Basics of macroscopic properties of adsorbed protein layers,
formed at air-water interfaces,
based on molecular parameters

Peter A. Wierenga
Promotoren:  Prof. Dr. M.R. Egmond  
Hoogleraar in Toegepaste Enzymologie, Universiteit Utrecht

Prof. Dr. Ir. A.G.J. Voragen  
Hoogleraar in de levensmiddelenchemie, Wageningen Universiteit

Co-promotor:  Dr. H.H.J. de Jongh  
Projectleider, Wageningen Centre for Food Science

Promotiecommissie:  Dr. David Clark  
DMV Campina, Nederland

Prof. Dr. S. Damodaran  
University of Massachussettes, U.S.A.

Prof. Dr. Ir. M.A. Cohen Stuart  
Wageningen Universiteit

Dr. W.J.H. van Berkel  
Wageningen Universiteit

Dit onderzoek is uitgevoerd binnen de onderzoeksschool VLAG
Basics of macroscopic properties of adsorbed protein layers,
formed at air-water interfaces,
based on molecular parameters

Peter A. Wierenga
Wierenga, P.A.
Basics of macroscopic properties of adsorbed protein layers formed at air-water interfaces, based on molecular parameters
Thesis Wageningen University, The Netherlands 2005 – with summary in Dutch
ISBN: 90-8504-327-1
Abstract

P.A. Wierenga (2005), Basics of macroscopic properties of adsorbed protein layers formed at air-water interfaces, based on molecular parameters, PhD thesis, Wageningen University, The Netherlands.

In food industry, proteins are applied in foam and emulsions, where the macroscopic properties of the interfacial layer impart stability to the system. These properties depend on the molecular properties of the adsorbed protein. In this thesis the role of protein molecular properties (i.e. surface hydrophobicity, structural stability, surface charge) on the different parameters describing protein functionality (i.e. surface tension, elasticity, viscosity) was studied. To do this, proteins were chemically modified in respect to one property, followed by a characterization of the changes in surface functional behavior. Results showed that the adsorption kinetics is governed by a kinetic energy barrier, the height of which depends on the hydrophobic exposure and electrostatic net charge density of the protein. The adsorption kinetics were also described with a model based on ‘Random Sequential Adsorption’. Further experiments showed that the surface pressure at certain adsorbed amount depends on the apparent size of the adsorbed proteins, which can be increased by electrostatic charge or by unfolding. Unfolding, was only observed if the kinetics of unfolding were faster than the rate of adsorption. A study on rheological properties of the interface showed no significant contribution of reactive sulfhydryl groups, which indicates that the adsorbed protein layer should be considered as a densely packed system, rather than a gelled system. The combination of these findings show that for all properties of adsorbed protein layers, these layers can be described as a system of non-associated particles.

Additionally, a series of experiments was performed in which the molecular parameters, the interfacial behavior and foaming behavior of different proteins were studied to obtain insight in the influence of the studied properties at planar-air water interface on foam formation and stability. These results show that the foam formation is limited by the same kinetic barrier as the adsorption at static planar air-water interfaces. In conclusion, the work illustrates that most of the interfacial phenomena studied are governed by colloidal interactions between the proteins and the interface, or between adsorbed proteins.
Voorwoord

Voordat u vol goede moed begint met het lezen van mijn proefschrift wil ik een kort woord tot u richten. Toch is het schrijven van dit voorwoord misschien wel het moeilijkste wat ik tijdens mijn AIO periode heb gedaan. Dit komt waarschijnlijk omdat, zoals ook in het wetenschappelijk onderzoek, het meestal makkelijker is om te beschrijven wat er is gebeurd, dan hoe of waarom juist datgene gebeurt. Waarom, is dan ook de vraag die mij vaak gesteld is, is dit onderzoek uitgevoerd, en waarom heb ik het gedaan?

Mijn drijfveer is voornamelijk nieuwsgierigheid. In het dagelijks leven worden we voortdurend omringd door objecten en producten die door mensen gemaakt zijn. Dat deze producten bestaan, en dat ze zijn zoals ze zijn, komt omdat er over nagedacht is. Toch is voor veel consumenten niet bekend hoeveel wetenschappelijke kennis en technologisch inzicht ten grondslag ligt aan deze producten. In dit opzicht is de levensmiddelensector misschien wel één van de meest opvallende sectoren in de wetenschap. Juist omdat we elke dag in aanraking komen met levensmiddelen zijn ze voor ons zo gewoon, toch is er een grote hoeveelheid wetenschap en technologie ontwikkeld, om deze producten te maken wat ze zijn.

Het onderwerp van mijn onderzoek, ‘eiwitadsorptie aan grensvlakken’ klinkt nogal abstract. Toch komen we toepassingen van deze kennis tegen in diverse systemen; in relatief simpele producten zoals sladressing, instant soep, ijs, chocolademousse en bier. Daarnaast speelt eiwitadsorptie een rol in veel meer processen, die niet gerelateerd zijn aan voedingsmiddelen. Tijdens mijn onderzoek heb ik bijvoorbeeld gebruik gemaakt van literatuur van mensen die onderzoek doen naar bloed en aderverkalking, afvalwaterverwerking, lenzenreinigingsmiddelen en aardolie winning. Dit is zomaar een greep uit de diversiteit van onderwerpen waar het proces van eiwitadsorptie een zeer belangrijke rol in speelt. Tijdens mijn studie kwam ik erachter dat er ondanks veel onderzoek nog steeds veel vragen waren over dit proces. Juist dat het proces in zo veel gebieden bestudeerd werd en er toch nog zoveel vragen bestonden heeft mijn interesse in dit onderwerp gewekt. De vraag waar ik in mijn onderzoek mee ben begonnen was of we de adoptie van eiwitten (en de gevolgen die dit heeft voor het oppervlak waaraan ze adsorberen) kunnen begrijpen op basis van de ontwikkelingen op het eiwit.

Ik hoop dat ik hiermee duidelijk heb kunnen maken wat de reden is van het in dit proefschrift gepresenteerde onderzoek. Het zou natuurlijk mooi zijn om hier af te sluiten met de conclusie en de uitkomsten van mijn onderzoek, maar helaas zult u daarvoor toch door moeten lezen.
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ</td>
<td>Surface tension</td>
<td>[mN/m]</td>
</tr>
<tr>
<td>Π</td>
<td>Surface pressure</td>
<td>[mN/m]</td>
</tr>
<tr>
<td>Γ</td>
<td>Adsorbed amount of protein</td>
<td>[mg/m²]</td>
</tr>
<tr>
<td>Ω</td>
<td>Apparent surface area of adsorbed proteins</td>
<td>[m²]</td>
</tr>
<tr>
<td>θ</td>
<td>Fraction of surface area covered by proteins</td>
<td>[-]</td>
</tr>
<tr>
<td>C_b</td>
<td>Bulk concentration of protein</td>
<td>[mg/mL]</td>
</tr>
<tr>
<td>D_b</td>
<td>Bulk diffusion constant</td>
<td>[m²/s]</td>
</tr>
<tr>
<td>ΔE_barrier</td>
<td>Activation energy for adsorption</td>
<td>[kJ/mole]</td>
</tr>
<tr>
<td>k_adsorb</td>
<td>Adsorption constant</td>
<td>[kJ/mole]</td>
</tr>
<tr>
<td>M_w</td>
<td>Molecular weight</td>
<td>[kDa]</td>
</tr>
<tr>
<td>π</td>
<td>Pi (mathematical constant)</td>
<td></td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
<td>[s]</td>
</tr>
<tr>
<td>A</td>
<td>Surface area</td>
<td>[m²]</td>
</tr>
<tr>
<td>E_d</td>
<td>Dilatational modulus</td>
<td>[mN/m]</td>
</tr>
<tr>
<td>γ</td>
<td>Strain applied in surface shear</td>
<td>[-]</td>
</tr>
<tr>
<td>E_young</td>
<td>Elastic Youngs modulus used in surface shear</td>
<td>[mN/m]</td>
</tr>
<tr>
<td>σ_ss</td>
<td>Steady-state surface shear stress</td>
<td>[mN/m]</td>
</tr>
<tr>
<td>ΔG_unf</td>
<td>Free energy of unfolding</td>
<td>[kJ/mole]</td>
</tr>
<tr>
<td>ΔE_unf</td>
<td>Activation energy of unfolding</td>
<td>[kJ/mole]</td>
</tr>
<tr>
<td>θ</td>
<td>Wetting angle</td>
<td>[°]</td>
</tr>
</tbody>
</table>

