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Competitive Adsorption of Water Soluble Plasma Proteins from Egg Yolk at the Oil/Water Interface

LARS NILSSON,*,† PETER OSMARK,§ CÉLINE FERNANDEZ,§ MARCUS ANDERSSON,† AND BJÖRN BERGENSTÅHL†

Division of Food Technology, Centre for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, S-221 00 Lund, Sweden, and Department of Experimental Medical Science, Division of Diabetes, Metabolism and Endocrinology, Molecular Endocrinology Group, Biomedical Centre, Lund University, S-221 84 Lund, Sweden

Water soluble plasma proteins were fractionated from hen's egg yolk, and the molecular weight and p*I* of the most abundant protein species were characterized with gel electrophoresis. The proteins were identified by mass spectrometry. The protein fraction was used to produce oil-in-water emulsions, both at various protein concentrations and at various pH values, and the surface load was determined through serum depletion. The competitive adsorption was studied through the determination of nonadsorbing species with gel electrophoresis. The results show that it was possible to form an oil-in-water emulsion for which droplet size and maximum surface load depended on the protein concentration and pH. Serum albumin and YGP40 adsorbed selectively at the oil/water interface throughout the pH range investigated, and for albumin the selectivity increased close to its p*I*. It is suggested that this selective adsorption is due to long hydrophobic stretches in the polypeptide chain, which are present in the selectively adsorbing species but absent in less adsorbing species.

KEYWORDS: Egg yolk proteins; livetin; emulsion; competitive adsorption; oil/water interface

INTRODUCTION

Food dispersions such as emulsions are often stabilized by proteins as proteins are surface active and abundant in most foodstuffs. The adsorption of proteins occurs at most interfaces and is the net result of interactions between the protein, the interface, and the solution (*1*). In the case of oil-in-water emulsions the adsorption is mainly due to hydrophobic interaction between the protein and the interface, and thus a high surface hydrophobicity of the protein should be an important property (*2*). Adsorbed proteins stabilize emulsions through electrostatic and steric stabilization. The steric stabilization is determined by the solubility of the protein in the aqueous phase, which in turn depends on protein charge. Thus, charge will determine both steric and electrostatic stabilization. To be an efficient emulsifying agent and stabilizer, a protein should also possess the ability to unfold and spread at the interface (*3*).

Proteins in food applications represent a mixture of protein species with different properties. As all proteins are surface active, they will tend to adsorb at interfaces, but when the amount of proteins present in a solution is large in comparison to the amount of interfacial area, the adsorption process becomes competitive. This can ultimately lead to only certain species present in a protein fraction being found at the interface. The protein that gives the highest reduction of the free energy of the system, that is, the most surface active species, will dominate the interface at equilibrium. An early and well-known example of interfacial displacement is the so-called Vroman effect, which describes the selective adsorption of blood serum proteins on glass surfaces. Vroman and Adams (*4*) showed that over time the composition of the adsorbed layer changed, with larger species replacing the smaller ones. This exchange is attributed to the higher adsorption energy of the large proteins. Both competitive adsorption and displacement will occur in food emulsions, and several authors have studied competitive adsorption between proteins at the oil/water interface (*5*−*8*). However, as reaching equilibrium can be a very slow process, other factors such as relative concentration and transport to the interface from the bulk solution will also influence the final composition of the interfacial layer. It has been shown that a less surface active protein can prevent the adsorption of a more surface active species (*9*).

When adsorption has occurred, proteins can unfold and spread at the interface to optimize their configuration from a thermodynamical point of view (*9*−*11*). During this step displacement of one protein by another may occur, although protein adsorption is to a large extent considered to be irreversible.

Egg yolk proteins are important in many disperse food systems as they are able to provide stabilizing effects at low pH, which is uncommon for proteins commonly used in dispersions. This is attributed to the fact that egg yolk contains

* Corresponding author (e-mail lars.nilsson@food.lth.se; telephone +46 46 2229670; fax +46 46 2224622).
† Division of Food Technology.
§ Division of Diabetes, Metabolism and Endocrinology.

