JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Impact of a Treatment with Phospholipase A_2 on the Physicochemical Properties of Hen Egg Yolk

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Changes in physicochemical properties of egg yolk were investigated after a treatment with phospholipase A_2 (PLA_2), where phospholipids are converted in lyso-phospholipids. Protein solubility and protein denaturation before and after modification by PLA_2 was monitored as well as the functionality of egg yolk by means of interfacial tension. Enzymatic treatment showed a significant impact on the properties of egg yolk with regard to protein solubility and denaturation behavior. To gain a closer insight, egg yolk was separated in its water-soluble fraction called plasma and the insoluble granules. Both fractions were separately modified by PLA_2. The granule fraction shows a higher protein solubility, and the plasma proteins show very high heat stability after modification by PLA_2. Hypotheses regarding related changes in the low-density lipoprotein (LDL) particles are discussed. Results suggest that significant differences in the functional properties of untreated and PLA_2-modified egg yolk do not primarily result from the existence of lyso-phospholipids but from structural changes in egg yolk granules and LDL particles.

KEYWORDS: Egg yolk; PLA_2; protein denaturation; LDL; granules; plasma; solubility

INTRODUCTION

Hen egg yolk is an excellent food emulsifier and therefore widely used in many applications ranging from bakery to production of cold sauces and salad dressings. Fresh egg yolk contains about 48−50% total dry matter, of which 80% is the water-soluble plasma fraction and 20% is present in the form of insoluble granules. Plasma contains 85% low-density lipoproteins (LDLs) and globular glycoproteins known as α-, β-, and γ-livetins. LDL, which apoproteins are called lipovitellenins, is the main constituent of egg yolk. It represents about 68% of its total dry matter (*1*). Granules contain 16% phosvitin, a phosphoprotein, and 70% high-density lipoprotein (HDL), which apoproteins are called lipovitellins. LDL (12%) can be found in insoluble granule aggregates. The complex between HDL and phosvitin is held together by phosphocalcic bridges. Granules can be dissociated when increasing the ionic strength to values above 0.3 M because of the rupture of phosphocalcic bridges (*2, 3*).

Industrial egg yolk has to be pasteurized to ensure microbiological safety. However, only low temperature−time combinations can be applied for pasteurization because of egg yolk's high total dry matter, high protein content, and tendency to block heat exchangers because of heavy fouling. Nowadays, phospholipase A_2 (PLA_2) is used in the egg industry to improve the heat stability of egg yolk and enhance its functionality (*4*). PLA_2 cuts the acyl group in position 2 of the triglyceride and converts the phospholipids into lyso-phospholipids, which show a higher solubility in water and therefore improve the emulsifying properties of egg yolk in o/w emulsions. Such modified egg yolk does not gel even under severe heat treatment and can therefore be pasteurized at higher temperatures. There is, however, hardly any report about the properties of egg yolk or egg yolk fractions that were enzymatically modified by PLA_2. One possible mechanism for the improved heat stability of egg yolk through PLA_2 treatment is suggested by Mine (*5*), but only changes of LDL particles and not the impact of phospholipase treatment on whole egg yolk are discussed. Modifications of LDL particles as a result of the treatment with phospholipase A_2 was reported for LDL of human blood (*6–8*). In general, the models on the structure of human blood LDL are transferred to hen egg yolk LDL, and the lipid core model is assumed to be essentially correct. Only minor differences in structures of LDL apoproteins from human and hen's egg are considered (*9*).

The emulsifying properties of egg yolk are closely related to the protein fraction. LDL is considered to be the main contributor to the emulsifying properties of egg yolk (*10*). Inside LDL, mainly proteins but also phospholipids are molecularly responsible for the emulsifying activity. Still little is known, however, about the structural and functional behavior of LDL and the changes of the physicochemical properties of egg yolk as a result of enzymatic treatment by PLA_2. The modification is supposed to influence its functionality in a wide range of properties. It is essential to characterize the properties of PLA_2-modified egg yolk for a better understanding and to be able to steer the reaction in a more targeted manner.

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10.1021/jf703641e CCC: $40.75
Published on Web 05/20/2008

The objective of this study was therefore to characterize egg yolk treated with PLA_2 in comparison to natural egg yolk. Discussing differences between fractions of egg yolk (plasma and granules), untreated and treated with PLA_2, should provide a better understanding of the mechanisms leading to high heat stability and improved emulsifying properties.

MATERIALS AND METHODS

Preparation of Egg Yolk Suspension 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 egg yolk was manually separated from the white and carefully rolled on a paper towel to remove all albumen from the vitellin membrane to ensure that no egg white protein was mixed up with the egg yolk. The membrane was then cut with a scalpel blade, the content of the yolk collected and gently homogenized by stirring with a glass rod in a beaker cooled with ice. One part of the fresh egg yolk was kept, and the rest was fractionated into its main fractions according to the method described by McBee and Cotterill (*11*). The egg yolk was at first diluted (1:2, w/w) in an isotonic sodium chloride solution (0.17 M NaCl) and stirred gently for 1 h before centrifugation at 10000*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 in 0.17 M NaCl solution. Both the plasma and washed granule fraction were then recentrifuged using the conditions described above.

Enzymatic Treatment. An aliquot of each suspension (whole egg yolk, plasma, and granules) was incubated with PLA_2 (trade name: Lecitase 10L, Novozymes, Bagsvaerd, Denmark) at 55 °C for 3 h. At the beginning of incubation, the pH of suspensions was 6.10 ± 0.03. After incubation, the pH was 5.85, 5.70, and 5.95 for egg yolk, plasma, and granules, respectively.

The concentration of enzyme was calculated on a total dry matter basis for the whole egg yolk, plasma, and granule fractions. Egg yolk was incubated at a total dry matter of 44%, diluted in an isotonic salt solution containing 0.17 M NaCl; i.e., 0.4 μL of enzyme/g of egg yolk suspension (lecitase activity of 10.000 units/mL) was added. Plasma had a total dry matter of 22% after centrifugation; therefore, 0.2 μL of enzyme/g of plasma solution was added. The granules were diluted after centrifugation to obtain the same total dry matter as plasma, and the same concentration of enzyme was added. After incubation, egg yolk, plasma, and granule suspensions were immediately cooled down in an ice−water bath and stored at 4 °C.

Chemical Analysis. Total protein content of egg yolk, plasma, and granule fraction was measured using a nitrogen gas analyzer system (model FP-528, LECO, Mönchengladbach, Germany). Total crude protein is calculated from the nitrogen content of the feed material. A conversion factor of 6.25 was used for all egg yolk samples (*12*). Results were expressed in grams of proteins/100 g of dried matter.

