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New Insights into the Structure of Apolipoprotein B from Low-Density Lipoproteins and Identification of a Novel YGP-like Protein in Hen Egg Yolk

PASCALE JOLIVET,*,†,‡ CÉLINE BOULARD,†,‡ THIERRY CHARDOT,†,‡ AND MARC ANTON§

INRA and AgroParisTech, UMR 206, Chimie Biologique, F-78850 Thiverval-Grignon, France, and INRA, UR 1268, Biopolymères, Interactions, Assemblages, F-44316 Nantes, France

Apoproteins of low-density lipoproteins (LDL) and soluble proteins (livetins) contained in hen egg yolk plasma have been demonstrated as being essential to the interfacial and emulsifying properties of yolk. The knowledge of their structure is necessary to better understand these properties. Purified protein fractions were separated by SDS-PAGE or 2D-PAGE and identified through the LC-MS/MS of their trypsin peptides. Hen blood apolipoprotein B gives rise to nine different apoproteins in LDL after maturation and proteolysis. Among these apoproteins, two protein fragments appeared to be less accessible to proteases and could be enriched in β-sheets and firmly associated with lipids. Plasma soluble proteins were constituted by ~45% of yolk immunoglobulins with a high heterogeneity of the variable regions of both heavy and light chains, 41% of glycoproteins constituted by YGP42 and YGP40, 14% of albumins, and one new minor protein we called YGP30, showing 75% similarity to YGP40.

KEYWORDS: Hen egg yolk; low-density lipoproteins; plasma proteins; LC-MS identification; structural proteomic strategy; enzymatic deglycosylation; proteinase K

INTRODUCTION

Hen egg yolk proteins are important in many emulsified food systems as they are able to provide stabilizing effects at low pH, which is unusual for proteins commonly used in dispersions. This is due to the fact that yolk contains protein species with a broad pI range of more than four pH units (*1–3*). Although yolk contains other surface active components (such as phospholipids, cholesterol, and cholesteryl esters), the proteins are believed to play a major role in emulsions. Yolk consists mainly of 68% low-density lipoproteins (LDL), 16% high-density lipoproteins (HDL), 10% livetins, and 4% phosvitins (*4*). Very recently, an exhaustive composition of egg yolk proteome was established that identified 119 proteins with many minor proteins as proteases, protease inhibitors, antioxidative enzymes, or some egg white and eggshell proteins (*5*). Searching the principal contributor to yolk emulsifying properties, numerous authors have separated yolk into its main fractions: plasma and granules. Plasma contains mainly LDL and livetins, whereas granules contain HDL and phosvitins. Large similarities were observed between emulsifying properties of yolk and plasma, whereas emulsions made with granules behaved very distinctly (*6–8*), indicating that yolk emulsifying properties reside in plasma. The emulsifying activity of LDL from egg yolk was confirmed (*2, 9, 10*), the protein part of LDL exerting a driving contribution in the formation and stability of emulsions. A study of competitive adsorption between egg yolk lipoproteins and milk proteins showed that LDL predominate at the oil/water interface and displace substantially whey proteins and caseins (*11, 12*). The surface activity and adsorption characteristics of water soluble plasma proteins also were studied at the oil/water interface (*1, 2*).

LDL are spherical particles of ~35 nm diameter consisting of a core of triglycerides, cholesterol, and cholesteryl esters, surrounded by a monolayer of phospholipids in which are embedded amphipathic apoproteins (*13*). These proteins are derived from maternal blood serum proteins that are synthesized in the hen liver, internalized by growing oocytes via receptor-mediated endocytosis, and partially matured through proteolysis. Very low-density lipoproteins (VLDL) of hen blood are the precursors of LDL of egg yolk (*14*). VLDL exist in the blood of immature pullet, and their production in liver is considerably increased during sexual maturity due to estrogen secretion. VLDL contain mainly two apoproteins: apo VLDL II and apo B. Apo VLDL II is the only apoprotein from blood lipoproteins to be transferred to yolk in large amounts without any modification and is then called apovitellenin I (*15*). Apovitellenin I is a small homodimer with disulfide-linked subunits of 9 kDa (*15*). Blood hen apo B is a 500 kDa protein constituted by only one unit and highly similar to the human apolipoprotein

* To whom correspondence should be addressed. Tel.: 33 1 30 81 54 67; fax: 33 1 30 81 53 73; e-mail: jolivet@grignon.inra.fr.
† INRA, Chimie Biologique.
‡ AgroParisTech, Chimie Biologique.
§ INRA, Biopolymères, Interactions, Assemblages.

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B-100 precursor, a major protein component of atherogenic LDL. Human apolipoprotein B-100 has a predicted pentapartite structure composed of three amphipathic α-helical domains alternating with two amphipathic β-strand domains, NH_2-α_1-β_1-α_2-β_2-α_3-COOH (*16, 17*). The pentapartite structure is a common supramolecular feature of apolipoprotein B-100 observed in nine vertebrate species (*16*). A similar pentapartite structure for hen apo B was very recently hypothesized (*18*). During its transfer into the yolk, blood hen apo B enzymatically is cleaved by endogenous cathepsin D, resulting in the production of apo B fragments (*18–20*). These fragments have a better capacity to take part in a protein network on the surface of LDL and help to stabilize them. A less random structure than that in hen blood VLDL has been observed (*19*). Specific lipid and apolipoprotein interactions are necessary for proper particle formation (*21, 22*).

Livetins, proteins found in the soluble phase of yolk that are not intimately associated with lipids (*19*), are constituted of immunoglobulins (γ-livetin), serum albumin (α-livetin), and glycoproteins (β-livetin), and it is suggested that they are translocated blood constituents (*23*). However, the relative proportions of the proteins occurring in egg yolk plasma are not the same as in blood serum (*23*). Glycoproteins are derived from the C-terminal domain of vitellogenins enzymatically split with cathepsin D and are released into the yolk plasma before or during compartmentation of the lipovitellin−phosvitin complex in the yolk granule (*24*).