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proteins species with a broad p*I* range (*12*, *13*). Although the yolk consists of other surface active components such as phospholipids and cholesterol, the proteins are believed to play a major role in emulsions, and the interaction between all the surface active constituents is likely to influence the emulsification properties (*7*, *14*). The general characterization of egg yolk proteins has to our knowledge been proceeding since the early 1900s (*15*) to the present (*16*), but it is mainly in recent years that the role of the yolk proteins in emulsions has been studied (*7*, *14*, *17*−*19*). Furthermore, egg yolk proteins in gels and emulsions have more recently been reviewed by Kiosseoglou (*20*). Mine studied the adsorption of egg yolk at the oil/water interface as a function of pH and oil volume fraction (*19*) and found that the constituents of the high-density lipoprotein fraction adsorbed preferentially. The author also found that neither phosvitin, from the granules, nor livetin, from plasma, adsorbed at the interface. It was also concluded that the low-density protein fraction was not a major contributor to the emulsification. Le Denmat et al. studied the adsorption of egg yolk plasma and granules and found that plasma proteins were present at the interface both at pH 3 and at pH 7 and at both 0.15 and 0.55 M NaCl (*14*). However, the composition of the adsorbed protein layer depended on the ionic strength and pH. This shows that the adsorption of egg yolk proteins is complex and that further work is needed in this area.

The aim of this study is to investigate the surface activity and adsorption characteristics in oil-in-water emulsions of a naturally occurring mixture of proteins fractionated from egg yolk. The protein fraction used was the α−*β*-livetin fraction from egg yolk plasma. Furthermore, any competitive adsorption of the protein species present in the fraction will be investigated and adsorbing species identified.

MATERIALS AND METHODS

Protein Fractionation and Characterization. Hen's eggs were purchased from a local supermarket. The eggs were manually broken, and the yolk and the white separated. The yolk was then carefully rolled on a filter paper to remove remaining albumen according to the method of Le Denmat et al. (*14*). The rolled yolks were mixed, and the proteins were then fractionated by stepwise precipitation with NaCl and centrifugation according to the method of McBee and Cotterill (*21*). The protein fractions were analyzed by two-dimensional polyacrylamide gel electrophoresis to investigate the molecular weight, p*I*, and relative amount of the different protein species. For two-dimensional polyacrylamide electrophoresis samples were focused on an 11 cm pH 3−10 NL ReadyStrip (Bio-Rad) at 8 kV for 24 h in 9.5 M urea, 2% CHAPS, 1% DTT, and 1% IPG buffer 3−10 ampholyte in a Protean IEF Cell (Bio-Rad). Subsequently, the strip was equilibrated and alkylated according to the Bio-Rad ReadyStrip instruction manual and mounted on a 10% SDS−polyacrylamide gel for protein separation.

Spots representing the most abundant proteins were cut from the gel and identified by mass spectrometry after trypsin digestion.

MS Analysis. Gel spots were manually cut out, digested with modified porcine trypsin (20 ng/μL) (Promega, Madison, WI) according to the protocol supplied by Amersham Bioscience, and spotted on MALDI target plates. MALDI-TOF spectra were acquired in a data-dependent mode on a MALDI-HT (Waters, Sollentuna, Sweden). MALDI spectrum processing and database searches ($p < 0.05$) were performed using the PIUMS software (*22*, *23*). Sequence information was obtained by LC-MS/MS spectra acquired on a Qtof Ultima (Waters, Sollentuna, Sweden). MS/MS database searches were performed using Mascot Daemon (Matrix Science, www.matrixscience.com).

Emulsions. Buffers containing equal parts (10 mmol/L each) of lactic acid, acetic acid, imidazole, and Tris to a total concentration of 40 mmol/L were prepared in range of pH from 2.8 to 8.0. The ionic strength was adjusted in the above buffers by the addition of NaCl. An additional buffer at pH 9.0 and 8 mmol/L was prepared and used for the dissolution of the freeze-dried protein sample. The protein stock solution was gently stirred for 1 h and centrifuged at 10000*g* for 15 min to remove any insoluble material.

