

Role of lysophospholipids on the interfacial and liquid film properties of enzymatically modified egg yolk solutions

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ABSTRACT

This study aims to clarify and explain the similarities and differences in the behavior of adsorption layers of native egg yolk (EY) and enzymatically modified egg yolk (MEY) at a soybean oil-water interface. For this purpose, the interfacial tension and the surface dilatational modulus of EY and MEY solutions are measured and compared. The interactions between two adsorption layers, formed from these solutions on an oil-water or air-water interface, are also studied by optical observations of thin foam and emulsion films, formed in a capillary cell. The chemical composition, the electrophoretic mobility of the molecular aggregates, and the rheological properties of the egg yolk solutions are also characterized. Adsorption layers formed from MEY solutions display a faster rate of adsorption, lower dilatational surface moduli and higher equilibrium surface tension. The enzymatic modification of egg yolk also leads to formation of much thinner foam and emulsion films and to faster film thinning. The observed differences between EY and MEY are explained by assuming that the interfacial properties of MEY are governed mostly by the lysophospholipids and oleic acid, which appear as reaction products of the enzymatic modification of EY. The latter assumption is unambiguously proven by chemical analysis of the MEY solutions and by deliberate addition of lysophospholipids and oleic acid to the non-modified EY solutions. Even at relatively low concentrations, the lysophospholipids and oleic acid change the interfacial and film properties of the EY solutions, making them very similar to those of the enzymatically modified egg yolk.

1. Introduction

1.1. Egg yolk composition – whole egg yolk, plasma and granules

Egg yolk (EY) is a widely used natural ingredient in food emulsions due to its nutritional and organoleptic properties, combined with excellent functional properties as it can act as an efficient emulsifier, coagulating and gelling agent. Mayonnaise and cakes are just two examples of popular egg yolk-based food products.

EY contains approximately 50% water, 35% lipids and 15% proteins (Mine, 1998). It is a complex system, both in composition and structure, and contains a large number of non-soluble polydisperse particles (granules) which are dispersed in clear yellow fluid (plasma). The plasma contains 77–81% of the total dry matter, 90% of the yolk lipids, including all carotenoids, and 50% of the yolk proteins (Anton, 2013).

The dry matter of plasma consists of low-density lipoproteins (LDL)

and soluble proteins (15% livetins). LDLs are composed of 11–17% protein and 83–89% lipid, of which 74% is neutral lipid and 26% phospholipid (Anton, 2013). LDLs are spherical nanoparticles (17–60 nm) with a lipid core of triglycerides and cholesterol esters, surrounded by a monolayer of phospholipids and proteins (Anton, 2013; Anton et al., 2003; Cook & Martin, 1969; Jolivet, Boulard, Chardot, & Anton, 2008). LDLs are dispersible in aqueous solutions (regardless of pH and ionic conditions). Phospholipids are an essential factor for the stability of the LDL structure, because the association forces are essentially hydrophobic (Anton, 2013; Saari, Powrie, & Fennema, 1964). Some cholesterol is included in the phospholipid film, increasing its rigidity.

The granules account for ≈22% of the yolk's dry matter, ≈50% of the yolk proteins, and ≈7% of the yolk lipids (Anton, 2013; Anton & Gandemer, 1997; Dyer-Hurdon & Nnanna, 1993). They contain 70% high-density lipoproteins (HDL) and 16% phospholipids linked by

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phosphocalcic bridges, and 12% LDL. Granules consist of globular complexes, ranging in diameter from 0.3 to 2 μm (Chang, Powrie, & Fennema, 1977). At low ionic strength, granules form mainly non-soluble HDL-phosvitin complexes with a very compact, poorly hydrated structure which is weakly accessible to enzymes and ensures an efficient protection against thermal denaturation and heat gelation (Anton, 2013; Castellani, Guerin-Dubiard, David-Briand, & Anton, 2004; Causeret, Matringe, & Lorient, 1991). At an ionic strength $> 0.3\text{ M}$ NaCl, the phosphocalcic bridges are disrupted, because the monovalent sodium ions replace the divalent calcium ions. Under such conditions, the solubility of the granules reaches 80% because phosvitin is a soluble protein and the HDL also behaves like soluble proteins (Anton, 2013; Anton & Gandemer, 1997; Cook & Martin, 1969). Complete disruption of granules occurs when the ionic strength reaches 1.71 M NaCl. In their soluble state (high ionic strength or neutral pH) phosvitin and HDL are not in a molecular form – they are assembled in micelles of 100–200 nm diameter that can spread and adsorb on the oil-water interface (Anton, 2013; Anton, Nau, & Lechevalier, 2008).

The majority of the studies related to the interfacial properties of egg yolk include interfacial tension measurements, determination of the solubility levels of the proteins, and the surface protein loads, as well as identification of the proteins adsorbed on the oil-water interface under different conditions - protein concentration, ionic strength and pH (Anton, Beaumal, & Gandemer, 2000; Anton & Gandemer, 1997; Daimer & Kulozik, 2008, 2010; Le Denmat, Anton, & Beaumal, 2000).

LDL particles are considered to be the main contributor to the interfacial properties of the egg yolk because of their quantitative dominance, high solubility, and high level of hydrophobic constituents (Daimer & Kulozik, 2008; Mizutani & Nakamura, 1984). LDL particles break down when they come into contact with an air-water or oil-water interface (Anton, 2013; Martinet, Saulnier, Beaumal, Couthaudon, & Anton, 2003). The apoproteins present on the LDL surface initially anchor at the interface which provokes unfolding of the protein and subsequent destabilization of the initially formed surface layer of the LDL particles (Anton, 2013; Jolivet, Boulard, Chardot, & Anton, 2009). Phospholipids and proteins spread on the interface, the neutral lipids are released on the interface and dissolve into the oil phase. Thus, a strong interfacial film is created by phospholipids and proteins, which leads to a decrease of the interfacial tension and an increase of the oil droplet stability in emulsions (Anton, 2013; Anton et al., 2003; Dauphas, Beaumal, Riaublanc, & Anton, 2006; Dauphas et al., 2007a, b; Martinet et al., 2003).

1.2. Impact of enzymatic modification on the physico-chemical properties of EY

Different methods for modification of the egg yolk have been explored, with the aim to improve its performance as emulsifier. One very effective modification is the enzymatic treatment with phospholipase A2 (PLA₂). This lipase cuts the acyl group in position 2 of the triglyceride and leads to hydrolysis of the phospholipids to lysophospholipids (Daimer & Kulozik, 2008; Dutilh & Groger, 1981).

Such modified egg yolk does not gel even under severe heat treatment, viz. it is heat-stabilized. The enzymatic action also causes structural changes in the egg yolk, related to the change of the chemical profile of the system. Daimer and Kulozik (2008) detect a significant increase of the protein solubility in the whole egg yolk after enzymatic modification, especially of the granule proteins. The granules' disruption is proposed to explain the latter observation. The authors report that the average size of the granules is reduced significantly (from ca. 2 μm down to 0.9 μm). However, the modified EY still contained some fraction of granules with the native larger size, which indicated that the enzymatic treatment did not lead to complete dissociation of the granules, in contrast to the complete dissociation observed at high ionic strengths. The partial disruption of the granules probably leads to release of water-soluble proteins, which is detected as increased solubility

of the granule proteins. Some authors suggest that the observed changes in the granular structure could also be due to modification of the LDL particles (12% of the granules), thus leading to a partial disruption of the granular aggregates (Daimer & Kulozik, 2008; Mine, 1997).

Daimer and Kulozik (2008) showed that, at low ionic strength and natural pH, the oil-water interfacial tension of EY is higher than that of modified egg yolk (MEY). Under these conditions, the adsorption kinetics is slow for EY, whereas constant interfacial tension is reached much faster with MEY. Daimer and Kulozik (2009) showed that the interfacial protein load on the oil-water interface is higher for modified egg yolk, after treatment with PLA₂. According to these authors, granular fragments in the enzymatically modified egg yolk could cover the oil-water interface more completely than the original granules in the untreated egg yolk. They suggested also that the adsorption mechanism of LDL particles changes after enzymatic modification, due to structural changes. The LDL micelles adsorb on the surface but do not break and remain compact, thus occupying a smaller fraction of the interface.

At high ionic strength and natural pH, i.e. when the solubility of the proteins from granules is significantly increased, Daimer and Kulozik (2009) showed that the surface protein load for EY is higher than that for MEY. In their study with gel-electrophoresis, Daimer and Kulozik (2010) showed that, under these conditions, the electrophoretic profiles of the two egg yolks are very similar and the majority of the proteins, both from the plasma and the granules, adsorb on the interface. Nevertheless, the LDL apoproteins are predominant in the adsorption layer since their IEP is close to neutral, $\approx 6.5\text{--}7.3$. Decreasing pH leads to adsorption layers which are rich in HDL-apoproteins for both EY and MEY. For MEY, the main protein at the interface is 31 kDa HDL-apoprotein, whereas for EY the mass distribution of the proteins at the interface is more balanced. Note that some of these results do not agree with the conclusions for EY made by other authors (Anton, 2013; Le Denmat et al., 2000). Thus, according to Le Denmat et al. (2000), around 65% of the adsorption layer consists of plasma proteins (LDL) at acidic pH, regardless of the ionic strength. At neutral pH, the adsorption layer consists of similar proportions of plasma and granule proteins. Under these conditions, the solubility of the granules is higher and the presence of micelles facilitates the adsorption of proteins from granules.