As the proteins contained in the plasma (apoproteins of LDL and soluble proteins) have been demonstrated as essential with respect to their interfacial and emulsifying properties, and as the competition between these proteins and their interfacial structures is not known presently, it was necessary to better understand their native structure in relation to their adsorption at the oil/water interface. It has been suggested that the competitive adsorption of proteins from total egg yolk could be described from their primary structure (*1, 2*). Because of the complexity of protein adsorption, knowledge of the secondary structure, flexibility, and lipid−peptide interactions also is required. Consequently, in this study, we used proteomic and structural proteomic approaches to obtain more information about the structure of the major apoprotein present in LDL fractions (apolipoprotein B or apo B) and of some soluble plasma proteins.

MATERIALS AND METHODS

Protein Fractionation. Hen's eggs were purchased from a local supermarket. They were manually broken, and the yolk and the white were separated. The yolk was carefully rolled on filter paper to remove remaining albumen and chalazes and was deposited in a funnel. The vitellin membrane was then perforated to collect egg yolk in a beaker cooled in iced water. Plasma proteins and the LDL fraction were purified from egg yolk as previously described (*9*). The yolk was fractionated into plasma and granules by stepwise precipitation with 0.17 M NaCl and centrifugation according to the method of McBee and Cotterill (*25*). Soluble plasma proteins were precipitated after the addition of ammonium sulfate (40%), stirring for 1 h at 4 °C, and centrifugation (10000*g* for 45 min at 4 °C) and were dissolved in 50 mM Tris-HCl buffer (pH 7). The supernatant was dialyzed (molecular mass cutoff of 8000 Da) against Milli-Q grade water overnight and then spun (10000*g* for 45 min at 4 °C). The floating material containing LDL was diluted in 50 mM Tris-HCl buffer (pH 7). Proteins were quantified with the Folin–Ciocalteu reagent (*26*) using bovine serum albumin as the standard.

SDS-PAGE. Aliquots of protein fractions were diluted in a dissociation buffer consisting of 0.250 M Tris-HCl (pH 6.8), 40% v/v glycerol, 20% v/v 2-mercaptoethanol, 8% w/v SDS, and 0.02% w/v bromophenol blue. Electrophoresis was run under 40 mA on 4−12% ready-to-use NuPAGE bis-tris gels (Novex) using 50 mM MES NuPAGE buffer (pH 7.3). The gel was stained with Coomassie blue (G-250) according to Neuhoff et al. (*27*) and briefly was washed with water before densitometric measurements and protein band excision. Molecular masses were estimated with Mark 12 standard from Novex. The gel was scanned (300 dpi) using an Epson Perfection 1200 photoscanner and the resulting file was analyzed using the Image Quant (version 4.2a) software (Molecular Dynamics). Plasma proteins also enzymatically were deglycosylated prior to electrophoresis. An aliquot of plasma proteins (100 μg) was treated at 37 °C overnight with 10 U of *N*-glycosidase F (Roche Diagnostics) in the presence of 80 mM sodium phosphate buffer (pH 7.5), 10 mM EDTA, 0.1% v/v Triton, and 0.02% w/v SDS (100 μL total volume).

2D-PAGE. The protein sample was adjusted to 2 mg mL^{-1} with 10 mM Tris buffer (pH 7.6). A total of 200 μg was precipitated with acetone overnight at −20 °C. After the pellet was collected by centrifugation (20 min, 5000*g*, 4 °C), proteins were resuspended in 100 μL of isoelectric focusing (IEF) buffer (8 M urea, 2 M thiourea, 2% w/v CHAPS, 2% v/v Triton X-100, 50 mM DTT, and 0.5% v/v ampholyte 3−10) as in ref *28*. For 2D-PAGE, the sample was focused on an 11 cm pH 3−10 Immobiline DryStrip (GE Healthcare). Passive rehydratation was performed with the same IEF buffer. After sample cup loading (IPGphor 3 system, GE Healthcare), the focusing method was activated (300 V for 1 h, 1000 V for 1 h, 6000 V for 1 h, and 6000 V for 15 000 Vh). Before the second dimension, the immobiline polyacrylamide gel strip was reduced and alkyled with DTT (5 mg mL^{-1}) and iodoacetamide (22.5 mg mL^{-1}) in equilibration buffer (50 mM Tris (pH 6.8), 6 M urea, 30% v/v glycerol, and 2% w/v SDS) for 10 min, rinsed in SDS running buffer, and placed onto a 10% acrylamide gel. The strip was held in place with 0.5% (w/v) low-melt agarose in SDS running buffer. The electrophoresis was performed at 60 mA/gel for 2 h. Following SDS-PAGE, the gel was washed 3 times in deionized water for 10 min and stained with PageBlue protein staining solution (Fermentas Life Sciences).

Identification of Proteins. Protein bands stained with Coomassie blue were excised from the polyacrylamide gel and stored at −20 °C. Before trypsin digestion, gel slices were washed for 5 min with 150 μL of ultrapure water, dehydrated for 15 min with 150 μL of acetonitrile (ACN), and dried by vacuum centrifugation. Proteins were reduced, alkylated, and in-gel trypsin digested as previously reported (*18*). The resulting peptides were extracted successively with 5% formic acid, then ACN/H_2O (50:50), and finally ACN. Combined extracts were dried, and samples were dissolved in 1% HCOOH and 5% ACN before liquid chromatography−mass spectrometry analysis.

HPLC was carried out with a Spectra System equipment (Thermo Separation Products) comprising a SCM1000 vacuum membrane degasser, P4000 gradient pumps, and a manual injector. Volumes of 10 μL of samples were loaded onto a reversed-phase Biobasic C18 column (2.1 mm × 150 mm, 300 Å pore size, and 5 μm film thickness). The column was eluted at a flow rate of 0.2 mL min^{-1} at room temperature with 5% v/v solvent B (ACN + 0.1% v/v HCOOH) in solvent A (H_2O + 0.1% v/v HCOOH) for 2 min and then with a linear gradient of B in A from 5 to 45% over 40 min and then 45−95% over 5 min before re-equilibration. Eluent from the column was introduced in the ESI source of a Thermo Electron LCQ Deca ion-trap mass spectrometer operating in positive ion mode. Instrumental parameters were capillary temperature 280 °C, capillary voltage 30 V, spray voltage 4.5 kV, sheath gas flow 80 au, and auxiliary gas flow 5 au. Mass spectra were acquired scanning from *m*/*z* 200 to 2000. MS/MS experiments were carried out using a normalized collision energy of 35 au. Peptide ions were analyzed using the data-dependent triple-play method as follows: (i) full MS scan, (ii) ZoomScan (scan of the major ions with a higher resolution to determine their charge), and (iii) MS/MS of these ions.