Two different series of emulsions were prepared. In the first series of experiments the pH was kept constant at 7.0 and the protein concentration was varied. In the second set the protein concentration was kept constant while the pH was varied between 2.8 and 8.0. In all cases the required pH was obtained by the addition of the appropriate buffer. Oil-in-water emulsions were then prepared with 2.5% (v/v) MCT oil (Miglyol 812, Sasol, Witten, Germany) by mixing with an Ultra Turrax for 3 min followed by homogenization in a valve homogenizer at 15 MPa. The valve homogenizer used has been described in detail elsewhere (*24*). The ζ-potential of the emulsions was then measured with a Zetasizer 4 (Malvern Instruments Ltd., Malvern, U.K.), and the area-weighted average droplet diameter of the emulsion droplets was determined with laser diffraction (Coulter LS 130, Beckman Coulter, High Wycombe, U.K.).

The adsorbed amount was determined through serum depletion, and the emulsions were separated in two steps by mild centrifugation to avoid coalescence until a clear subnatant was obtained. The first separation step was carried out at 3400*g* for 15 min and the second step at 10000*g* for 15 min. After the separation of the two phases, the tubes were vortexed to ensure that no sediment was formed. The total protein content was determined according to the Bradford method (*25*). The determination was carried out both in the subnatant of the separated emulsion sample and in the reference sample containing the same initial protein concentration but without a dispersed phase. The reference sample was treated in the same way as the emulsion sample and thus went through the same steps of homogenization and centrifugation. Any variation in the Bradford analysis due to varying sensitivity to different protein species was thus minimized by assuming that the sensitivities are equal for the sample and the reference. The adsorbed amount of protein is obtained from the difference between the amount in the reference sample containing no disperse phase and the amount in the subnatant after separation of the emulsion (eq 1), which assumes that all protein species have the same response in the Bradford analysis.

$$c_{\text{adsorbed}} = c_{\text{ref}} - c_{\text{sub}} \tag{1}$$

The surface load (Γ) is obtained by relating the adsorbed amount to the specific surface area of the emulsion (eq 2)

$$\Gamma = \frac{c_{\text{adsorbed}} d_{32}}{\varphi 6} \tag{2}$$

where d_{32} is the area-weighted average droplet diameter and φ is the dispersed phase volume fraction. The non-adsorbing species in the emulsification experiments were determined with one-dimensional SDS−polyacrylamide electrophoresis (*26*).

Statistical Analysis of the Emulsion Experiments. The adsorption isotherm obtained at constant pH was converted to a linearized form of the Langmuir isotherm according to eq 3

$$\frac{1}{\Gamma} = \frac{1}{\Gamma_{\max}} + \frac{1}{c_{\text{eq}}} \cdot k \tag{3}$$

where Γ is the surface load, $\Gamma_{\max}$ is the maximum surface load, and c_{eq} is the apparent equilibrium concentration in the bulk solution. The random error in $1/\Gamma_{\max}$ was obtained from eq 4.

$$\text{SSQ} = \sqrt{\frac{\sum_i \left(\frac{1}{\Gamma_i} - \frac{1}{\hat{\Gamma}_i}\right)^2}{n-2}} \tag{4}$$

The average error in $\Gamma_{\max}$ could then be determined from the average error in Γ. The standard deviation between replicates of the ζ-potential was determined with single-factor ANOVA ($n = 2-6$).

