Bioactive Egg Compounds

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With 30 Figures

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Preface

The hen egg is one of the most versatile foods. It contains high-quality proteins and lipids, as well as valuable minerals, carbohydrates, and vitamins. Eggs are also widely used in the food industry due to their multifunctional properties (e.g., foaming, gelling, and emulsifying). Of course, the primary aim of the laying hen is not to produce high-value human food but to give rise to new life. Therefore, avian eggs contain the basic elements for life, and many of the egg compounds have so-called biological activity. For example, almost all the albumen proteins are antimicrobial, thus protecting the developing embryo. Consequently, hen eggs are very good potential sources of raw materials for health-promoting, so-called functional foods, as well as for the traditional food and pharmaceutical industries.

In 2002 a COST action called "Multidisciplinary Hen Egg Research" was founded that involves experts in different branches of egg research from 13 countries. The most important result of the COST action is the exchange of expert knowledge between the participating countries and research groups.

Since the COST action has joined together leading European scientists in egg research, the idea was born (in 2004) to write a book about latest results/concepts in bioactive egg compounds and their possible use in the food, pharmaceutical, and cosmetic industries.

The book comprises 33 chapter written by authors from 13 European and non-European countries and is divided in three parts.

Part I contains 17 chapters concerning the composition and the extraction of yolk, albumen, and shell compounds, respectively. We have particularly paid attention to updating the data, notably by the contribution of our own research results. We have opted to present, in the majority of cases, the structural aspect of the egg compounds, and of their interactions in relation to their function. Lastly, very recent data coming from our research regarding minor compounds are given exposure in the chapters dealing with proteases, antiproteases, lipocalins, clusterin, etc.

In summary, this part is not an "umpteenth" presentation of the composition of the egg, but an updated document, presenting original results seen from unique angles.

Part II, with 5 chapters, is concerned with the role of eggs in human nutrition. The nutritive properties of eggs are evaluated in detail, with discussions of the importance of the egg-contained macro and micronutrients, the

presence of functional substances, and the bioavailability of nutrients. Further chapters deal with improvements in the nutritive value of eggs, namely, by the enrichment in omega-3 fatty acids, vitamins, and selenium, and the implications embodied therein for daily nutrient intake and human health. The role of eggs as one of the main food allergens is also examined, including the factors that determine the allergenic properties of egg proteins and the possibilities for making hypoallergenic egg products.

Part III comprises 11 chapters divided in two subparts, and as in part 1 the contents of the chapters are mainly based on the experiences of the authors themselves. In particular, the use of specific bioactive egg compounds for human beings is introduced. For example, possible application of egg white compounds with antibacterial (and perhaps also antiviral) activity, or of egg-white-derived peptides with antihypertensive activity, with the prospect that a functional food fortified with these peptides may be produced. Further subjects are the application (at present and in the future) of specific chicken egg yolk antibodies in human and veterinary medicine, the potency of ion-binding proteins as nutraceutical (ovoceutical), and new and interesting fields of lecithin application.

The chapters of a second subpart are mainly focused on biotechnological aspects of egg use: for example, new methods for egg protein fractionation, the use of phospholipid-based liposomes/emulsions in pharmaceutics and cosmetics, the use of specific yolk components for cryoprotection of spermatozoa, and the usefulness of egg-protein-based films and coatings as biodegradable packaging material. Finally, results are presented on nanotechnology in egg research and news is given on avidin–biotin biotechnology.

The editors would like to express our thanks to all the authors who contributed their expertise and knowhow to the success of this book. Furthermore, we thank Springer for the patient and trustful cooperation during the processing and realization of the project. Finally, we hope to present an interesting and stimulating book that makes a contribution to understanding and disseminating the state of art in research on bioactive egg compounds.

