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Efficient Analysis of Egg Yolk Proteins and Their Thermal Sensitivity Using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis under Reducing and Nonreducing Conditions

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The multiple functional properties of egg yolk are mostly influenced by its complex protein composition. The high lipid content of egg yolk as well as the low solubility of delipidated egg yolk lipoproteins make analysis by conventional chromatographic or electrophoretic techniques a difficult task. This work describes a method to profile egg yolk proteins after delipidation with acetone using sodium dodecyl sulfate polyacrylamide gel electrophoresis on precast 8−18% T polyacrylamide gradient gels. Twenty bands were obtained for the whole egg yolk profile with molecular weights ranging between 5 and 221 kDa. The bands were identified based on their molecular weight and by comparison with isolated egg yolk subfractions. The dissociation behavior under reducing and nonreducing conditions provided additionally helpful information for identification and characterization of the yolk proteins. The method presented is very well suited for assaying the thermal sensitivity of whole yolk and its components and thus for the characterization of heat treatment processes.

KEYWORDS: egg yolk; protein analysis; SDS−PAGE; thermal denaturation

INTRODUCTION

Egg yolk (EY) is a complex system showing valuable functional properties, notably in the formation and stabilization of gels and emulsions, and is thus applied for a large variety of industrial food products. EY is a natural oil-in-water emulsion containing 52% dry matter, of which fat represents about 65%, proteins about 31%, the remaining 4% being carbohydrates, vitamins, and minerals (*1*, *2*). All lipids of EY are associated with proteins to form lipoproteins, which are commonly classified in low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Structurally, EY is composed of 68% LDL, 16% HDL, 10% livetins, and 4% phosvitins (*3*). There is little agreement between different studies concerning the molecular weight (MW) of the apoproteins of LDL. There are reports of up to 18 polypeptides ranging mostly beween 15 and 180 kDa (*4*−*8*), and sometimes higher MWs up to 225−240 kDa (*9*−*12*). Bernardi and Cook (*13*) have shown that the HDL fraction of EY, also called lipovitellin, consists of two forms that they called α- and β-lipovitellin. Kurizaki et al. (*14*) studied the composition of the apoproteins of α- and β-lipovitellin (called apovitellin) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) and isolated eight polypeptides present in different amounts in α- and β-apovitellin. Recent studies have shown that the apoproteins of HDL consist of five major polypeptides between 32 and 105 kDa, this latter being the main one (*7*, *8*, *15*).

Livetins are lipid-free globular glycoproteins and represent about 10% of EY total solids. They are water soluble and correspond to blood serum proteins of the chicken. The mean MW of α-, β-, and γ-livetins were reported to be 80, 45, and 150 kDa, respectively, by Martin et al. (*16*) and their relative proportion in yolk to be 2:5:3 by Bernardi and Cook (*13*). The γ-livetins are immunoglobulins (IgY) and EY is an important source for polyclonal antibodies, if chicken have been immunized (*17*). Phosvitin is a phosphoprotein which represents about 10% of all lipid-free EY proteins. There is controversy regarding its MW as measured by SDS−PAGE, but it is generally accepted to be around 45 kDa.

Fresh EY contains insoluble structures called granules, which can be easily separated by centrifugation from the soluble phase, called plasma. The plasma of EY is made of 85% LDL and 15% livetins, while the granules are constituted of about 70% HDL, 16% phosvitin, and 12% granular LDL, which are highly similar to plasma LDL (*3*). Chromatographic techniques are less suitable than electrophoresis for the analysis of EY proteins, because of the high lipid content of EY and the low solubility of delipidated EY proteins. Nevertheless, a progress review on the applications of liquid chromatography to egg proteins analysis has been presented by Awade (*18*).

The SDS−PAGE technique has improved greatly over the years, particularly through the introduction of gradient gels, which allow a better resolution over a wider range of MW. Delipidation of EY lipoproteins prior to SDS−PAGE electro-

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phoresis has been shown to improve the electrophoretic mobility (*9*, *10*), particularly for large-sized lipoproteins (*6*). However, it appears that this advantage has been mostly neglected by other authors recently applying SDS−PAGE who have not delipidated the EY samples prior to analysis. Consequently, their protein profiles were deteriorated by considerable smearing and fairly pour resolution. Electrophoresis of EY proteins was generally conducted under reducing (R) conditions, for example by addition of 2-mercaptoethanol (2-ME) to the dissociation buffer (*5*, *7*−*9*, *11*, *19*, *20*). The induced dissociation of disulfide bridges and structural modifications (uncoiling) can lead to a modified electrophoretic mobility. However, there are only incomplete reports about the impact of different R conditions on the electrophoretic pattern of EY proteins. Although the superior proteomic tools as 2D-PAGE (*21*) and automated spot identification by matrix-assisted laser desorption−ionization mass spectrometry (MALDI-MS) or liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) (*22*) have become the most accepted techniques for protein identification they are not routineously applied for food protein identification or monitoring. The mere identification of EY proteins by their mobility in SDS−PAGE is complicated by the lack of commercially available purified protein standard, but defined EY fractions can be prepared based on difference of density and solubility according to a procedure published by McBee and Cotterill (*23*), based on earlier work from Bernardi and Cook (*13*).