RESULTS

The water-soluble fraction of yolk protein, often referred to as α−*β*-livetin, was isolated according to the description under

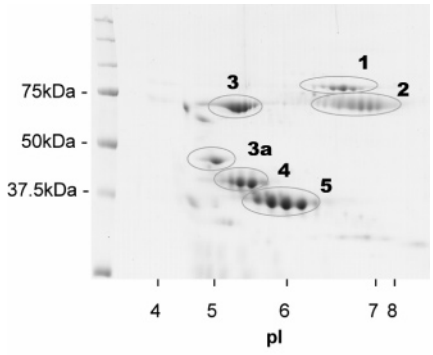

Figure 1. Two-dimensional polyacrylamide gel of soluble egg yolk plasma protein. The six most prominent protein bands, marked by a ring, were identified by mass spectrometry as follows: 1, ovotransferrin or conalbumin; 2, immunoglobulin G; 3, serum albumin or α-livetin; 3a, truncated serum albumin (aa 1–aa 410); 4, yolk plasma glycoprotein YGP42; 5, yolk plasma glycoprotein YGP40. The presence of several spots for most of the proteins is due to differential phosphorylation.

Table 1. Five Dominant Protein Species in the Egg Yolk Livetin Fraction As Determined by 2D SDS-PAGE and Subsequent Mass Spectrometry

spot	protein	obsd mol mass (kDa)	obsd p*I*	theor mol mass[a] (kDa)	theor p*I*[a]
1	ovotransferrin (conalbumin)	80	6.5–7	75.8	6.69
2	IgG, heavy chain[b]	65–70	6.5–8	(60–70)	(6–7)
3	serum albumin (α-livetin)	65	5–5.7	67.2	5.35
4	YGP42	40	5.3–5.8	31.4	5.88
5	YGP40	35	5.5–6.3	31.0	6.16

[a] Not taking into account post-translational modifications, e.g., glycosylation or phosphorylation. [b] The theoretical values for IgG are only approximate due to the inherent immunoglobulin heterogeneity.

Materials and Methods. The fraction was characterized with two-dimensional polyacrylamide gel electrophoresis and mass spectrometry.

The results of the two-dimensional polyacrylamide gel electrophoresis are shown in **Figure 1**. Five groups of spots were clearly identified, the multiple spots in each group being due to different degrees of phosphorylation. The molecular masses of the proteins range from about 37 to 80 kDa, and p*I* values vary between 5 and 8 (**Table 1**). Samples were cut from the gel for protein identification with MS, and the results of the identification are included in **Table 1**.

Subsequently, emulsions were formed with MCT oil and different amounts of the protein fraction, and the particle size of the emulsions was determined with light diffraction. The emulsions were then separated, and the amount of non-adsorbed protein was determined. The results from the emulsion experiments at a constant pH are shown in **Figures 2** and **3**. **Figure 2** shows that it is possible to obtain an oil-in-water emulsion with the protein fraction and that the emulsion surface area increases linearly with the initial protein concentration. The adsorption isotherm at pH 7 (**Figure 3**) shows that the surface load (Γ) first increases as the apparent equilibrium concentration of the proteins increase until a plateau is reached at ≈1.5 mg/m^2 with a random error of 0.4 mg/m^2. A similar series of emulsification experiments was carried out in which the amount of protein was kept constant and the pH was varied between 2.8 and 8.0. The non-adsorbing species were in both series examined by SDS−polyacrylamide electrophoresis. The SDS-PAGE gel of the non-adsorbing species is shown in **Figure 4a**,

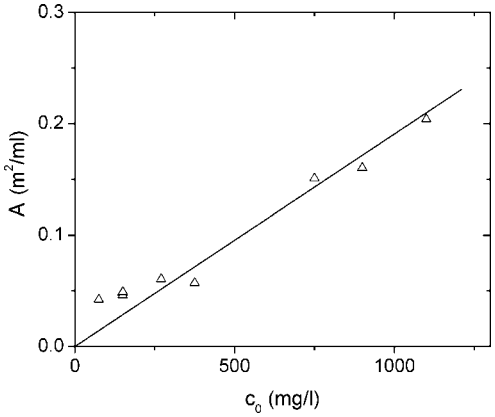

Figure 2. Results of the emulsification experiments shown as emulsion surface area (*A*) versus initial bulk protein concentration (c_0) at pH 7. The line was obtained through linear regression (correlation coefficient = 0.986).