Lipid content of each sample was determined after extraction by chloroform/methanol (1:1, v/v) using a method adapted from Hallermayer (*13*). To 1 g of sample, 14 mL of chloroform/methanol were added and mixed before centrifugation at 4000*g* during 5 min at 4 °C. The upper organic phase was collected. The bottom phase was treated again as described above as a washing step. The upper phases are combined, diluted with 14 mL of 0.58% NaCl solution, and left at room temperature for 1 h. The bottom phase containing lipids and chloroform were carefully decanted, and solvents were evaporated with nitrogen. Lipid content was estimated by weighing lipid extract after solvent evaporation. Results were expressed in grams of lipids/100 g of dried matter.

Extracted lipids were weighed in vials and were dissolved in a mixture of *n*-hexane/2-propanol (80:20) containing 0.01% butylhydroxytoluene (BHT) and directly used for phospholipid determination.

Phospholipids were analyzed by high-performance liquid chromatography (HPLC, Merck-Hitachi, Germany) equipped with a light-scattering detector (PL-ELS 1000, Polymer Laboratories, Darmstadt, Germany) after a method described in DGF Standard Method F-I 6a(07).

Separation was achieved using a Li Chrospher 100 Diol 5 type column (250 × 4 mm) with precolumn (20 × 4 m) packed with spherical microparticles (5 μm) of Diol-bounded silica.

Eluent A was a mixture of *n*-hexane, 2-propanol, acetic acid, and triethylamine (81.42:17:1.5:0.08). Eluent B was a mixture of 2-propanol, water, acetic acid, and triethylamine (84.42:14:1.5:0.08) (HPLC grade, LiChrosolv Merck, Germany).

The gradient elution program started with 95% eluent A and 5% eluent B. The flow rate was 1 mL/min. A total of 20 μL of the sample and standard solutions dissolved into *n*-hexane/2-propanol (80:20) was injected in the column at ambient temperature. The instrument parameters used were nitrogen flow, 1.0 L/min; detector nebulizer temperature, 50 °C; and evaporation temperature, 120 °C.

Two phospholipids classes were found in notable amounts, phosphaditylcholine (PC) and phosphaditylethanolamine (PE). Results were expressed in grams of phospholipids/100 g of dried matter.

The amount of free fatty acids (FFA) was determined using an enzymatic color test (NEFA C, Wako Chemicals, Neuss, Germany). The concentration of FFA was calculated for oleic acid (MW of 282 g/mol).

Degree of Hydrolysis. Degree of hydrolysis was calculated on the one hand by the reduction of PC and PE and, on the other hand, by the amount of FFA after PLA_2 treatment. The calculation by FFA was performed to verify, if this could be an appropriate approach to determine, the degree of hydrolysis instead of the more complex and time-consuming determination of PL composition.

The reduction of PC and PE is calculated as given in eq 1

$$\text{degree of hydrolysis} = \frac{(\text{PC}+\text{PE})_{\text{untreated}} - (\text{PC} + \text{PE})_{\text{PLA}_2\text{-treated}}}{(\text{PC} + \text{PE})_{\text{untreated}}} \times 100\% \quad (1)$$

For the calculation based on FFA, the amounts of PC, PE, and FFA were expressed in millimoles/100 g of dried matter. The molar concentration of FFA was determined by the molecular weight of oleic acid (282 g/mol).

Assuming that 1 mol of FFA is released by 1 mol of PC or PE, the degree of hydrolysis can be calculated as follows (eq 2).

$$\text{degree of hydrolysis} = \frac{\text{FFA}_{\text{PLA}_2\text{-treated}} - \text{FFA}_{\text{untreated}}}{(\text{PC} + \text{PE})_{\text{untreated}}} \times 100\% \quad (2)$$

Thermal Treatment of Egg Yolk, Plasma, and Granules. Heat treatment of all suspensions was carried out at a protein content of 20 mg/mL. All native suspensions were transferred into thin, 20 cm long stainless-steel tubes, with an inner diameter of 16 mm and a wall thickness of 2 mm to ensure rapid heating and cooling profiles. Each tube holds a volume of about 45 mL. Suspensions not treated with PLA_2 were only heated at 74 °C, whereas suspensions treated with enzyme were heated at 74 and 84 °C for various holding times ranging from 2 to 180 min. The tubes are heated from room temperature to 74 or 84 °C in a water bath set at 76 or 86 °C, respectively, and then rapidly transferred and held in a water bath at 74 or 84 °C for the total duration of the treatment. The heating time was 60 s in the 76 °C water bath and 85 s in the 86 °C water bath. The holding time is counted from the time that the solution reaches the desired heating temperature. Once the heating time was reached, the tubes were immediately transferred into an ice−water bath and cooled down to room temperature rapidly.

Measurement of Protein Solubility. After treatment, the protein solubilities of egg yolk, plasma, and granules were assessed by measuring the protein content of the whole sample and the supernatant of the same sample after centrifugation. Each sample was diluted to a protein content of 0.8 mg/mL, and the dispersions were allowed to equilibrate under mild agitation for 1 h at 20 °C.

Dilution was performed in a 0.01 M imidazole buffer at pH 6.5 and 0.15 M NaCl. Dilution of granule solutions for the determination of protein solubilities at different pH values were performed in a first step by dilution of the granules with an aqueous NaCl solution (not buffered)

containing either 0.15 or 0.55 M NaCl. The pH was then adjusted with 0.1 and 0.01 M HCl or NaOH with an accuracy of pH ±0.01.

An aliquote of each suspension was kept for the total protein content measurement (P_t), and the rest was centrifuged twice at 19.000 g for 20 min. The insoluble matter formed a sediment at the bottom of the tube. The supernatant contained the soluble matter, and an aliquot was taken to analyze the protein content (P_s). The protein content was determined using the BCA protein assy kit (bicinchoninic acid−protein assay kit, Sigma-Aldrich, Germany). The solubility S was determined as given in eq 3 below.

$$\text{solubility } S = \frac{P_s}{P_t} \times 100\% \quad (3)$$

Measurement of Protein Denaturation. The method of determining the denaturation degree of the proteins relies on the fact that thermally denatured egg yolk proteins form insoluble aggregates, which can easily be separated by centrifugation. The principle of this method therefore is based on determining the protein solubility as described above by assuming that soluble egg yolk proteins are native, while insoluble ones are denatured. For this assumption to be valid, the protein solubility has to be measured under environmental conditions at which all egg yolk proteins are soluble in their native state. This is only true under conditions where granules are dissociated, because the native granules are insoluble despite being native. In this study, egg yolk samples were suspended in a 0.1 M phosphate/NaOH buffer at a pH of 12, containing 0.85 M NaCl, to obtain a total protein concentration of about 0.8 mg/mL. The samples were then left to equilibrate under mild agitation at 20 °C for 1 h prior to centrifugation. Then, the protein solubility was measured as described above. The degree of protein denaturation D_P was calculated as indicated in eq 4.

$$\text{denaturation } D_P = 100 - \left(\frac{P_s}{P_t} \times 100\%\right) \quad (4)$$

Particle Size Measurement of a Granule Suspension. The particle size distribution of the untreated and enzyme-treated granule suspensions were measured by photon correlation spectroscopy using a N4+ particle size analyzer (Beckman Coulter, Krefeld, Germany).