Lysine
C_{10:0}-SU Succinylated capric acid
ANSA 8-anilino-1-naphtelenesulfonic acid
S-AMSA 8-anilino-1-naphtelenesulfonic acid
OPA Ortho-phthaldialdehyde
SDS Sodium Dodecyl Sulphate
CD Circular Dichroism
IRRAS Infrared Reflection Adsorption Spectroscopy
# Contents

**Abstract**  
**Voorwoord**  
**List of symbols and abbreviations**  
**Contents**  

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
</table>
| Chapter 1| General introduction  
1.1 *Adsorbed protein layers, a definition of the research area*  
1.2 *Adsorbed layers in food products*  
1.3 *From proteins to foam*  
1.4 *Current status of theory*  
1.4.1 Initial adsorption  
1.4.2 Adsorption at partially filled surfaces  
1.4.3 Development of surface pressure  
1.4.4 Surface reology  
1.4.5 Comparison of concepts  
1.5 *Aim of the current research*  
1.6 *Experimental techniques*  
1.6.1 Formation of adsorbed layers  
1.6.2 Properties of adsorbed layers  
1.6.3 Protein modification  
1.7 *Outline of the thesis*  | 1 | 3 | 3 | 5 | 5 | 13 | 13 | 19 |
| Chapter 2| Protein exposed hydrophobicity reduces the kinetic barrier for adsorption of ovalbumin to the air-water interface | 23 |
| Chapter 3| Quantitative description of the relation between protein net charge and protein adsorption to air-water interfaces | 43 |
| Chapter 4| The adsorption and unfolding kinetics determines the folding state of proteins at the air-water interface and thereby the equation of state | 66 |
| Chapter 5| Importance of physical vs. chemical interactions in surface shear rheology | 83 |
| Chapter 6| Intermezzo: Rendering a colloidal particle surface layer into a gelled protein system | 102 |
| Chapter 7| Calculation of protein adsorption rates to air-water interfaces based on molecular parameters; a predictive tool for foaming capacity? | 112 |
Chapter 1

General introduction
1.1 Adsorbed protein layers, a definition of the research area

The positioning of research on adsorbed protein layers may well be as challenging as the research itself. The reason is that proteins occur in all biological systems and will adsorb to many different types of interfaces. The adsorption leads to changes in the chemical, physical and/or mechanical properties of the interface, thereby affecting systems ranging from ship hulls to blood, from contact lenses to food systems. However, a distinction must be made between adsorbed layers at liquid-liquid or gas-liquid interfaces and solid-liquid interfaces. The adsorption at both types of interfaces will be governed by similar mechanisms, but there are also certain differences. Since a solid typically has a higher density and heterogeneity than a liquid or a gas, the interactions of adsorbed proteins with that interface will be different. This difference in interaction is observed in the lateral diffusion of adsorbed proteins, which is two orders of magnitude higher at liquid than at solid interfaces.\textsuperscript{1,2} Little is known about the extent to which findings for adsorption at solid interfaces can be extrapolated to the air-water interface. Therefore, we will not discuss many of the results and theories from studies on solid interfaces.

The present work is focussed on interfacial protein layers formed at liquid interfaces, since these govern the formation and stabilisation of emulsions and foam. The common theme of many studies on this subject has been to distinguish the contributions of system parameters (e.g. pH, T) from interfacial properties (e.g. solid, liquid, charged or not) and from protein functionalities (e.g. charge, folding stability). As will be shown further in the text, there are still many questions as to the influence of protein functionalities. To understand the role of the ingredient (proteins), it is necessary to identify those parameters that adequately describe the interfacial layer in terms that can be related to molecular properties of the proteins. Before we further define the type of research performed, we will discuss how we came to the research question.

1.2 Adsorbed layers in food products

Foam is the general description of any system containing dispersed air-bubbles. Examples of foamed food products are bread, ice cream, meringue and beer. While all these products have quite different structures, the formation and stabilisation of air bubbles is essential for good product characteristics. The properties of the foamed product depend on processes that act on
different length scales as depicted in figure 1.1. At the macroscopic level the relevant parameters are (1) bulk properties (aqueous, viscous, gelled), (2) the volume fraction of air that is incorporated and (3) the size distribution of the air-bubbles. At a microscopic level the interactions between the adsorbed layers of air bubbles are important. Processes at this level can be related to changes in the texture of foam over time, due to instability processes. The most important instability processes are: Oswald ripening, coalescence, and drainage (or creaming). These processes have been described in detail elsewhere.\(^3\)-\(^5\) The mesoscopic level, between micro- and molecular can be defined as the level at which properties of a single interfacial layer are studied. Parameters to describe these properties are the adsorbed amount of protein at the interface, the surface pressure and the response of the interface to deformation as described by the elastic and viscous moduli of the interface in dilatation or shear.

**Figure 1.1. The study of protein foam, going from the product scale (cm) to interactions at the interface (nm scale)**

Understanding why foam can be formed with excellent properties in some cases, while in another case no foam is formed, or foam with poor stability has been the aim of much research. In literature, possible relationships between the foam formation and stability and different macroscopic and mesoscopic parameters have been mentioned,\(^3\)-\(^6\)-\(^9\) but a quantitative relation was not established. For reviews on this topic we refer to the work of Lucassen-Reynders\(^10\), Dickinson\(^11\) and Izmailova.\(^12\) In recent years the tendency has been to extrapolate the knowledge of pure protein system to the description of multi-component systems.\(^13\)-\(^17\) The problems encountered in understanding observed phenomena in such complex systems indicate that the current description of adsorbed protein layers is still not sufficient. One reason for this might be that the system behaviour at macroscopic and mesoscopic levels are basically dependent on processes occurring at the molecular level. At this level the specific molecular characteristics of proteins (such as charge, size, hydrophobicity) are important.
parameters. In other words, the challenge is to see in what respect molecular characteristics of proteins really define the observed interfacial properties.

1.3 From proteins to foam
Even under conditions where proteins are soluble in the aqueous phase, they will accumulate at the interface to reach concentrations (in the order of 200 mg/mL) that can be many times higher than the bulk concentrations (f.e. 0.1 mg/mL). Although the adsorbed amount that is reached upon saturation of the layer is similar for most globular proteins, the rate at which saturation is reached can differ quite markedly. A general overview of the steps leading to the formation of adsorbed layers is given in figure 1.2. The suggested order of these steps is only hypothetical; they may overlap or occur at the same time. To understand the formation and stabilisation of interfacial layers, the importance and role of each of these steps in the overall process needs to be understood. In the next section the current state of knowledge on each of these steps will be discussed.

1.4 Current status of theory
The description and interpretation of phenomena related to protein adsorption has a long history. In addition, much research has been performed on the adsorption and adsorbed layers of low molecular weight surfactants. A good review of models describing such systems has been given by Chang and Frances. However, the behaviour of surfactants typically differs
from that of proteins, therefore we will focus here on theories for protein adsorption. The theories used are often descriptive in nature and few of the concepts encountered have been translated in formulas that allow a quantitative description of the system. In this part an overview will be given of the main ideas, ordered according to the steps in figure 1.2. More detailed information will be given in the relevant chapters of this thesis.

