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Protein Components of Low-Density Lipoproteins Purified from Hen Egg Yolk

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To identify apoproteins present in purified low-density lipoproteins from hen egg yolk in relation with their emulsifying properties, they have been separated by SDS−PAGE. We identified two different proteins by liquid chromatography−tandem mass spectrometry analysis of the peptides obtained by the trypsin digestion of protein gel bands. Apovitellenin I was identified as a monomer and a dimer. Its amino acid sequence was totally confirmed, and molecular mass determination by liquid chromatography−mass spectrometry showed that it did not present post-translational modifications but only a slight heterogeneity by the loss of one or two amino acids at the C-terminal part of the protein. Apolipoprotein B was identified into seven bands corresponding to fragments resulting of a processing of the hen blood apo-B protein. The identity of the fragments was determined by the observation of the sequence coverage by trypsin peptides and the sequence alignment with homologous human blood apolipoprotein B-100.

KEYWORDS: Hen egg yolk; low-density lipoproteins; LC−MS identification; apovitellenin I; apolipoprotein B

INTRODUCTION

Hen egg yolk consists mainly of 68% low-density lipoproteins (LDL), 16% high-density lipoproteins (HDL), 10% livetins, and 4% phosvitins (*1*). Yolk proteins (soluble or embedded in lipoprotein structures) are derived from maternal serum proteins which are synthesized in the hen liver, internalized by growing oocytes via receptor-mediated endocytosis, and partially processed. Very low-density lipoproteins (VLDL) of hen blood are the precursors of LDL of egg yolk (*2*). VLDL exist in 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-B is a 500 kDa protein constituted by only one unit and highly similar to the human apolipoprotein B-100 precursor. During its transfer into the yolk, hen apo-B is enzymatically cleaved, resulting in the production of apo-B fragments (*3*, *4*). The only apoprotein from blood lipoproteins to be transferred to yolk in large amount without any modification is apo-VLDL II, called apovitellenin I in the yolk. Apovitellenin I is a small homodimer with disulfide-linked subunits of 9 kDa (*5*, *6*). Apart from apovitellenin I, there is a lack of knowledge concerning the exact identification of the other apoproteins of LDL and specially the correspondence between LDL and blood lipoproteins. Furthermore, there are still gaps in the knowledge of the exact maturation mechanism of apoprotein precursors.

First, authors (*7*, *8*) attributed emulsifying properties of yolk to a phospholipid−protein complex. 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 have been observed between emulsifying properties of yolk and plasma, whereas emulsions made with granules behaved very distinctly (*9*−*11*), indicating that yolk's emulsifying power was situated in the plasma. More recently, it has been specified that the exceptional emulsifying activity of egg yolk was clearly due to LDL (*12*, *13*), and that the protein part of LDL exerted the driving contribution in the formation and stability of emulsions made with LDL from yolk (*13*).

LDL are spherical particles of about 35 nm in diameter, consisting of a core of triglycerides, cholesterol, and cholesteryl esters, surrounded by a monolayer of phospholipids in which apoproteins are embedded (*14*). It is commonly supposed that LDL micelles break down when they come into contact with the interface. The lipid core coalesces with the oil phase, and apoLDL and phospholipids spread at the interface (*15*−*17*). The adsorption of proteins and phospholipids at the oil−water interface forms a film that allows the stability of emulsions.

Direct adsorption of LDL proteins is not easy because of the insolubility of these proteins in water or in aqueous buffer. So

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the interactions between proteins and lipids in LDL are essential to transport surfactant proteins and phospholipids in a soluble form (LDL micelle) at the vicinity of the interface and then to release them at the interface (*13*). The main hypothesis is that apoproteins serve as the first anchorage of LDL at the interface, and then the subsequent denaturation leads to the destructuration and spread of LDL at the interface. This stresses the importance of the nature of the apoproteins in the mechanism of LDL interfacial adsorption and the stabilization of yolk emulsions.

As the protein part of LDL has been demonstrated as essential to understand their interfacial and emulsifying properties, and this protein synthesis and maturation have been partly studied, it was of interest to identify more clearly in this study all the apoproteins present in LDL fractions in relation with their maturation.

MATERIALS AND METHODS

Purification of LDL from Egg Yolk and Extraction of LDL Proteins. LDL were purified from egg yolk as previously described (*12*). The LDL solutions were extracted with ether−ethanol according to Anton et al. (*12*) to obtain a protein mixture which could be submitted to SDS−PAGE for identification.