Samples of granule suspensions were measured under environmental conditions, where granules are in their native, aggregated form or in a totally dissociated form. A granule suspension was taken and a few drops were suspended in a measurement cuvette filled with the corresponding buffer (0.1 M phosphate/NaOH buffer at a pH of 12, containing 0.85 M NaCl, or 0.1 M imidazole buffer at a pH of 6.5, containing 0.15 M NaCl). All measurements were carried out at 20 °C. Great care was taken to avoid the presence of interfering particles, such as dust. All cuvettes were rinsed with dust-free water immediately before use. The dilution buffer was filtered twice using a 0.2 μm filter cartridge to remove any dust particle from it. The light scattered at a 90° angle from the laser was analyzed over a period of 600 s and interpreted assuming a unimodal distribution of particles according to a method by ref *14*.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed under nonreducing conditions on commercially available "EXCEL" gradient gels (8−18% T), according to the procedure described by Guilmineau et al. (*15*). As a first step, all samples were assessed in terms of the protein concentration using a nitrogen analyzer as described above, so that samples with known protein concentration were treated further. Samples were delipidated with acetone, and protein was recovered as powder and dissolved in a 0.05 M Tris-acetate dissociation buffer containing 1% (w/v) SDS, 8 M urea, and 0.05 mg/mL bromophenol blue as tracker dye (Merck, Darmstadt, Germany). Samples were gently shaken for at least 12 h to ensure complete dissolution, then heated at 100 °C for 10 min, and allowed to cool at room temperature before centrifugation at 10000*g* for 5 min. An amount of 5 μL of the supernatant was transferred to the gel. 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 to 50 mA. Proteins were stained without fixation using PhastGel Blue R (Amersham Bioscience, Freiburg, Germany) in 10% acetic acid. Molecular weights (MW) were determined by a MW standard (wide molecular weight range, Sigma-Aldrich, Steinheim, Germany) using image analysis (ImageMaster 1D Elite version 4.00, Amersham Pharmacia Biotech, Uppsala, Sweden).

The relative amount of soluble protein based on the total protein was calculated by surface area × pixel intensity by data analysis, which allows for the calculation of the volume of the band. The volumes of the bands with total protein (V_t) and the bands with soluble proteins (V_s) were determined, and the solubility level was calculated according to eq 5.

$$\text{solubility level} = \frac{V_s}{V_t} \times 100\% \quad (5)$$

Results for solubility levels are given in **Table 3**. The table is classified in different levels, starting with below 10% solubility, in increments of 20% levels, and ending with above 90% solubility. At solubility levels below 10%, bands were detected by the known pixel position of the band in the reference sample; therefore, their detection is not significantly reproducible.

Interfacial Tension at the Oil−Water Interphase. Interfacial tension was measured with a drop volume tensiometer model TVT2 (Lauda Dr. R. Wobser GmbH and Co. KG, Lauda Königshofen, Germany) in a dynamic mode. In this method, a droplet of the egg yolk suspension was formed at the tip of a capillary tube at a controlled velocity inside a given volume of commercial sunflower oil. The interfacial tension between the immiscible phases was calculated from the volume of the droplet formed as it detached from the capillary. The velocity of the droplet formation was varied to control the age of the interface at the time when the droplet detached. This allowed us to obtain a plot of the interfacial tension as a function of the age of the interface. Measurements were carried out at 20 °C.

Statistical Analysis. Three replicates were carried out. Protein solubility, protein denaturation, and particle size of granules were measured twice each time. The parameters were subjected to a one-way analysis of variance using Statgraphics software (Statistical Graphics Corporation, Rockville, MD), with a confidence level of 95% ($p < 0.05$). A multiple range test was used to determine which means are significantly different from each other.

RESULTS AND DISCUSSION

Composition of Egg Yolk, Plasma, and Granules before and after Treatment with PLA_2. To characterize the hydrolysation of phospholipids by PLA_2, the amount of phospholipids (PC and PE) and lyso-phospholipids (LPC and LPE), as well as the amount of free fatty acids (FFA), was determined.

The composition of egg yolk, plasma, and granules before (not treated) and after incubation with PLA_2 (PLA_2-treated) is given in **Table 1**. Before incubation, means egg yolk and both fractions are in their natural conditions without any treatment. The composition of untreated egg yolk, plasma, and granules confirmed values reported by other authors (*16*–*18*). Dried egg yolk (100 g) contains 30.8 g of proteins and 64.3 g of lipids, which consist of 16.2 g of phospholipids. The PL are composed of 12.9 g of PC, 2.9 g of PE, 0.2 g of LPC, and 0.1 g of LPE.

No data for the composition of egg yolk, plasma, and granules hydrolyzed by PLA_2 was found in the literature until now. The total protein and lipid content after PLA_2 treatment was not different from egg yolk, granules, and plasma before incubation, unlike the composition of lipids. The amount of LPC and LPE before incubation is very low, 0.1−0.2 g/100 g of dried matter in egg yolk, granules, and plasma. After hydrolysis, the amount of PC and PE is decreased because of the cutoff of one fatty acid from PC and PE by the action of PLA_2 increasing the amount of LPC and LPE. Accordingly, a higher amount of free fatty acids was measured in all samples after enzymatic treatment. In 100 g of dried egg yolk treated with PLA_2, 10.8 g of phospholipids was found, consisting of 2.5 g of PC, 0.1 g of

Table 1. Composition of Egg Yolk, Granules, and Plasma before (Untreated) and after Incubation with PLA_2 (PLA_2-Treated) (g/100 g of Dried Matter)[a]

	egg yolk		granules		plasma	
	untreated	PLA_2-treated	untreated	PLA_2-treated	untreated	PLA_2-treated
proteins	30.8 a	30.8 a	60.0 b	60.2 b	23.1 c	22.9 c
lipids	64.3 a	66.5 a	33.8 b	34.1 b	72.4 c	73.7 c
phospholipids (PL, lyso-PL)	16.2 a	10.8 b	11.7 c	7.7 d	16.3 a	12.1 c
phosphaditylcholine (PC)	12.9 a	2.5 b	9.2 c	3.4 b	13.2 a	4.9 d
phosphaditylethanolamin (PE)	2.9 a	0.1 b	2.2 a	0.6 c	2.8 a	0.4 c
lyso-phosphaditylcholine (LPC)	0.2 a	6.0 b	0.2 a	2.5 c	0.2 a	5.4 b
lyso-phosphaditylethanolamine (LPE)	0.1 a	2.2 b	0.1 a	1.2 c	0.1 a	1.4 c
free fatty acids (FFA)	0.2 a	5.3 b	0.1 a	3.0 c	0.5 a	4.6 b

[a] Each value is a mean of three determinations. In one row, means with different letters differ significantly ($p < 0.05$).