Protein identification was performed with Bioworks 3.1 software using a *Gallus gallus* protein sequence database extracted from the nr database downloaded from the National Center for Biotechnology Information (NCBI) FTP site. No enzyme specificity was set for the query. The database-searching algorithm Sequest uses cross-correlation (Xcorr) and delta correlation (dCN) functions to assess the quality of

the match between tandem mass spectrum and amino acid sequence information in a database. The output data were evaluated in term of (i) trypsin nature of peptides in the case of trypsin digestion, (ii) Xcorr magnitude up to 1.5, 2, and 2.5 for, respectively, mono-, di-, and tricharged peptides to minimize false positives, and (iii) dCN higher than 0.1. Sequence coverage of a protein or a protein fragment was calculated taking into account all amino acids identified by LC-MS/MS and matched to the amino acid number of protein or fragments. Three independent experiments comprising all four experimental steps (purification, electrophoresis protein separation, enzymatic digestion, and LC-MS/MS analysis) were carried out to obtain maximal coverage.

LDL Digestion with Proteinase K. Proteinase K was used to remove and analyze all protein regions located outside of the particle membrane (*29*). Purified LDL sample (5 mg of protein) was diluted in 1 mL of 80 mM $Na_2HPO_4/NaHPO_4$ (pH 7.5), 5 mM $MgCl_2$, and 250 mM sucrose and incubated at 4 °C for 30 min after the addition of proteinase K (Roche Diagnostics) in 10 mM Tris-HCl buffer (pH 7) to obtain a 1:50 mass/mass ratio of enzyme to substrate. LDL were isolated as floating material by microcentrifuging at 15000*g* for 40 min at 4 °C and discarded. The lower phase contained a mixture of peptides obtained from the digestion of domains accessible by proteinase K. This mixture was cleaned using a Vydac BioSelect solid phase extraction cartridge filled with C18 reversed-phase material before LC-MS/MS analyses. The steps of conditioning, sample loading, washing, and elution were carried out according to the manufacturer's protocol. Fractions eluted with 30% v/v ACN and 60% v/v ACN were combined, evaporated to dryness, and dissolved in 1% v/v HCOOH and 5% v/v ACN. Analysis of peptides was performed as described previously.

RESULTS

The proteins contained either in the purified LDL fraction or in the plasma fraction were first separated by SDS-PAGE and identified through LC-MS/MS of their trypsin peptides.

Identification of Apolipoprotein B from LDL Fraction and New Insights in Maturation Pattern. The electrophoretic pattern of the LDL fraction was obtained in a highly reproducible way as previously described (*18*), showing major bands ranging from 8 to 200 kDa and identified as apovitellenin I (bands 8 and 9) and fragments from hen apolipoprotein B (**Figure 1**A). Investigation of the NCBI database showed that the predicted sequence of the apolipoprotein B precursor protein highly similar to the human apolipoprotein B-100 precursor and previously registered as XP_419979 in 2005 (*18*) was replaced in 2006 by a new protein sequence including 427 supplementary amino acids and referenced as NP_001038098 (*5, 30*). This new predicted sequence (**Figure 2**) presents a higher identity (49%) and similarity (69%) to human apo B-100 than the previous one (46 and 64%, respectively). The new sequence presents three insertions (highlighted in yellow in **Figure 2**), the first of 94 amino acid residues between K_{1963} and Y_{1965}, the second of 321 amino acid residues between F_{2674} and I_{2675}, and finally, 13 amino acid residues are included in the N-terminal part of the protein, between Q_{20} and D_{21}, presumably in the signal peptide of the precursor protein. LC-MS/MS analysis of trypsin peptides obtained through in-gel digestion of protein bands showed a very good total coverage of the new sequence of the protein (77.7%). The mean coverage of the two new domains was still better (83%), indicating without ambiguity the accuracy of the new sequence. It was then possible to know more exactly the sequences of the different fragments previously described (*18*). The seven protein bands heavier than 55 kDa corresponded in fact to nine different fragments of hen blood apo B (**Table 1** and **Figure 2**). In bands 1−3, there was the N-terminal moiety of apoB ($f_{43-2123}$), which could be cleaved in two lighter fragments, $f_{43-1297}$ and $f_{1284-2123}$. These fragments correspond to apo 1, apo 2, and apo 3 previously described in ref *18*. Apo 2 could lead to a lighter fragment, apo 4 (f_{93-972}). The C-terminal

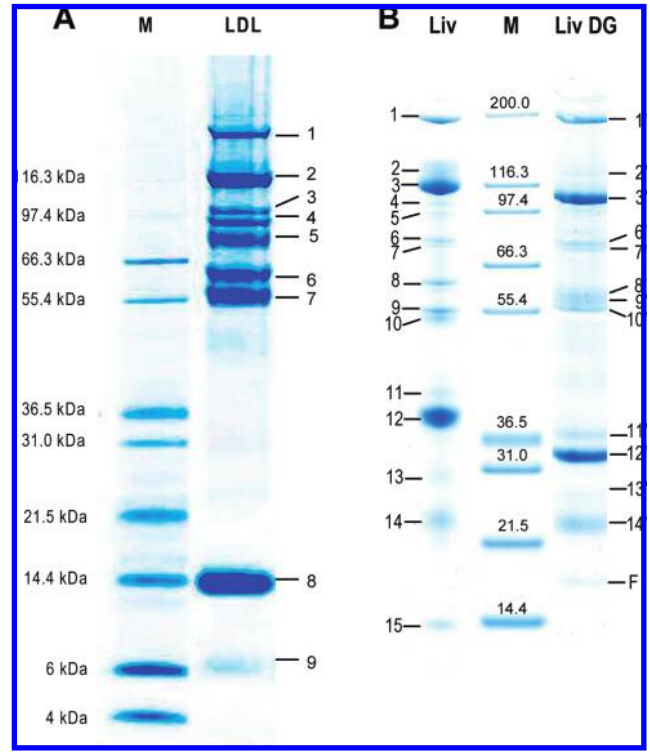

Figure 1. SDS-PAGE of proteins from purified egg yolk LDL (**A**) and soluble plasma fraction (**B**). Proteins (10 μg) were separated on 4−12% bis-tris gels. Plasma proteins (Liv) also enzymatically were deglycosylated prior to electrophoresis (Liv DG) with *N*-glycosidase F (band F). After deglycosylation, identical protein bands are indicated. Gels were stained with G250 Coomassie blue. The molecular mass marker was Mark 12 from Novex (M).