Apoprotein of near 15 kDa, the lower molecular mass protein, was separated from the other apoproteins by gel filtration (Ultrogel ACA 34, Sepracor/IBF, Villeneuve-la Garenne, France) using 0.05 M Tris-HCl buffer (pH 8.2) containing 0.5% SDS as eluent. Fractions containing apoprotein of 15 kDa were detected by recording optical density (280 nm), and the purity of protein was checked by SDS−PAGE. Fractions were lyophilised.

SDS−Polyacrylamide Gel Electrophoresis. Proteins from LDL preparations were precipitated with 3 vol of cold acetone at −20 °C overnight. The pellet was dried and resuspended in a dissociation buffer consisting of 0.250 M Tris-HCl (pH 6.8), 40% glycerol, 20% 2-mercaptoethanol, 8% SDS, and 0.02% bromophenol blue. Electrophoresis was run under 100 V for 100 min on 4−12% ready to use NuPAGE bis-tris gels (Novex, San Diego, CA) using 50 mM MES NuPAGE buffer (pH 7.3). Gel was stained with Coomassie blue (G-250) according to Neuhoff and Harold (*18*) leading to a clear background not requiring destaining. Molecular masses were estimated with Mark 12 standard from Novex. Gel was briefly washed with water before densitometric measurements and protein bands excision.

Gel was scanned (300 dpi) using an Epson Perfection 1200 Photo scanner, and the resulting TIFF file was analyzed using the Image Quant (version 4.2a) software (Molecular Dynamics).

Identification of Proteins of the LDL. 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 for 30 min with 150 μL of 10 mM dithiothreitol (DTT) and 0.1 M NH_4HCO_3 at 56 °C and alkylated in the dark with 100 μL of 55 mM iodoacetamide and 0.1 M NH_4HCO_3 for 20 min at room temperature. The gel pieces were washed in 0.1 M NH_4HCO_3, dehydrated with ACN, and vacuum-dried. Then, proteins were digested overnight at 37 °C with sequencing grade trypsin (EC 3.4.21.4, Roche Diagnostics, Meylan, France) at the concentration of 12.5 mg L^{-1} in the presence of 25 mM NH_4HCO_3 and 5 mM $CaCl_2$. 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 before liquid chromatography−mass spectrometry analysis.

HPLC was carried out with a Spectra System equipment (Thermo Separation Products, Riviera Beach, FL) comprising an SCM1000 vacuum membrane degasser, P4000 gradient pumps, and an AS3000 autosampler. Volumes of 10 μL of samples were loaded onto a reversed-phase VydacC18 column (3.2 × 250 mm, 300 Å pore size, 5 μm film thickness). The column was eluted at room temperature with 5% of solvent B (ACN and 0.1% HCOOH) in A (H_2O and 0.1% HCOOH) for 2 min and then with a linear gradient of B in A from 5 to 45% over 40 min, then 45 to 95% over 5 min before re-equilibration. The flow rate was 0.5 mL min^{-1}. After elution from the column, a 1/5 split introduced 100 μL min^{-1} 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; 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 higher resolution to determine their charge), (iii) MS/MS of these ions.

Protein identification was performed with Bioworks 3.1 software using a *Gallus gallus* protein sequence database extracted from 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 a cross-correlation (Xcorr) and delta correlation (dCN) functions to assess the quality of the match between a tandem mass spectrum and amino acid sequence information in a database. The output data were evaluated in terms of (i) trypsin nature of peptides; (ii) Xcorr magnitude up to 1.7, 2.2, and 3.3 for mono-, di-, and tri-charged peptides, respectively, to minimize false positives; and (iii) dCN higher than 0.1. Only proteins identified with at least three peptides were considered.

Determination of Molecular Mass of Apoprotein of 15 kDa. An aliquot of apoprotein of 15 kDa (1 mg) was dissolved for 30 min at 37 °C in guanidinium buffer (0.2 M Tris-HCl, 6 M guanidinium chloride, and 2 mM EDTA, pH 8.5) before the addition of DTT (0.06 M). Both reactions of reduction for 240 min and alkylation with vinyl pyridine (0.18 M) for 90 min were carried out at 37 °C in the dark. Reactions were stopped with acidification to pH 2 using formic acid, and the sample was dialyzed with 0.1 M HCOOH in centrifugal filter devices (Ultrafree-MC, Millipore Corporation, Bedford, MA). Protein was dried and dissolved in a minimal volume of ACN−H_2O (50:50, v/v) containing 0.1% HCOOH before liquid chromatography−mass spectrometry analysis.