EY is known for its ability to increase viscosity and form a gel upon heating (*2*, 24, *25*). Most of these rheological properties have been attributed to the LDL of egg yolk, which apoproteins unfold during heating and interact with each other to form hydrophobic bonds (*26*). But the role and thermal sensitivity of all other constituents of EY is not well understood. Modification of the EY proteins due to heat has been studied with electrophoretical technique but always using native PAGE (*25*, *27*−*30*). However, this technique does not allow the analysis of the LDL, which are too large to enter the gel (*23*, *27*, *29*), and the HDL resolve in only two diffuse zones. The resolution of bands in native PAGE is not equivalent to that obtained under dissociating conditions of SDS−PAGE (*28*, *29*). This lack of resolution as well as some confusion regarding the identification of the bands obtained in native-PAGE are the main reasons for discrepancies in the heat sensitivity of EY proteins (*25*, *27*−*30*). For example, β-livetin has been reported to be very sensitive to heat (*27*), moderately sensitive (*28*) or even highly heat stable (*25*, *29*). In a similar way, if isolated phosvitin has been shown to be thermally stable up to 100 °C (*31*), there are inconsistencies regarding the thermal sensitivity of phosvitin in egg yolk. It was shown to be either denatured around 69−72 °C (*28*) or not affected by heat (*29*, *30*).

We here describe the application of an enhanced resolution SDS−PAGE method for monitoring EY proteins using standardized commercially available gradient gels and isolated protein fractions from fresh egg yolk. Special attention has been paid to delipidation of proteins prior to analysis as well as to obtain differential profiles comparing the mobility obtained under fully and nonreducing (NR) conditions. This work was aimed at establishing a basic protocol for qualitative and quantitative analysis of EY proteins using SDS−PAGE in order to study the functional properties of egg yolk proteins and their thermal sensitivity.

MATERIALS AND METHODS

Material Sources. Tris(hydroxymethyl)-aminomethane, Tris(carboxyethyl)-phosphin (TCEP), urea, bromophenol blue, and aluminum nitrate were obtained from Merck (Darmstadt, Germany). Acetic acid and acetone were obtained from Neolab (Heidelberg, Germany). β-Lactoglobulin AB from bovine milk and sodium alginate were obtained from Sigma-Aldrich (Steinheim, Germany). γ-Livetin was obtained from ICN Biomedicals (Costa Mesa, CA). SDS and the dialysis tubing (Spectra/Por MWCO 6000−8000) were obtained from Serva (Heidelberg, Germany). Sodium chloride was obtained from Baker (Deventer, The Netherlands). ExcelGel SDS Gradient 8−18% polyacrylamide precast gels and PhastGel Blue R Coomassie tablets were obtained from Amersham Biosciences (Freiburg, Germany). Regenerated cellulose membranes RC-55 and medium-flow filter paper (No. 6041/2) were obtained from Schleicher & Schuell (Dassel, Germany).

Egg Yolk Preparation and Fractionation. Freshly laid eggs from "Lohman Tradition" hens were collected from the University's research farm (Thalhausen) and used within 48 h after collection. Each EY was manually separated from the white and carefully rolled on a paper towel to remove all albumen from the vitellin membrane. The membrane was then cut open with a scalpel blade and the content of the yolk collected and gently homogenized by stirring with a glass rod in a beaker cooled with ice. A sample of the fresh EY was kept as a reference for the whole EY sample (WEY), and the rest was fractionated according to the method described by McBee and Cotterill (*23*). The EY was first suspended (1:2 w/w) in an isotonic saline solution (0.17 M NaCl) and stirred gently for 1 h before centrifugation at 10 000 g for 45 min at 10 °C. The supernatant plasma was collected, and the sedimented granules were washed by resuspending them in twice their volume of 0.17 M NaCl solution. Both the plasma and washed granule fractions were then recentrifuged using the conditions described above. An aliquot of each fractions was delipidated using the method explained below and introduced to SDS−PAGE analysis.

Heating of the Egg Yolk. An aliquot of EY was diluted (1:5, w/w) in a 1% (w/v) NaCl solution, and the resulting suspension was heated in a water bath at 74 °C for 15 min. The solubility of proteins in the nontreated and in the heated samples was assessed using the method published by Morr et al. (*32*) with the following modification: Both nonheated and heated samples were diluted to 3.2 mg/mL with a 0.1 M glycine/NaOH buffer (pH 9; 0.56 M NaCl). After 1 h of equilibration at 20 °C under mild agitation, the samples were centrifuged at 14 000 g for 30 min in order to separate the insoluble proteins. The supernatant containing the soluble proteins was filtered on paper and a known amount of β-lactoglobulin (β-lg) dissolved in 0.17% NaCl (usually 1 mg β-lg to 5 mL of supernatant) was added to each sample prior to delipidation, as an internal standard for SDS−PAGE.

Preparation of the Phosvitin Fraction. The washed granules were dissociated in about 10 times their sample volume of 0.8 M NaCl solution, allowed to equilibrate for 1 h at 20 °C under mild agitation and dialyzed overnight against bi-distilled water. The retained desalted suspension was centrifuged at 20 000 g for 30 min, and the supernatant obtained, containing the phosvitin faction was delipidated with acetone (see procedure described below).