1.4.1 Initial adsorption

As a new interface is created, no proteins are present in the adsorbed state and the concentration of proteins as a function of distance to the surface is equal for all distances. According to Ward and Tordai\(^\text{19}\) proteins close to the interface will then adsorb immediately, resulting in a decrease of the effective protein concentration in the sub-surface layer, just below the interface (figure 1.3). The increase of the adsorbed amount of protein (\(\Gamma\) in mg/m\(^2\)) in time (\(t\) in s) is then limited by diffusional transport to the interface and can be calculated from equation 1.1:

\[
\frac{d\Gamma}{dt} = C_b \sqrt{\frac{D_b}{\pi \cdot t}}
\]

Where \(D_b\) [m\(^2\)/s] and \(C_b\) [mg/m\(^3\)] are the diffusion coefficient and concentration of protein in the bulk respectively. Based on this equation, different authors have since used a plot of \(\Gamma\) (or even \(\Pi\)) against \(\sqrt{t}\) to illustrate the diffusion-limited character of the adsorption process. This has lead to the common assumption that initial adsorption, in the absence of an appreciable surface pressure is not limited by any energy barrier. The absence of a detectable energy barrier, combined with the assumed irreversible character of adsorption has lead to a deep-rooted idea that the transition from dissolved to adsorbed state is due to unfolding (or denaturation) of the protein chain.\(^\text{20,21}\) It has been suggested by MacRitchie that ‘conclusions reached about proteins that do not readily unfold are doubtful’.\(^\text{22}\) Based on this line of reasoning, it has been suggested that the faster adsorption at oil-water interfaces than at air-water interfaces is due to more rapid unfolding of proteins at the oil-water interface.
1.4.2. Adsorption at partially filled surfaces

As adsorption continues the rate of adsorption is found to decrease. This has been attributed to the development of two forces: (1) the existing surface pressure ($\Pi$)\textsuperscript{a}, and (2) the electrostatic repulsion between adsorbed and adsorbing proteins. To account for these forces a correction of equation 1.1 has been proposed by MacRitchie:\textsuperscript{22}

\[
\frac{d\Gamma}{dt} = k_{\text{adsorb}} C_b e^{-(\Pi A + q\Psi)/kT}
\]

Where $k_{\text{adsorb}}$ is the adsorption rate constant (not further defined by the author), $\Pi A$ is the energy needed to create an area ($A$) to let the protein adsorb; $q$ is the net charge on the protein and $\Psi$ the wall potential of the interface. Authors that used this equation have reported that the values calculated for $A$ (100-175 Å\textsuperscript{2}) were independent of protein size.\textsuperscript{22-25} This apparent discrepancy was explained by reasoning that apparently only a small part of a protein needed to attach at the interface to allow the whole protein to adsorb.\textsuperscript{26} An alternative model was proposed by Guzman \textit{et al.}\textsuperscript{27} Here the filling of the interface is described by the Langmuir term (1-$\overline{A}\Gamma$) where $\overline{A}$ is the average area of the protein. In contrast to the Ward and Tordai equation this model does not assume infinite medium and irreversible adsorption:

\[
\frac{d\Gamma}{dt} = k_{\text{adsorb}} e^{-AE_s/kT} C_{\text{subsurface}} (1 - \overline{\Gamma}) - k_{\text{desorb}} e^{-AE_s/kT} \overline{\Gamma}
\]  

\textsuperscript{a}The surface pressure is calculated as the decrease in surface tension of the empty interface due to adsorption of proteins, and will be discussed in detail in section 1.4.3.
However, they have not given a quantitative description for the calculation of the energy barriers and rate constants of adsorption and desorption ($\Delta E_a$, $\Delta E_d$, $k_{\text{adsorb}}$ and $k_{\text{desorb}}$). Another model based on the reversible equilibrium between dissolved and adsorbed proteins was proposed by Al-Malah et al.\textsuperscript{28}

$$\Gamma = V_p C_{eq} \left( \frac{1}{A_p} \right) e^{-W_a A_c/RT} + V_p C_B$$ \hspace{1cm} (1.4)

Here $V_p$ and $A_p$ are the partial volume and area respectively, $C_{eq}$ the equilibrium concentration in bulk, and $W_s = \gamma_{sw} + \gamma_{pw} - \gamma_{ps}$ the work of adhesion of a protein to the interface.

Both latter models are based on the reversible equilibrium nature of the adsorption process, but this reversibility has been matter of debate.

Equilibrium between the bulk and interface requires that adsorption is reversible and that desorption can occur in short time-scales. Such reversibility of protein adsorption under static (interfacial) conditions has been studied by changing the bulk concentration under an adsorbed interfacial layer. These studies show that the exchange of adsorbed proteins with the bulk solution under such static conditions is either non-existent, or very slow.\textsuperscript{22,29-31} Desorption was also found to occur when low-molecular weight surfactants are added to the bulk phase. Displacement of the adsorbed proteins by adsorption of these surfactants lead to higher desorbed amount than changing the bulk solution,\textsuperscript{32,33} but was also found to be a slow process. These results illustrate that the adsorption is in reversible, but that the high activation energy for desorption is an indication that this reversibility does not lead to an instantaneous equilibrium between the adsorbed amount and the bulk concentration.

An alternative to the empirical models mentioned above, is the dynamic and statistic model of random sequential adsorption.\textsuperscript{34-37} This model is based on irreversible random adsorption of spherical particles on a homogeneous interface, although changes have been proposed to account for lateral diffusion, and deviations from the spherical shape. A great benefit of this model is that, in contrast to the above models, it does not need any (presumed) information about fitting parameters.

1.4.3. Development of surface pressure

To understand the effect of protein adsorption on surface pressure ($\Pi$), first a short comment will be made on the surface tension ($\gamma$) and characteristics of the clean air-water interface.
The most basic property of an air-water interface is the existence of a surface tension. For pure liquid interfaces the surface tension is defined as the excess free energy due to the difference between the interaction energies of molecules in the bulk phase and at the interface. To calculate this excess free energy, often the Gibbs convention is used, which defines the location of the interface. A good definition of the interface is not as easy as one would at first glance expect. Starting from the air-phase and moving to the aqueous phase, the density and composition gradually change until the conditions of the bulk solution are reached. This is illustrated in figure 1.4, where the chemical potential of air and water is drawn as a function of the distance to the interface. One can understand that for ice the transition would be more clearly defined than for water at 20 °C, which is still sharper than at 100 °C; at 20 °C the surface roughness is found to be approximately 4 Å. To enable a thermodynamic description of the interface, the Gibbs convention is used, which defines the interface as a plane (with no volume) separating the two bulk phases. All the excess energy is located in this interface.

Figure 1.4. Schematic representation of the air-water interface with adsorbed proteins, in the right panel, the chemical potential of air, water and protein is given as a function of the distance to the interface.

The surface tension for pure liquid interfaces is defined as the change in free energy with a change in surface area. The change in free energy is the result of the fact that as the interface is expanded, water molecules move from the bulk environment to the interface, and in the reverse direction upon compression. This is an instantaneous and fully reversible process. As soon as proteins adsorb at this interface a new description is necessary. Upon compression and expansion, the adsorbed proteins will come closer together and further apart, but will not exchange with the bulk phase as quickly as water molecules.
Rather than using the surface tension of adsorbed protein layers, often the surface pressure ($\Pi$) is used. The surface pressure is calculated as the difference between the measured surface tension and the surface tension of a pure air-water interface ($\gamma_0$).

$$\Pi = \gamma_0 - \gamma_{\text{measured}}$$  \hspace{1cm} (1.5)

The analysis of $\Pi$-time curves has often been used as a tool to monitor the adsorption behaviour of proteins. In literature three regimes have been distinguished in the $\Pi$-time curve (figure 1.5). These regimes have been denoted gaseous, liquid and solid (or condensed liquid) based on the interactions between adsorbed proteins.\textsuperscript{39,40} This leads to the relation between $\Pi$ and $\Gamma$, often referred to as the surface equation of state (see refs. 41-44 for reviews on this topic). The basic equation is a 2-dimensional variant of the gas-law (or as used to describe osmotic pressure), based on interactions between hard-particles:

$$\Pi = R T \Gamma$$ \hspace{1cm} (1.6)

Figure 1.5. Three regimes of the $\Pi$-time curve, I- gaseous state, II- liquid state, and III- solid (or condensed liquid) state, figures below the graph represent the interface as seen from above

Although corrections can be made to equation 1.6 to include a finite size of protein molecules, the agreement of hard-particle theory is limited to the gaseous regime (low $\Pi$ and $\Gamma$).\textsuperscript{31} De Feijter and Benjamins have discussed the applicability of the hard particle concept in relation
to the alternative soft-particle theories. Fainerman et al. have proposed a model, based on the concept of soft-macromolecules, where the surface area taken up by each protein decreases with increasing adsorption. This model should reflect well-known differences between proteins and ordinary surfactants, such as the shape of the adsorption isotherm. The ‘equation of state’ is than written as:

$$-\frac{\Pi \omega_0}{RT} = \ln(1 - \omega \Gamma) + (\omega - \omega_0)\Gamma + a(\omega \Gamma)^2$$

Here $\omega_0$ and $\omega$ are the molar areas of solvent and protein molecules respectively, $\Gamma$ is the total adsorbed amount, and $a$ an intermolecular interaction constant. This model uses a polymer description of the protein chain, and the assumption that segments of the protein can desorb if the surface pressure increases. The importance of protein unfolding is also stressed by Damodaran et al. who mentioned that proteins that retain their globular structure cannot cause an increase of $\Pi$, even in a saturated monolayer because such a protein layer will remain in a gaseous state. In contrast to these polymer models, a colloidal approach to the calculation of the surface pressure is given by Rasanov et al. in an article where they also review other existing models. Their colloidal approach does, however, not take into account the net-charge of the adsorbed molecules, which should be included if the approach is used for the description of adsorbed protein layers.