The HPLC gradient for protein separation was slightly different from the peptide gradient described before: 10% of solvent B in A for 2 min and a linear gradient of B in A from 10 to 70% over 30 min, then 70% of B was maintained over 13 min before re-equilibration. Mass spectra were acquired scanning from *m*/*z* 600 to 2000 using the same mass spectrometer parameters without ion fragmentation in the trap.

RESULTS

The proteins contained in the LDL fraction were separated by SDS−PAGE (**Figure 1**). The electrophoretic patterns were obtained in a highly reproducible way and showed nine major bands ranging from 8 to 190 kDa with apparent molecular masses near 8, 15, 55, 62, 73, 96, 98, 118, and 190 kDa, respectively (**Table 1**). These bands were identified through the analysis of their trypsin peptides with LC−MS/MS and the interrogation of Swiss-Prot or NCBI databases. Surprisingly, only two apoproteins were identified from purified LDL after interrogation of databases. Apovitellenin I was recovered in bands 8 and 9, and a predicted protein similar to human apolipoprotein B-100 precursor was recovered in all the bands ranging from 55 to 190 kDa (bands 1−7).

Apovitellenin I. The first amino acid sequence of apovitellenin I from hen egg yolk has been determined using both automatic and manual procedures by Dopheide and Inglis (*19*). The sequence of the mature protein (82 amino acids, 9331 Da) was completely recovered with trypsin peptides analyzed in the two protein bands 8 and 9 and then verified by this indirect manner. So these two protein bands are identified as apovitellenin I.

The apparent molecular mass of the protein from band 8 (15 kDa) was higher than its theoretical average molecular mass (9331 Da). The protein was also digested by trypsin without

Table 1. Identified Proteins in LDL Purified from Hen Egg Yolk

band	apparent molecular mass (kDa)	identified protein[a] (accession number)	fragment	molecular mass of fragment[b] (kDa)	amino acid number of fragment[b]	sequence coverage of fragment (%)[b]
1	>150	XP_419979	55–2017	218.57	1963	75.2
2	118	XP_419979	55–1244	132.37	1190	77.5
3	98	XP_419979	1245–2017	86.21	773	71.7
4	96	XP_419979	80–838	84.42	759	77.5
5	73	XP_419979	3594–4195	70.36	601	66.4
6	62	XP_419979	2036–2575	64.44	540	77.8
7	55	XP_419979	2960–3321	39.77	362	74.0
8	15	P02659		9.33	82	100
9	8	P02659		9.33	82	100

[a] Identified protein and accession number were obtained with Bioworks 3.1 research using a *Gallus gallus* protein sequence database extracted from nr database downloaded from the NCBI site (htpp://www.ncbi.nlm.nih.gov/). [b] Molecular mass of the 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 amino acids identified by LC–MS/MS related to amino acid number of fragments.

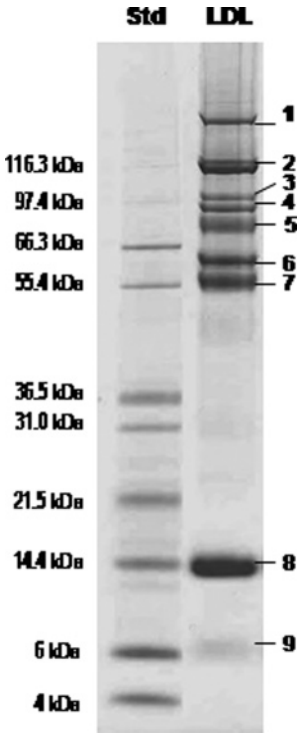

Figure 1. SDS–PAGE of proteins from purified egg yolk LDL. Proteins have been separated on 4–12% NuPAGE gel. Gels have been stained with G250 Coomassie blue. Molecular mass marker was Mark 12 from Novex (Std).

prior reduction and alkylation of cysteine residue (data not shown). In this case, apovitellenin I was identified with a sequence coverage of 88% instead of 100%, and the peptide $N_{77}LCYTK_{82}$ containing a cysteine residue was missing in the peptide mixture analyzed by LC−MS/MS. In the contrary, a peptide with a molecular mass of 1479 for the mono-charged ion and 740 for the doubly charged ion was recovered at 12.71 min. It could correspond to the sequence $[(NLCYTK)_2 - 1]$. The mass spectra obtained after fragmentation (**Figure 2**) confirmed this hypothesis (loss of K, TK, YTK, NL, NLCYTK).