Turku, Madrid,
Nantes, Berlin
September 2006

Rainer Huopalahti, Rosina López-Fandiño,
Marc Anton, Rüdiger Schade

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Mark Anton

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Chapter 1 Composition and Structure of Hen Egg Yolk

Marc Anton

1 Introduction

Unlike mammals, the embryos of birds are not fed by the mother during their development and have no possibility of elimination of metabolic waste. Consequently, the egg yolk provides vital nutrients (proteins, lipids, vitamins, and minerals) that are extremely well metabolized by the chicken embryo. Egg yolk is also a very attractive source of nutrients for humans: Its coefficient of digestive use is comparable to that of milk, and the biologic value of proteins in the egg is even superior to that of milk proteins (Bourgeois-Adragna 1994). Besides, hen egg yolk is a multifunctional ingredient widely used in many food products such as mayonnaise, salad dressings, cakes, pasta, creams, etc. Indeed, it possesses emulsifying, gelling, coloring, aromatic, and antioxidant properties. Each constituent of yolk possesses peculiar physical and chemical characteristics responsible for its own functional properties. Environmental conditions (pH, ionic strength, competition) and preservative treatment (heating, freezing, drying) can influence and modulate these functional properties. Finally, due to its original role as an embryonic chamber, yolk contains many constituents essential for life. Thus yolk represents a major source of active principles usable in medical, pharmaceutical, cosmetic, nutraceutical, and biotechnological industries.

2 Composition

Yolk makes up about 36% of the weight of the fresh whole hen egg. The dry matter of the freshly laid yolk varies from 50 to 52% according to the age of the laying hen and the duration of preservation. A transfer of water from the white to the yolk takes place during the storage of eggs (Kiosseoglou 1989; Thapon and Bourgeois 1994; Li-Chan et al. 1995). The compositions of fresh and dry yolks are presented in Table 1: the main components are lipids (about 65% of the dry matter) and the lipid to protein ratio is about 2:1.

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Table 1. Composition of hen egg yolk, from Powrie and Nakai (1986)

	Fresh yolk (%)	Dry yolk (%)
Water	51.1	–
Lipids	3.6	62.5
Proteins	16.0	33.0
Carbohydrates	0.6	1.2
Minerals	1.7	3.5

Lipids of yolk are exclusively associated with lipoprotein assemblies. They are made up of 62% triglycerides, 33% phospholipids, and less than 5% cholesterol. Carotenoids represent less than 1% of yolk lipids, and give it its color. Proteins are present as free proteins or apoproteins (included in lipoprotein assemblies). The interactions between lipids and proteins result in the formation of lipoproteins (low and high density), which represent the main constituents of yolk. So, on the basis of its dry matter, yolk is constituted of five major constituents: 68% low-density lipoproteins (LDL), 16 % high-density lipoproteins (HDL), 10% globular proteins (livetins), 4% phosphoprotein (phosvitin), and 2% minor proteins (Table 2; Powrie and Nakai 1986).

In the fatty acid composition of lipids, based on a standardized feed of hens, about 30–35% is of saturated fatty acids (SFA), 40–45% of monounsaturated fatty acids (MUFA), and 20–25% of polyunsaturated fatty acids (PUFA). The main fatty acids are oleic acid (C18:1, 40–45%), palmitic acid (C16:0, 20–25%), and linoleic acid (C18:2, 15–20%; Kuksis 1992). However, this composition is subject to strong variations, in particular according to the nature of fatty acids ingested by the hen (Posati et al. 1975; Anton and Gandemer 1997).

Table 2. Partition of hen egg yolk constituents, from Powrie and Nakai (1986)

	Yolk dry matter (%)	Yolk lipids (%)	Yolk proteins (%)	Lipids (%)	Proteins (%)
Yolk	100	100	100	64	32
Plasma	78	93	53	73	25
LDL	66	61	22	88	10
Livetins	10	–	30	–	96
Others	2	–	1	–	90
Granules	22	7	47	31	64
HDL	16	6	35	25	75
Phosvitin	4	–	11	–	95
LDLg	2	1	1	88	10

Dietary fatty acids particularly modify the proportions of PUFA and MUFA, whereas SFA proportions are slightly affected.

