal sequence determination by Edman reaction turned out to be impossible. This behavior suggested an acetylation of the N-terminal residue, similarly to ovalbumin (*13*).

These results then constitute the first evidence of ovalbumin gene Y presence in the hen egg white, thus confirming its expression in oviduct as related by Colbert et al. (*14*). Quantification of ovalbumin gene Y has also been attempted by IEF gel image analysis. Because of the staining saturation for A1 and A2 ovalbumin bands (di- and monophosphorylated forms, respectively), ovalbumin gene Y was quantified respect to A3 (no phosphorylated form) ovalbumin band. The intensity of the gathered three ovalbumin gene Y bands was 4.3 higher than that of the A3 ovalbumin band intensity. Considering that A3 ovalbumin represents roughly 3% of whole ovalbumin (*15*), ovalbumin gene Y would represent 13% of whole ovalbumin. This estimated ovalbumin:ovalbumin gene Y concentration ratio was consistent with the previously indicated extent of hormonal responsiveness of these two closely related genes, i.e., 100:10 (*14*).

Ovalbumin gene Y displayed polymorphic patterns on IEF and 2D PAGE gels. Because of their pI differences, posttranslational modifications such as phosphorylation and/or glycosylation have been considered.

Searching for Phosphorylation Sites in Ovalbumin Gene Y Sequence. The homology between ovalbumin and ovalbumin gene Y, the existence of different phosphorylated forms of ovalbumin (*16*), and the putative phosphorylated sites predicted from ovalbumin gene Y sequence (*17*, at http://www.cbs.dtu.dk/) led us to consider that the three ovalbumin gene Y forms could have different phosphorylation levels. To explore this assumption, enzymatic dephosphorylation by acid phosphatase was performed. To increase the ovalbumin gene Y concentration

Table 1. Identification of Ovalbumin Gene Y on IEF and 2D PAGE Gels: Peptide Molecular Masses Observed with LC-MS Analysis (IEF Bands B1, B2, and B3) and with MALDI-TOF Analysis (2D PAGE Spots S1, S2, and S) vs Ovalbumin Gene Y Theoretical Peptide Molecular Masses (Mass Accuracy < 0.5 ppm)

ovalbumin gene Y peptide position	11–20	11–20[a]	21–47[a]	57–85	111–123	124–136	125–136	137–143	144–152
peptide theoretical MW	1336.57	1352.55	3083.62	3158.41	1542.77	1517.7	1389.53	746.78	1116.6
				peptide MW observed					
IEF									
B1		1352.39		3158.48	1542.29	1517.69	1389.74		
B2		1352.09			1542.29	1517.84	1389.59	746.6	
B3		1352.39		3157.98	1542.29	1517.6			
2D PAGE									
S1				3157.36	1542.76	1517.75	1389.67		
S2	1336.55	1352.54	3083.49	3157.36	1542.76	1517.76	1389.65		1116.58
S3				3157.32	1542.82				

ovalbumin gene Y peptide position	187–195	230–255	230–255[a]	259–264	278–287[a]	281–285	354–362	363–372	373–384	protein coverage (%)
peptide theoretical MW	1066.52	2829.5	2846.29	736.82	1284.68	646.79	1159.26	1262.48	1412.61	
				peptide MW observed						
IEF										
B1			2846.73	736.7		646.7	1158.89		1412.24	31
B2				736.7		646.4	1159.19	1262.39	1412.69	21
B3				736.7		646.7	1159.0	1262.69	1412.69	27
2D PAGE										
S1	1066.5		2845.43				1159.56	1262.66	1412.73	31
S2	1066.5	2829.42	2845.4		1284.64		1159.56	1262.66	1412.73	45
S3			2845.41					1262.72	1412.79	23

[a] Peptide with Met oxidation.

for an easier reading of the gel, an ovalbumin gene Y enriched fraction was used as the substrate instead of the whole egg white. This fraction still contained low ovalbumin quantities. **Figure 2** shows that an extensive 24 h enzymatic reaction made the