The band 8 corresponded to apovitellenin I as a homodimer with an inter disulfide bond $C_{79}-C_{79}$, whereas the band 9 corresponded to the monomer. Upon scanning of the SDS−PAGE gel and image analysis, an approximate quantification on the basis of dyed protein bands intensity was obtained, and it appears that the monomer represents only 18% of the protein.

The molecular mass of apovitellenin I was directly determined with electrospray ionization mass spectrometry. A multiply charged mass spectrum was obtained (**Figure 3**), showing, after deconvolution, an intense single peak with an experimental mass of 9436 which corresponded to the theoretical mass of apovitellenin I (9331) with its cysteine residue alkylated with vinylpyridine (+105). Minor peaks appeared at 9273 and 9216 Da, suggesting the loss of the C-terminal tyrosine (9436-163) or the loss of the dipeptide glycine−tyrosine (9436-220) and thus the existence of a slight heterogeneity of apovitellenin I.

Hen Apolipoprotein B. All the other bands seen in the gel referred to the same protein sequence deduced from an annotated genomic sequence and highly similar to the human apolipoprotein B-100 precursor. This protein registered in NCBI data bank (accession number XP_419979) is a very high molecular mass protein (475.836 kDa) consisting of 4204 amino acids and must correspond to hen blood apo-B. The molecular mass of the mature protein is not known.

The study of the precise localization of trypsin peptides analyzed by LC−MS/MS unambiguously indicated that the 7 protein bands heavier than 55 kDa corresponded in fact to 7 different fragments of hen blood apo-B (apo 1 to apo 7). The identified fragments are reported in **Table 1**. There is the N-terminal moiety of apo-B ($f_{55-2017}$, apo 1) which could be cleaved in two lighter fragments, apo 2 and apo 3 ($f_{55-1244}$ and $f_{1245-2017}$). Apo 2 could lead also to a lighter fragment, apo 4 (f_{80-838}). The C-terminal part of the protein ($f_{3594-4195}$) was recovered in apo 5. Finally, the internal part of the protein was present as two protein fragments which corresponded to $f_{2036-2575}$ (apo 6) and $f_{2960-3321}$ (apo 7). All these fragments were identified with very high sequence coverage (more than 66%). With only these seven fragments, 86% of the sequence of apo-B was recognized.

It was noted that apo 7 corresponded to a protein band with an apparent molecular mass estimated by SDS−PAGE (55 kDa) higher than the molecular mass deduced from the sequence identified with trypsin peptides (40 kDa, **Table 1** and **Figure 1**). In fact, the trypsin digestion of the protein region comprised between $Ser_{2786}-Lys_{2959}$ leads to either very short and undetected peptides or very large peptides with too few ionisable amino acids to be analyzed by mass spectrometry in our experimental conditions. So, this part of the protein was not detected in our experiment, but it must belong to apo 7. This fact was confirmed by the approximate quantification of each apo-B fragment relative to the entire protein on the basis of their band intensity measured by gel scanning and image