1.4.4. Surface reology

Surface reology of adsorbed protein layers should be divided between dilatational and shear stress measurements. Not merely because different measurement techniques and deformation is applied, but more because in literature the explanation of results from both types of experiments are based on different concepts.

The dilatational elasticity is measured by sequential expansion and compression of the interface and registration of the change in surface pressure with surface area. The elastic modulus ($E_d$ [mN/m]) is defined as:

$$E_d = \frac{d\Pi}{d\ln A}$$

Generally, this dependence is explained in terms of molecular adsorption and desorption processes, or on adsorption and desorption of protein segments at the interface. The latter explanation uses the idea that an adsorbed protein has adopted a ‘loop-train’ configuration at
the interface. The trains are segments of the chain that are adsorbed at the interface, while the loops protrude into the aqueous phase.

Shear rheology is determined by deformation of the interface at constant surface area. The interpretation of observed elastic and viscous behaviour is commonly interpreted in analogy with three-dimensional protein gels. The assumption is made that the adsorbed protein layer can be described as a continuous network.

1.4.5. Comparison of concepts

In the discussion above it is illustrated that different concepts are used for the description of phenomena related to adsorbed protein layers. The adsorption is, for example, described in analogy to polymer models. The development of Π is described either based on soft-particle interactions, or hard-particle interactions. The soft particle theory is related to the polymer description, where the soft character of the particle is due to desorption of segments of the polymer chain. In contrast, the hard-particle models represent the proteins as hard spherical particles. In the description of surface shear rheology, neither of these models is used. In this case, often the interfacial layer is no longer viewed as a collection of individually adsorbed proteins. Rather, the interfacial layer is described as a continuous, gelled system. This concept seems to disagree with the concept used in dilatational rheology, where adsorption and desorption of segments or even whole proteins is often used as the explanation of the response of Π to deformation of the interface.

The disadvantage of such diverse concepts is that it becomes difficult to describe the overall process of interfacial layer formation, from initial adsorption to the development of surface shear elasticity, in a single model. Furthermore, the unfolding of proteins and the rapid adaptation of protein conformation at the interface are dominant factors in these models, while known properties of proteins such as charge and hydrophobicity are rarely included. This omission may be the reason that several common observations are not easily explained by these concepts. At this place we will just mention, but not discuss them, as this will be dealt with later in this thesis.
As the pH is changed, the charge of proteins is changed. Typical observations show increased rate of development of $\Pi$ with time and an increase of the total adsorbed amount at saturation.\textsuperscript{56-59}

The rate of adsorption at oil-water is typically higher than at air-water interfaces.\textsuperscript{60}

Even for interfaces that normally show good surface shear elasticity, displacement of adsorbed proteins has been found after addition of low molecular weight surfactants to the system.\textsuperscript{32,33}

1.5. \textit{Aim of the current research}

The current literature provides explanations for observed phenomena that are difficult to combine in one complete conceptual model. The research performed in this thesis was mainly aimed at providing an understanding of the mesoscopic parameters used in the description of adsorbed layers (adsorption rate, surface pressure, etc.). This understanding should be based on the known chemical and physical properties of the proteins used in the experiments. Furthermore, the relation between molecular and mesoscopic properties should be quantified where possible, to allow other researchers to compare their observations with this model. To study this relation, chemical modification has been used in this work to specifically alter one molecular parameter of a protein. With this technique different sets of modified protein variants could be obtained; the modifications used will be discussed at the end of this chapter. The modified proteins were thoroughly characterized with respect to chemical and physical properties, to assure that apart of the selected property the modified protein was similar to the unmodified protein. Subsequently, the surface functional characteristics of the modified proteins were determined, using the techniques discussed below.

1.6. \textit{Experimental techniques}

1.6.1 \textit{Formation of adsorbed layers}

Techniques that give direct information on the adsorbed amount of proteins are the spectroscopic techniques applied in reflective mode; such as neutron reflection, ellipsometry and InfraRed Reflection Adsorption Spectroscopy. These techniques are all based on the change in refractive index of the interface as protein adsorbs. This change is a direct quantitative measure of the adsorbed amount. However, the adsorbed amount is more readily calculated from ellipsometry results than from neutron reflection results. The latter is more
often used to provide details on the density profile of protein perpendicular to the interface. The spectral resolution of IRRAS makes it discriminative to the presence of non-protein components (if they absorb at a different part of the mid-IR spectrum). Also, information can be obtained on the secondary structure of the adsorbed protein, due to the sensitivity of the amide I region (1700-1600 cm\(^{-1}\)) to secondary structure content. However, due to the low signal intensity –especially at the initial stages of adsorption- the time to collect each spectrum is in the order of 1 to 2 minutes, which in many cases is not fast enough to accurately follow adsorption in time. Neutron reflection data also has limited time-resolution, since the time needed to collect the data is in the order of 30 minutes to 2 hours, making this technique only suitable for the determination of the adsorbed layer under static conditions. Ellipsometry does not have this disadvantage; data points can be taken at time intervals of several seconds. Therefore, in this study the adsorption kinetics have been studied with ellipsometry, while neutron reflection spectroscopy and IRRAS have been applied to obtain information on the static state of the interfacial layers in certain specific systems. Ellipsometry measures the change in ellipticity of polarised light after reflection from the interface. This change is related to the refractive index and thickness of the interfacial layer, from which the adsorbed amount of protein can be calculated. For a further, thorough description of the technique and its uses, we refer to refs. 61-64.

1.6.2. Properties of adsorbed layers

In this study the surface pressure has been measured with two techniques; the Wilhelmy plate tensiometry and automated drop tensiometry. The Wilhelmy plate technique is based on the measurement of the force exerted on a plate suspended from a tensiometer and placed exactly at the interface. The meniscus surrounding the plate exerts a force; this force divided by the circumference of the plate yields the surface pressure. A benefit of this set-up is the fact that the registration is fast (0.1 s time resolution) and reproducible. Further, if the measurements are conducted in a Langmuir trough, it can easily be combined with techniques such as ellipsometry, or IRRAS. Another technique to measure the surface pressure in time, is automated drop-tensiometry (ADT), where a bubble of air is formed at the tip of a syringe with a computer controlled plunger. The shape of the air-bubble is recorded by a digital camera, and via the Laplace equation the surface tension can be calculated from this shape.\(^65\) The ADT measurements do not require the placement of a probe at the interfacial layer, and is not sensitive to the contact angle between the syringe needle and the bubble interface.
Therefore, it is expected to give a more absolute measure of the surface tension, but we have found no evidence of disagreement between results from both techniques (results not shown).

While the ADT is not easy to combine with techniques that measure the adsorbed amount of proteins (such as ellipsometry and IRRAS), it is a perfect tool to measure the dilatational rheological properties of the interface. A good description of this application is given by Benjamins et al.\textsuperscript{65} During such experiments the surface area of the bubble is subjected to a sinusoidal deformation (compression-expansion) leading to differences in the surface pressure. By simultaneous measurement of the surface deformation and the resulting surface pressure, the elastic and viscous response of the interface can be calculated. In the current work we have applied a constant rate and amplitude of deformation, to allow comparison of the dilatational properties of different samples.

Additional information on the surface layer can be obtained from surface shear rheology. These techniques measure the mechanical stress in an interface as a response on deformation at constant surface area. Small deformational shear can be applied by small sinusoidal rotations of a probe at the interface.\textsuperscript{66} From such measurements information is obtained on the Young’s modulus of the interface (defined as the ratio between applied strain and resulting stress). If larger deformation is applied, the network will show a transition from the initial elastic response to a more viscous response. This type of measurements can be performed with the use of an equivalent to the Couette rheometer applied in the characterization of three-dimensional gelled systems (see also chapter 5). From the initial part of the stress-strain curves, information can be obtained on the elasticity of the interface. As the strain is increased, the stress increases, until the system yields and a steady state-surface shear stress is reached. As discussed above, the results of such measurements are often interpreted in analogy to three-dimensional shear rheology.