The major triglyceride fatty acids comprise most of the fatty acid total. The glycerol of the triglycerides is mainly esterified by palmitic acid in position 1, by oleic and linoleic acids in position 2, and by oleic, palmitic, and stearic acids in position 3 (Kuksis 1992).

Phospholipids are amphiphilic molecules that contain one hydrophilic head group: phosphoric acid + alcohol, amino acids or polyol, and one hydrophobic group: two fatty acids. Phospholipids of yolk are very rich in phosphatidylcholine (PC): 76% of total phospholipids (three-fold higher than natural soy phospholipids). Phosphatidylethanolamine represents 22% of the phospholipids. Phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), cardiolipins (CL), lysoPC, and lysoPE are present in very low amounts. PUFA represent 30–40% of the fatty acids phospholipids, whereas SFA account for 45%, and MUFA for 20–25% (Kuksis 1992). Phosphatidylcholine contains important quantities of ω3 fatty acids as the nonpolar part. Choline forms the polar part and is an important nutrient in brain development, liver function, and cancer prevention (Gutierrez et al. 1997), routinely added to commercial infant formulas as an essential nutrient. Consumption of phosphatidylcholine increases plasma and brain choline levels and accelerates neuronal acetylcholine synthesis. It has been demonstrated that consumption of yolk phospholipids tends to alleviate the symptoms of Alzheimer disease (Juneja 1997).

Cholesterol is the sterol found in egg yolk. It results partly from the hen feed and partly from synthesis in the liver during the elaboration of lipoproteins. It represents approximately 5% of total lipids in free (85–90%) or in esterified (10–15%) form (Bitman and Wood 1980). The free cholesterol participates in the structure of LDL. Cholesterol esters are present in the lipid core of LDL and contain 35% oleic acids, 33% palmitic acids, 12% linoleic acids and 11% stearic acid (Kuksis 1992).

Carotenoids are the natural pigments of hen egg yolk.. They confer it its yellow color, which can go from a very pale yellow to a dark brilliant orange. They are far from plentiful in eggs, but economically important because the color represents a quality criterion. They are mainly carotene and xanthophylls (lutein, cryptoxanthin, and zeaxanthin). The total concentration of lutein and zeaxanthin is ten times greater than that of cryptoxanthin and carotene, combined (Shenstone 1968). Carotene is the main carotenoid found in common hen feeds corn and alfalfa. After ingestion this compound is largely oxidized to form the xanthophylls.

Consequently yolk is a plentiful source of lipids, some of which are particularly suitable for nutrition and health. Particularly, omega 3 fatty acids (ω3), mainly found in phospholipids, are considered to be an essential nutrient for brain function and visual acuity in humans (Maki et al. 2003). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the main ω3 fatty acids. They are particularly useful for pregnant and nursing mothers for optimizing

growth of their infants, especially as breast milk is currently low in ω3 due to the modern diet of most mothers.

3 Macrostructure

Yolk is a complex system with several particles in suspension in a clear yellow fluid (plasma) that contains proteins. The main types of particles are spheres, profiles, and granules. Spheres are minor components (1% of yolk dry matter) and have a diameter between 4 and 150 µm (Romanoff and Romanoff 1949). They appear as tightly packed drops of lipids and lipoproteins (Chang et al. 1977). Profiles are round particles of 12–48 nm diameter and are considered as low-density lipoproteins (Martin et al. 1964). Granules consist in circular complexes ranging in diameter from 0.3 µm to 2 µm (Chang et al. 1977). Consequently, yolk can be easily separated into two fractions after a dilution (two times) with 0.3 M NaCl and a centrifugation at 10,000 g (30 min) according to the method of McBee and Cotterill (1979): a dark orange supernatant called plasma and a pale pellet called granules are separated (Fig. 1). Yolk dilution helps to decrease its viscosity, thus permitting motion and separation of particles.