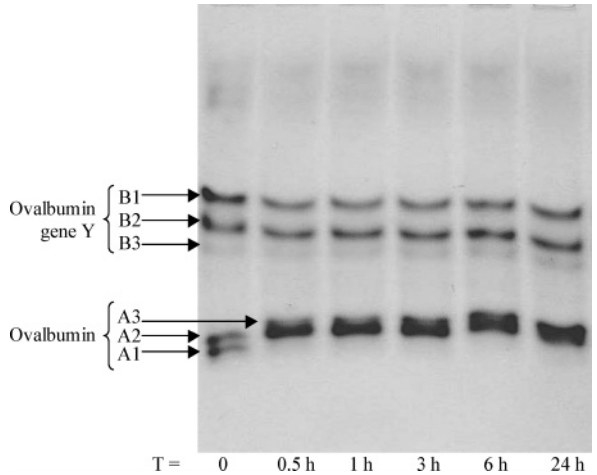

Figure 2. IEF analysis of ovalbumin gene Y enriched fraction during acid phosphatase reaction; the gel was stained with Coomassie blue.

ovalbumin A1 band (with the fastest mobility) weaker and the A2 band, and finally the A3 band, stronger. This evolution demonstrated that the enzymatic dephosphorylation of ovalbumin was effective. On the other hand, simultaneously, no modification of the ovalbumin gene Y bands was observed, indicating that no dephosphorylation was achieved for these components. This result suggested that ovalbumin gene Y does not contain phosphorylated amino acids. This conclusion was reinforced by the immunoblotting tests. Whereas ovalbumin positively reacted with anti-phosphoserine antibodies, no reaction was obtained for ovalbumin gene Y (**Figure 3b**). In the same way, no reaction was observed with ovalbumin gene Y against anti-phosphothreonine or against anti-phosphotyrosine (**Figure 3c**,**d**). Nevertheless, positive reactions were obtained with ovalbumin against these two antibodies, whereas no experimental evidence of phosphorylation of threonine or tyrosine has been published for this protein, even if putative phosphorylation sites on threonine and tyrosine could be predicted. However, the negative response concerning ovalbumin gene Y was unambiguous.

Searching for Glycosylation Sites in Ovalbumin Gene Y Sequence. Variations in glycosylation states are usually visualized using IEF and 2D PAGE, because of the potential resulting variations in pI values. Typical pictures of aligned spots along the first dimension (IEF) were obtained (*18*), which were very comparable to the picture obtained with ovalbumin gene Y (**Figure 1c**). Moreover, putative N-glycosylation sites are predicted for ovalbumin gene Y, the most likely being Asn 293 (http://www.cbs.dtu.dk/). Finally, this assumption was confirmed by PAS staining after IEF (**Figure 1b**), as well as ovalbumin, ovalbumin gene Y positively reacted to this dye reagent, which is specific for glycosylated proteins (*11*).

To explore if different glycosylation states could explain the three isoforms observed on IEF and 2D PAGE gels, an enzymatic deglycosylation was performed. As for the dephosphorylation reaction, an ovalbumin gene Y enriched fraction was used as the substrate instead of the whole egg white. As expected, the deglycosylation reaction modified the mobility of ovalbumin gene Y during IEF (**Figure 4**). The positions of deglycosylated forms were lower in the gel, indicating more acidic pI values for the deglycosylated ovalbumin gene Y, suggesting the anionic character of the linked glycosylated groups. However, even after deglycosylation, three bands of ovalbumin gene Y were still visible on IEF gel. Consequently,