Fractionation of the Plasma Proteins. To an aliquot of the plasma, solid NaCl was added to finally contain 10% NaCl (w/v) and was then centrifuged at 120 000 g for 6 h at 10 °C (Ultracentrifuge Beckman L7-65, Germany). The floating layer of crude LDL was resuspended in a 10% NaCl solution and recentrifuged in the same conditions for further purification. The recovered LDL fraction was dialyzed overnight against bi-distilled water prior to delipidation (see procedure described below). The LDL-free subnatant of the plasma ultracentrifugation was transparent and very slightly yellow: it was recovered, diluted by addition of about half its volume of a 10% NaCl solution, and recentrifuged in the same conditions for further purification. The clear solution containing the α- and β-livetins (*23*) was dialyzed overnight against bi-distilled water prior to delipidation. The sticky translucent sedimented pellet of γ-livetin was resuspended in an excess of 10% NaCl solution and recentrifuged. The recovered γ-livetin pellet was dialyzed overnight against bi-distilled water prior to delipidation.

The soluble plasma proteins were additionally isolated by precipitation of the lipoproteins with sodium alginate, based on the optimal conditions identified by Hatta et al. (*33*). One volume of pure fresh EY was diluted in 7 volumes of 0.1% sodium alginate solution in water. The pH was adjusted to 6.1 by addition of 0.1 N HCl and the suspension

allowed to equilibrate for 1 h at 20 °C before centrifuging it at 10 000 g for 15 min. A small amount of the recovered clear supernatant (ca. 5 mL) were prepared with acetone as described below.

Delipidation of EY Protein Fractions. Samples of either whole EY or isolated fractions were mixed with excess acetone at 4 °C (typically 10 volumes of acetone for 1 volume of aqueous solution). The combined lipid extraction/protein precipitation was carried out for 1 h at 4 °C under mild agitation. The protein precipitate was then collected on a 0.45 μm regenerated cellulose membrane. The membrane carrying the protein powder deposit was allowed to dry under a ventilation hood while being flushed with nitrogen to limit the contact with oxygen. The dried protein fractions were stored frozen for up to 8 weeks at −40 °C in a glass jar flushed with nitrogen and analyzed according to the procedure described below.

SDS−PAGE. Commercially available "EXCEL" gradient gels (8−18% T) were used for all experiments. The powdered protein samples recovered from the delipidation with acetone were dissolved in a 0.05 M Tris−acetate dissociation buffer containing 1% (w/v) SDS, 6 M urea, and 0.05 mg/mL bromophenol blue (tracker dye), at a concentration of 4 mg of powder per mL of buffer. Samples were gently shaken for a period of at least 12 h to ensure complete dissolution, then heated in boiling water for 5 min and allowed to cool at room temperature. For samples treated under R conditions, 10 μL of a freshly prepared 20% aqueous TCEP solution was added per milliliter of sample. Samples were then centrifuged at 10 000 g for 5 min, and 5 μL of the supernatant was transferred onto 7 × 4 mm^2 cellulose acetate membrane filter pieces, which were placed closely aside each other at a distance of 1 cm from the cathode. Inclusion of urea as well as sample application via cellulose acetate membranes greatly improved band sharpness of egg proteins as was experienced during SDS−PAGE analysis of whey proteins and caseins (*34*). Electrophoretic separation was performed in a Multiphor II system (Pharmacia Biotech, Amersham Biosciences, Uppsala, Sweden) applying a maximum voltage of 600 V and a current gradient increasing from 15−50 mA. At the end of the run, when the bromophenol blue marker has approached the anode strip by 3 mm, proteins were stained without fixation using PhastGel Blue R in 10% acetic acid. For the detection of phosvitin, which is not stained by Coomassie blue (*35*), 0.1 M aluminum nitrate was added to the staining solution. Destaining was performed with 10% acetic acid solution. MW were determined by a MW standard (SigmaMarker Wide Molecular Weight Range, Sigma-Aldrich, Steinheim, Germany) using image analysis (ImageMaster 1D Elite ver. 4.00, Amersham Pharmacia Biotech, Uppsala, Sweden). As a consequence of the lack of appropriate pure egg protein standards or extinction coefficients for Coomassie staining of individual egg proteins, relative protein composition of egg yolk fractions were estimated from band volumes (pixel intensity × band area). Protein composition was expressed as relative volumes, i.e., volume of a single band related to the sum of all band volumes of the lane.

RESULTS

In this chapter, the results of EY protein profiling by SDS electrophoresis on ready-made high-resolution polyacrylamide gradient gels are reported. The MWs reported in this study were averaged from three repetitive determinations ($n = 3$) carried out by the same person on three different days using the same electrophoresis system to analyze whole EY from different eggs of the same flock of hen. The average relative standard deviation was 2.8% of the measured MW and the maximum standard deviation was observed at 4% for a band with MW 122 kDa. For clarity of result presentation, the discussion of the results is compiled in detail in the "discussion" chapter.