Granules represent 19–23% of yolk dry matter, accounting for about 50% of yolk proteins and 7% of yolk lipids. The dry matter content of granules is about 44%, and they contain about 64% proteins, 31% lipids and 5% ash (Dyer-Hurdon and Nnanna 1993 ; Anton and Gandemer 1997) Lipids of granules are 60% triglycerides, 35% phospholipids, and 5% cholesterol. They are mainly constituted by HDL (70%) and phosvitin (16%) linked by phosphocalcic bridges between the phosphate groups of their phosphoseryl residues

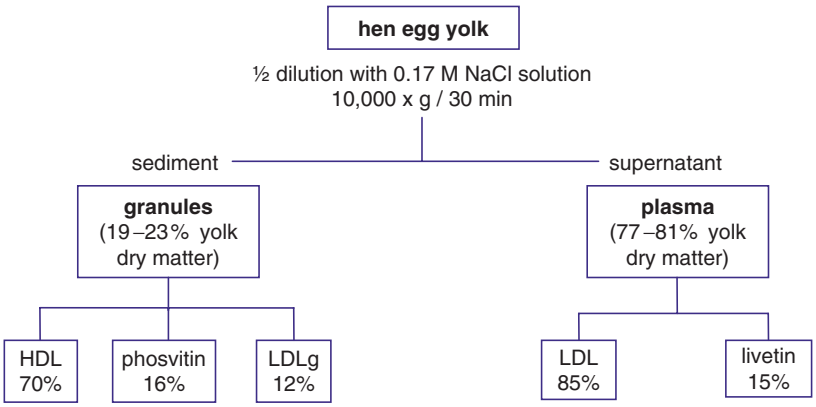

Fig. 1. Fractionation of yolk into granules and plasma

(Burley and Cook 1961 ; Saari et al. 1964). LDL (12%) are included in the granular structure (Table 2). At low ionic strength, granules mainly form insoluble HDL–phosvitin complexes linked by phosphocalcic bridges as HDL and phosvitin contain a high proportion of phosphoserin amino acids able to bind calcium (Causeret et al. 1991). The numerous phosphocalcic bridges make the granule structure very compact, poorly hydrated, weakly accessible to enzymes, and lead to an efficient protection against thermal denaturation and heat gelation.

At an ionic strength over 0.3 M NaCl, the phosphocalcic bridges, are disrupted because monovalent sodium replaces divalent calcium. In such conditions, the solubility of granules reaches 80% because phosvitin is a soluble protein and HDLs behave like soluble proteins (Cook and Martin 1969 ; Anton and Gandemer 1997). Complete disruption of granules occurs when ionic strength reaches 1.71 M NaCl. Acidification or alkalization likewise cause the disruption of granules and the solubilization of these constituents. At acidic pH the increase in number of the positive charges (NH_3^+) induces electrostatic repulsion and results in disruption of the granules constituents. At basic pH, carboxylate groups are deacidified leading to the repulsion of negative charges (COO^-), again causing disruption.

Plasma comprises 77–81% of yolk dry matter and is composed of 85% LDL and 15% livetins (Burley and Cook 1961; Table 2). It forms the aqueous phase where yolk particles are in suspension. It accounts for about 90% of yolk lipids (including nearly all the carotenoids), and 50% of yolk proteins. Plasma contains about 73% lipids, 25% proteins, and 2% ash. Lipids of plasma are distributed thus: 70% triglycerides, 25% phospholipids, and 5% cholesterol.

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Chapter 2 Low-density Lipoproteins (LDL) or Lipovitellenin Fraction

Marc Anton

1 Introduction

Low density lipoproteins are the main constituents of yolk: about 2/3 of the total yolk dry matter. They are mainly located in plasma but a small portion is included in granules (LDLg). LDLs of yolk are similar to very low-density lipoproteins of chicken blood (Nakamura et al. 1977) and are synthesized in the liver of the laying hen. They are transported with the blood to the ovary where they are transferred to the yolk without much change in structure and composition (Holdsworth and Finean 1972). LDLs constitute about two-thirds of the lipids present in hen egg yolk. The nomenclature of the LDL is complicated, some authors calling them lipovitellenin, while others use this latter term to designate only the protein part of LDL after extraction with ether. We prefer calling them low-density lipoproteins of hen egg yolk.