Table 2. Molar Concentration (mmol/100 g of Dried Matter) of PC, PE, and FFA and Calculated Degrees of Hydrolysis for Egg Yolk, Granules, and Plasma

		egg yolk			granules			plasma		
	MW (g/mol)	not treated	PLA_2-treated	degree of hydrolysis (%)	not treated	PLA_2-treated	degree of hydrolysis (%)	not treated	PLA_2-treated	degree of hydrolysis (%)
phosphaditylcholine (PC)	770	16.8	3.2	84[a]	11.9	4.4	65[a]	17.1	6.4	67[a]
phosphaditylethanolamin (PE)	725	4.0	0.1		3.0	0.3		3.9	0.6	
free fatty acids (FFA)	282	0.7	18.8	87[b]	0.4	10.6	69[b]	1.8	16.3	69[b]

[a] Calculated by the reduction of PC plus PE, according to eq 1. [b] Calculated by the increase of FFA, assuming that 1 mol of FFA is released by 1 mol of PC/PE, according to eq 2.

PE, 6.0 g of LPC, and 2.2 g of LPE. The amount of PL in treated egg yolk, granules, and plasma (10.8, 7.7, and 12.1 g, respectively) is less than in untreated egg yolk, granules, and plasma (16.2, 11.7, and 16.3 g, respectively). This is due to the fact that the lyso-PL weigh less than PL and come more into account after hydrolysation. Accordingly, this amount is now present in the form of free fatty acids (i.e., 5.3 g in 100 g of dried matter of treated egg yolk).

The determination of the degree of hydrolysis by the increase of FFA (87% in egg yolk and 69% in plasma and granules) was in accordance with the values determined by PL reduction of 84, 67, and 65% for egg yolk, plasma, and granules, respectively. The calculation of FFA by the molecular weight of oleic acid (282 g/mol), although the composition of FFA is not only composed of oleic acid, is in good accordance with values calculated by the reduction of PL during hydrolysis. Therefore, measuring the amount of FFA is an appropriate method to control the degree of hydrolysis after enzymatic treatment of egg yolk, plasma, or granules.

PL in egg yolk was hydrolyzed to 84%. No complete hydrolysis was achieved in egg yolk because of the pH value at which enzymatic treatment was carried out. The pH value of egg yolk suspensions was kept at pH 6.1 and was not changed to the optimal working pH of the enzyme (pH 8.0−8.5). When the pH was changed to 8.0, a higher degree of hydrolysation could be achieved but it would also have meant a change in the natural environment of the egg yolk.

The degree of hydrolysis for plasma and granules is 67 and 65%, respectively, based on the reduction of PL. Surprisingly, lower degrees of hydrolysation were found than in egg yolk, despite the same incubation conditions used (55 °C for 3 h). The enzyme dosage was calculated on total dry matter, and egg yolk and plasma show the same amount of PL in 100 g of dried matter; therefore, the enzyme dosage per PL is identical for egg yolk and plasma. Granules have less PL in 100 g of dried matter; accordingly, the enzyme dosage per PL was even higher. However, the same hydrolysation degree for plasma and granules was found. This leads to the assumption that the total dry matter of the solutions must have influenced the conversion of PL into LPL. The total dry matter can effect the enzyme reaction because molecules are able to move more flexible in higher diluted solutions and contact between enzyme and substrate (PL) is more unlikely. Therefore, the enzyme converts relatively less PL in the same time in higher diluted solutions. Further trials have to concern dependencies of the enzymatic reaction to evaluate the kinetics of the reaction (**Table 2**).

Solubility of Proteins from Enzyme-Treated Egg Yolk, Plasma, and Granules. Solubility of egg yolk proteins is significantly impacted by the solubility of granule proteins, which are insoluble in natural environmental conditions of egg yolk. Plasma proteins are completely soluble independent of the environmental conditions as reported by Anton and Gandemer (*16*). In natural conditions, i.e., without any use of PLA_2, egg yolk shows a protein solubility of 70% (**Figure 1**). After the egg yolk was treated with enzyme, the protein solubility increased to almost 100%. As mentioned above, this increase must be due to a higher solubility of proteins formerly present in the granules. Granules in their native state show a low protein solubility of 30% at pH 6.5 and 0.15 M NaCl. With the same environmental conditions but treated with PLA_2, granules reach a protein solubility over 80%. It has to be strongly emphasized that a treatment with PLA_2 does specifically modify phospholipids and is not associated with a direct modification of proteins. Nevertheless, the observed effect of PLA_2 on the solubility of egg yolk indicates that structural changes of dense insoluble granules develop after modification of phospholipids, whereby granule proteins, trapped in HDL before modification, become soluble.

Thus far, only structural changes in LDL particles are reported, i.e., the cutting-off of one fatty acid from the phospholipids. This is related to the formation of a heat-stable complex between lyso-PL and LDL apoproteins as discussed by Mine (*5*). However, nothing is found in the literature about the impact of PLA_2 on whole egg yolk or even granules. An explanation of the results shown in **Figure 1** is presented and discussed below.

A similar effect like the dissociation of granules at high ionic strength can be taken into account. It is likely that calcium−phosphate bridges between HDL and phosvitin, together forming the granules, are affected, because PLA_2 is catalyzed by calcium

Table 3. Solubility Level of 4 HDL Apoproteins and 2 LDL Apoproteins with and without Treatment of Granules by Means of PLA_2 (Peak Numbers 1−6 Belong to Peaks Detected in **Figure 4**)

Treatment:			without Enzyme						with Enzyme					
Protein category	MW [kDa] (peak)	Protein name	Solubility level [%]						Solubility level [%]					
			< 10	11-30	31-50	51-70	71-90	> 90	< 10	11-30	31-50	51-70	71-90	> 90
LDL-apo-proteins	17 (1)	Apovitellenin I					75						90	
	122 (6)	Apovitellenin Va	5							25				
HDL-apo-proteins	110 (5)	Apovitellin 3+4		12							45			
	31 (2)	Apovitellin 8		15							50			
	47 (3)	Apovitellin 7	5*							15				
	78 (4)	Apovitellin 5+6	3*									55		

*Below the detection limit in electrophoretogram (see **Figure 4**).