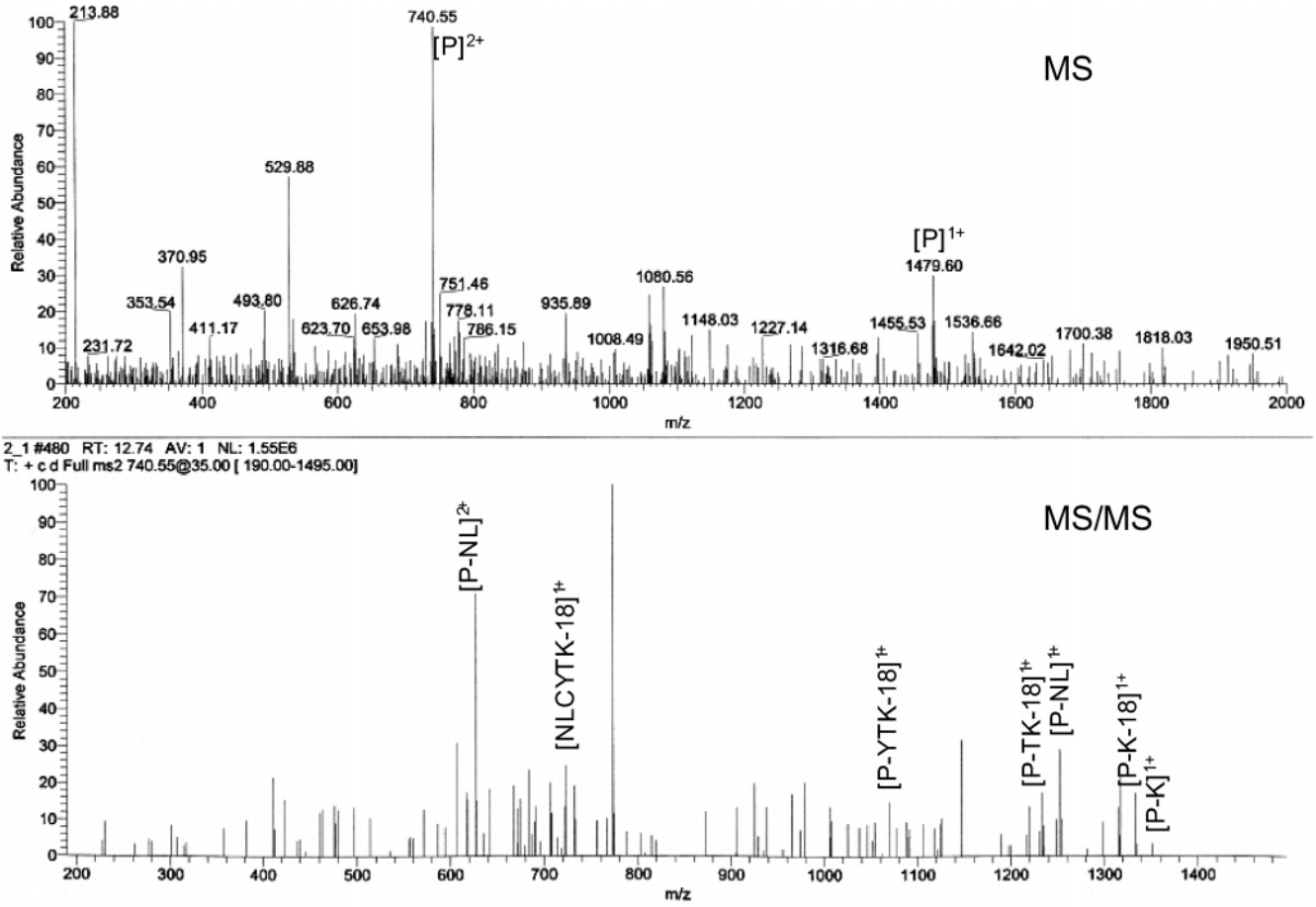

Figure 2. Mass spectrum (MS) of the peptide P eluted at 12.71 min in the case of apovitellenin I digested by trypsin without reduction and alkylation of cysteine residue showing the presence of mono- and doubly charged ions. Fragmentation (MS/MS) of doubly charged ion (740 Da) gave some information about the sequence of the peptide P, showing the loss of K, TK, YTK, and NL and the presence of the fragment NLCYTK. Some of these fragments were associated with the loss of water (– 18).

analysis. The N-terminal moiety of the protein (apo 1 to apo 4), apo 5, apo 6, and apo 7, respectively, represent 46, 14, 13, and 9% of the length of the total sequence and 43, 14, 18, and 24% of the relative intensity of electrophoresis bands. This would indicate in particular that the sequence of apo 7 (362 amino acids) was underestimated through the identification of trypsin peptides analyzed by LC−MS/MS.

DISCUSSION

Hen Blood Apo-B Is Maturated. The main apoprotein of hen blood LDL is apo-B, and this protein gives rise to different apoproteins after maturation and proteolysis. Cathepsin D or a cathepsin D-like pepstatin A-sensitive protease could be responsible for apo-B degradation inside the chicken oocyte, whereas other proteases, such as thrombin and kallikrein, are unable to generate a pattern of proteolytic fragments similar with that present in egg yolk (*20*−*22*). The proteolytic action of cathepsin D has been associated both with the endosomal targeting and with yolk formation (*23*). Burley et al. (*4*) have proposed that apo-B is split up because blood and yolk lipoproteins have different functions. The blood lipoproteins have short half-lives and must be readily available, whereas those in yolk have to remain stable for long periods before they are needed by the embryo. Moreover, as the yolk lipoproteins have to resist to the effects of atmospheric oxygen, blood apo-B is split up into smaller fragments that are better able to take part in a protein network on the surface of the particles and thus help to stabilize them (*4*).