part of protein ($f_{3826-4573}$) or apo 5 was recovered in band 5. Finally, the internal part of the protein was present in bands 6 and 7. All these fragments were identified with very high sequence coverages (75−89%), higher than those previously reported due to the more accurate protein sequence predicted from a genomic sequence. The presence of two fragments with different pI values in bands 6 and 7 was verified after separation of purified LDL proteins through two-dimensional polyacrylamide gel electrophoresis (data not shown). The fragment $f_{2142-2681}$ exactly corresponds to apo 6, and fragment $f_{3373-3748}$ is very similar to apo 7. On the contrary, a new fragment $f_{2720-3212}$ was identified. This fragment could not be previously identified since it possesses the major insertion of 321 new amino acid residues, which represents 65% of the fragment sequence.

Location of Apo B at the Surface of LDL. A protease protection assay was conducted to analyze protein topology and relative localization of exposed and protected domains of LDL apoproteins using a method derived from Wu et al. (*29*). The purified LDL sample was treated with proteinase K at neutral pH and low temperature (4 °C) to identify peptides from all hydrophilic domains and α-helix domains. In **Figure 2**, apo B protein domains not identified through LC-MS/MS analysis and then resistant to proteinase K digestion are in red, while the rest of the protein was highly covered through proteomic analysis. It is possible to distinguish two such domains: $E_{1349-2023}$ (675 amino acids, 74.6 kDa) and $S_{2750-A3422}$ (673 amino acids, 75 kDa). The first is approximately equivalent to the fragment present in band 3 (apo 3), and the second contains the new fragment present in band 6 plus a 160 amino acid sequence identified with a very low coverage (5.6%) through

Figure 2. Protein sequence of hen apolipoprotein B (NCBI accession number NP_001038098). The three insertions of new amino acid residues with regard to the previous sequence (XP_419979) are highlighted in yellow. Apolipoprotein B maturation into apo 1 to apo 7 is indicated as deduced from this work (fragments are delimited by ⌈ and ⌉) or from previous results (*18*) (fragments are then delimited by ⌊ and ⌋). The two domains ($E_{1349-2023}$ and $S_{2750-A3422}$), which were not accessible to proteinase K digestion, are in red. The pentapartite structure NH_2-α_1-β_1-α_2-β_2-α_3-COOH previously hypothesized (*18*) and verified in this work also is indicated.

Table 1. Identified Fragments of Apo B[a] in LDL Purified from Hen Egg Yolk

band[b]	obsd molecular[c] mass (kDa)	fragment	theoretical molecular mass of fragment[d] (kDa)	amino acid number of fragment[d]	sequence coverage of fragment (%)[d]	theoretical pI[d]	fragment[e] as in ref *18*
1	>150	43–2123	231.47	2080	88.5	8.29	apo 1
2	118	43–1297	139.63	1255	89.3	7.57	apo 2
3	98	1284–2123	93.49	840	87.4	8.82	apo 3
4	96	93–972	97.63	880	87.9	7.65	apo 4
5	73	3826–4573	86.17	748	77.4	6.15	apo 5
6	62	2142–2681	64.44	540	86.1	7.11	apo 6
6		2720–3212	55.47	493	74.8	8.84	new
7	55	2142–2681	64.44	540	86.1	7.11	apo 6
7		3373–3748	41.28	376	83.8	9.28	apo 7

[a] Apo B sequence referenced as NP_001038098 was obtained with Bioworks 3.1 research using a *G. gallus* protein sequence database extracted from the nr database downloaded from the NCBI site (htpp://www.ncbi.nlm.nih.gov/). [b] Band number as in **Figure 1A**. [c] Molecular mass was estimated from the electrophoretic mobility of protein bands. [d] Theoretical molecular mass and pI of identified protein fragments were calculated from their amino acid sequence using SWISS-PROT tools (htpp://expasy.ch/sprot/). Sequence coverage was calculated taking into account all amino acids identified through three experiments by LC-MS/MS and matched to amino acid number of fragments. [e] Names of fragments as reported in ref *18* are indicated.

LC-MS/MS after trypsin in-gel digestion due to the presence of several basic amino acid clusters (as KTKFDKYK, RTLKLPRFR, TRKRGLK...) and the existence of long and poorly ionizable peptides (longer than 3000 Da). Both domains are very basic with pI 9.14 and 9.32, respectively. The distribution of hydrophilic and hydrophobic domains can be seen through the representation of Kyte−Doolittle hydropathy plots (*31*) in **Figure 3**. The coherent distribution of hydrophobic blocks in the amino acid sequence suggested membrane-bound protein behavior (*31*).

Soluble Plasma Proteins. The plasma fraction (Liv) was first characterized by 1-D SDS-PAGE (**Figure 1B**). Two major protein bands near 40 and 110 kDa (bands 12 and 3) were visible with many minor ones. All visible protein bands were cut from the gel for protein identification with LC-MS/MS (**Table 2**). The major presence of livetins was observed with α-livetins (serum albumin) identified in bands 9, 10, and 12, *β*-livetins in bands 11−13, and *γ*-livetins in bands 3, 8, and 14.