SDS−PAGE Profile of EY, Granules, and Plasma Proteins. The complexity of the whole EY as well as of the granules and plasma fractions obtained after SDS−PAGE is shown in **Figure 1**. There are no overlapping band between polypeptides of granules and plasma, which facilitates their identification within the total EY profile containing 20 bands having a MW ranging from 5 to 221 kDa (**Figure 1**, lanes a and a′). Five out of these twenty bands could clearly be attributed to proteins of the granules (i.e., HDL apoproteins and phosvitin, see **Figure 1**, lanes c and c′) with apparent MW ranging between 110 and 31 kDa. It should however be noticed that the gel in Figure 1 is stained without aluminum nitrate so that phosvitin is not stained. The remaining 15 bands were clearly located in the EY plasma protein profile (i.e., LDL apoproteins and livetins, see **Figure 1**, lanes b and b′, and **Figure 2**, lane a). The bands corresponding to the granular LDL (LDL_g) did not appear in the granule profile, which is probably due to the low concentration of LDL_g in granules. However, a faint band with an apparent MW of 17 kDa (corresponding to the Apovitellenin I) was visible in the granule profile (**Figure 1**, lanes c and c′).

Because of the lack of appropriate protein standards, we used the relative volume of individual bands in relation to the total volume of the summarized protein population of the whole lane for monitoring compositional effects. Visual observations indicated that the color uptake of phosvitin (see the 59-kDa band on **Figure 1**) is much lower than that of the other EY proteins, which confirmed earlier reports of this problem (*35*). Despite

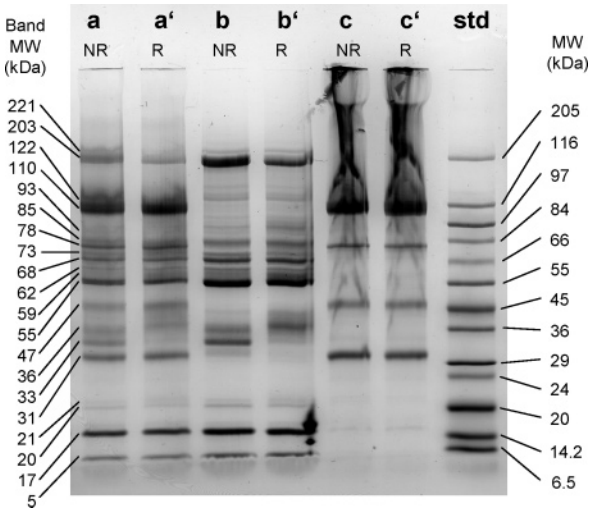

Figure 1. SDS–PAGE profile of delipidated proteins of EY fractions in NR and Rconditions: (**a** and **a′**) whole EY; (**b** and **b′**) EY plasma; (**c** and **c′**) EY granule; (std) MW standard.

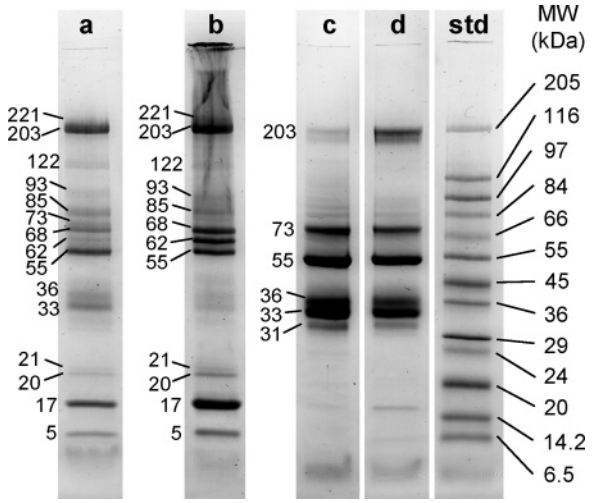

Figure 2. SDS–PAGE profile of delipidated proteins of hen's EY fractions in NR conditions: (**a**) EY plasma; (**b**) LDL fraction of plasma; (**c**) α- and β-livetin fraction of plasma; (**d**) supernatant after sodium alginate precipitation of EY. Std = MW standard.

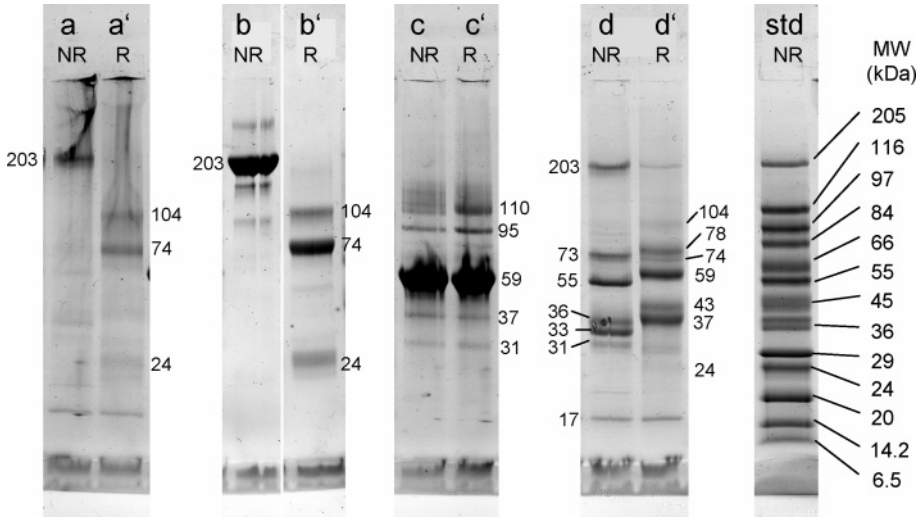

Figure 3. SDS–PAGE profile of EY protein fractions in NR and R conditions: (**a** and **a′**) prepared γ-livetin; (**b** and **b′**) commercial γ-livetin; (**c** and **c′**) prepared phosvitin; (**d** and **d′**) fraction containing both α- and β-livetin. Std = MW standard.

this deviating property, phosvitin was taken into account for the relative quantification. However, the band corresponding to phosvitin represents only 1.3% of the total volume of the EY profile, which does not reflect the amount of that protein in EY (ca. 10% w/w of total protein).