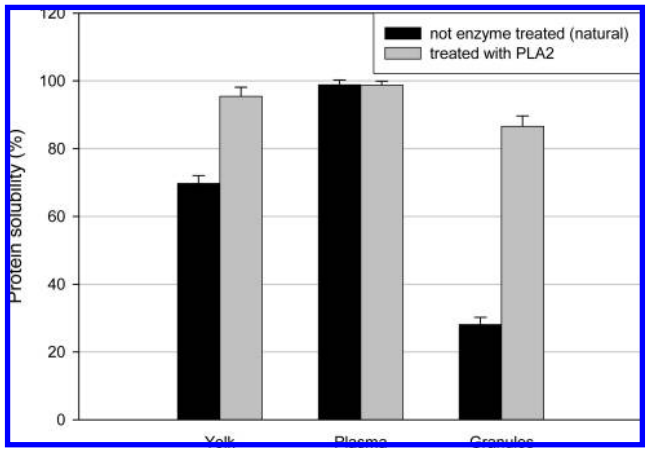

Figure 1. Impact of a treatment with PLA_2 on the solubility of proteins from whole egg yolk, plasma, and granules at pH 6.5 and 0.15 M NaCl, the natural environmental conditions of egg yolk ($n = 3$).

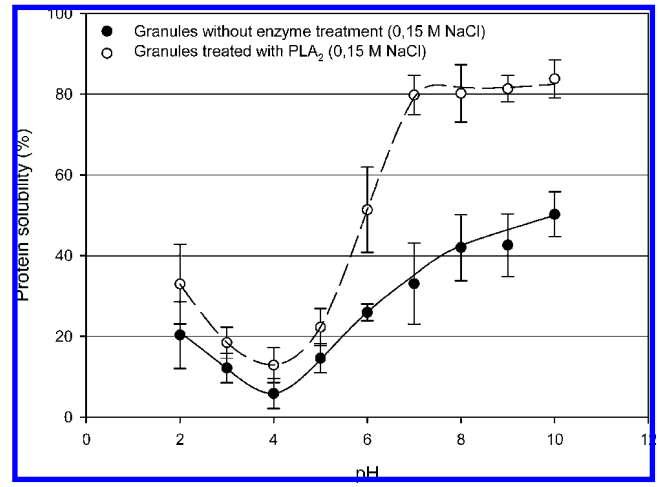

Figure 2. Impact of a treatment with PLA_2 on the solubility of proteins from granules at various pH and 0.15 M NaCl concentration ($n = 3$).

ions (*19*). Changes in granular structure can also be due to the modification of the LDL particles (12% of the granules), leading to a disruption of the aggregated structure. The mechanism of solubilization is not clear at the moment and will be discussed further below.

As **Figure 1** demonstrates, the total protein solubility of egg yolk mainly depends upon the solubility of granule proteins. Hence, the solubility of granules was monitored at various pH and at two concentrations of NaCl, 0.15 and 0.55 M.

Solubility of Proteins from Enzymatic-Treated Granules in Different Environmental Conditions. At natural pH of egg yolk, granules are insoluble because of the low ionic strength (pH 6.5; 0.15 M). **Figure 2** presents the protein solubility level of granules at various pH at 0.15 M NaCl concentration. Granules are natural (not treated with enzyme, ●) or treated with PLA_2 (○) before pH was adjusted and solubility was measured. When the pH is increased above 6.5, granules become more soluble, because of increased electrostatic repulsion between proteins forming the granules (*17*). At pH below 3.5, the solubility increases, because pH gets close to the isoelectric point (pI) of phosphoseryl residues. Therefore, negative charges are weakened, and their affinity for cations is reduced (*20*).

At pH 2−5, the granules treated with PLA_2 show no remarkable difference in protein solubility in comparison to nontreated granules. As the pH is further increased, solubility of proteins from enzyme-treated granules increases stronger than that from natural granules. Enzymatically treated granules reach a high solubility of about 80%, although the ionic strength is low (0.15 M NaCl; **Figure 2**).

Figure 3 presents granules diluted at high ionic strength (0.55 M NaCl) at various pH with or without enzymatic treatment. At high ionic strength, granules show an increased protein solubility because of the disruption of phosphocalcic bridges, where sodium ions replace calcium ions. Also, at high ionic strength, granules treated with enzyme show higher protein solubility than nontreated granules at pH above 4.

Above pH 5, the proteins in enzyme-treated granules are almost completely solubilized, whereas granules without enzyme treatment only have 60% soluble proteins. The remarkable increase in solubility can be explained by the fact that, at high salt concentrations, the tendency to aggregate is very strong only below or near the isoelectric point of proteins (IEP ∼ 4). Above the IEP (pH > 4), positive charges lead to electrostatic repulsion and attractive forces are weakened.

To further explain the observations shown in **Figures 2** and **3**, the solubilization of lipovitellenins of HDL was assessed by SDS−PAGE. **Figure 4** presents the electrophoretogram obtained for the electrophoretic patterns using image analysis. SDS−PAGE

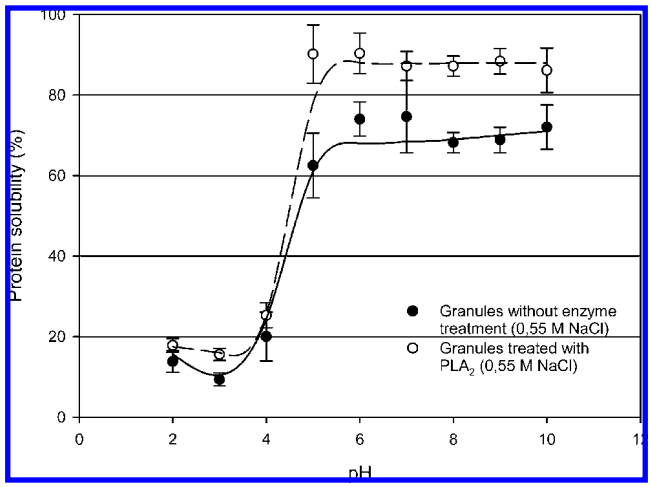

Figure 3. Impact of a treatment with PLA_2 on the solubility of proteins from granules at various pH and 0.55 M NaCl concentration ($n = 3$).