Albumin. Chicken serum albumin appeared in bands 9 and 10 with an apparent molecular mass between 54−55.4 kDa. The sequence of the mature protein corresponds to a 592 amino acid protein with a 67.2 kDa theoretical molecular mass, so albumin had a greater electrophoretic mobility in SDS-PAGE gel than the expected mobility. A very high coverage of the sequence was obtained in our experiments (82.6%) with fragments detected by mass spectrometry distributed all over the entire sequence. This result suggested a condensed structure of albumin with 17 supposed disulfide bridges and not the loss of some protein regions. A minor truncated form of albumin also was recovered in band 12 with a 40.5 kDa observed molecular mass close to the theoretical mass (44.3 kDa). This truncated form originated from the loss of the C-terminal domain of the protein.

β-Livetins. Bands 11 and 12 contained proteins corresponding to the C-terminal regions of minor vitellogenin I and major vitellogenin II, respectively. Vitellogenins I and II are large proteins (1912 or 1850 amino acids) that are cleaved by cathepsin D into four fragments: the N-terminal regions termed lipovitellins I, the phosphoseryl-rich domains termed phosvitin, lipovitellins II, and C-terminal regions termed YGP42 and

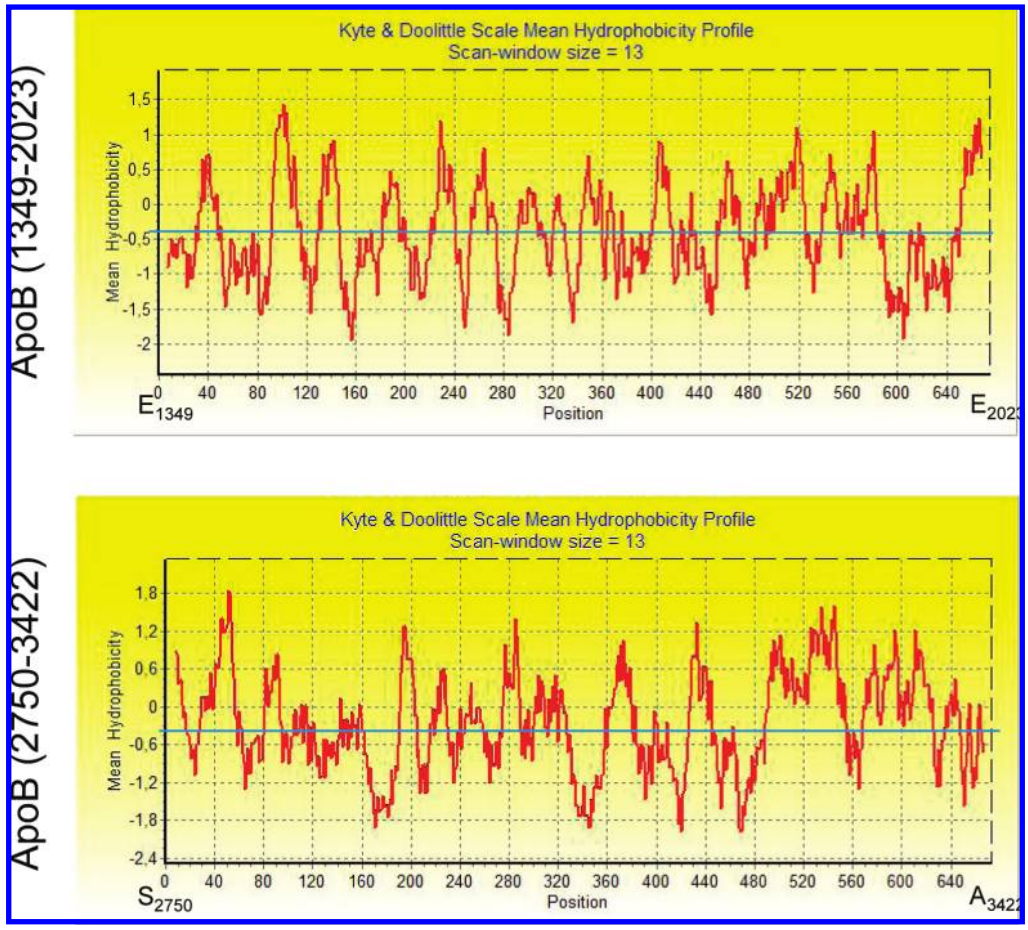

Figure 3. Kyte−Doolittle hydropathy plots of the two hen apo B domains not accessible to proteinase K digestion ($E_{1349-2023}$ and $S_{2750}-A_{3422}$) with a window of 13 amino acids (*31*). The positive direction of the *y*-axis is hydrophobic, and the negative is hydrophilic. A horizontal blue line was added at −0.4, which is the hydropathy attributed to the glycine residue in the Kyte−Doolittle algorithm.

Table 2. Identification of Soluble Plasma Proteins or Fragments from Hen Egg Yolk

band[a]	obsd molecular[b] mass (kDa)	NCBI accession number[c]	identified protein[c]	theoretical molecular mass[d] (kDa)	amino acid number of protein or fragment[d]	peptide nb[c]/coverage (%)[d]	theoretical pI[d]
1	190	NP_001038098	apoB(43−2123)	231.47	2080	107/56.4	8.29
2	130	NP_001038098	apoB(43−1297)	139.63	1255	21/22.3	7.57
3	115	S00390	Ig heavy chain[e]	45.38	426	19/46.7	7.63
4	105	NP_001038098	apoB(1284−2123)	93.49	840	13/23.4	8.82
5	97	NP_001038098	apoB(93−972)	97.63	880	2/4.1	7.65
6	81	NP_001038098	apoB(3826−4573)	86.17	748	43/46.0	6.15
7	78	BAE16337	ovotransferrin	75.78	686	2/3.2	6.69
8	64	NP_001038098	apoB(2142−2681)	64.44	540	11/19.8	7.11
8	64	NP_001038098	apoB(2720−3212)	55.47	493	21/51.3	8.84
8	64	S00390	Ig heavy chain[e]	45.38	426	15/36.1	7.63
9	55	NP_001038098	apoB(2142−2681)	64.44	540	36/51.3	7.11
9	55	NP_001038098	apoB(3373−3748)	41.28	376	20/59.3	9.28
10	54	NP_990592	albumin	67.19	592	68/82.6	5.35
11	44	NP_001004408	YGP42	31.45	285	31/64.2	5.88
12	40	NP_990592	truncated albumin	44.30	387	37/65.1	5.25
12	40	NP_001026447	YGP40	30.98	284	56/71.8	6.16
13	30	XP_422371	YGP30	31.46	283	25/55.5	7.55
14	24	A21177	Ig light chain[e]	11.35	103	8/81.5	6.09
15	14	NP_990814	apovitellenin 1	9.33	82	15/81.7	9.21