SDS−PAGE Profile of EY Protein Subfractions and Impact of R Conditions. The difference between profiles obtained under R and NR conditions for plasma, granules, and EY is shown in **Figure 1** and for α-, β-, and γ-livetin and phosvitin subfractions in **Figure 3** and referred to in the following paragraphs.

EY Livetins. Under NR conditions, γ-livetin gives one band with an apparent MW measured at about 203 kDa (see **Figure 3**, lane a, for γ-livetin isolated using McBee and Cotterill method (*23*) and lane b for commercial γ-livetin). In R conditions, this band dissociates in three bands measured at 104, 74, and 24 kDa, with the 74 kDa band representing 60−70% of the volume of these bands (**Figure 3**, lanes a′ and b′). The structure of γ-livetin has been studied in detail because of its immunological properties (*1*): it is known as the immunoglobulin Y (IgY), which is made of two symmetrical heavy chains (MW 65−70 kDa) and two light chains (MW 21−25 kDa) all linked by disulfide bridges. The bands observed on **Figure 3** correspond to the heavy (74 kDa) and light (24 kDa) polypeptidic chains as well as the association of one light and one heavy chain (measured at 104 kDa).

The EY fractionation technique applied in this study resulted in incomplete separation of α- and β-livetins only. Unfortunately there are no purified standards commercially available for these proteins, which renders their formal identification particularly difficult. The isolated fraction containing both α- and β-livetins clearly shows the presence of 4 polypeptides having MW of 73, 55, 36, and 33 kDa (see **Figure 2**, lane c). Under R conditions, a shift of the position of all four bands corresponding to the α- and β-livetins is observed (**Figure 3**, lanes d and d′). This shift corresponds to an increase of the apparent MW of about 5 kDa. This can be interpreted as a slight increase of the hydrodynamic volume of the protein after reduction. This effect generally occurs with proteins of globular structure, which unfold after reduction and adopt a more loose three-dimensional conformation, leading to a slower migration through the gel compared to the original nonreduced structure.

The pattern obtained for soluble proteins of EY after precipitation of lipoproteins by addition of sodium alginate confirms the presence of the 5 major polypeptides corresponding to the livetins (**Figure 2**, lane d).

LDL Apoproteins (Also Called Apovitellenins). The pattern of EY plasma (**Figure 1**, lane b) contains both apovitellenins and livetins. On the basis of observation of the main bands obtained for the isolated LDL fraction (**Figure 2**, lane b) and the bands associated to the various livetins, we identified 12 bands containing an apoprotein of LDL: bands 221, 203, 122, 93, 85, 68, 62, 55, 21, 20, 17, and 5 kDa. It should be noted that bands at 203 and 55 kDa not only contain an apovitellenin but also the γ-livetin and part of the α-livetin, respectively. The five most important in terms of quantity are the bands with MW of 203, 122, 68, 55, and 17 kDa. Observation of the plasma protein profile in R conditions (**Figure 1**, lane b′) revealed that the bands corresponding to LDL apoproteins are not affected by R conditions. These results partly confirm measurements published earlier, but the lack of agreement between authors regarding the name and MW of bands obtained from SDS−PAGE of EY proteins makes it difficult to compare results from different authors. This was highlighted again by Mine (*9*) and will be discussed in the "Discussion" chapter.

HDL Apoproteins (Also Called Apovitellins). The four major polypeptides corresponding to the apovitellins are clearly visible in **Figure 1** (lanes c and c′) although some "smearing" in the high MW region occurred, which was attributed to incomplete delipidation of the HDL of granules. The MW of the apovitellins were measured at 110, 78, 47, and 31 kDa. These values are consistent with those reported by Kurizaki et al. (*14*), later confirmed by Itoh et al. (*10*), and more recently Anton et al. (*15*) and LeDenmat et al. (*7*). The MW of HDL apoproteins has been reported with more consistency between authors than that of LDL apoproteins. In **Table 1**, we used the numbering given by Kurizaki et al. (*14*) to name the four major apovitellins. It appears that R conditions do not affect the SDS−PAGE profile of any of the four apovitellins (**Figure 1**, lanes c and c′). Furthermore, it appeared that protomers or undissociated oligomers with apparent MW at or above the exclusion limit of the polyacrylamide gel matrix (i.e., >400 kDa) seemed to form a precipitate on the gel surface and to prevent a constant ion transport into the gel, leading to an increased "smearing" effect (**Figure 4**, lanes a and c). To maintain an acceptable reproducibility it was therefore decided to analyze only the supernatant of completely soluble proteins after centrifugation instead of the crude extract.