1.6.3. Protein modification

Of all surface-active molecules, proteins are probably the most complex. They are built-up of a sequence of amino acids that contain acidic, basic, aliphatic or aromatic residues. Cysteine is of special importance, since this amino acid contains a free sulfhydryl group that can react to form inter- or even intramolecular disulfide bridges. In a protein the amino acids are connected through peptide bonds that are quite flexible, giving rise to differences in the secondary and tertiary structure of proteins. This may lead to differences in surface charge,
exposed hydrophobicity, and in the stability of the protein globular fold. To obtain insight in the role of a singular property on the interfacial behaviour of the protein, a comparison should be made between proteins that differ in this respect. Quite a number of studies have endeavoured to reach this goal by comparing two native or wild type proteins. But as is shown schematically in figure 1.6 (proteins Y and Z), two different proteins will typically have different values for more than one property. In this example, conclusions on the role of exposed hydrophobicity are hindered by the fact that there is also a difference in chemical reactivity.

To circumvent this problem enzymatic, genetic, or chemical modification can be used (see refs. 67-69). The advantage of genetic modification is that single amino acids can be altered, for instance to change a negatively charged residue to a positively charged one. However, difficulties in obtaining high-level expressions of multiple modified proteins, and in obtaining correctly folded proteins are hard to overcome. Chemical and enzymatic modification is more often based on the coupling of chemical groups to a side-chain residue of the protein chain. We found that chemical modification was an easy tool to obtain different types of modified proteins. With careful control over the reaction conditions, changes in the conformational state of the protein can usually be avoided. Especially at lower degrees of modification, specific properties of the protein can be altered (proteins Z¹, Z², Z³ in figure 1.6) without affecting the other properties. The modification can also be performed on quite large amounts of protein (in the order of grams), which enables a thorough characterization of the modified proteins. Typically, chemical modification is not very specific and a heterogeneous mixture of proteins with a range of degrees of modification will be obtained. However, using different chromatographic techniques, the modified proteins can be separated into batches of homogenously modified proteins. The reactivity of lysine groups is often used to introduce different types of groups in the protein, such as the reaction of different anhydrides to the lysine group.⁷⁰,⁷¹
Comparing proteins Z and Y

Comparing protein Z with modified variants

A. Electrostatics
B. Exposed hydrophobicity
C. Chemical reactivity
D. Bulkiness
E. Conformational stability

Figure 1.6. Illustration of the difference between comparing different native proteins (Y and Z) and comparing a native protein with chemically modified variants (Z₁, Z₂ and Z₃)

The availability of different specific modifications, and the high yield of modified proteins are the most important reasons why this technique has been used to provide a toolbox full of proteins with different functionalities for the study of surface behaviour.

In the presented work three chemical modifications have been used: (1) caprylation, to increase the exposed hydrophobicity, (2) succinylation, to increase the net charge and (3) thiolation to increase the possibility of covalent interactions via SS cross-linking. The reaction schemes are given in figure 1.7.
Several authors have reported drastic changes in the protein structure as a result of modification. For ovalbumin it has been reported that such changes were mainly the result of excessive modification. Batra et al. found that modification of up until 36% of the lysine groups of ovalbumin only little effects were observed in tertiary structure. The secondary structure was found to be stable until 75% (14 out of 20) lysine groups were modified. From this and our results, we concluded that observations of significant changes in protein structure reported by others is likely due to the high degrees of modifications that have been used, less mild reaction conditions, or a lower structural stability of the protein used.

Chemical modification is not typically food grade and cannot be applied for the production of ‘new’ protein ingredients for food. However, the use of chemical modification enables the production of well-defined systems that can be used for a mechanistic study of the formation and stabilisation of adsorbed protein layers. The knowledge that is obtained in this way can then be used to understand the phenomena encountered in food systems. Furthermore, this knowledge might help the selection of proteins for applications in foods based on their molecular properties. Finally, there are several enzymatic methods available that can be used to modify proteins in much the same way as was done by chemical modification, although the cost of such methods is typically higher than of chemical modification.
1.7. Outline of the thesis

The present work will first describe the kinetics of protein adsorption, in relation to the hydrophobic (chapter 1) and electrostatic (chapter 2) properties of the protein. Furthermore, the contribution of electrostatic interactions on the development of surface pressure is discussed (chapter 2). In chapter 3, it is described how unfolding of proteins can affect the exerted surface pressure and which intrinsic molecular parameters determine this unfolding process. Subsequently the characteristics (surface pressure, dilatational elastic modulus) of the saturated adsorption layer are discussed, with a description of the role of chemical cross-linking on the surface shear rheological behaviour (chapter 4) and the effects of applied stress (chapter 5). Chapter 5 is focussed on the shear properties of adsorbed protein layers formed under static conditions. However, during formation of foam and emulsions the interfacial layers will be subjected to high mechanical stresses. Therefore, in chapter 6 we consider the differences that may occur as a result of large deformation of the interface. For adsorbed layers the evidence shows that the interfacial layer can be described as a jammed system. Only after compression or other large stresses are applied, the proteins start to aggregate and form a continuous network.

While chapters 2-6 aim to provide an understanding of the relation between protein molecular parameters and mesoscopic properties of the interface, such as surface pressure, in the final chapter of this thesis, we describe a series of experiments where different proteins were characterized on their chemical and surface functional properties. Based on the obtained insight in the relevance of the molecular parameters of these proteins a prediction is done of their adsorption behaviour. In addition, the development of surface pressure in time, elastic modulus and foam formation and stability are measured for these proteins. In the general discussion (chapter 8) the main theme is whether a polymer description or a colloidal approach of protein adsorption is more in line with experimental evidence provided in this thesis.

References
Adsorbed Protein Layers

General Introduction

Chapter 2

Protein exposed hydrophobicity reduces the kinetic barrier for adsorption of ovalbumin to the air-water interface

Abstract

Using native and caprylated ovalbumin, the influence of exposed hydrophobicity on the kinetics of protein adsorption to the air-water interface is studied. First, changes in chemical properties of the protein upon caprylation were characterised followed by measurement of the adsorption kinetics. No change in the molecular structure of ovalbumin was observed upon caprylation. However, aggregation of the protein was observed when more than 3 capryl chains were coupled per protein. A batch of caprylated ovalbumin with an average coupling of 4 capryl chains per protein was separated into a monomeric and an aggregated protein fraction. The exposed hydrophobicity of the monomeric and the aggregated species was measured using 8-anilino-1-naphtelenesulfonic acid (ANS) fluorescence. The exposed hydrophobicity of the monomeric fraction was significantly higher than the non-modified protein. The changes in adsorption kinetics were studied by measuring the increase in surface load ($\Gamma$) and in surface pressure ($\Pi$) as a function of time (t) using an ellipsometer and a Wilhelmy plate respectively. It was found that the increase of surface load in time (even at low surface coverage) is much lower than the value that was calculated from diffusional transport. This shows that the adsorption of native ovalbumin is barrier limited. The adsorption kinetics of the caprylated protein follow the calculations from diffusional transport more closely, which shows that the energy barrier for adsorption of caprylated ovalbumin is much lower than for the native protein. The surface pressure at a certain surface load ($\Pi-\Gamma$) was not affected by the modification, indicating that the effect of increased hydrophobicity is limited to the adsorption process.
Chapter 2

Introduction

Protein adsorption to the air-water interface leads to the formation of an interfacial layer that changes the physical and mechanical properties of that interface. This interfacial layer is necessary to form and stabilize foam from protein solutions upon aeration. While the concentration of protein in the adsorbed surface layer and the resulting surface pressure -in equilibrium- are comparable for many protein solutions, large differences are found in their ability to form and/or stabilize foam. For instance, ovalbumin is a protein that has surface rheological properties that are comparable to that of β-lactoglobulin\(^1\). However, it was found that no foam could be formed with ovalbumin at protein concentrations between 0.01 and 3.0 mg/mL, while for β-lactoglobulin such concentrations were sufficient to obtain foam\(^1\). This poor foaming behavior of ovalbumin was suggested to be related to slow adsorption kinetics, which in turn are determined by the molecular properties of the protein.