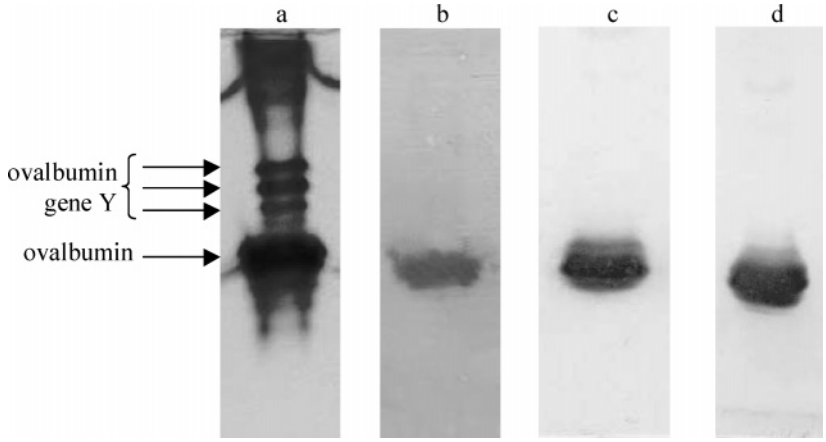

Figure 3. IEF analysis of hen egg white proteins: Gels were either stained with Coomassie blue (**a**) or transferred prior to immunodetection using anti-phosphoserine (**b**), anti-phosphothreonine (**c**), and anti-phosphotyrosine (**d**) antibodies.

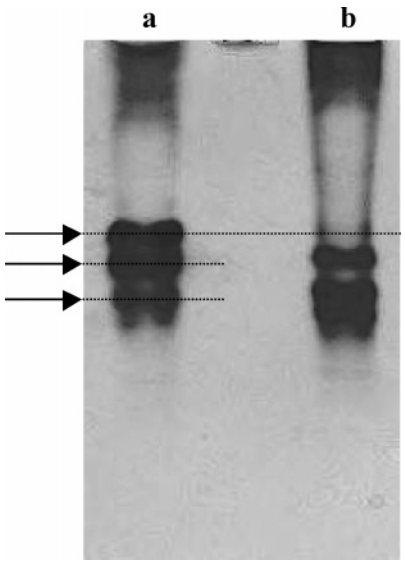

Figure 4. IEF analysis of ovalbumin gene Y enriched fraction before (**a**) and after (**b**) enzymatic deglycosylation; the gel was stained with Coomassie blue; the arrows indicate the three initial ovalbumin gene Y bands.

the variations of glycosylation states could not explain the three isoforms observed.

Finally, the present results conclusively proved the presence of ovalbumin gene Y in hen egg white. This information is consistent with the previous studies concerning the ovalbumin gene family. Heilig et al. (*19*) demonstrated that the X, Y, and ovalbumin genes are all expressed in the oviduct. The presence of ovalbumin gene Y in hen egg white enabled us to be more precise: Ovalbumin gene Y, as well as ovalbumin, is secreted by the magnum, which is the part of oviduct where all the egg white components are produced. Three isoforms of ovalbumin gene Y have been identified, with different pI values. Unlike ovalbumin, and despite putative phosphorylation sites, ovalbumin gene Y is not phosphorylated. However, according to the predictions, ovalbumin gene Y is glycosylated, like ovalbumin. These posttranslational modifications could not explain the polymorphism observed on IEF gels. In the present study, each electrophoresis analysis was performed with one egg, then corresponding to one hen. Consequently, the assumption of genetic variations could not be considered alone to explain the polymorphism, because in that case, we should observe only a maximum of two isoforms. An explanation could be alternative splicing processes leading to casual exon skipping events, such as a mechanism being elsewhere displayed (*20*, *21*). Because an extensive amino acid sequencing of the three isoforms has not been achieved, this assumption cannot be confirmed. In other respects, because of the genetic proximity of ovalbumin, ovalbumin gene Y, and ovalbumin gene X, the present results led us to think that the third component of this gene related family, that is ovalbumin gene X, is very likely to be another minor protein of hen egg white. However, no evidence of its presence in hen egg white is available at the moment.

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LITERATURE CITED

(1) Li-Chan, E.; Nakai, S. Biochemical basis for the properties of egg white. *Crit. Rev. Poult. Biol.* **1989**, *2*, 21−58.

(2) Mine, Y. Recent advances in the understanding of egg white protein functionality. *Trends Food Sci. Technol.* **1995**, *6*, 225−232.

(3) Kato, A. Interactions of egg white proteins. In *Ingredients Interactions, Effects on Food Quality*; Gaonkar, A., Ed.; Marcel Dekker Inc.: New York, 1997; pp 357−375.