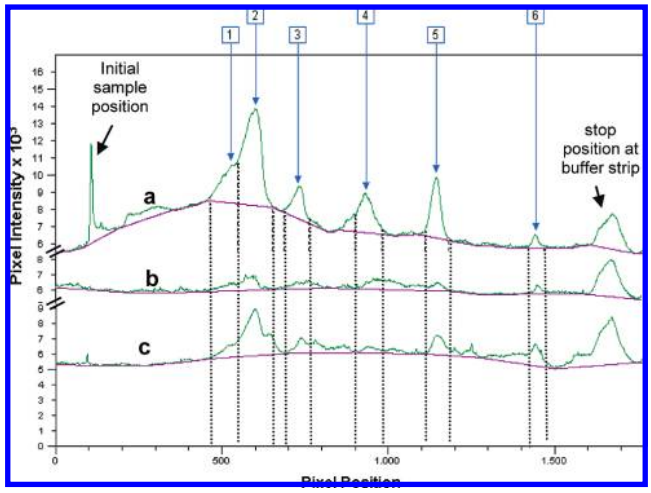

Figure 4. Electrophoretogram obtained for granule proteins using image analysis: (a) whole granules of egg yolk, (b) soluble granules without enzyme treatment, and (c) soluble granules after an enzymatic treatment with PLA_2.

was performed with the whole granule fraction to assess all proteins present in the granules as well as the supernatant after centrifugation of the granule fraction to assess the soluble proteins of granules. To obtain the soluble proteins, granules were centrifuged and the sediment containing the insoluble matter was removed. The supernatant with the soluble proteins was used for electrophoresis. **Figure 4** shows the detection of four of the main granule proteins (peaks 2−5) according to Guilmineau et al. (*15*). Two main LDL apoproteins were detected (peaks 1 and 6), resulting from the LDL particles contained in the granules. The protein designation and molecular weight belonging to the respective peaks are given in **Table 3**.

Electrophoretogram line a shows the peaks obtained for the whole granule fraction. Electrophoretogram lines b and c show the peaks for soluble proteins from granules untreated or treated with PLA_2, respectively. Peaks in line c appear clearly but less intense than peaks in line a. Peaks in line b also appear clearly, except for peak numbers 3 and 4, which are broader and less distinct. Those peaks were assigned to the known pixel position of the corresponding reference protein in line a. The peaks had no clear separation from the baseline. However, a band volume was calculated by fitting a Gaussian peak between the peak borders obtained in line a, and the solubility level was calculated from there. The levels obtained for peaks 3 and 4, namely,

Table 4. Median Particle Size of Granules ($n = 4$)

	median particle size (nm)
granules, natural conditions: pH 6.5; 0.15 M NaCl	2196.4 ± 91.9
granules after treatment with PLA_2: pH 6.5; 0.15 M NaCl	894.5 ± 173.0
granules, dissociated in Na−P buffer: pH 12; 0.55 M NaCl	125.3 ± 27.7

apovitellin 7 and apovitellin 5 plus 6, respectively, are very close to zero, i.e., close to or below the detection limit.

Overall, a clear difference between line b (untreated sample) and line b (PLA_2-treated sample) can be seen in **Figure 4**. Evidently, all peaks in line c have a higher intensity than in line b. Therefore, the major conclusion is that proteins from granules show higher solubilities after the enzymatic treatment as summarized by the data shown in **Table 3**.

Changes of the Egg Yolk Granules Structure Because of Treatment with PLA_2. The results of protein solubility indicate a modification of granule aggregates because of the enzymatic treatment. Native granules in natural environmental conditions of egg yolk (pH 6.5; 0.15 M NaCl) are known to have a size of 0.3−2 μm when insoluble (*21*). Natural environment means the pH and the ionic strength of egg yolk is not changed and therefore represents the original structure of fresh egg yolk. These dense structures are disrupted at high ionic strengths. Therefore, the particle size of granule suspensions was measured at natural environmental conditions, at conditions where granules are dissociated by high pH and high ionic strength and after an enzymatic treatment (**Table 4**). **Table 4** represents the median particle size obtained. Results show the typical particle size for the dense aggregated structure of insoluble granules of 2 μm and very small particle size for granules dissociated by high ionic strength. In natural environmental conditions of egg yolk, enzyme treatment leads to a breakdown of granule structure but not to a complete dissociation. The particle size of enzyme-treated granules ranges between the natural and dissociated granules. The mean diameter in this case is close to 900 nm. This indicates that the treatment with PLA_2 alone does not lead to a complete dissociation of granules like the high ionic strength.

Denaturation Characteristics of Enzyme-Treated Egg Yolk. As already reported by different authors, egg yolk treated with PLA_2 possesses a high heat stability (*4, 5*). However, no systematic study was performed to quantify the obtained heat resistence by means of denaturation degree of proteins. Hence, this study reveals the impact of heat treatment on PLA_2-modified egg yolk by assessing the protein denaturation after heating at two different temperatures and different heating times. To obtain high denaturation degrees up to 80% in nonenzyme-treated egg yolk, plasma, and granules without causing gelation, all suspensions were diluted to a protein concentration of 20 mg/mL, accordingly, a 8 times dilution of egg yolk. A severe heat treatment was of interest to point out the heat stability of the enzyme-treated egg yolk. Although the enzyme was not inactivated after incubation, the egg yolk was highly diluted and enzyme activity is very low at temperatures above 70 °C anyway. Therefore, the observed effects can be attributed only to the incubation time of egg yolk, plasma, and granules with PLA_2.

Figure 5 presents the denaturation degree of proteins of untreated and PLA_2-treated egg yolk at the used heating conditions.

Egg yolk was heated at 74 °C without prior enzyme treatment, leading to a protein denaturation of 30% in 2 min and to a further

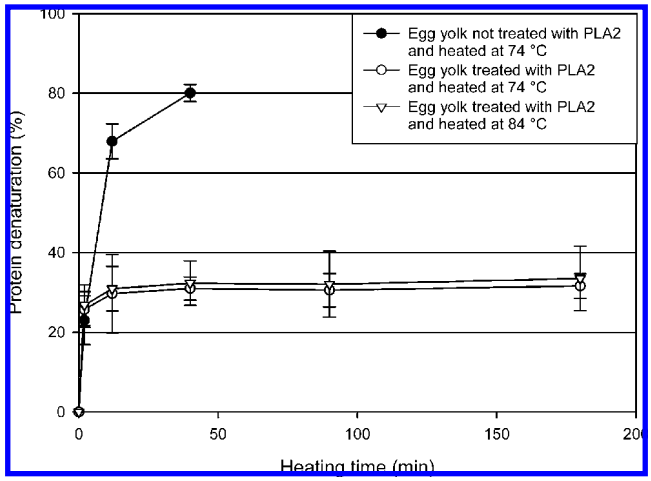

Figure 5. Denaturation of proteins in whole egg yolk without enzymatic treatment, heated at 74 °C for 0−40 min, and after treatment with PLA_2, heated at 74 and 84 °C for 0−180 min ($n = 3$).