A thorough description of protein adsorption to air-water interfaces is given by MacRitchie and Alexander\(^2,3\) and Graham and Philips.\(^4\) Adsorption starts with diffusion of proteins from the bulk to the interface. Close to the interface the protein can go from the ‘dissolved’ to the adsorbed state. The main driving force for this adsorption is the decrease of exposure of hydrophobic groups to the aqueous medium.\(^5\) The transition from the dissolved to the adsorbed state is assumed to occur without an energy barrier. This means that all proteins adsorb as soon as they come into contact with the surface, and that the adsorption is only limited by diffusional (or convectional) transport of proteins to the surface.\(^4-6\) Only at higher surface load the protein needs to overcome an energy barrier, related to the work required to adsorb against the existing surface pressure and the chance for the adsorbing protein to arrive at an empty location at the interface.\(^2,7\) However, in some cases indications were found that the adsorption of proteins was slower than predicted from diffusional transport, although this was not directly attributed to the existence of an energy barrier to adsorption. For instance, Damodaran et al. studied the adsorption of BSA variants in different states of unfolding and found that all unfolded variants adsorbed faster than the native protein.\(^8\) Their conclusion was that the differences in the rates of adsorption and desorption had to be related to solvent-solute interactions. A similar conclusion was made by Beverung et al.,\(^9\) who studied the adsorption of ovalbumin at the heptane/water and the air-water interface. They observed a large difference in the induction times for the surface pressure of the heptane/water and the air-water interface (10s and 1000s respectively). This difference was proposed to be due to
the greater affinity of hydrophobic groups of ovalbumin for the heptane than the air. From the above-mentioned results already an indication was obtained for the role of exposed hydrophobicity on adsorption.

Other studies on the role of protein hydrophobicity were based on the comparison of the adsorption of different proteins. Due to the fact that the proteins studied differed in other structural properties as well (e.g. electrostatic charge, size), it appeared to be difficult to derive conclusions on the influence of only the hydrophobicity on adsorption kinetics.\textsuperscript{10, 11}

A more controllable approach is to chemically modify proteins to increase the exposed hydrophobicity. If the reaction conditions are chosen well, not only the primary, but also the other structural properties of the native and the modified protein will be the same. In this way, experimental results can be specifically attributed to the chemically modified property. A commonly used technique to increase protein exposed hydrophobicity is the reaction of N-hydroxy-succinimidic esters of fatty acids with accessible amino groups of a protein.\textsuperscript{12-15} Using this technique, fatty acids of different chain lengths have been successfully coupled to different proteins. The extent of acylation is controlled by varying the ratio of protein to activated fatty acid. A review on the interfacial properties of such modified biomolecules is given by Magdassi \textit{et al.}\textsuperscript{16} When chemical lipophilization is used to study processes at the interface, care must be taken to avoid unwanted changes in protein chemical and structural properties, such as unfolding or aggregation of the protein.\textsuperscript{12,15,17,18}

Using chemical modification Baszkin \textit{et al.}\textsuperscript{19} showed that an increase in exposed hydrophobicity of human immunoglobulin G resulted in a faster adsorption. This was based on the measurement of the increase of surface pressure in time, since the actual increase of surface load was only measured for the native protein. This directly shows the importance of a good experimental setup in the study of interfacial properties of proteins. In this chapter simultaneous measurements of surface load and surface pressure, during the adsorption of native and chemically modified ovalbumin to a clean air-water interface, were used to determine the direct influence of protein exposed hydrophobicity on the adsorption kinetics.
Materials and Methods

Isolation of ovalbumin from hen eggs. Ovalbumin was isolated as described by Vachier et al.\textsuperscript{21} and Takahashi et al.\textsuperscript{20} from fresh hen eggs (less than 3 hrs old) that were obtained from the Department of Animal Sciences (Wageningen University and Research Centre). Egg white was manually separated from the yolk and pooled. The egg white was diluted two times with 50 mM Tris (pH 7.5) containing 10 mM β-mercaptoethanol. The solution was gently stirred for 6 hours at 4 °C. After centrifugation the supernatant was diluted two times with 50 mM Tris (pH 7.5). Subsequently DEAE Sepharose CL-6B was added to the albumin solution (9 g DEAE per 100 mL) and stirred gently overnight at 4 °C. The material was transferred onto a glass filter (G2) and subsequently washed with distilled water (1.8 L per 500 g DEAE) and 0.1 M NaCl (0.5 L per 500 g DEAE). The bound ovalbumin was eluted from the ion-exchange material with 0.15 M NaCl. The eluate was dialysed, lyophilised and stored at −20 °C. The purity of the ovalbumin was found to be 98 ±0.5 % as determined by densitometric analysis of SDS-PAGE gels.

Lipophilisation of ovalbumin with succinimidic esters of capric acid.

Esterification of capric acid with N-hydroxy-succinimide. To increase the reactivity of capric acid toward protein amino groups, the fatty acid was first esterified with N-hydroxysuccinimic acid as described by Lapidot et al.\textsuperscript{22} Esterification was realised by reaction of equimolar amounts of fatty acid and N-hydroxysuccinimide in anhydrous tetrahydroxyfuran, in the presence of 1,3-dicyclo-hexylcarbodiimide for 36 hrs at room temperature. The precipitated side-product dicyclohexylurea was removed by filtration and the filtrate was dried in a vacuum evaporator. In order to remove other impurities, the succinimidic ester of capric acid (C\textsubscript{10:0}-SU) was redissolved (1 g in 5 mL) in 60 % ethanol in H\textsubscript{2}O at 60 °C and recrystallized by cooling down to room temperature. The yield of the esterification reaction was approximately 70 %. The purity and identity of the activated capric acid was confirmed (>97 % purity) using thin layer chromatography, mass spectrometry and nuclear magnetic resonance spectroscopy (results not shown).

Modification of ovalbumin. Activated capric acid (C\textsubscript{10:0}-SU) was covalently coupled to the free primary amino groups of ovalbumin as described by Liu et al.\textsuperscript{23} In order to obtain
ovalbumin with different degrees of modification, four batches were prepared that differed in the added amount of C_{10:0}-SU. Ovalbumin was dissolved at a concentration of 8.5 mg/mL in 250 mL 100 mM sodium carbonate buffer pH 8.5. C_{10:0}-SU acid was dissolved at concentrations of 0.4, 1.4, 2.8 and 8.0 mg/mL in 40 mL DMSO and subsequently added to the ovalbumin solution. This gave end concentrations of 0.2 mM ovalbumin and 0.2, 0.7, 1.4 and 4 mM activated capric acid. The mixtures were incubated for 18 hrs at 40 °C. After incubation the mixtures were dialysed against water and after lyophilization stored at –20 °C.

**Chemical characterization**

*Detection of primary amino groups.* Primary amino groups in native and caprylated ovalbumin were detected using *o*-phthalic-dialdehyde (OPA) as described by Church et al.\textsuperscript{24} The OPA reagent is freshly prepared by dissolving 40 mg OPA in 1 mL methanol, followed by the addition of 25 mL 0.1 M sodium borate, 200 mg 2-(dimethylamino)-ethanethiol hydrochloride (DMA) and 5 mL 10% SDS. The total volume is adjusted to 50 mL with H\textsubscript{2}O. Samples were prepared (in triplicate) by adding 65 µL of a 0.1 mM protein solution to 3 mL of the reagent solution. After addition of the reagent solution the samples was left to equilibrate for 2 minutes. The presence of alkyl-iso-indole derivatives formed after reaction of OPA with free amino groups, was measured by the absorbance at 340 nm. To calculate absolute amounts of primary amino groups per protein molecule a calibration curve was measured using leucine as a reference compound.