(4) Desert, C.; Guerin-Dubiard, C.; Nau, F.; Jan, G.; Val, F.; Mallard, J. Comparison of different electrophoretic separations of hen egg white proteins. *J. Agric. Food Chem.* **2001**, *49*, 4553−4561.

(5) Nau, F.; Guerin-Dubiard, C.; Desert, C.; Gautron, J.; Bouton, S.; Gribonval, J.; Lagarrigue, S. Cloning and characterization of HEP21, a new member of the uPAR/Ly6 protein superfamily predominantly expressed in hen egg white. *Poult. Sci.* **2003**, *82*, 242−250.

(6) Nau, F.; Guerin-Dubiard, C.; Desert, C.; Gautron, J.; Carre, W.; Lagarrigue, S. Identification of three minor hen egg white proteins. *Xth European Symposium on the Quality of Eggs and Eggproducts*, Saint Brieuc, France, 23−26th September 2003; Vol. III, pp 36−42.

(7) Heilig, R.; Muraskowsky, R.; Kloepfer, C.; Mandel, J. L. The ovalbumin gene family: Complete sequence and structure of the Y gene. *Nucleic Acids Res.* **1982**, *10*, 4363−4382.

(8) Guerin-Dubiard, C.; Pasco, M.; Hietanen, A.; Del Bosque, A.; Croguennec, T. Hen egg white fractionation by ion exchange chromatography for biological activity studies. *J. Chromatogr. B* Submitted for publication.

(9) O'Farrel, P. H. High-resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **1975**, *250*, 4007−4021.

(10) Görg, A.; Postel, W.; Gunther, S. The current state of two-dimensional electrophoresis with immobilized pH gradients. *Electrophoresis* **1988**, *9*, 531−546.
(11) Kapitany, R. A.; Zebrowski, E. J. A high-resolution PAS stain for polyacrylamide gel electrophoresis. *Anal. Biochem*. **1973**, *56*, 361−369.
(12) Towbin, H.; Staehelin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4350−4354.
(13) Thompson, E. O. P.; Fisher, W. K. A correction and extension of the acetylated amino terminal sequence of ovalbumin. *Aust. J. Biol. Sci*. **1978**, *31*, 443−446.
(14) Colbert, D. A.; Knoll, B. J.; Woo, S. L.; Mace, M. L.; Tsai, M. J.; O'Malley, B. W. Differential hormonal responsiveness of the ovalbumin gene and its pseudogenes in the chick oviduct. *Biochemistry* **1980**, *19*, 5586−5592.
(15) Kitabatake, N.; Ishida, A.; Doi, E. Physicochemical and functional properties of hen ovalbumin dephosphorylated by acid phosphatase. *Agric. Biol. Chem.* **1988**, *52*, 967−973.
(16) Perlman, G. E. Enzymatic dephosphorylation of ovalbumin and plak-albumin. *J. Gen. Physiol*. **1952**, *25*, 711.
(17) Blom, N.; Gammeltoft, S.; Brunak, S. Sequence- and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol.* **1999**, *294*, 1351−1362.
(18) Davidsson, P. Combination of 2-D gel and liquid-phase electrophoretic separations as proteomic tools in neuroscience. *BioRadiations* **2002**, *108*, 26−30.
(19) Heilig, R.; Perrin, F.; Gannon, F.; Mandel, J. L.; Chambon, P. The ovalbumin gene family: Structure of the X gene and evolution of duplicated split genes. *Cell* **1980**, *20*, 625−637.
(20) Ferranti, P.; Addeo, F.; Malorni, A.; Chianese, L.; Leroux, C.; Martin, P. Differential splicing of pre-messenger RNA produces multiple forms of mature caprine alpha(s1)-casein. *Eur. J. Biochem*. **1997**, *249*, 1−7.
(21) Miranda, G.; Mahé, M. F.; Leroux, C.; Martin, P. Proteomic tools to characterize the protein fraction of Equidae milk. *Proteomics* **2004**, *4*, 2496−2509.

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