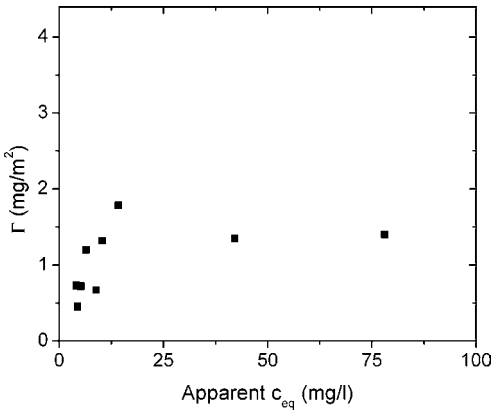

Figure 3. Adsorption isotherm at pH 7 shown as the protein surface load (Γ) versus the apparent equilibrium protein concentration (c_{eq}). The random error in Γ was determined to be 0.4 mg/m^2.

and the profiles of the intensity of the three lanes are given in **Figure 4b**. In general, at low initial protein concentrations all protein is depleted from the aqueous phase and adsorbs at the oil droplets, but as the protein concentration is increased toward saturation of the interface, a notable selectivity in the adsorption occurs. At pH 7 a clear preferential adsorption of two of the dominant protein bands is observed (**Figure 4**). These were identified as serum albumin (protein 3) and yolk plasma glycoprotein YGP40 (*27*) (protein 5).

Figure 5 shows the results from the experiments where the protein concentration was kept constant and pH was varied. In **Figure 5a** the emulsion surface area dependence on the pH is shown, and the plot shows that at pH 4.5 there is a minimum in the surface area. On the other hand, this pH corresponds to a maximum (≈3.7 mg/m^2) in the plot of Γ versus pH, which is shown in **Figure 5b**. At higher or lower pH, that is, when the proteins have a higher charge density, the adsorbed amount decreases. **Figure 5c** shows the ζ-potential versus pH for the emulsions, and the results show that the isoelectric point of the droplets lies somewhere between pH 4.0 and 4.5. At lower pH the droplets are positively charged and above they are negatively charged. The standard deviation between replicates ($n = 2-6$) of the ζ-potential was determined to be 1.9 mV. Significant degradation of some of the proteins was observed below pH 4 (**Figure 6**), which made it difficult to determine the selective adsorption below this pH. Above pH 4 the tendency observed at pH 7 is seen in the entire interval. Serum albumin seems to

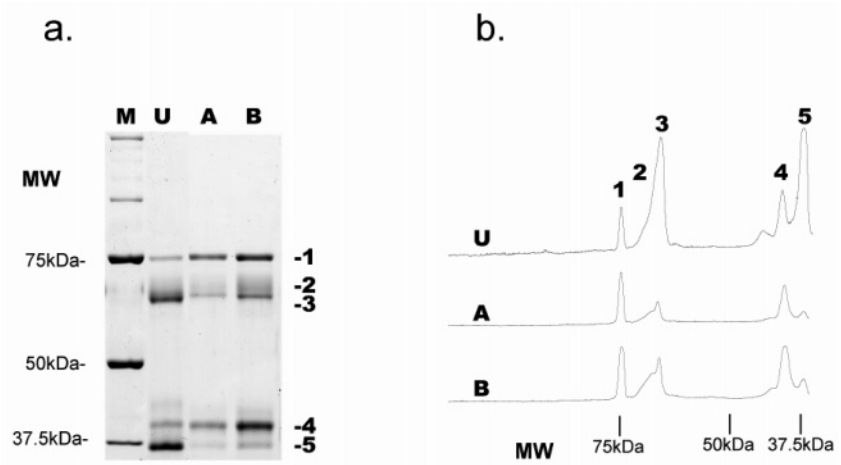

Figure 4. (**a**) SDS-PAGE of soluble egg yolk proteins before and after emulsification: lane M, molecular weight marker; lane U, unemulsified; lanes A and B, non-adsorbed subnatant after emulsification at apparent c_{eq} of 40 and 80 mg/L, respectively. For clarification, the integrated lane profiles for lanes U, A, and B are displayed in (**b**), where the intensity has been normalized after the least changing protein band, which is marked 1 (ovotransferrin). The numbering of the protein bands is the same as in **Figure 1**.

adsorb much more efficiently at pH 4−5, which is slightly below its p*I*. The glycoprotein YGP40 adsorbs efficiently at any pH with a slight increase at pH 6−8, which is just above its p*I*. Immunoglobulin G adsorbs slightly more at pH 8, which is close to its p*I*.