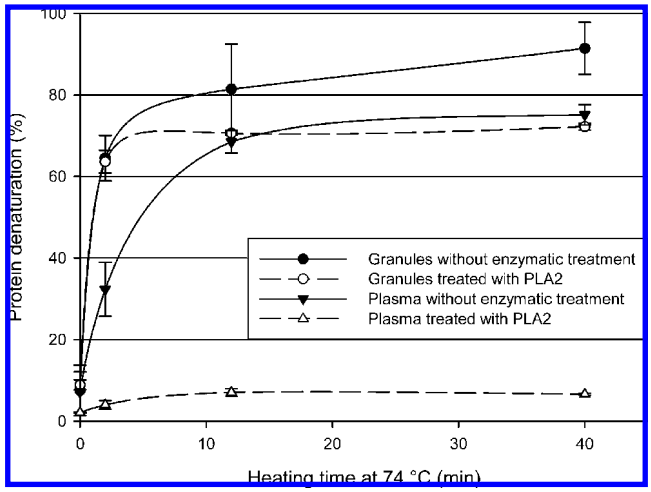

Figure 6. Denaturation of proteins in plasma and granule fraction without enzymatic treatment, heated at 74 °C for 0−40 min, and after treatment with PLA_2, heated at 74 °C for 0−40 min (pH 6.5; 0.15 M NaCl).

increase of denaturation up to 80% in 40 min of heating. This result is in agreement with Guilmineau and Kulozik (*22*). Natural egg yolk was not heated further, because the egg yolk suspension began to gel. Egg yolk treated with PLA_2 was heated at 74 °C and, because no gelling occurred, similar to the untreated egg yolk, additionally at 84 °C up to 180 min. PLA_2-treated egg yolk shows the same protein denaturation degree at 74 and 84 °C. After 2 min of heating, a denaturation degree of 30% is reached, similar to natural egg yolk (not treated with enzyme). Any further heating showed no remarkable increase in protein denaturation. Even after a heating time of 180 min, a denaturation degree of 40% was not exceeded. While 80% of all proteins were denatured in natural egg yolk, the PLA_2 treatment led to a degree of protein denaturation of only 40%. This means that at least 60% of all egg yolk proteins are heat-stable under these conditions when the egg yolk was treated by PLA_2.

To gain more insights related to the contribution of different egg yolk constituents to this effect, both main fractions were treated separately.

Denaturation Characteristics of PLA_2-Treated Plasma and Granules. Figure 6 presents the protein denaturation of granule and plasma fraction when untreated or treated with PLA_2 and heated at 74 °C up to 40 min. Granules treated with PLA_2 show a lower protein denaturation degree as granules without PLA_2

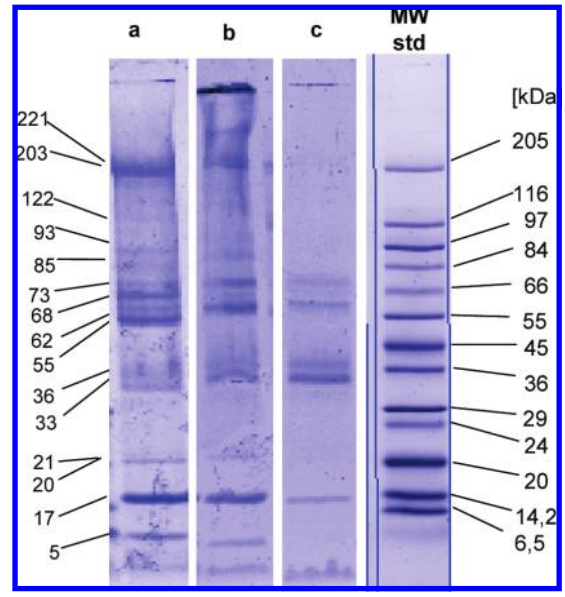

Figure 7. SDS−PAGE profile of plasma proteins: (a) all plasma proteins, (b) soluble proteins of enzyme-treated plasma after 12 min of heating at 74 °C, and (c) soluble proteins of plasma (no enzyme treatment) after 12 min of heating at 74 °C.

modification after heating for 12 min. Plasma proteins show nearly complete heat stability when treated with PLA_2. The heat stability is due to the heat stability of LDL apoproteins as described by Mine (*5*). Mine suggests a heat-stable complex formed between LDL apoproteins and lyso-PL. Studies on human blood LDL demonstrate that PLA_2 treatment leads to a reduction of LDL particle size from 22.8 ± 0.3 to 21.0 ± 0.3 nm because of the loss of free fatty acids, resulting in a closer and tighter packing of the surface (*8*). The enhanced structural rigidity of the micelles surface precludes particle fusion (*7*). This observation can be transferred to the effect that no aggregates are formed during heat treatment of the egg yolk plasma. Because the LDL particles are not likely to aggregate because of their increased surface rigidity induced by lyso-PL, no insoluble aggregates can form during heat treatment and, therefore, the plasma proteins remain soluble.

Because granules contain 12% LDL, the lower denaturation degree of granule fraction after enzymatic treatment can be explained by the heat stability of LDL apoproteins.

In **Figure 7**, the SDS−PAGE profiles of the plasma proteins are depicted. Protein bands in lane a represent all plasma proteins; lane b represents soluble plasma proteins after heating, when plasma was modified by PLA_2 prior to heating; and lane c shows soluble plasma proteins after heat treatment when no PLA_2 modification was performed.

It is obvious that protein bands in lane b appear stronger than protein bands in lane c. After heat treatment (74 °C, 12 min), only five bands are clearly visible in nontreated plasma (68, 55, 36, 33, and 17 kDa). Bands with a MW of 36 and 33 kDa appear most clearly and are referred to livetins of plasma. In lane b, the whole plasma profile can be seen, similar to lane a, although it looks slightly smearing. The smearing is probably an effect of lyso-phospholipids, which are present after enzymatic modification. Because of their hydrophilic structure, they cannot be removed as easily by delipidation with acetone compared to other lipids.

Interfacial Tension. The modification of phospholipids is likely to have an impact on the functionality of egg yolk because of structural changes in granule and plasma proteins. The higher

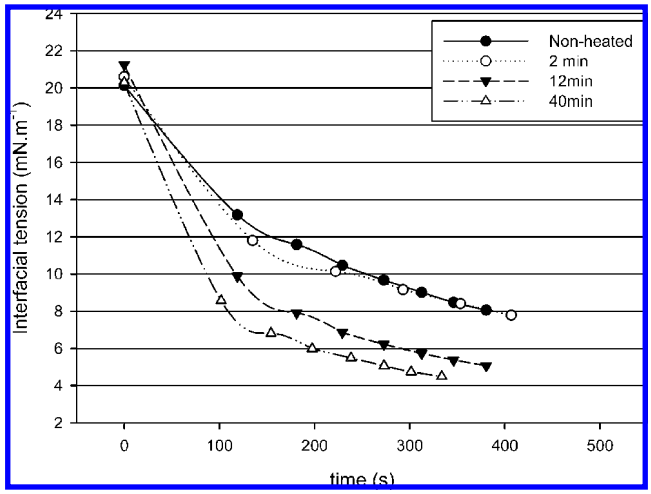

Figure 8. Interfacial tension decrease at the interface between sunflower oil and an aqueous suspension containing egg yolk heated for various times at 74 °C (without enzymatic treatment).