2 LDL Structure and Composition

LDLs are spherical particles (17–60 nm in diameter with a mean of about 35 nm) with a lipid core in a liquid state (triglycerides and cholesterol esters) surrounded by a monofilm of phospholipid and protein (in this circumstance called apoprotein; Cook and Martin 1969 ; Evans et al. 1973; Fig. 1). LDLs are soluble in aqueous solution (whatever the pH and ionic conditions) due to their low density (0.982). Phospholipids take an essential part in the stability of the LDL structure because association forces are essentially hydrophobic (Burley 1975). Some cholesterol is included in the phospholipid film, increasing its rigidity.

LDLs are composed of 11–17% protein and 83–89% lipid, out of which latter 74% is neutral lipid and 26% phospholipid (Martin et al. 1964). Their population is composed of two sub-groups: LDL_1 (10×10^6 Da) and LDL_2 (3×10^6 Da). LDL_1 represent 20% of total LDL and contain twice the amount of proteins as LDL_2 (Martin et al. 1964). Chemical compositions of both LDL fractions are

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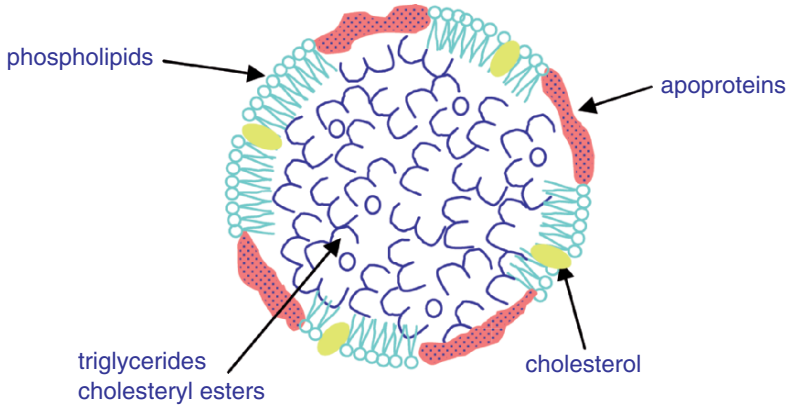

Fig. 1. Schematic representation of yolk LDL

similar but the proportions between apoproteins and lipids change, and size increases with lipid content. From a technofunctional point of view, it has been largely proved that LDLs are the main contributors to the exceptional emulsifying properties of egg yolk. This capacity is clearly due to the LDL structure, through interactions between amphiphilic apoproteins and phospholipids. This assembly of phospholipids and apoproteins allows transport of these insoluble amphiphilic species through the aqueous phase until the interface is reached. The lipoprotein disruption at the oil–water interface, already presumed in several studies concerning LDL emulsifying properties, is now confirmed. The interfacial films of LDL are constituted by a blend of apoproteins and phospholipids that assure both a decrease in interfacial tension and resistance to rupture. Formation and stability of food emulsions made with yolk is thus permitted.

An electron micrograph (TEM and Cryo-TEM) of LDL negatively stained with 2% sodium phosphotungstate is shown in Fig. 2. Particles of LDL appear spherical, with heterogeneous sizes between 20 and 60 nm diameter (average 35–40 nm). Contiguous particles have flattened edges; these polygonal shapes could be the result of particle aggregation due to dehydration on grids or to a vacuum in the electron microscope during analysis. The same sample of LDL was analyzed by photon-correlation spectroscopy (results not shown) and gave an average particle diameter of 35 nm, in agreement with the average value obtained from the electron micrograph. However, we observed some structures of about 200 nm diameter surrounded by aggregated LDL. It is possible that these structures are very low density lipoproteins as observed by Martin et al. (1964) or merged LDL as noted for human plasma LDL (Ala-Korpela et al. 1998).