DISCUSSION

The results show that the α−*β*-livetin fraction as obtained with the method of McBee and Cotterill (*21*) contains five dominant protein species, which vary in molecular weight and p*I* (**Table 1**). Furthermore, the results show that it is possible to produce an emulsion with the α−*β*-livetin fraction of egg yolk. The droplet size of the emulsion depends on the amount of protein added, and the emulsion surface area increases linearly with concentration. Linear regression of the results gave a correlation coefficient of 0.986, and the standard error in the emulsion surface area was determined to be 0.01 m^2/mL. The linearity and positive slope of the plot in **Figure 2** show that the proteins in the α−*β*-livetin fraction are working as emulsifiers. At pH 7.0 the surface load increases with the protein concentration and reaches a plateau at ≈1.5 mg/m^2 with a random error of 0.4 mg/m^2. This value is comparable to what can be expected from a protein with high solubility in the water phase, which adsorbs in monolayers at the interface (*28*, *29*). At pH 7.0 the gel analyses show a pronounced selectivity in protein adsorption from the protein fraction with a preference for YGP40 (protein 5) and serum albumin (protein 3). The simplest assumption is that the most hydrophobic protein should adsorb selectively. However, these proteins are all water soluble and do not display any significant difference in overall hydrophobicity. A second possible explanation is the molecular weight as high molecular weight gives more potential sticking possibilities (per molecule), which should favor adsorption. However, the results show adsorption of both one high molecular weight species and one with low molecular weight, depending on pH, excluding the possibility that the molecular weight has an overall influence on the competitiveness in the system. A third alternative could be kinetics. In this system with comparatively small molecules adsorbing we would expect diffusion to determine the adsorption rate. However, this hypothesis would also suggest that the competitiveness should follow the molecular weight. The fourth possibility would be that the competitiveness is controlled by the distribution of hydrophilic and hydrophobic domains in the protein molecule. Both YGP40 and serum albumin contain long contiguous stretches of low hydrophilicity, as can be seen from the Kyte−Doolittle plots (*30*) in **Figure 7**. A coherent hydrophobic block in the amino acid sequence could be an important property in the adsorption selectivity as proteins that are adsorbed can unfold and spread at the interface (*9*−*11*) and the rate of conformational changes depends on surface load, temperature, pH, and ionic strength (*31*).

The emulsifying activity of proteins shows a weak positive correlation with protein surface hydrophobicity (*32*). The emulsifying activity is also strongly related to the ability to unfold at the interface, and partially unfolded proteins often have higher emulsifying activities, which is related to a higher surface hydrophobicity and greater flexibility (*33*, *34*). The amount of spreading and the time scales depend on the protein structure, the solubility, and the interface itself. The time scale for this interfacial spreading ranges from 10 s for flexible proteins such as *β*-casein to 10^3 s for globular proteins (*35*), but times as short as 10^{-2} s have been reported for bovine α-lactalbumin (*36*).

The importance of the hydrophobic block character for the adsorption of other surface active macromolecules such as triblock copolymers has been shown by several authors (*37*, *38*). The results of these studies show that a longer coherent block of monomers with a high affinity for the interface tends to give greater surface activity and higher surface loads. Griffiths et al. has studied the role of copolymer architecture on the adsorption at interfaces (*39*). The authors found that a cyclic triblock copolymer (in which the two hydrophilic blocks were covalently bound together) had adsorption behavior surprisingly similar to that of a linear triblock copolymer of the same composition. This is indeed an interesting result, which could to some extent be compared to the adsorption of proteins. Furthermore, the heterogeneity in the distribution of hydrophobic substituents in modified celluloses has been shown to influence the clouding behavior of such derivatives (*40*). Thus, the distribution of hydrophobic groups plays a large role in determining the amphiphilic character and surface activity of water soluble macromolecules, and we suggest that the Kyte−Doolittle plots in **Figure 7** reflect this concept.