Homology of Hen Apo-B with Human ApoB-100. Hen blood apo-B presents high identity (46%) and homology (64%) with human apolipoprotein B-100 (apoB-100), the major protein component of the atherogenic low-density lipoprotein. This protein has 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 (*24*, *25*). Two dense clusters of helixes are located in the middle and at the C-terminal end of apoB-100, precisely between residues 2103−2560 and 4061−4338, respectively. These two domains correspond closely to the two major apoB-100 lipid-associated domains (*24*).The N-terminal part α_1 (the first 800 amino acid residues) represents a globular domain associated with triglyceride transfer protein to create a lipid transfer pocket required for lipoprotein assembly (*25*, *26*). The pentapartite structure is a common supramolecular feature of apolipoprotein B-100 observed in nine vertebrate species (*25*).

The Structure of Hen Apo-B: A Hypothesis. Through sequence alignment with human apoB-100, we searched α helical and β strands domains of hen egg LDL apo-B and the localization of cleavage sites with cathepsin D by homology (**Figure 4**). Cathepsin D is an aspartic endopeptidase. Like pepsin and gastricsin, this protease has broad peptide bond specificity. However, it preferentially attacks peptide bonds containing hydrophobic amino acids such as Phe−Phe, Phe−Tyr, and Leu−Tyr. The exact cathepsin D cleavage sites on human apoB-100 and blood chicken apo-B are unknown (*22*), but we are looking preferentially for these potential cleavage

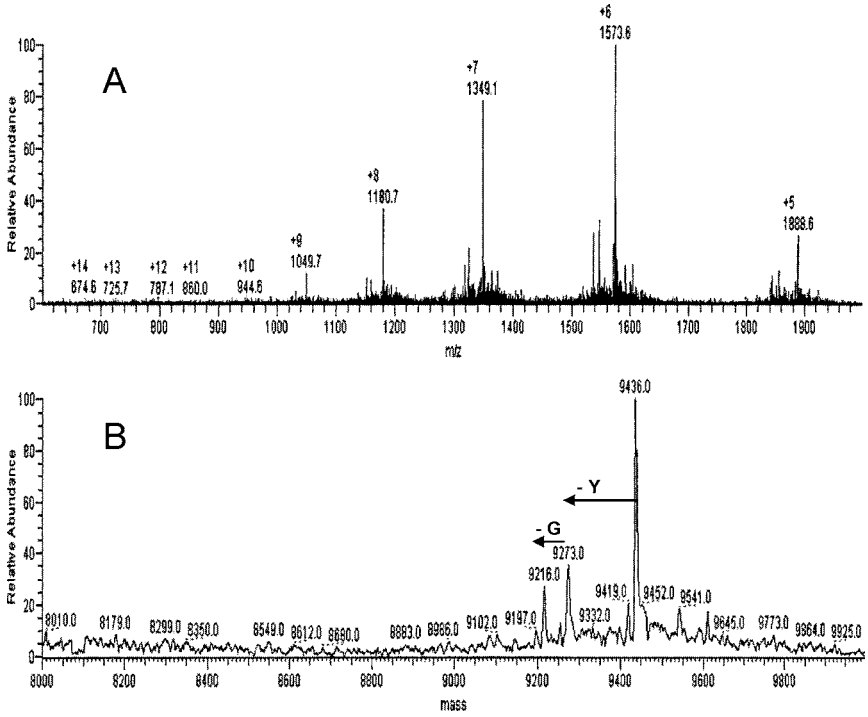

Figure 3. Electrospray ionization mass spectrum of the entire apovitellenin I after reduction and alkylation with vinyl pyridine: experimental raw data of multiply charged ions (**A**) and deconvoluted spectrum to calculate protein average mass (**B**).