Table 1. Relative Volume (RV) and Standard Deviation (SD) for the RV of the 20 Protein Bands Observed in the SDS–PAGE Analysis of Delipidated EY Proteins in NR Conditions ($n = 5$)

MW (kDa)	relative band volume (%)	SD (%)	protein name
221	2.9	1.3	apovitellenin VIa[a]
203	8.7	3.4	γ-livetin + apovitellenin VI[b]
122	7.7	2.5	apovitellenin Va[b]
110	21.4	8.3	apovitellin 3 + 4[c]
93	0.6	0.3	apovitellenin Vb[a]
85	1.6	0.7	apovitellenin V[b]
78	4.5	1.8	apovitellin 5 + 6[c]
73	1.5	0.7	α-livetin
68	3.6	1.4	apovitellenin IV[b]
62	1.0	0.4	apovitellenin IIIa[b]
59	1.3	0.6	Phosvitin
55	10.7	1.3	α-livetin/apovitellenin III[b]
47	4.8	1.8	apovitellin 7[c]
36	2.9	0.8	β-livetin
33	4.8	1.1	β-livetin
31	7.6	1.6	apovitellin 8[c]
21	0.3	0.1	apovitellenin IIa[a]
20	1.2	0.5	apovitellenin II[b]
17	9.6	2.1	apovitellenin I[b]
5	3.3	0.6	apolipoprotein CII[d]

[a] LDL apoproteins named by the authors, based on Burley and Sleigh (*38*). [b] LDL apoproteins named after Burley and Sleigh (*38*). [c] HDL apoproteins named after Kurizaki et al. (*14*). [d] LDL apoproteins named after Bengtsson et al. (*39*).

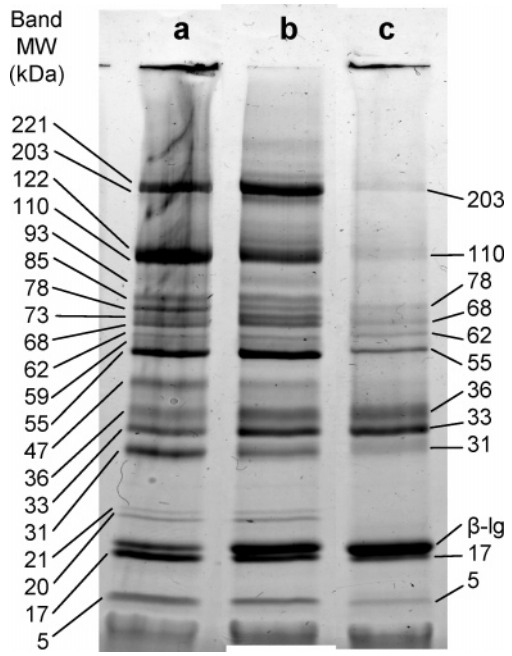

Figure 4. Electrophoretic profile of the (**a**) total and (**b**) soluble proteins of native egg yolk as well as (**c**) the soluble proteins of heat-treated EY (heating at 74 °C for 15 min) in NR conditions.

Phosvitin. The isolated phosvitin fraction could be stained with Coomassie blue only upon addition of 0.1 M aluminum nitrate, which was used to stain the gel presented in **Figure 3** only. It showed a single band at 59 kDa (see **Figure** 3, lanes c and c′), in the same position as that observed with commercially purified phosvitin (data not shown). The electrophoretic mobility of phosvitin was not affected by R conditions. This reflects the absence of cysteine residues in the molecule and thus the lack of formation of intra- or intermolecular disulfide bridges (*2*).

Impact of a Thermal Treatment on EY Protein Solubility. The loss of solubility of EY proteins during thermal treatment is shown in **Figure 4**. There are minimal differences between the profiles obtained for the total proteins (lane a) and soluble proteins present in the supernatant of saline buffer extraction (lane b). This reflects the fact that most proteins are soluble in the saline buffer used for the extraction (pH 9, 0.56 M NaCl) as long as they are in their native state. This is important in order to validate the correlation between proteins' insolubility and their denatured state. Comparing the profile obtained for the native sample (lane b) to that obtained after thermal denaturation (lane c), it can be seen that the EY proteins remaining soluble after heating (74 °C, 15 min) can be divided into three classes. First, the heat-stable proteins at MW of 36 and 33 kDa, which profile remains unchanged. Second, some moderately heat stable proteins, which are partially affected by the heat treatment occurring at MW 203, 110, 78, 68, 62, 55, 31, 17, and 5 kDa, respectively. And finally thermolabile proteins, which completely lose their solubility when heated at 74 °C for 15 min: 221, 122, 93, 85, 73, 47, 21, and 20 kDa.

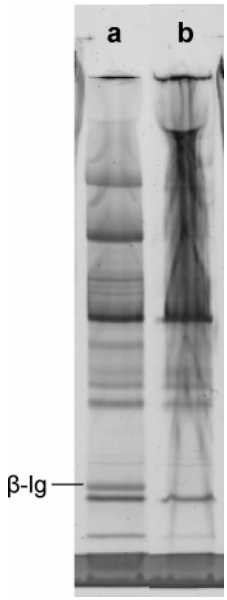

Figure 5. Electrophoretic profile of the total proteins of native egg yolk (**a**) after delipidation with acetone and (**b**) without any prior delipidation (β-lg was added after delipidation only in the delipidated sample).