The adsorption experiments at various pH values show that there is a minimum in the emulsion area versus pH (**Figure 5a**) at pH 4.5 which is accompanied by a high surface load of

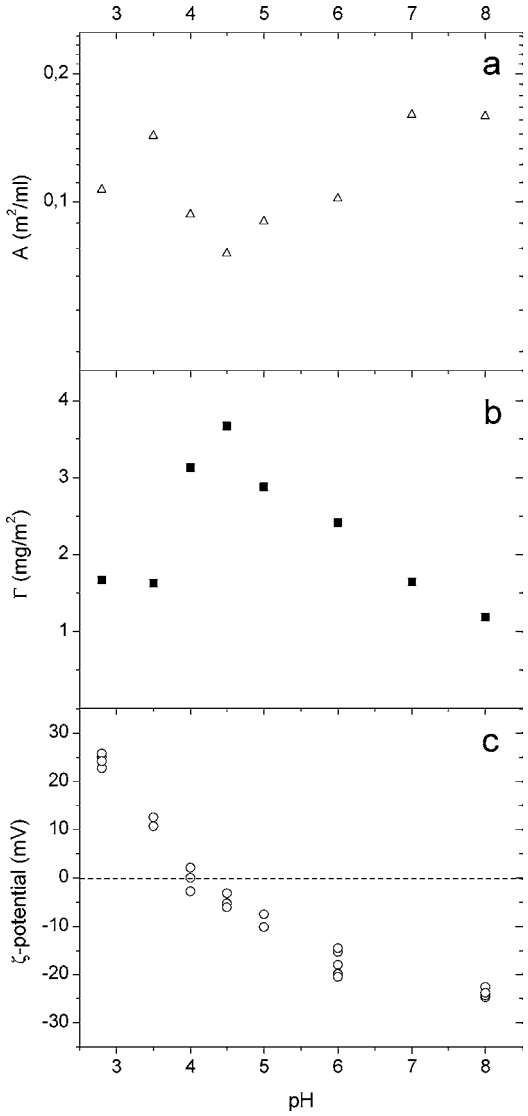

Figure 5. Adsorption experiments at various pH values and constant initial bulk protein concentration (1000 mg/L): (**a**) emulsion surface area (*A*) versus pH; (**b**) adsorption isotherm, that is, protein surface load (Γ) versus pH; (**c**) ζ-potential of the emulsion droplets versus pH. The standard deviation between replicates ($n = 2$–6) of the ζ-potential was determined to be 1.9 mV with single-factor ANOVA.

≈3.8 mg/m² (**Figure 5b**). This is due to the fact that the isoelectric point of the emulsion droplets occurs around this pH, which is shown by the ζ-potential in **Figure 5c**. This deviates slightly from the p*I* determined for the proteins with 2D gel electrophoresis, where it ranged between 5 and 6.3 for the selectively adsorbing species, albumin and YGP40. However, the ζ-potential and thus the isoelectric point of the droplets could depend on the orientation, in relation to the interface, of the charged groups of adsorbed proteins. This could explain the slight deviation between protein p*I* and ζ-potential. The ζ-potential results do, however, correspond well with the results for droplet size and surface load (**Figure 5**). To act as an efficient emulsifier it is important that a substance is sufficiently surface active to be able to lower the surface tension. It is also important that the substance is able to provide some form of stabilization of the droplets immediately after they are formed to prevent coalescence. As the stabilizing effect of proteins depends, to a large extent, on the steric and electrostatic repulsion between protein-covered interfaces, it is expected that