Most of the studies cited above barely consider the role of lysophospholipids on the interfacial properties of the modified egg yolk and focus on the role of proteins. However, Mel'nikov (2002) hypothesized that it is rather likely that the lysophospholipids adsorb preferentially on the interface and, therefore, the role of proteins on the interfacial properties could be lower in MEY systems. The comparison of the two types of egg yolk in Mel'nikov (2002) shows that the interfacial tension at the EY solution-oil interface is lower compared to MEY solution-oil – an opposite trend to the results reported by Daimer and Kulozik (2008). Most probably the reason for this difference is related to the different concentrations of egg yolk used in the two studies – 1.12% egg yolk in the work of Daimer and Kulozik (2008) and 0.01% in the study of Mel'nikov (2002). Mel'nikov (2002) showed also that the interfacial tension was less sensitive to pH, the surface dilatational modulus was lower, and the relative viscous contribution in this modulus was higher for the MEY-SFO interface, under the conditions studied. All these changes indicate that, indeed, the role of lysolipids on the interfacial properties could be significant in the MEY systems, but no conclusive results have been obtained to verify this hypothesis.

Several papers report that the presence of lysophospholipids in other protein systems induce heat stability and significant change in the interfacial properties (Lilbaek, Fatum, Ipsen, & Sorensen, 2007; Tran Le et al., 2007, 2011) which are similar to those observed with enzymatically modified egg yolk. Thus, Tran Le et al. (2007) showed that heat-induced milk protein aggregation is suppressed in the presence of lysophospholipids, due to lysophospholipids-protein interactions. Lysophospholipids were found to stabilize the unfolded state of denatured whey proteins and to minimise further the whey protein aggregation

and interaction with casein micelles. A similar effect was observed when using hydrophilic small-molecular-weight surfactants (Tran Le et al., 2011). Lilbaek et al. (2007) studied the surface properties of milk and whey after enzymatic hydrolysis of the milk phospholipids and observed a transition from protein-dominated to surfactant-dominated interfacial behavior.

One should note that most of the studies, discussed above, are carried out with simplified systems, such as egg yolk solutions of low concentrations and single oil-water interfaces. Therefore, it is difficult to relate directly the results from these model laboratory experiments to the properties of food emulsions. From this viewpoint, the investigation of the properties of EY and MEY at conditions closer to food emulsions would be very useful to clarify whether the results from the model experiments can be used to explain the emulsion properties.

1.3. Aims of the study

The major aim of the current study is to compare systematically the properties of the adsorption layers, formed from solutions of native egg yolk and enzymatically modified egg yolk on the oil-water interface. More specifically, the role of reaction products from the enzymatic modification (lysophospholipids and oleic acid) on the interfacial properties is investigated, under conditions relevant to food emulsions.

2. Materials and methods

2.1. Preparation of EY and MEY solutions

Non-modified egg yolk and egg yolk modified by enzymatic treatment with phospholipase A2 (PLA2) were supplied by Bouwhuis Enthoven (Raalte, the Netherlands), contained 8% sodium chloride, and were stored at 4 °C. The solutions used for characterization of the surface properties contained 22.5 wt% egg yolk. Sodium chloride ($\geq 99.8\%$ active compound, Sigma), sugar (from grocery store) and ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA, $\geq 99.0\%$ active compound, Sigma) were added in levels, typical for mayonnaise type of dressings. In two series of experiments, we added 1-Palmitoyl-2-hydroxy-SN-glycero-3-phosphocholine (lyso-phosphatidylcholine, LPC, 99.8% active compound, NOF corporation) and oleic acid (OAc, 85% active compound, Tokyo Kasei Kogyo) into EY solutions. In the first series the concentrations were 0.8 wt% LPC (0.485 mmol in the solution) and 0.45 wt% OAc (0.485 mmol in the solution) corresponding to 64% transformation of the phospholipid (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) to LPC, while in the second series these concentrations were 1 wt% LPC (0.604 mmol in the solution) and 0.57 wt% OAc (0.604 mmol in the solution) corresponding to 80% transformation of the phospholipid.

Deionized water, purified by Elix 3 system (Millipore, USA), was used for solution preparation. The pH of the initial EY and MEY solutions was adjusted in several series of experiments to $\text{pH} \approx 3.8$ by adding a solution of 12 wt% acetic acid. The oil used in all experiments is soybean oil (SBO), purified from polar contaminants by multiple passes through a chromatography column filled with Florisil adsorbent. The measured mass density and the viscosity of SBO at 25 °C were 0.92 g/cm³ and 49.1 mPa s, respectively.

2.2. Chemical analysis of EY and MEY composition

To determine the protein composition of the studied yolks we used a standard SDS-PAGE protocol, described in details in Appendix A. The identification of the obtained protein bands was carried out using molecular weight standards and literature data provided by Guilmineau, Krause, & Kulozik, 2005 and Daimer & Kulozik, 2010.

The lipid profile of the studied egg yolks was determined by gas chromatography (GC). To simplify the analysis and its interpretation, the yolks were first fractionated into polar lipids-rich and non-polar

lipids-rich fractions by modifying the procedure by Juneja (1997). First, a 0.96 wt % dispersion of EY or MEY in methanol-water at 17:1 v/v ratio was prepared. The obtained dispersion was homogenized by hand, then sonicated three times for 5 min in an ultrasonic bath (total sonication time of 15 min). Afterwards, the sample was stored overnight at 4 °C. Then, the dispersion was centrifuged for 1 h at 4500 rpm at 4 °C. The clear solvent phase (composed mainly of methanol) and the precipitate were carefully separated using a syringe and preserved for further treatment and analysis. The precipitate was dispersed in 5 mL chloroform. The obtained dispersion was sonicated three times for 5 min in an ultrasonic bath and then centrifuged for 1 h at 4500 rpm. The clear solvent phase (chloroform) was transferred into a glass vial. In this way, a chloroform phase was obtained, containing mainly non-polar lipids (triglycerides) and methanol phase, which contained predominantly polar lipids (phospholipids and fatty acids). The concentrations of the polar and non-polar lipids in the two fractions were then determined by GC. The analytical protocol is described in details in the Appendix material.

2.3. Measurement of the size and electrophoretic mobility of egg yolk aggregates

The ζ -potential and the size of the aggregates in the aqueous solution of 0.1 wt% EY and MEY, in presence of 5 mM NaCl, was measured on a Zetasizer Nano ZS instrument (Malvern, UK). The isoelectric points (IEP) of the studied egg yolks were also determined by measuring the ζ -potential of the aqueous solutions of 0.1 wt% EY and MEY at different pH values, adjusted by acetic acid.

2.4. Rheological characterization of solutions

The viscosity of 12 mL solutions of non-modified and modified egg yolks, without acetic acid (natural pH), was measured on a Brookfield DV II Pro Viscometer with S00 spindle. The viscosity of each system was measured at 3 different speeds of rotation, with the highest and lowest speed differing by a factor of two. There was no trend of increasing or decreasing the viscosity with increasing the speed of rotation, which reflects the Newtonian behavior of these solutions. Therefore, these results represent the real solution viscosity.

The rheological properties of 2 mL of the initial aqueous phases with added acetic acid (pH 3.8) were characterized on a rotational rheometer (Bohlin Geminin, Malvern UK). For all experiments, a cone and plate geometry with diameter of 60 mm was used. All measurements were conducted at 20 °C and the sample was left to equilibrate for 120 s at the measurement temperature before each experiment. Rheological tests were performed in steady deformation via the following protocol: the shear rate was varied stepwise in a logarithmic scale, from 0.2 to 1000 s⁻¹. The delay time for each step was 2 s and the integration time was 3 s. The shear stress was monitored as a function of the shear rate.

2.5. Interfacial tension at the oil-water interface

The interfacial tension between the aqueous phase and the purified soybean oil was measured by drop shape analysis (DSA) on instrument DSA100m (Krüss, Germany) at room temperature (25 ± 1 °C). A water droplet of $\approx 12 \mu\text{L}$ was formed in bulk oil phase and the changes in the drop shape and the related interfacial tension variations were monitored for 15 min.

2.6. Measurement of the surface dilatational modulus

The surface dilatational modulus was measured on a DSA100m instrument, equipped with Oscillating Drop Module (ODM module DS3260). Sinusoidal oscillations of the area of pendant drops were applied and the changes in the area and in the interfacial tension of the drops were measured by using drop shape analysis. The total surface

dilatational modulus, G_S , the surface dilatational storage and loss moduli, G_{ST} and G_{LOSS} , were determined by the procedure described in (Alexandrov, Marinova, Danov, & Ivanov, 2009; Russev et al., 2008). The relation between these moduli is given by the following equation:

$$G_S = ((G_{ST})^2 + (G_{LOSS})^2)^{1/2} \quad (1)$$

Preliminary experiments with solutions of modified and non-modified egg yolk were performed at different concentrations and pH values. The results showed that the moduli of these systems did not change significantly with the amplitude of deformation (for all systems), but were affected by the period of deformation. Therefore, in the experiments presented below, the amplitudes of deformation were between 1 and 5%, and the period of oscillation was varied (2, 5 and 10 s). The oscillatory deformation was applied 5 min after drop formation.