The differential thermal sensitivity of egg yolk proteins is a characteristic that is expected to have a particularly important influence on their functional properties. This matter will be dealt with in a subsequent publication.

DISCUSSION

Methodological Aspects of EY Protein Profiling by SDS−PAGE. Because of the high lipid content of EY, delipidation of the protein samples prior to electrophoresis is one of the essential measures when attempting to analyze EY proteins at high resolution. Among the few studies reporting profiles of delipidated egg yolk proteins, only one was concerned with whole EY (*10*), whereas the others were limited to the LDL apoproteins (*4*, *6*, *9*). Throughout all these studies, a mixture of chloroform and methanol in a 2:1 ratio was used for delipidation. In this study we have for the first time applied a combined protein delipidation/precipitation procedure with acetone prior to SDS−PAGE analysis. This resulted in a decreased "smearing" effect and an improved resolution of the protein bands, as can be seen on **Figure 5**.

A linear polyacrylamide gradient from 8 to 18% is required to profile the broad MW spectrum of proteins present in egg yolk (5−221 kDa) at high resolution. The controlled manufac-

turing of these gels provides a high-quality reproducible separation.

In contrast to other studies published on EY protein analysis, no alcohol was included in the staining solution. Thus the diffusion and washing out of lipophilic proteins precipitated in dilute acetic acid was minimized, which favors reliable quantification of EY proteins. However, staining of phosvitin required the addition of 0.1 M aluminum nitrate to the staining solution. We have noticed that by this addition concomitant precipitation of the PhastGel Blue R dye occurred, which impaired the staining of the residual proteins. For this reason, it was decided not to include aluminum nitrate for all the experiments, except for the gel presented in **Figure 3**. A double-staining procedure (i.e., PhastGel Blue R with and without aluminum nitrate) might be the most appropriate procedure when phosvitin staining is required.

Effect of R Conditions on Apovitellins and Livetins. To our knowlegde, the impact of R conditions on the electrophoretic mobility of the HDL apoproteins has never been reported before, even though those proteins are expected to form intra- or intermolecular disulfide bridges as they contain 1.6−2 mol % cysteine residues (*2*). This indicates that the thiol groups of the cysteine are not accessible for inter or intra disulfide cross-linking reactions.

R conditions also had no impact on the SDS−PAGE profile of the LDL apoproteins. However, apovitellenin I is reported to be a homodimer consisting of two 9-kDa protomer linked by a disulfide bond (*4*). Dissociation of this dimer by addition of 2-ME was reported to be uneffective; only performic acid oxidation resulted in complete dissociation (*4*, *6*). Although the R potential of TCEP is superior to 2-ME by magnitudes, we also did not succeed to dissociate the dimer even in the presence of 6 mol/L urea as an unfolding additive in the sample buffer. This fact strongly suggests that the intermolecular disulfide bridge is perfectly protected in a hydrophobic environment, which becomes accessible for oxidative cleavage only upon distortion by concentrated formic acid.

Apovitellenins II, III, IV, and VI contain 4.9, 0.4, 0.31, and 0.7 mol % cysteine residues, respectively (*2*). Our results indicate that, in these LDL apoproteins, the thiol groups of the cysteine are not accessible for intermolecular (or even possibly intramolecular) disulfide linking as their hydrodynamic volume and thus the electrophoretic migration of the SDS complex is not influenced by reduction.

To our knowledge Burley and Vadehra (*36*) are the only ones, prior to this study, reporting on the behavior of livetins on SDS−PAGE in NR conditions, whereas the direct comparison with R conditions was never reported. Livetins with apparent MWs of 73, 55, 36, and 33 kDa are known to be rich in cysteine (*3*) so that the formation of disulfide bonds can be expected. The slight shifts in their MW observed upon R conditions suggests that some intramolecular disulfide bonds are present in these proteins, which contribute to their compact globular structure.

Variability of MW Reported for EY Proteins. The controversy between the data for the apparent molecular weight obtained in this study to previously published data will be discussed in the following paragraph, whereas agreeing results have already been highlighted in the Results section.

Livetins. The original work from Martin et al. (*16*) gave a MW of 80 and 45 kDa for the α- and β-livetins, respectively, while Burley and Vadehra (*36*) later measured 70 and 42 kDa for these proteins under NR conditions. On the basis of these results, we assume that the lighter polypeptides at 33 and 36 kDa (**Figure 2**, lane c) correspond to β-livetins and that the heavier ones at 55 and 73 kDa correspond to the α-livetins. Two distinct bands are also clearly separated in the α-livetin profile obtained with SDS−PAGE by Burley and Vadehra (*36*), which is consistent with our observations. By use of isoelectric focusing, Ternes (*37*) reported at least 25 bands for livetins focusing between pH 4.3 and 5.5, which suggests that the α- and β-livetin fractions identified by Martin et al. (*16*) are far more heterogeneous than observed in SDS−PAGE.