[a] Band number as in **Figure 1B**. [b] Molecular mass was estimated from electrophoretic mobility of protein bands. [c] Protein identification was obtained with Bioworks 3.1 research using a *G. gallus* protein sequence database extracted from the nr database downloaded from the NCBI site (htpp://www.ncbi.nlm.nih.gov/). The number of peptides recovered for each protein is indicated in column 7 (pept nb). [d] Theoretical molecular mass and pI of identified protein or fragments were calculated from their amino acid sequence using SWISS-PROT tools (htpp://expasy.ch/sprot/). Sequence coverage was calculated taking into account all amino acids identified through three experiments by LC-MS/MS and matched to amino acid number of fragments. [e] For immunoglobulin, only both constant regions of heavy and light chains were considered for determination of molecular mass, theoretical pI, and sequence coverage.

YGP40, respectively. The protein sequences of YGP42 and YGP40 appear in **Figure 4** (*5, 24*). YGP42 is a 285 amino acid protein with a calculated molecular mass of 31 446 Da. YGP40 contains 284 amino acids for a mass of 30 978 Da. These two proteins present 34% identity and 52% similarity and are considered as cysteine-rich proteins containing, respectively, 6.3

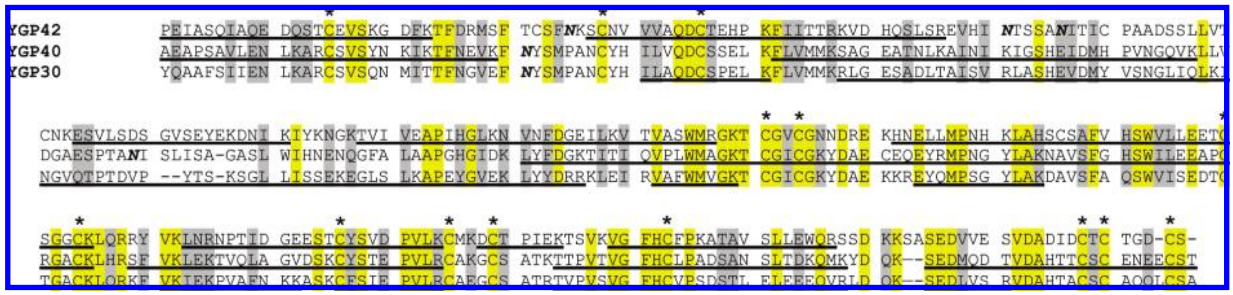

Figure 4. Sequence alignment of the two glycoproteins YGP42 and YGP40 identified in a plasma fraction with the newly hypothesized YGP30. Gray and yellow highlighting indicates similar and identical residues, respectively. Peptides identified through LC-MS/MS analysis are underlined. Asparagine residues that could be potential glycosylation sites are in bold and italics. Asterisks indicate the positions of the 14 highly conserved cysteine residues.

and 5.3% cysteine residues. Moreover, 14 cysteine residues strictly are conserved in these sequences (**Figure 4**). It was noticed that both theoretical molecular masses were lower than the apparent masses (44.5 and 40.5 kDa, respectively) because YGP42 and YGP40 are known to be glycosylated (*24*). YGP42 contains three potential N-glycosylation sites at positions 35, 71, and 76, while YGP40 contains two at positions 31 and 99.

To verify the sequence of the N-terminal domain of YGP42 and YGP40, it is not sufficient to consider only trypsin peptides from LC-MS/MS analysis, but it is necessary to observe all peptides obtained after digestion. Cathepsin D, which generates YGP42 and YGP40 from vitellogenins, is an aspartic proteinase with a broad peptide bond specificity, even though it preferentially attacks peptide bonds between hydrophobic amino acids such as F−F, F−Y, and L−Y. In our experiments, we were able to obtain the N-terminal peptide of the two proteins and verify that vitellogenin 1 is cleaved between L_{1627} and P_{1628} to give YGP42 and that vitellogenin 2 is cleaved between F_{1566} and A_{1567} to give YGP40 (*24*). YGP40 was obtained with a high coverage of the protein (71.8%), and YGP42 was covered at 64.2%. In both cases, the peptides containing the potential N-glycosylation sites were not identified. To verify the glycosylation sites, plasma soluble proteins were deglycosylated with *N*-glycosidase F, subsequently separated by SDS-PAGE, and identified through their trypsin peptides (**Figure 1**B). Among all the protein bands, only bands 11 and 12 had their mobility largely affected after deglycosylation, indicating a decrease in their molecular mass by ~6500 Da. New peptides were identified (T_{24}−K_{51} and L_{88}−F_{118} for YGP40 and E_{67}−K_{93} for YGP42) without post-translational modifications on N_{31}, N_{99}, N_{71}, and N_{76}. Moreover, deglycosylation with *N*-glycosidase F converted the asparagine residue involved in the glycosylation site to an aspartate residue (mass difference of +1). The replacement of N_{31}, N_{99}, N_{71}, and N_{76} with aspartate was verified through the mass of the new peptides and their fragmentation mass spectra.