2.7. Observation of thin liquid films in capillary cell

Thin liquid films of millimeter diameter were formed and observed in a capillary cell using the method of Scheludko, 1967. The films were formed from a biconcave drop of aqueous solution, placed in a short capillary, by sucking out this solution through a side orifice. For emulsion films, the space around the capillary is filled with oil phase. The emulsion films were observed in reflected light for 60 min using an optical microscope Axioplan (Zeiss, Germany), equipped with a long-distance objective Zeiss Epiplan $20 \times /0.40$, CCD camera (Sony) and digital video recorder. The foam films were observed for 15 min in a closed cell (to prevent the water evaporation from the film surfaces), and for additional 15 min in a cell which was open to the external atmosphere. The evaporation of water from the foam films in open capillary cell effectively leads to higher capillary pressure ($\sim 10^5$ Pa) which pushes the film surfaces against each other (Kralchevsky & Nagayama, 2001). After an equilibrium film thickness was reached, the films were observed for additional 5 min before closure to check for their stability.

3. Results and discussion

3.1. Impact of enzymatic treatment on the chemical composition of EY

Protein composition. The results from the electrophoretic analysis of the proteins in EY and MEY are presented in Fig. 1. One sees that the protein composition of both yolks is the same. The various proteins were identified on the basis of literature data (Daimer & Kulozik, 2010) and by using standard proteins with known molecular weight as a reference.

Lipid composition. EY contains lipids with different structure and properties: non-polar lipids, such as triglycerides and cholesterol, and polar lipids, such as phospholipids, lysophospholipids, and fatty acids. The results from the gas chromatography analysis of the native and enzymatically modified egg yolks are compared in Fig. 2. Some significant differences in the polar lipid content of the two types of yolk are observed: EY contains a significant amount of phospholipids and very low amount of fatty acids, whereas MEY contains a large amount of oleic acid and much less phospholipids. These differences reflect the enzymatic modification of MEY, during which the phospholipids are (partially) hydrolyzed to lysophospholipids and fatty acids.

These results are in very good agreement with the results by Daimer & Kulozik, 2008, who showed that the major difference in the chemical compositions of EY and MEY is the decrease of the phospholipids and the increase of lysophospholipids and fatty acids after the enzymatic treatment. As the major phospholipid in EY is POPC (palmito-oleyl phosphatidylcholine), the main fatty acid produced in the hydrolysis is the oleic acid.

The other major lipid components in EY and MEY are cholesterol and triglycerides, which have similar concentrations in both yolks. The total lipid profile of EY and MEY is presented in Table 1. The results for

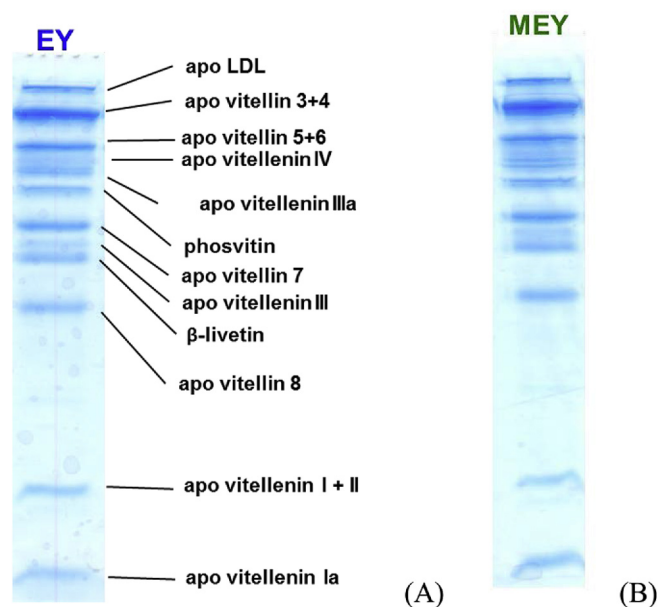


Fig. 1. Images of scanned gels from gel-electrophoretic analysis of (A) EY and (B) MEY.

the concentration of the lipid components in EY agree well with the literature data (Powrie & Nakai, 1985) according to which EY contains around 66% triglycerides, 28% phospholipids, 5% cholesterol and a minor amount of other lipids.

3.2. Electrokinetic and rheological properties of the solutions

The effect of enzymatic treatment on the bulk properties of the solutions was also investigated. The pH of the non-modified and modified EY was 6.1 and 5.9, respectively. Addition of the solid components (salt, sucrose, and EDTA) led to a slight decrease of pH of the solutions by ≈ 0.1 unit. From the ζ -potential measurements, shown in Fig. 3, the isoelectric points (IEP) of the two egg yolk solutions were determined: for MEY IEP ≈ 5.1 and for EY it is ≈ 5.4 . Apo-LDL isoelectric region is between pH 6.5 and 7.3 (Kojima & Nakamura, 1985; Le Denmat et al., 2000; Nakamura, Hayakawa, & Sato, 1977), the isoelectric point of livetins varies between pH 4.3 and 5.5 (Le Denmat et al., 2000; Ternes, 1989) and the isoelectric point of phosvitin is around pH 4.0 (Le Denmat et al., 2000; Ternes, 1989). Apo-HDL have not been characterized precisely but their isoelectric region is expected to be in an “enlarged” neutral pH region. Overall, the IEP of the surface active proteins from egg yolk is considered close to the neutral pH 6–7 (Daimer & Kulozik, 2009). The IEP values determined in the current work are slightly lower, most probably due to the presence of added salt in the egg yolk studied (according to the EY supplier, the content is 92% egg yolk, 8% salt).

The ζ -potential of the aggregates in EY is slightly higher in the entire pH range studied when compared to those in MEY. At natural pH, the aggregates in both egg yolk solutions are negatively charged, whereas they are positively charged at acidic pH. One can see that the absolute values of the ζ -potential of the EY is higher than that of MEY at acidic pH, while at natural and alkaline pH the opposite trend is observed.

The results from the light scattering measurements (Table 2) showed that there is a significant difference in the size distribution of the aggregates in EY and MEY solutions. Both egg yolks have a bimodal size distribution. In the EY solution, the peaks are at ≈ 1400 nm and at ≈ 4500 nm in diameter, with area of the peaks by volume of 52% and 48% respectively. In contrast, significantly smaller aggregates are observed in MEY: the main peak is at ≈ 35 nm in diameter ($\approx 97\%$ area), while some aggregates with diameter ≈ 400 nm are also detected.

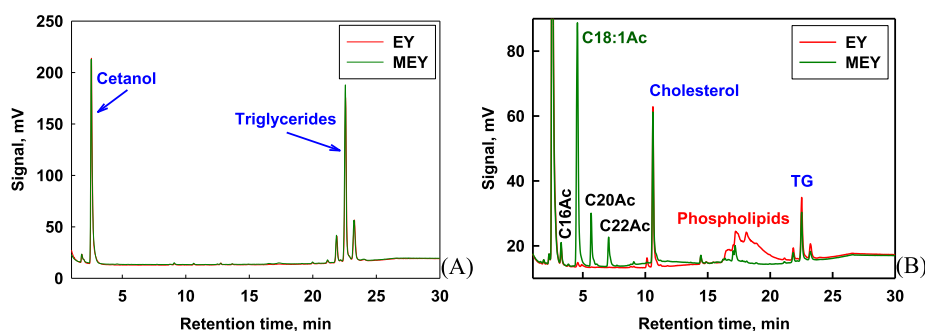


Fig. 2. Comparison of the gas chromatograms of EY (red line) and MEY (green line) from (A) chloroform and (B) methanol extract. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Composition of the lipids in the studied egg yolks. Two independent samples were analysed for both EY and MEY. Significant difference of $P < 0.05$, as determined by one-way ANOVA statistical analysis, is denoted by **.

Lipid content, %	EY	MEY
Neutral triglycerides	71.8 ± 2.5	78.6 ± 2.7
Free fatty acids	1.7 ± 0.5	12.1 ± 3.5**
Phospholipids	23.3 ± 0.6	5.1 ± 0.1**
Cholesterol	3.2 ± 0.1	4.2 ± 0.1

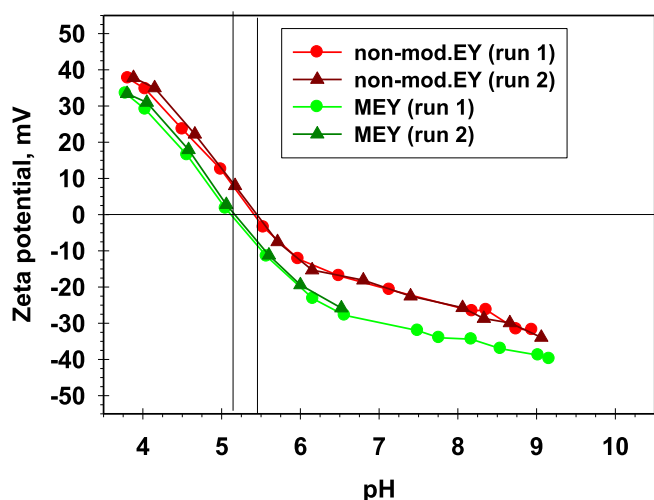


Fig. 3. ζ -Potential of solutions of EY (red symbols) and MEY (green symbols), as a function of solution pH. Concentration of egg yolk: 0.1 wt %; no other additives in the solution. IEP \approx 5.1 for MEY and IEP \approx 5.4 for EY. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

These results are confirmed by optical microscopy (results not shown).