Recently we have observed LDL by cryomicroscopy and confirmed the findings on the structure of LDL with a less denaturing microscopic technique.

LDL structure is sensitive to technological treatments (Anton et al. 2003). When LDLs are heated for 10 min at 75 °C, a disruption and a subsequent

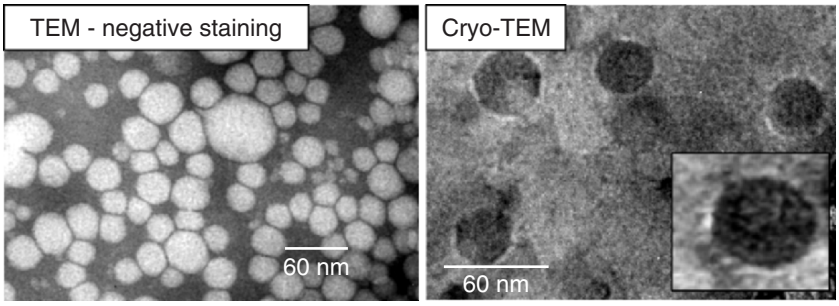

Fig. 2. Transmission electronic microscopy (TEM) and Cryo-TEM visualization of LDL

rearrangement of the fragments into big clusters occurs (average size about 300 nm). The treatment by high hydrostatic pressure (5,000 bars) also brought about a disruption followed by a rearrangement of the fragments into clusters, of about 90 nm diameter. Finally, the structure of LDL is not altered by passage through a high pressure homogenizer at up to 250 bars or through a rotor/stator homogenizer (Anton et al. 2003). This means that during the fabrication of mayonnaise, for example, the homogenization step did not disrupt its LDLs.

It has been suggested (Wakamatu et al. 1982) that LDLs form a gel during a freezing–thawing process due to the alteration of the interactions between phospholipids and apoproteins at their surface. Liberated apoproteins are thought to aggregate and form a gel (Kurisaki et al. 1981). However, temperature attained and medium conditions (LDL concentration, salts), as well as frozen and thawing kinetics, are important in this process. We have observed for LDL (1 mg protein/ml) frozen for 12 h at −80 °C and subsequently thawed at room temperature that this treatment did not visually change the structure of LDL. The time of storage at −80 °C was certainly too weak to induce destruction and gelation of LDL.

3 LDL Apoproteins

There are six major apoproteins of LDL. The main one accounts for more than 70% of the total; its molecular weight is estimated to be 130 kDa. The second apoprotein, with a molecular weight of 15 kDa, represents about 20% of the total. Four other minor apoproteins with molecular weights between 55 and 80 kDa have been identified (Fig. 3; Anton et al. 2003). The isoelectric point of all the apoproteins ranges from 6.3 to 7.5 (Kojima and Nakamura 1985). About 40% of the amino acids of apoproteins of LDL are hydrophobic (Tsutsui and Obara 1982). Consequently, these apoproteins are highly hydrophobic and flexible molecules. Recently, using circular dichroism,

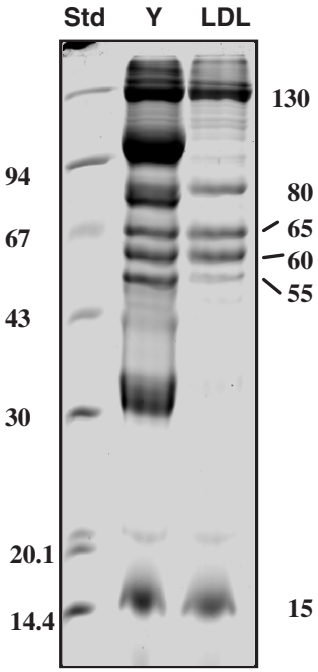

Fig. 3. SDS-polyacrylamide gel electrophoresis of yolk (*Y*) and *LDL* solution, gel with 10% acrylamide. Coomassie blue stain; low-molecular weight calibration kit was used as protein standard (*Std*)