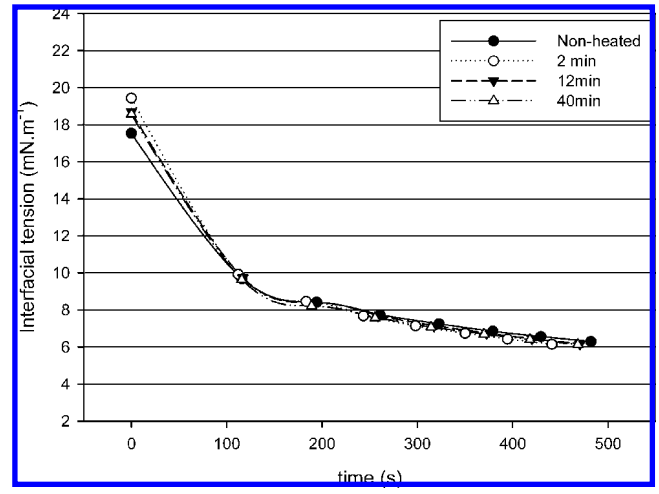

Figure 9. Interfacial tension decreases at the interface between sunflower oil and an aqueous suspension containing PLA$_2$-treated egg yolk heated for various times at 74 °C.

protein solubility of the granule fraction and the heat stability of the plasma fraction can be attributed to changes in protein conformation and structure. This was further assessed by measuring the interfacial tension. The interfacial tension provides information about the emulsifying capacity of egg yolk and is therefore a meaningful criterion of the functionality of untreated and PLA$_2$-treated egg yolk. **Figure 8** shows the decrease of interfacial tension between sunflower oil and egg yolk suspensions not treated by PLA$_2$. The suspensions were heated at 74 °C for 0−40 min. A faster reduction of interfacial tension is observed when heated egg yolk is used. The heat-treated samples also reach lower interfacial tension values. This indicates an improved emulsifying capacity of heated egg yolk. A minimum of heat-related structural changes in egg yolk seems to be required to achieve this effect, which is not the case after a heat treatment at 74 °C for 2 min. A strong effect in this regard can be observed after 12 and 40 min of heating time. The corresponding degrees of denaturation are 23, 68, and 80%. The contradiction between a poor solubility of proteins (corresponding to their higher denaturation degree) and the good interfacial activity is thought to result from the laminar flow conditions in the drop volume tensiometer and the extreme complexity of egg yolk, which contains different types of emulsifiers. The interface in a drop volume tensiometer is formed very slowly, and small molecules adsorb in priority because they diffuse and adsorb faster then aggregates. Therefore, the faster decrease of interfacial tension with a higher protein denaturation degree is expected to be due to molecules generated or activated by the heat treatment. The molecules that preferentially adsorb are not thought to be protein aggregates but other interfacial active compounds, such as phospholipids. Proteins aggregates are not likely to contribute to the adsorption kinetics of emulsifiers anymore because of their high molecular mass.

When the egg yolk suspension is first treated with PLA$_2$ and subsequently heated, no difference in interfacial tension can be observed between the unheated and heated samples (**Figure 9**). It is remarkable that the reduction of interfacial tension of PLA$_2$-treated egg yolk is located between the natural (without enzyme treatment) egg yolk without heating and egg yolk heated at 74 °C for 40 min.

This indicates that the emulsifying capacity of PLA$_2$-treated egg yolk is better than that of egg yolk not treated by PLA$_2$. However, it is inferior to the sample only heated at 74 °C for 40 min. Apparently, the aggregates can adsorb at the interface and are even more effective than proteins in their native state.

These results provide an interesting perspective on the possibility of a targeted use of the pasteurization step in the treatment of egg yolk. As the results clearly show, denatured egg yolk proteins have enhanced emulsifying properties (**Figure 8**) even better than egg yolk only treated by PLA$_2$ (**Figure 9**). Protein denaturation in enzyme-treated egg yolk (31% after 74 °C for 40 min) does not reach the same dimensions as in PLA$_2$-untreated egg yolk (80% after 74 °C for 40 min)

Further, the results indicate that the improved emulsifying properties of PLA$_2$-treated egg yolk may result from lyso-phospholipids, which have an increased emulsifying activity in o/w emulsions. Therefore, it is likely that lyso-phospholipids dominate at the interface and proteins are displaced, whereas protein adsorbs preferentially when phospholipids are present.

In conclusion, this study demonstrates that enzymatic treatment of egg yolk by PLA$_2$ has a significant impact on the structure and functional properties of egg yolk. Proteins are not changed by the action of PLA$_2$, but they are indirectly affected in their solubility and denaturation behavior. This appears as a surprising result, because the enzyme is reported to be specifically acting on phospholipids and not on proteins. An explanation for this effect includes the resulting lyso-phospholipids, which lead to a structural change of the LDL particles, thus preventing the particles from aggregation. The PLA$_2$-treated egg yolk therefore has a higher heat stability. The change of the LDL particles also has an impact on the structure of granules, which contain 12% LDL. The dense structure of native granules breaks up because of the PLA$_2$ treatment, resulting in higher protein solubility after modification. This is in agreement with a lower particle size of granules found after treatment with PLA$_2$, suggesting a breakdown of aggregates to a certain extent.

The high heat stability of plasma and the higher protein solubility of granules after modification with PLA$_2$ indicate changes in the natural structure and functionality of egg yolks as described by interfacial tension measurements.

ABBREVIATIONS USED

HDL, high-density lipoprotein; IEP, isoelectric point; LDL, low-density lipoprotein; MW, molecular weight; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PLA$_2$, phospholipase A$_2$; PL, phospholipid; LPL, lyso-phospholipid.

ACKNOWLEDGMENT

The authors thank Annette Bruemmer-Rolf for her valued contribution in the experimental phase of this work. Many thanks to Hermine Kienberger for carrying out phospholipid analysis.

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Received for review December 14, 2007. Revised manuscript received March 7, 2008. Accepted March 28, 2008. This research project was supported by the German Ministry of Economics and Technology (via AiF) and the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn), project AiF 14041 N.

JF703641E