According to Jolivet et al. (2009), the size of the granules could vary significantly, depending on the environmental conditions. At relatively low ionic strength and pH \sim 6, the granules exist in two forms: intermediate micelles with size 100–200 nm and aggregates with size in the range 1–8 μ m, which is in a good agreement with our results for EY. As for the aggregate size in MEY solution, the first peak seems to

Table 2

ζ -Potential and aggregate size in 0.1 wt% solutions of EY and MEY at natural pH. Ionic strength: 5 mM. Three independent measurements were performed for each sample. For both the ζ -potential and drop size, the values for EY and MEY differ significantly, with $P < 0.001$ as determined by one-way ANOVA statistical analysis.

Emulsifier	ζ -potential (mV)	Average diameter by volume, nm (1st peak)	Average diameter by volume, nm (2nd peak)	Area Peak 1%	Area Peak 2%
EY	-21.07 ± 0.4	1419 ± 302	4510 ± 544	51.8	48.2
MEY	-25.2 ± 0.6	35 ± 1	386 ± 60	96.6	3.2

correspond to the size of LDL particles, whereas the second peak could be due to partially disrupted granules. Daimer and Kulozik (2008) report that the size of the granules in native egg yolk at pH = 6.5 and 0.15 M NaCl is around 2 μ m, whereas the size of the granules decreases down to 900 nm after enzymatic treatment. The differences between their data and the results shown in Table 2 are most probably related to the different conditions at which the experiments are performed. Strixner and Kulozik (2013) found that the size of the particles in different granules sub-fractions varied between 0.84 and 4.87 μ m, which is in a good agreement with the results shown in Table 2 for non-treated EY.

Interestingly, no significant difference was observed between the rheological properties of the initial (non-diluted) solutions of EY and MEY at natural pH. This result indicates that the concentrated EY and MEY probably contain similar in size aggregates – such an assumption could explain the similar viscosities of the two egg yolks: \approx 3.08 ± 0.05 mPa s for EY solution and \approx 2.88 ± 0.05 mPa s for MEY solution at natural pH (both systems have Newtonian behavior). The light scattering results show that the dilution of these solutions causes significant structural changes and aggregate disruption in MEY.

Fig. 4 presents results for the rheological properties of EY and MEY solutions at acidic pH 3.8. One sees that EY solution has significantly higher η and exhibits a non-Newtonian behavior at acidic pH. Microscope observations in transmitted light revealed the presence of large aggregates in the solutions of both egg yolks at pH 3.8, which could explain the significant increase in the viscosity of the EY solution, but these aggregates did not show a significant effect on the rheological response of MEY solution.

3.3. Interfacial tension at SBO-water interface

As already explained, the enzymatic treatment causes structural changes in the egg yolk components. To check for the effect of enzymatic treatment on the interfacial properties, we first measured the interfacial tension of the studied egg yolk solutions at a SBO-water interface. We also studied the effect of pH by performing experiments at the natural pH of the respective solution and at pH = 3.8, adjusted by addition of acetic acid.

The results evidenced for faster interfacial adsorption of MEY solutions, at both pH values, see Fig. 5. The interfacial tension of the non-modified EY solutions reached a relatively constant value after a much longer time period. Under all conditions studied, MEY solutions

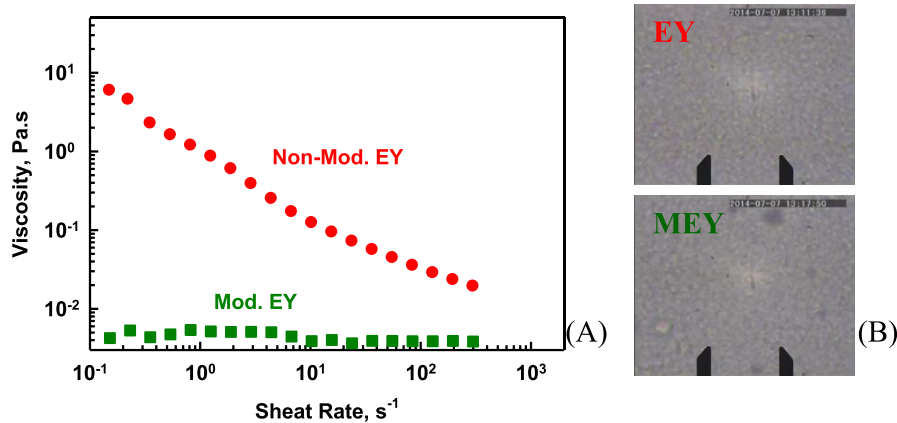


Fig. 4. (A) Viscosity of the solutions determined in steady shear deformation for non-modified (red circles) and modified (green squares) egg yolk at acidic pH 3.8. (B) Microscope images of the respective solutions – both solutions contain micrometer sized aggregates at this low pH. The distance between the bars is 20 μm . Solutions contain: 22.5 wt% yolk, salt, sucrose, EDTA and acetic acid. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

displayed significantly higher interfacial tension, compared to the non-modified EY. This result is unexpected because normally the low-molecular-mass surface active species, such as the lysophospholipids, are expected to decrease the interfacial tension of MEY solutions to lower values. As known from literature (Anton, 2013), strong and dense adsorption layers, composed of proteins and phospholipids, are formed at the interface in the case of non-modified EY. Therefore, the surface active components in MEY might not form such closely packed, dense adsorption layers at the interface, resulting in a higher interfacial tension. It is rather possible that the lysolipids could be attracted and bound to the aggregates in the bulk of the MEY solutions, thus decreasing their surface activity. Similar competition between the aggregation in the bulk solution and the adsorption of surfactants was reported to result in higher surface tension in the solutions of some polymer-surfactant mixtures (Taylor, Thomas, & Penfold, 2007).

Another interesting result was observed when varying the pH. For the solution of MEY, an increase in the interfacial tension was observed upon decrease of pH, in a good agreement with the results by Mel'nikov (2002). The interfacial tension of the MEY solution at pH 3.8 after 15 min is ≈ 11.8 mN/m, compared to interfacial tension ≈ 10.6 mN/m at natural pH. The opposite trend was observed for the non-modified EY: the interfacial tension decreased from ≈ 6.6 mN/m at natural pH to ≈ 5.4 mN/m at acidic pH. The observed different effect of pH for EY and MEY is probably related to the fact that the granules are already disrupted in MEY, due to the enzymatic modification, whereas pH has strong impact on the size of the aggregates and on the rheological properties for the non-modified EY. From the comparison of the curves for the non-modified EY at the two pH values, one sees much faster initial adsorption at acidic pH. Some of the researchers in this area (Anton & Gandemer, 1999; Mel'nikov, 2002) report that EY has a minimum in the interfacial tension at pH ≈ 6 (close to the IEP of LDL proteins), which is opposite to what we observe in Fig. 5. This discrepancy could be caused by the more complex composition of the

solutions studied by us: much higher concentration of yolk and salt, in combination with sugar, could lead to different interfacial properties from those reported in literature.

The observed lower interfacial tension in the initial stage of EY adsorption for the modified EY at natural pH is in a qualitative agreement with the results reported by Strixner et al. 2014, where they showed that the plasma separated from enzymatically modified EY has much lower interfacial tension, as compared to the plasma separated from native EY.

3.4. Surface rheological properties of EY and MEY at SBO-water interface

We studied the rheological properties of the adsorption layers, formed from EY and MEY solutions, by measuring the surface dilatational, storage and loss moduli. Typically, the adsorption layers formed from low-molecular-mass surfactants have low surface modulus (< 10 mN/m at low frequency), whereas the adsorption layers composed of proteins usually have higher modulus (> 30 mN/m), due to stronger interactions between the adsorbed molecules.

From the results shown in Fig. 6 one sees that the adsorption layers from MEY have significantly lower surface modulus compared to that of EY, regardless of the solution pH. A slight decrease in the surface modulus is observed upon decrease of pH for the EY systems. No such pH dependence of the surface modulus for MEY is observed, but the modulus decreases significantly upon increase of the oscillation period.