Anton et al. (2003) have observed, for a blend of the main apoproteins a double minimum at 208 and 222 nm, and a maximum at 191–193 nm, consistent with a considerable proportion of α-helix chains. In this study sodium dodecyl sulphate (SDS) was used to solubilize apoproteins and it is suggested that SDS could lead to an induction of α-helix structures (Montserret et al. 2000). But, in the case of membrane proteins or amphiphilic proteins like LDL apoproteins, SDS is known to mimic the hydrophobic environment existing in biological membranes without changing protein conformation.

Apoproteins of LDL are glycosylated on asparaginyl residues: about 1.3% hexose, 0.7% hexosamine, and 0.4% sialic acid (Nakamura et al. 1977).

Apoproteins extracted from LDL with ether–ethanol are very difficult to dissolve in aqueous buffers. A basic pH (pH 12) associated with a low ionic strength helps to attain 30% solubility.

Very-low density lipoproteins (VLDL) of hen blood are the precursors of egg yolk LDL (Evans and Burley 1987). While VLDLs exist in the blood of immature pullets, their production in hen liver is considerably increased with sexual maturity due to estrogen secretion. VLDLs contain mainly two apoproteins, apo-VLDL II and apo-B. Apo-B is a 500 kDa protein constituted by only one subunit 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. 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. Apart from apovitellenin I, there is a lack of knowledge concerning the exact identification of the other apoproteins of LDL, and specially about the correspondence between LDLs and blood lipoproteins. Furthermore, gaps still exist in the knowledge of the exact maturation mechanism of apoprotein precursors. Recently, Jolivet et al. (2006) have confirmed the existence in egg yolk of apovitellenin I either as a monomer or a homodimer of disulfide-linked subunits, and this homodimer is resistant to SDS. The monomer has a molecular weight of 9.331 kDa in spite of a slight heterogeneity, and its amino acid sequence has been totally confirmed through the analysis of trypsin peptides. Hen apo-B is known to be cleaved into several protein fragments by cathepsin D. The structural homology of hen apo-B with human apoB-100 has been verified, and sequence alignment could be an interesting tool for further the characterization of hen apo-B.

4 LDL Extraction

Initially, techniques of ultracentrifugation have been favored because the low density of these assemblies (0.98) allowed an efficient separation. However, this type of technique is extremely time consuming (several days of centrifugation) and the yield of extraction is very low (only few grams of LDL), prohibiting a scale-up of the method. Recently a new method of separation of LDLs allowing an efficient extraction has been finalized that can be extrapolated to industrial dimensions (Moussa et al. 2002). The principle of separation is the addition of ammonium sulfate (40% saturation) to eliminate livetins from the solution of plasma extracted from egg yolk. After 1 hour under stirring at 4 °C, the blend is centrifuged at 10,000 g. The pellet obtained containing γ and β livetins is avoided. The supernatant is then dialyzed against distilled water for 20 hours at 4 °C with frequent changes of baths. The dialysis, desalting samples, provokes the aggregation of LDL, which precipitate. An orange-colored dough containing 97% LDL is obtained. The yield of extraction is about 60%. This process is protected by a patent (Anton et al. 2001). The purity of 97% obtained can be increased further by the use of gel filtration chromatography. Thirty grams of LDL extract are dispersed in 50 ml Tris-HCl 50 mM pH 7 buffer, and 12 ml of this dispersion are injected into the column of Ultrogel AcA 34 (Pall, East Hills, NY, USA). This chromatography permits separation of the LDL from contaminating livetins, hence to recovery of pure LDL. This last step is efficient for refining LDL purification in the laboratory, but scaling-up is not feasible because of the low quantities treatable by this technique.