Additionally, a third similar protein was identified in band 13 that we named YGP30. Twenty-five peptides were obtained from a protein similar to vitellogenin 3 and indexed previously in the NCBI databank as XP_422371. This record was predicted by automated computational analysis and derived from an annotated genomic sequence using a gene prediction method. Recently, this record was removed from the nonredundant databank as a result of standard genome annotation processing. However, this protein obviously exists since its sequence is largely covered in our analyses (55.5%). YGP30 could be generated from maturation of vitellogenin 3. The existence of both minor vitellogenin 3 protein and gene was previously hypothesized (*32, 33*), but no more information about them has appeared to date. The vitellogenin 3 gene would be localized on chromosome 8 (geneID 424534) near the vitellogenin 1 (geneID 424547) and vitellogenin 2 (geneID 424533) genes. The protein sequence of YGP30 shows 58% identity and 75% similarity with YGP40 and 32% identity and 54% similarity with YGP42 (**Figure 4**). The molecular mass of the protein was calculated close to 31.4 kDa and is consistent with the apparent electrophoretic mobility of the protein, so it is not guaranteed that YGP30 is glycosylated despite the presence of one potential N-glycosylation site (N_{32}). This fact was verified after deglycosylation with *N*-glycosidase F, which revealed that the electrophoretic mobility of protein band 13 was barely affected (1500 Da). The N-terminal peptide was not unambiguously found. In fact, F−Y_1 is a potential cleavage site for cathepsin D, but the first identified peptide was I_{41}−K_{51}. YGP30 was also a cysteine-rich protein (4.9%).

Yolk Immunoglobulins. The heavy chains of immunoglobulins were recovered in bands 3 and 8, while the light chains were recovered in band 14. The heavy chains were composed of an N-terminal variable region and of a constant region. This constant region begins at A_{89} after the conserved VSS sequence, is 426 amino acids long, and presents a theoretical molecular mass of 45 383 Da (*34*). A total of 47% of the protein sequence of this constant region was verified in our work through trypsin peptides analysis. Besides the constant region, we have been able to identify 11 different protein sequences for the variable region of heavy chains (NCBI accession numbers BAA10002, BAA10011, BAA11098, BAA11100, BAA11101, BAA11102, BAA11104, BAA11105, BAE80132, BAE80138, and BAE80139). The determination of the molecular mass of immunoglobulin is rather challenging since the protein consists of two large heavy chain strands covalently attached to two smaller light chain strands by disulfide bonds with an inherent heterogeneity. When we used an average molecular mass of 13.3 kDa for the variable region of the heavy chain calculated from the 11 protein sequences identified through LC-MS/MS analyses, we obtained an average molecular mass of 58.7 kDa for the heavy chain subunit. Then, we hypothesized that protein band 3 corresponded to a dimer consisting of two heavy chains not completely dissociated by our electrophoresis experimental conditions, the monomer appearing in band 8. The light chains also were characterized with a variable domain and a constant one, both being near 103 amino acids long (*35*). The constant region was largely covered by proteomic analysis (81.5%), and 15 different sequences for the variable region of the light chain were identified (NCBI accession numbers BAA09982, BAB47279, BAB47288, BAB47289, BAB47296, BAB47328, BAB47334, BAB71866, BAB71874, BAB71878, BAB71883, BAB71889, BAB71893, BAB71903, and BAB71925). An average molecular mass of 21.8 kDa was calculated and was

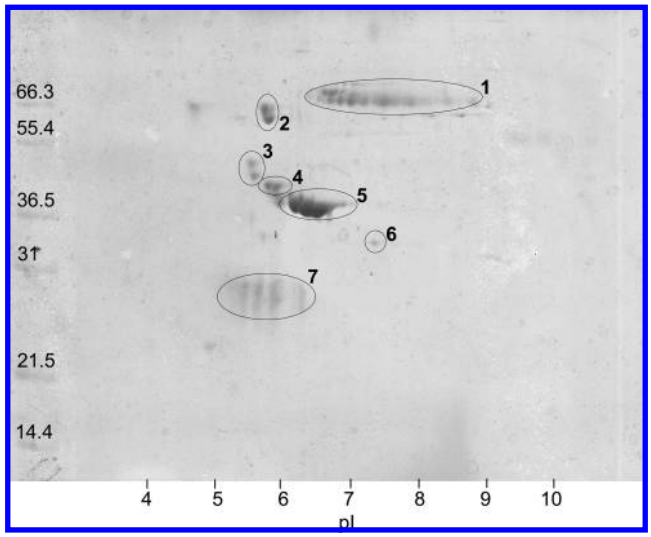

Figure 5. 2D-PAGE gel of soluble egg yolk plasma proteins. Seven groups of spots were identified as 1: heavy chains of immunoglobulin; 2: albumin; 3: truncated form of albumin; 4: glycoprotein YGP42; 5: glycoprotein YGP40; 6: putative YGP30; and 7: light chains of immunoglobulin.

consistent with the observed molecular mass from SDS-PAGE analysis.

The approximate quantification of each livetin relative to total proteins was calculated on the basis of their band intensity measured by gel scanning and image analysis. Albumins comprised near 14%, glycoproteins 41%, and immunoglobulins 45% of the total proteins. The results of the 2D-PAGE are shown in **Figure 5**. Seven groups of spots were clearly identified (from pI and molecular masses) corresponding to immunoglobulins (spots 1 and 7), albumins (2 and 3), and β-livetins (spots 4−6). The multiple spots in the groups are produced by protein heterogeneity or post-translational modifications. It was observed that YGP30 (spot 6) did not reveal any heterogeneity and exhibited a pI coherent with no phosphorylation state (ExPASy proteomics tools), and it is possible that this protein was not glycosylated.

Beside livetins, a slight contamination with LDL was visible as apovitellenin I (band 15 around 14.4 kDa) or apo B fragments (band 1 at 200 kDa and bands 2, 4−8, and 10 in the range of 55−120 kDa). Contrary to other previous works (*1, 5, 36*), we did not find ovotransferrin as a major soluble plasma protein. This protein was only detected with two peptides, indicating that its presence was minor due to contamination from egg white (*37, 38*) or eggshell membranes (*39*).