To better understand these results, the storage and loss moduli of the two egg yolk types were studied in more detail (Fig. 7). One sees that the interfacial storage modulus of non-modified EY is significantly higher than the loss modulus and the latter is close to zero. On the other hand, the two moduli have similar values when the adsorption layer is formed from MEY solution. From these observations, we can conclude that the adsorption layer formed in EY solutions has more pronounced elastic properties and behaves like a true protein-dominated

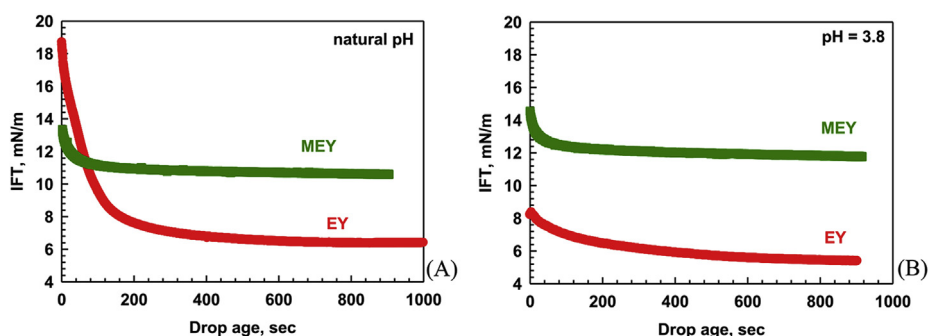


Fig. 5. Interfacial tension vs surface age for solutions of EY (red circles) and MEY (green squares): (A) natural pH; (B) pH 3.8. Solutions contain: 22.5 wt% yolk, salt, sucrose and EDTA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

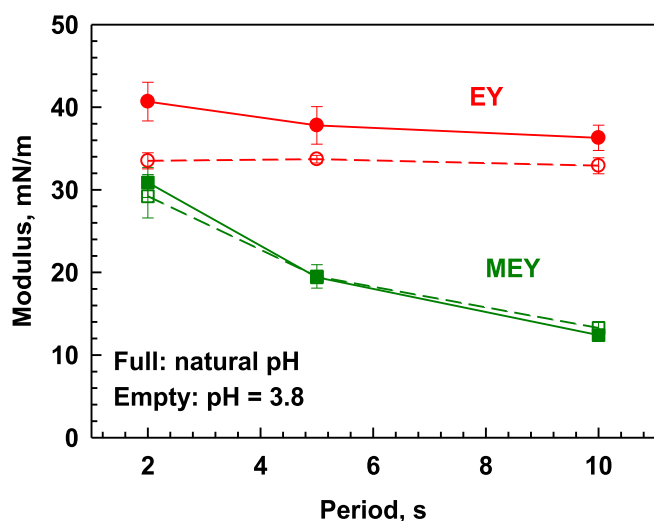


Fig. 6. Surface modulus vs period of deformation for solutions of EY (red circles) and MEY (green squares) at natural pH (full symbols) and acidic pH 3.8 (empty symbols). Solutions contain: 22.5 wt% yolk, salt, sucrose and EDTA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

viscoelastic layer. In contrast, the adsorption layer of MEY has viscoelastic rheological properties which resemble those of low-molecular-mass surfactants which indicates that the interfacial properties are dominated by the lysophospholipids and/or oleic acid in this system.

3.5. Behavior of emulsion and foam films, stabilized with EY and MEY

We performed microscope observations of the process of thinning of emulsion films. This method gives information about the properties of the film surfaces and about the stability of the aqueous films, formed between two oily phases, thus mimicking the film between two drops in O/W emulsions. From the equilibrium thickness of the film one can also elucidate the surface forces which stabilize the emulsions (electrostatic or steric). The behavior of foam films was also studied to obtain information about the properties of the adsorption layers.

In Table 3, images of emulsion and foam films, taken at different moments after film formation, are presented. Very clear differences between the two egg yolk types are seen for both foam and emulsion films. At natural pH, the EY solution stabilizes emulsion films which are non-homogeneous in thickness with trapped aggregates. This behavior is typical for most protein-stabilized emulsion films (Basheva, Gurkov, Christov, & Campbell, 2006; Dimitrova, Leal-Calderon, Gurkov, & Campbell, 2004). After ca. 1 h, the emulsion films reach a thickness of ≈ 100 nm and several thinner black spots are formed. For the solution of MEY, we observe faster film thinning. In this case some aggregates are also trapped inside the film, but these aggregates are gradually expelled from the center of the film into the meniscus region

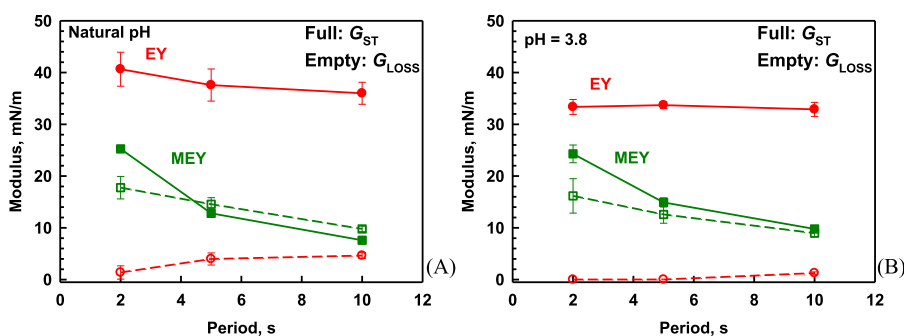
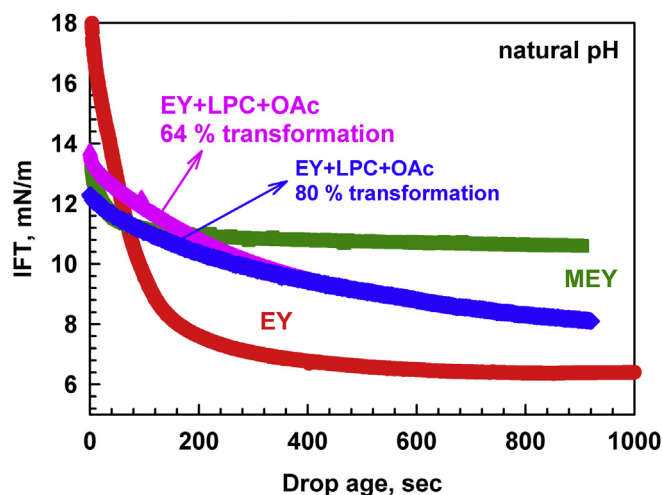


Fig. 7. Surface dilatational storage moduli (full symbols) and loss moduli (empty symbols) as function of the period of deformation. The red circles correspond to the solution of EY and the green squares correspond to MEY: (A) natural pH; (B) pH 3.8. Solutions contain: 22.5 wt% yolk, salt, sucrose and EDTA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3

Dynamics of thinning of liquid films, formed from solutions of EY and MEY: (A) Emulsion films at natural pH; (B) Foam films at natural and acidic pH. The distance between the two vertical bars on the images is 50 μ m.



surrounding the film, so that the film eventually becomes more homogeneous in thickness. The final result is a significantly thinner emulsion film, when compared to that of EY, as evidenced by the black color of the film. Therefore, the emulsion films stabilized by MEY behave similarly to films stabilized by low-molecular-mass surfactants, most probably due to the dominance of the lysophospholipids and oleic acid on the film surfaces.

The behavior of the foam films, formed at natural pH from the solutions of the two types of egg yolk, is qualitatively similar to that of the emulsion films. Again, significantly faster film thinning is observed for MEY: the equilibrium film thickness is reached after ≈ 3 min. These films remain stable even after opening the capillary cell to the atmosphere. In contrast, EY stabilizes thicker and non-uniform foam films. A particularly large difference is observed between the foam films of EY and MEY, formed at the lower pH. For MEY, the results are similar to those at natural pH, although even faster film thinning is observed – the equilibrium film thickness is reached after ≈ 2 min only. In the case of the native EY, we observe a very thick, non-homogeneous film with many aggregates trapped inside.

All results presented so far evidence for a significant difference in the interfacial properties of the native and enzymatically modified egg yolk. The native EY has surface properties and liquid film behavior which are typical for a protein-dominated system, whereas the properties of MEY resemble those of a low-molecular-mass surfactant. Similar results were reported by Lilbaek et al. (2007) who studied milk and whey after enzymatic modification of the phospholipids into lyso-PLs. The difference observed in the aggregate size might be explained by the suppression of the protein-protein attraction in the presence of lyso-PLs – as found by Tran Le et al. (2007), the heat-induced milk

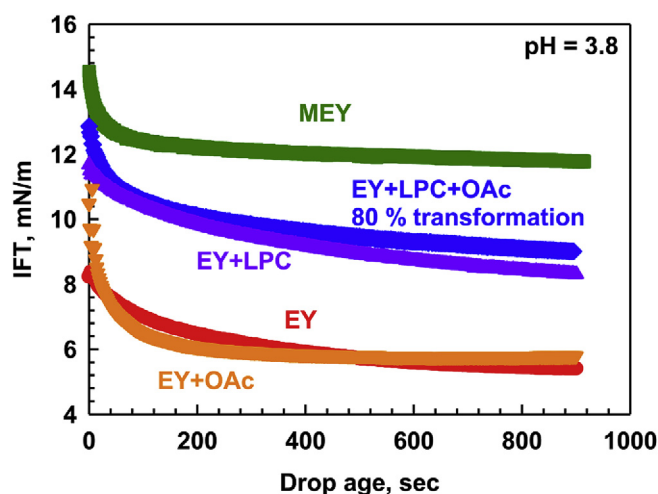


Fig. 8. Interfacial tension vs surface age of solution of 22.5 wt% EY (red circles), 22.5 wt% EY + 1 wt% LPC + 0.57 wt% OAc (blue diamonds), 22.5 wt% EY + 1 wt% LPC (purple triangles up), 22.5 wt% EY + 0.57 wt% OAc (orange triangles down), and 22.5 wt% MEY (green squares). pH = 3.8. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

protein aggregation is suppressed due to lysoPL-protein interactions.