*Quantification of protein surface hydrophobicity.* ANSA (8-anilino-1-naphtalenesulfonic acid) was used in a fluorimetric assay to quantify the protein exposed hydrophobicity of native and caprylated ovalbumin. Ovalbumin (2.3 µM) and ANSA (2.4 mM) were separately dissolved in 10mM sodium phosphate buffer at pH 7.0. The ANSA solution was titrated in aliquots of 10 µL to 1 mL ovalbumin solution, as described by Haskard\textsuperscript{25} and Alizadeh.\textsuperscript{26} The excitation wavelength was 385 nm and the emission spectrum was measured from 440-650 nm on a Perkin Elmer Luminescence Spectrometer LS 50 B with a scan speed of 120 nm/min. The excitation and emission slits were set at 5 nm and the measurements were done at 20 °C. The relative apparent hydrophobicity of each sample was expressed as the maximum area of the fluorescence spectrum in the range mentioned above, relative to the maximum area of ANSA fluorescence measured for native ovalbumin.
Determination of secondary structure. Samples were dissolved (0.1 mg/mL) in a 10 mM phosphate buffer at pH 7.0. Far-UV CD spectra (190-260 nm) were recorded 8-fold using a 1 mm quartz cuvette, on a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) and averaged. Spectra were measured both at 20 °C and at 90 °C, with a scan speed of 100 nm/min, a data-interval of 0.2 nm, a bandwidth of 1.0 nm and a response time of 0.125 s. All spectra were corrected for the corresponding protein-free sample and analyzed for the secondary structure estimates using a non-linear least squares fitting procedure with reference spectra as described by de Jongh et al.27

Evaluation of tertiary structure. Intrinsic fluorescence of the tryptophan and tyrosine residues of 0.1 mg/mL protein solutions in 10 mM phosphate buffer (pH 7.0) was measured on a Perkin Elmer Luminescence Spectrometer LS 50 B. The excitation and emission slit were set at 5 nm. The excitation wavelength was 295 nm or 274 to excite tryptophan or tyrosine residues respectively. The emission spectra were recorded from 300-450 nm with a scan speed of 120 nm/min. Each spectrum was the average of two scans and corrected for a protein-free sample.

Determination of quaternary structure. Protein samples were dissolved (5 mg/mL) in 10 mM phosphate buffer (pH 7.0) in the presence of 100 mM NaCl to avoid non-specific binding of the protein to the column material. This buffer was also used to equilibrate the Superdex 200 HR column (60x1cm, Amersham Pharmacia Biotech AB, Uppsala, Sweden). Samples of 0.2 mL were applied to the column and eluted at a flow-rate of 0.4 mL/min with the same buffer, while monitoring the eluate at 280 nm. The column was calibrated for apparent molecular mass determination by applying a mixture of ferritin (440 kDa), aldolase (158 kDa) and ovalbumin (43 kDa).

Adsorption kinetics measurements.

Adsorption of native and caprylated ovalbumin from bulk solution (0.1 mg/mL, phosphate buffer 10 mM pH 7.0) to the air-water interface was studied using a combination of a multiskop ellipsometer (Optrell, Germany) and a Langmuir trough (Riegler and Kirstein, Germany). The trough volume was 100 mL and the surface area could be varied from 192 cm² to 30 cm². In order to start the experiment with an interface essentially free of adsorbed
molecules, the interfacial layer was removed using a custom made suction device after which the clean interface was expanded to the maximum area in 40 s (from 30 to 190 cm$^2$). The adsorption of proteins from the bulk solution (at 20 °C) was measured using the ellipsometer and a Wilhelmy plate. A good description of the ellipsometry with further references is given by Graham and Philips.$^3$ The values for $\Delta$ and $\psi$ from the ellipsometer were used to calculate the adsorbed amount of proteins, using a 3-layer model, with $n_{\text{air}} = 1.000$ the, $n_{\text{protein solution}} = 1.3327$ and $dn/dc = 0.18^{28}$ (the refractive indices of air and water and the refractive index increment of the protein respectively). The angle of incidence was 50 °.

**Results**

**Lipophilisation of ovalbumin with succinimidic esters of capric acid.**

*Degree of modification.* Lipophilisation of ovalbumin was performed at four different protein to substrate (succinylated capric acid) ratios. The average number of capric acid chains that were covalently bound to the amino groups of ovalbumin was determined using a chromophoric assay (the OPA method), based on the selective reaction of ortho-phthalic-dialdehyde to amino groups. In figure 2.1 it is shown that the degree of modification increases with an increase in the reactant to substrate ratio. The degree of modification levels off at approximately 60 %, which corresponds to an average coupling of 12 capric acid chains per ovalbumin molecule. Batra *et al.*$^{29}$ reported that for ovalbumin 15 of the 20 lysine groups are easily modified via succinylation or acylation as a result of their solvent accessibility. That here only 12 groups were modified at the highest excess reagent concentration may have been due to aggregation of the proteins (as will be discussed later), resulting in a decreased accessibility of some of the lysine groups.
Figure 2.1 The degree of modification as function of the molar ratio of the added reactant (C_{10:0-SU}) to the total amount of accessible amino groups (NH\textsubscript{2}) in ovalbumin, as determined by the OPA method. The degree of modification is expressed as the % of the total number of amino groups per protein that has reacted to capric acid.

Other techniques that were used to measure the degree of modification are iso-electric focussing (IEF) and mass-spectrometry. Results of the IEF are shown in table 2.1. A small decrease in pI with increasing degree of modification is found (from 4.5 to 4.3). This is expected since the coupling with capric acid eliminates the positive charges of the amino groups of lysine. Calculation of the degree of modification based on the shift of pI is not accurate since the pI of the protein is close to the pK\textsubscript{a} value of the carboxylate groups and therefore rather insensitive to changes. A more direct technique to measure the covalent binding of capric acid to the protein is mass spectrometry. However, due to the low polarisability of the caprylated ovalbumin, no conclusive results could be obtained using this technique.

* Reproducibility was ±4%
Adsorption Kinetics of Ovalbumin

**Table 2.1. Controllability of the caprylation of ovalbumin and the changes in pI and relative hydrophobicity as function of the degree of modification**

<table>
<thead>
<tr>
<th>C_{10:0}-SU : LYS ratio [moles/mole]</th>
<th>Moles of capric acid/mole ovalbumin (±1)</th>
<th>Degree of modification [%] (±5)</th>
<th>pI (Measured)</th>
<th>Relative hydrophobicity* (±0.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4.51</td>
<td>1.0</td>
</tr>
<tr>
<td>0.5</td>
<td>2</td>
<td>12</td>
<td>4.48</td>
<td>2.4</td>
</tr>
<tr>
<td>1.75</td>
<td>5</td>
<td>23</td>
<td>4.45</td>
<td>3.5</td>
</tr>
<tr>
<td>3.5</td>
<td>8</td>
<td>40</td>
<td>4.38</td>
<td>6.0</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>60</td>
<td>4.3</td>
<td>8.0</td>
</tr>
</tbody>
</table>

*) The relative hydrophobicity is expressed as the maximal area under the fluorescence spectrum, where the value for non-modified ovalbumin is set at 1.

**Chemical characterization**

Exposed hydrophobicity. The relative exposed hydrophobicity of the proteins was measured by ANSA-fluorescence. The total fluorescence intensity of the probe depends on the polarity of its environment and increases with increasing hydrophobicity. ANSA was titrated to protein solutions in 10 µL aliquots and a fluorescence spectrum was recorded after each addition. The area under the fluorescence spectra is plotted against the added concentration ANSA in figure 2.2A. At low ANSA concentrations the fluorescence increases with the added amount, but the fluorescence signal levels off at higher ANSA concentrations. This maximum value of each titration curve is taken as a measure of the apparent hydrophobicity of the sample, since no indications of quenching are present in the data. The relative hydrophobicity (shown in figure 2.2B) is calculated by dividing the maximal fluorescence of each sample by that found for native ovalbumin. Similar results are obtained when the initial slope of the fluorescence against the concentration ANSA is taken as a measure of hydrophobicity (data not shown). The exposed hydrophobicity of caprylated ovalbumin is found to increase linearly with the number of capryl chains on the protein surface, with an exposed hydrophobicity of 8 times that of native ovalbumin for the sample with 60 % modification.
Figure 2.2 (A) Exposed hydrophobicity of modified and non-modified ovalbumin as measured by ANSA fluorescence, fluorescence intensity against added amount of ANSA (protein concentration was 2.3 µM, 20 °C, pH 7.0; (B) Relative hydrophobicity as a function of the degree of modification

To distinguish between the effects of the lipophilization process and the presence of coupled capryl chains on the protein, a sample of ovalbumin that was treated in the same way as the lipophilized protein without the addition of activated capric acid was included in the measurements. The exposed hydrophobicity of this non-modified protein was similar to that of the native protein, indicating that the changes in the hydrophobicity upon lipophilization are due to the presence of the coupled capryl chains.