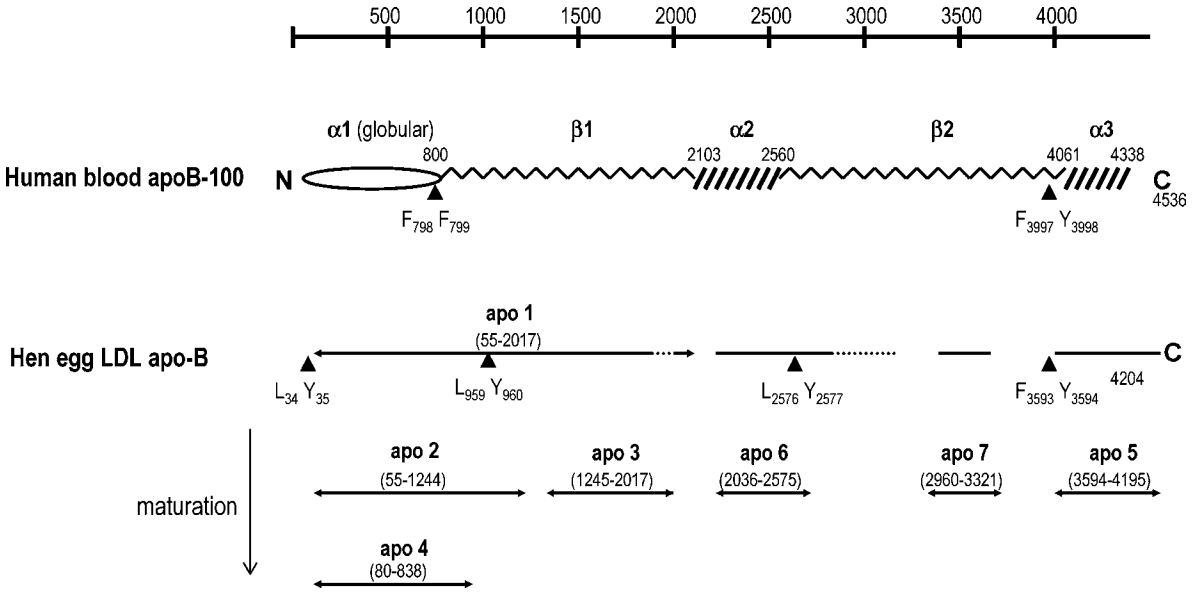

Figure 4. Sequence alignment of hen egg yolk apo-B with human apolipoprotein B-100. The schematic pentapartite structure of apoB-100, is composed of α helical domains alternating with β strand domains (NH_2-α_1-β_1-α_2-β_2-α_3-COOH) as suggested by ref *24*, is presented. Potential cleavage sites for cathepsin D were noted (▲). For egg apo-B, the peptides identified in this work by LC–MS analysis were indicated (↔). Blank indicates some regions of the protein where no sequence information was obtained by mass spectrometry analysis. Dotted lines indicate regions of human apoB-100 without equivalent regions in the case of egg apo-B.

sites when they did not belong to regions of the protein covered with identified trypsin peptides. We could observe that the different domains of the hypothesized tertiary structure of hen apo-B could correspond approximately to the different fragments observed on SDS−PAGE and obtained after proteolytic cleavage (**Figure 4**). So the N-terminal moiety of apo-B (apo 1) was composed of α_1 and β_1 domains. The α_1 domain of human apoB-100 is delimitated by a potential cathepsin cleavage site Phe_{798}−Phe_{799}. In the case of apo-B, Leu_{34}−Tyr_{35} and Leu_{959}−Tyr_{960} are potential cathepsin cleavage sites, and they could be considered to delimitate the α_1 domain of hen apo-B, approximately corresponding to apo 4. The internal part of apo-B (apo 6 and 7) was composed of α_2 and β_2 domains with a potential cleavage site at Leu_{2576}−Tyr_{2577}. Finally, the α_3 domain was the C-terminal part of apo-B (apo 5) with a potential cleavage site at Phe_{3593}−Tyr_{3594} peptide bond conserved in the sequence of human apoB-100.

In conclusion, this work led to the determination of major protein component of hen egg yolk LDL. The information obtained here is important since, to our knowledge, no proteomic investigation of egg yolk has been carried out previously. Only two different proteins were identified. The existence of apovi-

tellenin I either as a monomer or a homodimer of disulfide-linked subunits was confirmed. The monomer has a molecular mass of 9331 Da despite a slight heterogeneity, and its amino acid sequence has been totally confirmed through the analysis of trypsin peptides. We propose that hen apo-B is cleaved into several protein fragments by cathepsin D. The structural homology of hen apo-B with human apoB-100 was verified, and sequence alignment could be an interesting tool to carry on with the characterization of hen apo-B.

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