3.6. Effect of addition of LPC and OAc on the interfacial properties of EY

To explain the observed differences between the two egg yolk types discussed above, we assumed that the lysophospholipids (LPC) or/and fatty acids adsorb preferentially at the oil-water interface, partially displace the proteins, and thus play a dominant role in the interfacial properties of the MEY. To check this hypothesis, additional model experiments were performed in which we deliberately added LPC and oleic acid (the two main products of the enzymatic reaction) to the solutions of native EY, thus mimicking the composition of MEY. We studied two different concentrations of the additives which correspond to $\approx 64\%$ and $\approx 80\%$ transformation of the phospholipids in the native EY into lysophospholipids and fatty acids.

The results about the interfacial tension are shown in Fig. 8 as a function of time. One sees that the addition of LPC into EY at acidic pH leads to an increase of the interfacial tension and it becomes closer to that of MEY. In presence of oleic acid only, the oil-water interfacial tension is similar to the interfacial tension of the solution of the non-modified EY. The results from the oscillating drop method (Fig. 9) showed that the LPC modifies the surface moduli of the EY solution so that they become similar to those of MEY. We also found that EY + LPC resemble the storage and the loss moduli of the MEY solutions very closely (results not shown). Similar results are obtained also at natural pH (see Fig. B1 and B2 in Appendix B). These results indicate that the lysolipids play a key role in the determination of the oil-water interfacial properties of the studied egg yolk solutions.

In addition, we checked the effect of LPC and oleic acid on the emulsion and foam film behavior of the native EY. The respective images are shown in Table 4 and they confirm that the emulsion films from solutions of EY + LPC and OAc, at natural pH, behave similarly to the films formed from MEY solution – very thin homogeneous films were obtained. The same is true for the foam films in these systems.

The effect of LPC and oleic acid on the foam and emulsion films was studied at acidic pH as well. As shown in Table 4C, when oleic acid only is added to EY solutions, the behavior of the foam films resembles that of the film formed from MEY solution – fast film thinning and black film formation. On the other hand, when LPC only is added into the EY solution, the films thin very slowly and black spots are (eventually) formed, but they expand also slowly. This film behavior is very different

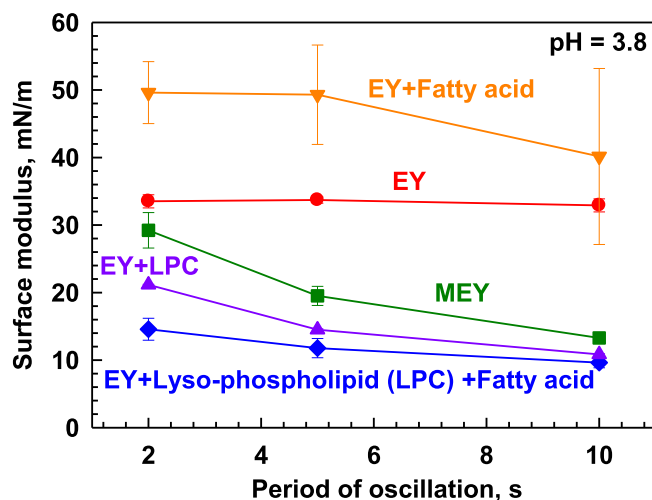
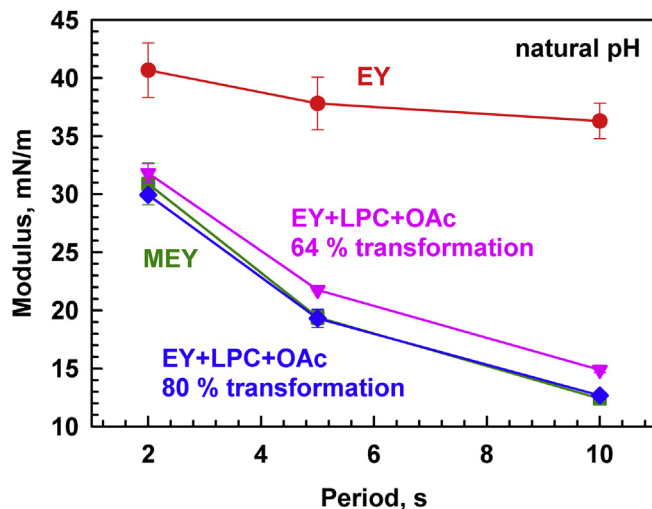


Fig. 9. Surface modulus vs period of deformation of solution of 22.5 wt% EY (red circles), 22.5 wt% EY + 1 wt% LPC + 0.57 wt% OAc (blue diamonds), 22.5 wt% EY + 1 wt% LPC (purple triangles up), 22.5 wt% EY + 0.57 wt% OAc (orange triangles down), and 22.5 wt% MEY (green squares). pH = 3.8. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 4

Dynamics of thinning of liquid films, formed from solutions of EY + LPC + OAc: (A) Emulsion films at natural pH; (B) Foam films at natural pH and (C) Foam films at pH = 3.8. The distance between the 2 bars on the images is 50 μm .



from the behavior of the foam film of MEY.

In conclusion, from the view point of the evolution of the foam films, the air-water interfacial properties of the MEY solution are governed predominantly by the oleic acid. It is possible that the lysophospholipids and oleic acid form mixed adsorption layers at the air-water interface, but we did not perform additional experiment to prove this hypothesis, because the oil-water interfacial properties are of major interest in the current study.

We encountered experimental difficulties in the formation of emulsion films from the studied model systems at acidic pH and we cannot present results from experiments with emulsion films performed at pH 3.8. Nevertheless, the obtained results on the interfacial tension and the surface moduli at the oil-water interface confirm that the interfacial properties of the enzymatically MEY are governed mainly by the lysophospholipids and the oleic acid, both formed in the process of lipolysis.

The above observations indicate that the role of the protein aggregates in the interfacial properties is significantly weakened in the presence of LPC and OAc. This trend was investigated further by measuring the ζ -potential and the size of the protein aggregates in EY solutions with added LPC and OAc. Similar to the interfacial properties, the solutions of EY + LPC + OAc reproduced well the results, obtained with MEY solutions (see Fig. 3 and Table 2). The ζ -potential of the aggregates is -23.1 ± 0.2 mV and -24.6 ± 0.9 mV for 64% and 80% degree of transformation, respectively. Both solutions showed a bimodal particle size and the peaks were located at ≈ 48 nm and at ≈ 312 nm in diameter for the solution with lower LPC concentration (peak area by volume is 82% and 18%, respectively). A further slight decrease in the aggregate size was observed at the higher concentration of LPC: the peaks in this system are located at ≈ 37 nm (83% peak area) and at ≈ 263 nm in diameter (17% peak area). One possible explanation for all these results is that the lysophospholipids and oleic acid stabilize the small aggregates, formed after disintegration of the granules at high ionic strength, thereby suppressing the intermolecular protein attraction and preventing the re-aggregation of the small aggregates upon dilution of the solution.

From all experimental results reported above we conclude unambiguously that the lysophospholipids and oleic acid, formed in the process of enzymatic EY modification, become the surface active species which dominate the interfacial and film behavior in the enzymatically modified egg yolk (MEY), while the properties of the native, non-modified egg yolk (EY) are governed by protein-lipid aggregates. This difference is schematically illustrated in Fig. 10.

4. Conclusions

After enzymatic treatment with phospholipase A2, the native egg yolk has significantly improved emulsifying properties. In the current paper we perform a detailed comparison of the interfacial properties of native and enzymatically modified EY and investigate the main reasons for the observed differences. The experimental conditions are chosen to mimic closely those in real food emulsions.

The obtained results show that the interfacial tension of the MEY solution is significantly higher, whereas the surface dilatational modulus is much lower, and the kinetics of interfacial adsorption is faster, when compared to the native non-modified EY. No significant effect of pH on the interfacial properties is observed for MEY in the pH range studied. In contrast, both the interfacial tension and the surface

modulus of the native EY solution decrease significantly upon lowering the solution pH. The emulsion and foam films, formed from MEY solutions at natural pH, thin faster and reach a much smaller thickness, compared to EY. In general, the interfacial and the film behavior of the EY solutions resemble those of protein-dominated systems, whereas those of the MEY solutions resemble those of low-molecular-mass dominated systems.

These results allow us to verify unambiguously the hypothesis that the lysophospholipids and the oleic acid, formed in the process of enzymatic EY modification, become the surface active species which govern the interfacial and film properties in the MEY. Indeed, we showed that the addition of lysophospholipids and oleic acid to native, non-modified EY leads to interfacial and film properties resembling closely those of MEY: higher interfacial tension, lower surface modulus, and thinner homogeneous films.