DISCUSSION

Hypothesized Pentapartite Structure of Hen Blood Apo B Is Confirmed. Identification of LDL apolipoproteins was better-defined with new sequences for hen apo B fragments, the pI of which varies from 6.1 to 9.3. With ~40% hydrophobic amino acid residues, apo B is thought to exist as a random coil structure or a β-sheet conformation (*40, 41*). In the present work, we used a structural proteomic approach to identify the protein domains remaining associated with the hen LDL particle after a step of proteolysis by proteinase K as was achieved by several authors with human apo B-100. It is thought that proteolytic activity is limited to the part of the protein protruding from the lipid layer surrounding the LDL particle. The peptides released after trypsin cleavage of human apo B-100 have been shown to have a high content of α-helix and little β-sheet structure (*42*), and the fraction of apo B-100 associated with the particle after extensive proteolytic treatment (blend of proteases with papain, proteinase K, and chymotrypsin) contained almost exclusively β-sheet structures (*43*). These results led to the conclusion that the β-sheet structures are less accessible to proteases. By using proteinase K in our experiments, it was possible to distinguish two domains of hen apo B not easily accessible to protease activity and presumably existing as β-sheet structures: $E_{1349-2023}$ and $S_{2750-}A_{3422}$ (**Figure 2**). We then confirmed the presence of two domains, β_1 and β_2, previously hypothesized (*18*) from the comparison of hen apo B with human apolipoprotein B-100. Both proteins have a pentapartite structure composed of three amphipathic α-helical domains alternating with two amphipathic β-strand domains, NH_2-α_1-β_1-α_2-β_2-α_3-COOH (*16, 17*). These two domains were characterized with very basic pI (9.14 and 9.32, respectively) and hydropathy plots showing hydrophobic behavior (**Figure 3**). They could be the anchoring point in egg LDL. It is very interesting to note that the pentapartite structure of hen blood apo B is maintained during its maturation. The tertiary structure of fragments of apo B from LDL is in the same conformation as that of the different protein regions in the precursor.

It was hypothesized that the β-sheet regions of human apo B-100 are the key structural areas for the integrity of human LDL particles and represent irreversibly lipid-associated domains due to a marked preferential orientation (*43, 44*). The schematic representation of a β-sheet−phosphatidylcholine interaction leading to the firm attachment of a phosphatidylcholine molecule to apo B-100 and to immobilization of the phosphocholine headgroup was described (*44*). Controversies persist concerning the adsorption mechanism of egg LDL at the oil/water interface. Some evidence suggests that LDL particles break down when they come into contact with the interface (*10*). The lipid core coalesces with the oil phase, and apolipoproteins and phospholipids spread at the interface. The disruption of LDL particles is attributed to a weakening of protein−protein interactions. The presence of β-sheet regions in hen apo B firmly associated with lipids suggests that they could participate in this mechanism due to their natural amphiphilicity with hydrophobic and hydrophilic sides (**Figure 3**).

Structure of Soluble Plasma Proteins Is Detailed. Among livetins, we observed that albumins (α-livetins) represented nearly 14%, glycoproteins (β-livetins) 41%, and immunoglobulins (γ-livetins) 45% of the total protein. If we consider that the contribution of glycoproteins was overestimated and that of albumins was underestimated due to the fact that band 12 contained both YGP40 and truncated albumin, our result fits well to the previous findings of Shepard and Hottle (*45*) reported by Williams (*23*). These authors observed that in hen egg yolk, α-livetins comprised 17.3%, β-livetins 32.7%, and γ-livetins 50.0% of the total soluble plasma proteins, whereas these proteins comprised 53.3, 18.3, and 28.3% of the total proteins in hen blood serum.

The immunoglobulin content of the yolk is always higher than that of hen serum (*23, 46*), so egg yolk is often used as a convenient and inexpensive source of polyclonal antibodies. Immunoglobulin from yolk (IgY) was observed in this work with a high heterogeneity due to the presence of variable regions of light and heavy chains. We were able to identify 11 different protein sequences for the variable region of heavy chains and 15 different sequences for the variable region of light chains. The high heterogeneity of immunoglobulins was previously noted (*1, 2, 5*). The presence of multiple N-glycosylation states favors this heterogeneity. A relative stability of immunoglobulin against physicochemical treatments was reported, showing a heat stability up to 70 °C for 15 min with nearly all antibody activity remaining, no affect of freezing and freeze-drying unless repeated several times, and stability at pH 4 or above (*47*). The molecular mass of yolk endogenous immunoglobulin estimated

in this work is lower than that of antibodies produced in egg yolk against exogenous proteins (*46, 48*).

Nilsson et al. (*1, 2*) observed that α- and β-livetin fractions can act efficiently as emulsifier and surfactant. They were able to produce an emulsion, the droplet size of which depends on the amount of protein added and the emulsion surface area increasing linearly with concentration. However, in competition with LDL, α- and β-livetins are not predominantly adsorbed at the oil/water interface (*2*). They noted that glycoprotein YGP40 adsorption was efficient at any pH with a slight increase at pH 6−8, which is just above its pI value. Albumin seemed to adsorb much more efficiently at pH 4−5, which is slightly below its pI. As these proteins are water soluble and not hydrophobic with high or low molecular masses, they hypothesized that the competitiveness in adsorption was controlled by the distribution of hydrophilic and hydrophobic domains in the protein molecules. Both YGP40 and albumin contain in their amino acid sequence some coherent hydrophobic blocks to adsorb and long contiguous stretches of low hydrophilicity to spread at the interface. If we consider the Kyte−Doolittle GRAVY (grand average of hydropathicity) score (*31*), YGP40 is characterized with a score of −0.233, exceeding the mean of soluble proteins (−0.4). GRAVY scores for YGP42 and albumin are rather low, −0.359 and −0.347, respectively. On the contrary, the new protein YGP30 has a higher GRAVY score than YGP40 (−0.157), indicating amphiphilic behavior. However, the low abundance of YGP30 does not favor its effect.

In this work, it was verified that hen blood apo B was enzymatically matured into nine protein fragments recovered in egg yolk LDL. Two fragments were highly enriched in β-sheets firmly associated with lipids and less accessible to proteinase K, suggesting a similar pentapartite structure as human apo B-100. A new minor protein we called YGP30 was identified, showing 75% similarity with the YGP40 glycoprotein. This protein exhibited amphiphilic behavior more pronounced than YGP40. From our results, it is not possible to conclude if YGP30 is glycosylated or not. Plasma constituents (LDL and livetins) are remarkably soluble in the common pH range of food emulsions. They can adsorb efficiently and competitively (with selectivity for LDL) at the interfaces due to their structure and composition, which combines amphipathic character and flexibility. Thus, these proteins can be used to prepare emulsions under a wide range of conditions, and their presence explains the excellent emulsifying properties of egg yolk.

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