LDL Apoproteins. Significant differences have been previously reported for the molecular weights of apovitellenins, which can partly be explained by the fact that some authors used delipidated proteins (*4*, *6*, *9*, *10*, *38*), whereas the others did not remove the lipids (*5*, *7*, *8*, *11*, *12*, *15*). Some inconsistencies were also noticed between subsequent publications from the same author. This was already highlighted by Mine (*9*), who reported across different publications a total of 9 apoproteins in LDL with MW of 225, 170, 82, 66, 60, 48, 40, 34, and 19 kDa in either delipidated (*9*) or nondelipidated samples (*11*, *12*). However, at least two of the bands we observed (i.e., at 122 and 5 kDa) were not mentioned in these studies.

Yamauchi et al. (*6*) refer to at least 18 polypeptides in EY LDL fraction and concluded that delipidation of the LDL prior to migration allowed large apoproteins to enter the gel while they were absent of the profile obtained without delipidation. However, their data were restricted to the apparent MW for five major apoproteins (135, 82, 71, 62, and 16 kDa) and three minor ones (150, 20, and 10 kDa), whereas the high molecular weight polypeptides (>150 kDa) were neglected.

In other recent studies (*5*, *7*, *8*), in which the lipids of LDL were not removed prior to SDS−PAGE analysis, the five major LDL proteins were reported to have MWs of about 140, 81, 73, 60, and 18 kDa, which is always slightly higher than what was measured in the present work after delipidation. However, they did not investigate the higher MW range (i.e., 203−221 kDa) as well as the lower range (5 kDa).

Burley and Sleigh (*38*) published a total apoprotein pattern of delipidated LDL, associating roman numbering for each of the major polypeptides identified (i.e., apovitellenin I−VI). As the observations for these proteins made in our study are highly congruent to their results, we decided to adopt their nomenclature (see **Table 1**: MWs 21, 93, and 221 kDa). For apovitellenin VI, the authors measured an apparent MW of 170 kDa. From comparing the relative intensities of the bands in the apoprotein pattern, we assume that the band migrating at 203 KDa in our experiments corresponds to apovitellenin VI.

In the present study, 12 apoproteins are resolved in the lane profile of LDL. It covers the high MW apoproteins (203−221 kDa), which are often overlooked because they are too large to enter the gel, particularly when the lipoproteins are not delipidated (*5*−*8*). Strikingly, even the smallest LDL protein, measured at 5 kDa in this study, is detected simultaneously, which corresponds to the apolipoprotein CII. To our knowledge, this small protein was only reported in one previous study (*39*), since it migrates within the dye tracker in the front in most published studies. The enhanced resolution of the EY profiles in gradient gels is clearly demonstrated with the apolipoproteins.

HDL Apoproteins. In the work of Kurisaki et al. (*14*) according to which we named the HDL apoproteins (**Table 1**), the authors mentioned a minor protein at 140 kDa (called apovitellin 1 and 2), which was not detected on our gel. This minor fraction may be a heterodimer made of one 110-kDa and one 31-kDa polypeptide and is dissociated under the conditions we used in this study.

Phosvitin. Abe et al. (*40*) have reported phosvitin to dissociate in several components with MW ranging between 18.5 and 60 kDa, with a major component at 45 kDa. They concluded that phosvitin was composed of several polypeptides that interacts or polymerizes in aqueous solution to form larger MW aggregates. Recent studies using SDS−PAGE have shown a single band with an apparent MW of 35−36 kDa for both commercial and laboratory-prepared phosvitin (*20*, *41*). In our study, however, we measured a single band at 59 kDa for phosvitin prepared in our lab (**Figure 3**, lanes c and c′) as well as for the commercially purified standard (data not shown). This difference might arise from the urea present in our sample buffer, which aids for complete unfolding of the (reduced) protein.

Modification of EY Protein Solubility during Heating. This work has confirmed that virtually all egg EY proteins in their native state are soluble in a buffer containing 0.56 M NaCl at a pH of 9. Only a small proportion of proteins from granules were found to be absent of the soluble protein profile of native EY proteins. Upon heating, proteins are denatured and thus become insoluble in the saline buffer. Only β-livetins seem to be unaffected by a heating of 15 min at 74 °C, which confirms earlier observations made using native PAGE (*29*). Proteins showing partial denaturation comprise three of the four major HDL apoproteins, whereas the thermolabile proteins are mostly LDL apoproteins and γ-livetin. Evidences of the greater heat sensitivity of plasma proteins over granule proteins were already published (*29*) although they were never reported in such details. It should be noted that the heat treatment was carried out on a suspension having a relatively low ionic strength (0.17 M NaCl) so that HDL and phosvitin were associated in the form of granules. This has been shown to contribute to the heat stability of granule proteins (*30*). Moreover, interactions during heating between different proteins within EY are very likely and lead to the formation of heterogeneous protein aggregates. Some heat-resistant proteins may therefore be seen as denatured because they lose their solubility by co-aggregation with denatured heat-sensitive proteins. The technique used allows measurement of the loss of solubility of EY proteins when heated in the presence of one-another. Their behavior may differ widely from their behavior when they are taken in isolation (*2*).

ABBREVIATIONS USED

EY, egg yolk; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MW, molecular weight; 2-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCEP, Tris(carboxyethyl)-phosphin; IgY, immunoglobuline Y; R, reducing; NR, nonreducing.

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