The latter conclusion is particularly important from the viewpoint of the practical applications of enzymatically modified EY as emulsifier and foamer in food systems. The methodology, used to analyze the systems studied here, could be applied for similar type of comparative analysis of other modified foamers and emulsifiers, such as milk and plant protein-lipid mixtures.

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Declaration of interest

None.

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Appendix A. Reagents for chemical analysis and analytical protocols

A.1. Protein analysis with SDS-PAGE Gel-electrophoresis

For the preparation of gels and to perform SDS-PAGE analysis, the following chemicals were used: glycine (product of Teocom, Bulgaria);

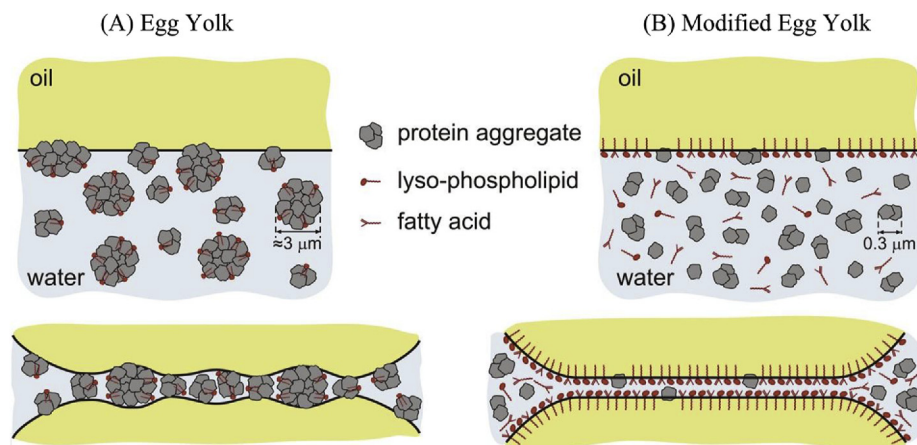


Fig. 10. Schematic illustration of the main conclusion of the current study: (A) The interfacial and film properties of the non-modified egg yolk (EY) are governed by the native protein-lipid aggregates, whereas (B) these properties of the enzymatically modified egg-yolk (MEY) solutions are governed mostly by the lysophospholipids and the oleic acid, formed in the process of enzymatic modification.

acrylamide-bisacrylamide (Merck); N,N,N',N'-tetramethylethylenediamine, TMED (Merck); ammonium persulfate, APS (Merck); SDS (Acros); Tris (hydroxymethyl)aminomethane (Sigma Aldrich), β -mercaptoethanol (Merck); bromophenol blue (Merck); ethanol (Chemax Pharma Ltd), glycerol (Teocom) and glacial acetic acid (Teocom). Mini-PROTEAN Tetra cell (advanced setup) of BioRad company was used with the experimental protocols as described in its user manual.

Discontinuous gels containing two consecutive layers (upper and bottom) were used. The upper (first) layer contains 3.5% of acrylamide and has lower pH 6.8. Its purpose is to “compress” the initial protein bands, thus forming a very narrow band at the entrance of the second layer (with higher pH 8.8) which contains 9% of acrylamide and is used for the actual protein separation. The separated protein bands were stained using the Coomassie Blue staining protocol of Laemmli (1970). Briefly, it consists of (1) staining the polyacrylamide gel as well as the protein bands in a dye solution; (2) destaining the gel to visualize the protein bands; (3) fixing the protein bands.

For analysis, 2 wt % egg yolk solutions were first diluted in the so-called “sample buffer” which contains TRIS buffer (0.5 M at pH 6.8) for adjusting pH, mercaptoethanol (reducing agent for breaking the -S-S- bonds in the protein molecules), SDS (for complete denaturing of proteins), glycerol (to adjust solution mass density) and dye (as front marker). The ratio of the sample buffer to the protein sample should be at least 2/1 to ensure appropriate pH of the analysed solution. The mixture of protein and sample buffer is then heated for 4 min at 95 °C to achieve complete protein denaturing. The total quantity of protein introduced in a single well was 6 mg/mL. At higher quantities, the gel becomes overloaded and the final protein bands are distorted and poorly visualized.

A.2. Gas chromatography (GC) analysis of lipids

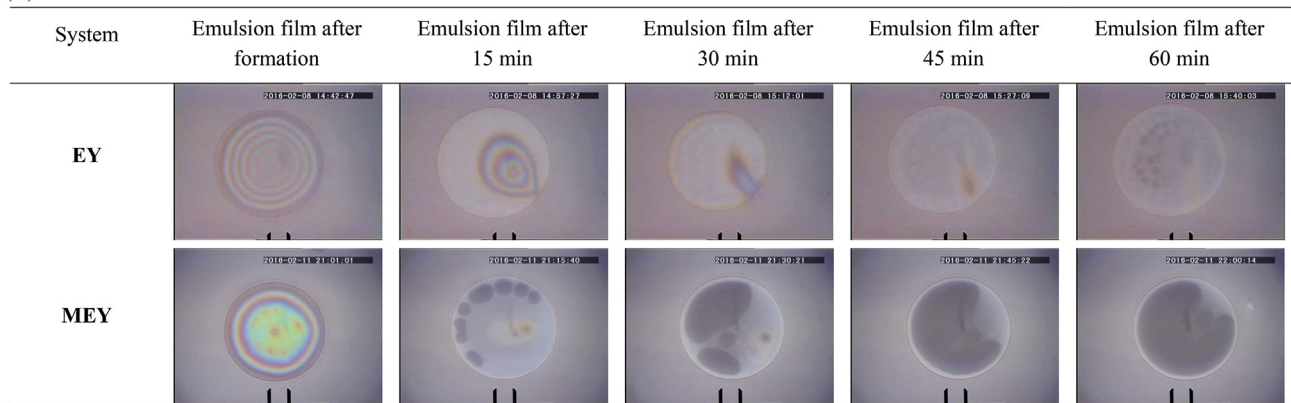
For the extraction and separation of the egg yolk components, methanol and chloroform were used. The standards used for determination of retention times in GC were: myristic acid, palmitic acid, stearic acid (97%, Acros), oleic acid, 1-oleyl glycerol, dipalmitin, triolein, 1-Palmitoyl-2-hydroxy-SN-glycero-3-phosphocholine, dipalmitoylphosphatidylcholine (DPPC). For derivatization of the samples prior their injection into the GC head, N,O-Bis(trimethylsilyl) trifluoroacetamide (derivatization grade, Supelco) and anhydrous pyridine were used. After derivatization, the samples were diluted with toluene. Unless specified otherwise, all materials described in this section were obtained from Sigma-Aldrich and are with purity $\geq 97\%$.

The obtained chloroform (non-polar lipids) and methanol (polar lipids) fractions were derivatized by using the following protocol: 4 mL of the methanol extract was evaporated in a vacuum dryer and the dry precipitate was dissolved in 400 μ L chloroform; for the chloroform extracts, we transferred 400 μ L extract to a 1 mL glass vial; afterwards, 100 μ L 1.8 wt % cetanol in chloroform, 200 μ L pyridine and 200 μ L BSTFA were added to each sample and the mixture was heated for 1 h at 60 °C; after cooling to room temperature, 75 μ L of each sample was diluted with 925 μ L toluene in GC vials and the samples were introduced in the GC autosampler.

The GC analyses were performed on a TRACE GC apparatus (ThermoQuest, Italy), equipped with an autosampler AS 2000. A capillary column Quadrex, USA, with the following specifications was used: 5% phenyl methylpolysiloxane, 10 m length, I.D. 0.53 mm, 0.1 μ m film thickness. A cold on-column injection was used, at a secondary cooling time of 0.3 min. The injection volume was 1 μ L. Before introducing the sample into the GC column, all solutions were derivatized to obtain the trimethylsilyl derivatives of the studied substances. The oven was programmed as follows: start at 110 °C, hold 2 min, ramp 1–325 °C at 10 °C/min, ramp 2–345 °C at 5 °C/min, hold 5 min. The flame-ionization detector (FID) temperature was set to 350 °C. The carrier gas was helium, set at a constant pressure flow mode (60 kPa). The detector gases were hydrogen and air, with nitrogen as a make-up gas. The secondary cooling gas was nitrogen with a purity of 99.99%. All other gases were of 99.999% purity. This procedure allows one to separate the TG, FA, MG, phospholipids, lysolipids and cholesterol in the samples studied. For proper quantification, we used standard solutions of the studied substances and cetanol as an internal standard.