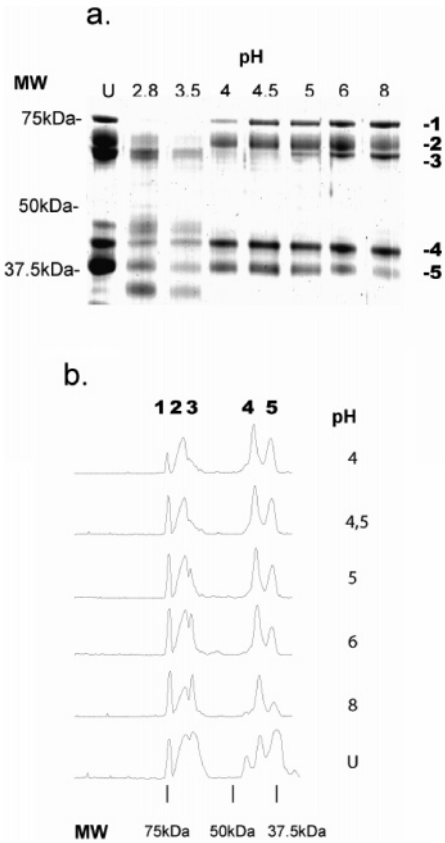

Figure 6. (**a**) SDS-PAGE of soluble egg yolk proteins before (lane U) and after emulsification at various pH values (noted above each lane). The integrated lane profiles are shown in (**b**). The numbering of the protein bands is the same as in **Figure 1**. The protein concentration (c_0) was 1 mg/mL.

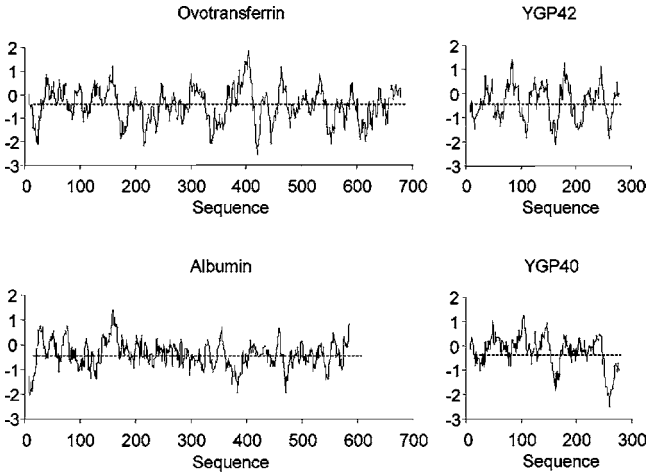

Figure 7. Kyte–Doolittle hydropathy plots of 4 of the 5 most abundant proteins in the egg yolk livetin fraction with a window of 15 amino acids (*30*). The positive direction of the *y*-axis is hydrophobic and the negative hydrophilic. No hydropathy plot is shown for IgG due to the large sequence variation. IgG, however, tends to consist of alternating hydrophobic and hydrophilic stretches as is the case for ovotransferrin. To facilitate comparison, a horizontal dotted line has been added at –0.4, which is the hydropathy attributed to the glycine residue in the Kyte–Doolittle algorithm.

the emulsion droplets become larger; that is, the emulsion surface area decreases when the protein charge density is low. The increase in surface load may be due to kinetic effects during the adsorption, which in turn could cause jamming at the

interface when many protein molecules compete for a small surface. This could limit the ability to spread at the interface and also cause orientational differences if they are asymmetrical (*41*).

Thus, we conclude that the water soluble plasma protein fraction of egg yolk constitutes a mixture of proteins with various properties. The most abundant proteins were characterized with 2D gel electrophoresis identified with mass spectrometry, and these powerful techniques give us the possibility to understand more of the adsorption behavior of the proteins. The oil-in-water emulsion's droplet size and maximum surface load depend on the protein concentration and pH. Serum albumin and YGP40 adsorbed selectively at the oil/water interface throughout the pH range investigated. We suggest that this selective adsorption is due to long hydrophobic stretches in the polypeptide chain which are present in the selectively adsorbing species but absent in the less adsorbing species. Egg yolk proteins constitute a fascinating area that deserves more attention as many aspects of their properties and functions in emulsions remain complex.

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