Appendix B. Oil-water interfacial properties of EY solution in presence of LPC and Oleic acid at natural pH

(A)



(B)

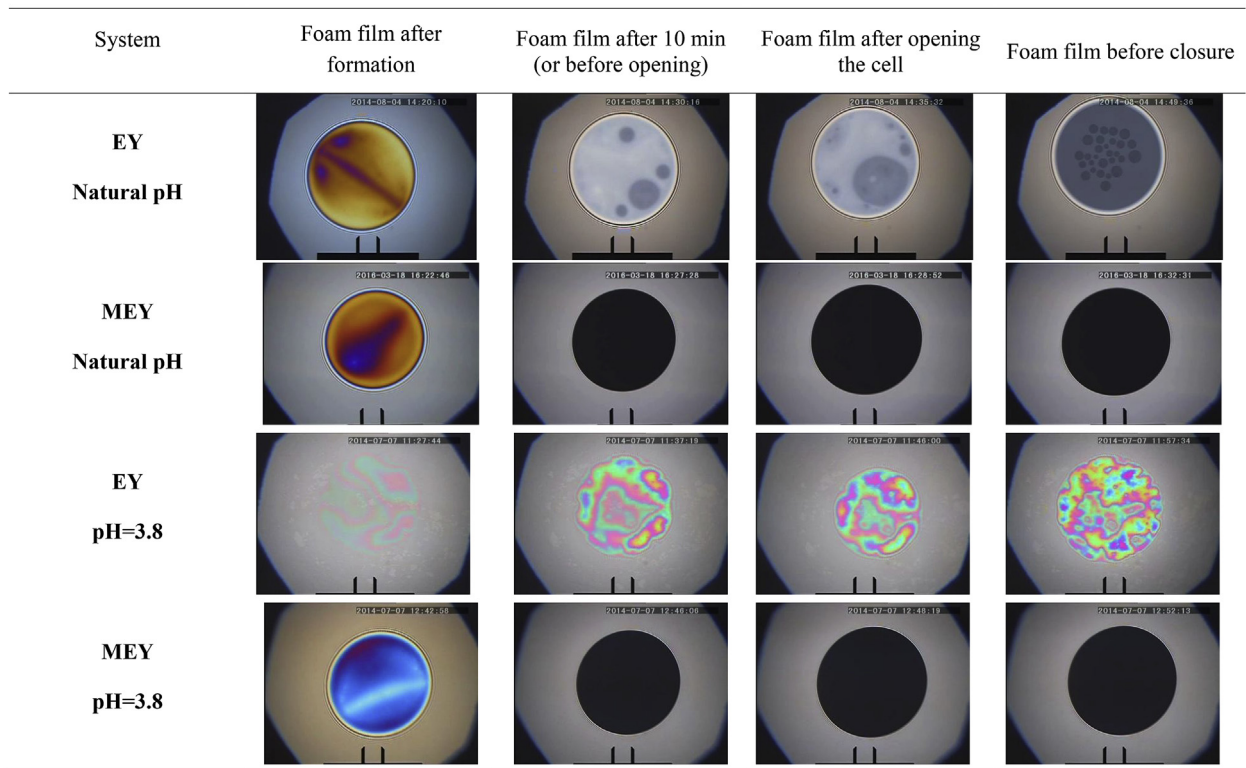
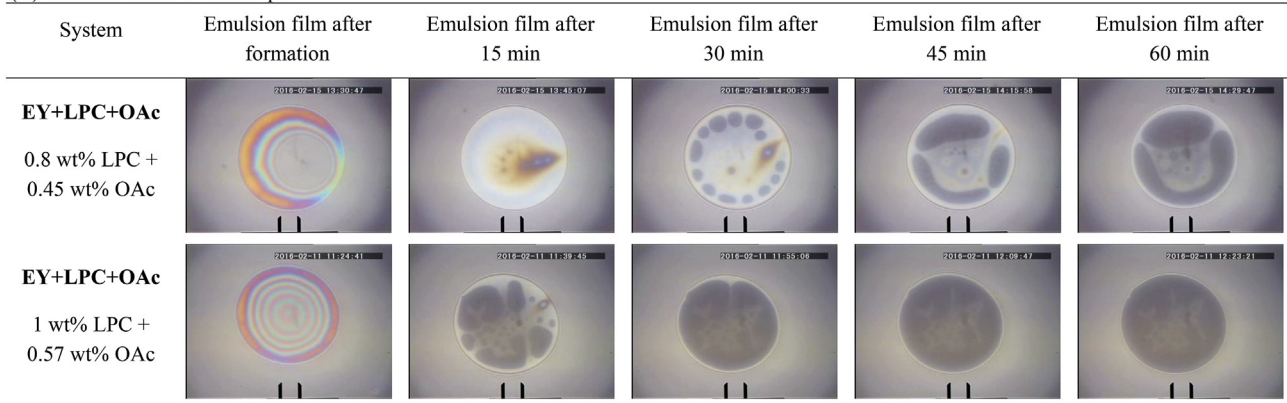
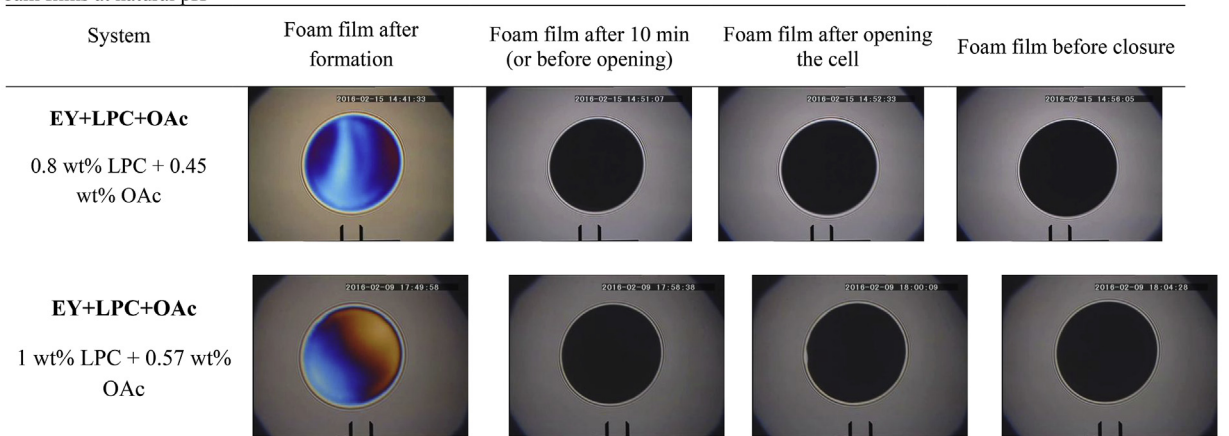


Fig. B.1. Interfacial tension vs surface age of solution of 22.5 wt% EY (red circles), 22.5 wt% EY + 0.8 wt% LPC + 0.45 wt% OAc (pink triangles), 22.5 wt% EY + 1 wt% LPC + 0.57 wt% OAc (blue diamonds) and 22.5 wt% MEY (green squares). Natural pH.

(A) Emulsion films at natural pH



(B) Foam films at natural pH



(C) Foam films at pH=3.8

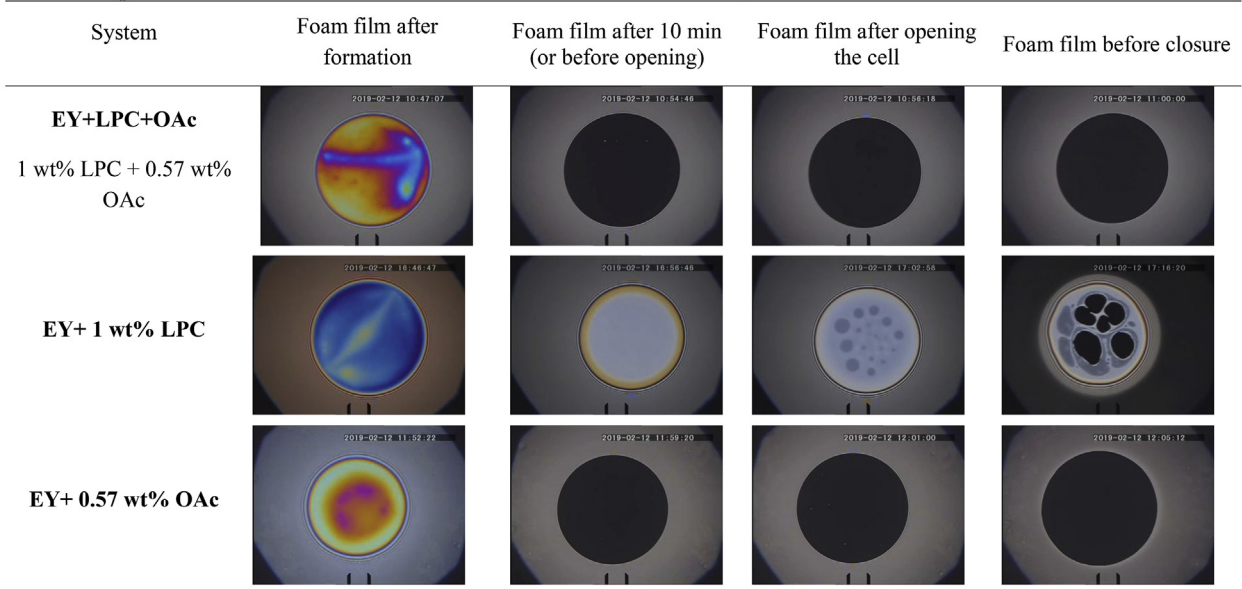


Fig. B.2. Surface modulus vs period of deformation of solution of 22.5 wt% EY (red circles), 22.5 wt% EY + 0.8 wt% LPC + 0.45 wt% OAc (pink triangles), 22.5 wt% EY + 1 wt% LPC + 0.57 wt% OAc (blue diamonds) and 22.5 wt% MEY (green squares). Natural pH.

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