Secondary and tertiary structure. To test the effect of the modification procedure on other properties of the protein, the structural properties of modified and non-modified ovalbumin were determined. At 20 °C no significant differences are found between the far-UV CD spectra of modified (up to 40 %) and non-modified ovalbumin (results not shown). Secondary structure estimates derived from spectral analysis indicate at a content of 15 % α-helix, 54 % β-structure (β-helix and β-turn) and 31 % random coil ±2 % for all ovalbumin variants. The intrinsic fluorescence of tryptophan and tyrosine residues in the protein also show no significant change upon caprylation, indicating that the tertiary fold of the protein is preserved (results not shown). Only the 60 % caprylated sample shows minor differences in far-UV CD and fluorescence spectra, suggestive for a loss of globular packing for a minor part of the protein.
Figure 2.3. Size exclusion (Superdex-200) chromatograms of caprylated ovalbumin (labels 1-4 represent caprylated ovalbumin batches 12%, 23%, 40% and 60% respectively), (500 µL, 0.1 mg/mL, pH 7.0), inset: the relative amount of aggregates and monomers as a function of the degree of modification.

Quartenary structure. In figure 2.3, the size-exclusion chromatograms of the ovalbumin samples with different degrees of modification are shown. The total amount of protein material, as calculated by integrating the area under the chromatogram, is constant for all samples, indicating that all material elutes from the column. With increasing degree of modification, the fraction of monomeric protein decreases, while the fraction of aggregated protein increases (as shown in the inset). When the average degree of modification exceeds 10 % (i.e. 3 capryl chains per protein), significant aggregation occurs. In the aggregated fraction, at least two distinct peaks can be identified. These correspond with trimeric and pentameric forms of ovalbumin. The maximum size of the higher aggregates does not exceed 600 kDa, which indicates that aggregation stops when approximately 15 proteins are present in the aggregate. To test whether the aggregation is an equilibrium process, both the monomeric and the aggregated fraction were pooled and re-applied to the column. No aggregation of the monomeric fraction was observed and no disintegration of the aggregates in the aggregated fraction was found (data not shown). This indicates that the aggregation is a non-equilibrium reaction under the conditions used here.
Selection of sample. To study the effect of increased exposed hydrophobicity on the adsorption kinetics of ovalbumin a batch with an average coupling of 5 capryl chains per protein was separated into a monomeric and an aggregated fraction. The (average) degree of modification is 4 and 7, and the relative hydrophobicity is 1.4 and 5.8 for the monomeric and aggregated fraction respectively. The relative hydrophobicity of the aggregated material is higher than would be expected based on the degree of modification. Most likely the oligomeric structure has a higher binding capacity for ANSA, compared to the monomeric material. The relative hydrophobicity of the monomeric fraction is comparable to that of for example β-lactoglobulin, measured using the same technique (data not shown).

![Graph](image)

**Figure 2.4:** Surface load (A) and surface pressure (B) vs. time for native (◊), monomeric caprylated (■) and aggregated caprylated ovalbumin (▲), 0.1mg/mL in 10 mM phosphate pH 7.0. Measurements were taken every 20 s, markers are shown to give an indication of the experimental error.

Adsorption kinetics. The adsorption kinetics for native ovalbumin and the selected caprylated sample described above were studied by measuring the increase of surface pressure (Π) and surface load (Γ) in time at the (cleaned) air-water interface of a protein solution (see figures 2.4A and B respectively). For native ovalbumin Γ starts to increase at t=0 s, while during the first 600 s no significant increase of Π is measured. The endpoint of this lag-phase (or lag-time) in the Π-t curve is reached when the surface load exceeds the so-called ‘overlap concentration’. For a wide range of proteins this overlap concentration (or critical surface load Γc) was found to be in the range of 0.5-1.5 mg/m². After the lag-phase a decrease in the rate of adsorption is found, while the surface pressure increases rapidly. The decrease in the rate of adsorption is attributed to the amount of work that is required for a protein to ‘clear’ enough surfacial area to adsorb. The sharp increase in surface pressure in this region is the result of the interactions between the adsorbed proteins. After approximately 2000 s the
surface load has reached its equilibrium value of 1.5 mg/m$^2$, while the surface pressure still increases from 10 mN/m to 17 mN/m between 2000 and 3000 s. This further increase in surface pressure at constant surface load is not yet fully understood, but has been related to changes in the intermolecular interactions, possibly due to conformational rearrangements$^b$ of the adsorbed proteins.$^{10,4}$

For caprylated proteins a drastic change in adsorption behavior is observed. At $t=0$ s, the surface load is already higher than $\Gamma_c$ found for native ovalbumin (i.e. >1.1 mg/m$^2$). Thus, the proteins adsorb so fast, that in the time between the cleaning of the surface and the start of the ellipsometric measurements (about 100 s) the same amount of protein adsorbed at the surface as in 600 s for native ovalbumin. The faster rate of adsorption of the caprylated ovalbumin is also demonstrated by the absence of the lag time in the $\Pi$-$t$ curve. At 1000 s, $\Gamma$ has reached its equilibrium value, also at 1.5 mg/m$^2$, while the surface pressure continues to increase, from 10 mN/m at 1000 s to 17 mN/m at 4500 s, comparable to the native protein.

![Figure 2.5. Surface pressure vs. Surface load curves (equation of state) of native (○) and monomeric (■) and aggregated caprylated (▲) ovalbumin, 0.1 mg/mL in 10 mM phosphate pH 7.0 (markers shown correspond to the datapoints shown in figure 2.4).](image)

The aggregated fraction showed results that are comparable to those of the monomeric fraction, although the rate of adsorption is slightly slower. When the results from ellipsometry and surface tensiometry are combined, a $\Pi$-$\Gamma$ curve can be drawn. This relation between surface load and surface pressure is also referred to as the ‘equation-of-state’ of the protein and provides information on interactions between adsorbed molecules. In figure 2.5 the results for native and both modified fractions are shown. The surface pressure starts to

---

$^b$ Literature indeed provides references that state that the increase of surface pressure at seemingly constant surface load is due to protein unfolding. However, in the general discussion we will show that this is not the case.
increase at a surface load of 1.1 mg/m² for all samples. Within the margin of error of the experiments all samples show a similar relation between $\Pi$ and $\Gamma$.

Discussion

In this study, capric acid was covalently bound to ovalbumin in order to increase the exposed hydrophobicity of the protein and thereby affect its adsorption kinetics. The extent of acylation can be controlled by varying the ratio of activated capric acid to ovalbumin in the reaction mixture. This control over the reaction has also been demonstrated in other studies.14,15,23 Four batches were prepared with increasing amounts of coupled capryl chains. No significant changes in secondary and tertiary structure were found, when up to 8 of the available 20 lysine groups were modified. However, aggregation was observed when more than three capryl chains were coupled to the protein. This aggregation has been reported to depend on the number of fatty acids per protein and the length of the fatty acid chains.12,15,17,18 One batch of modified ovalbumin was fractionated in a monomeric and an aggregated fraction. The (average) degrees of modification were 4 and 7, and the increases in exposed hydrophobicity were 1.4 and 5.8 for the monomeric and aggregated fractions respectively. These fractions, having a similar secondary and tertiary fold, were used in the adsorption kinetics experiments.

To relate the influence of the increased exposed hydrophobicity directly to the adsorption kinetics, the adsorption kinetics of native ovalbumin was studied and compared to the monomeric and aggregated caprylated proteins. According to Ward and Tordai33 the adsorption of proteins to the air-water interface, in the limit $\Gamma \to 0$, can be described by:

$$\Gamma(t) = C_b \sqrt{\frac{D_b t}{\Pi}}$$

(2.1)

where $D_b$ is the diffusion constant of the protein, $C_b$ the bulk concentration and $t$ the time. More recently models have been proposed that allow for multiple adsorption states,34 or for the charge and unfolding of the adsorbing proteins.35 However, we focus on the initial stages of adsorption where the adsorbed molecules have no interaction ($\Gamma \ll 1$ mg/m², $\Pi \ll 1$ mN/m), so the Ward and Tordai equation should be a good approximation.
Table 2.2. Apparent diffusion constant and calculated energy barrier for adsorption of native and caprylated ovalbumin.

<table>
<thead>
<tr>
<th></th>
<th>$D^*$ [m$^2$/s]</th>
<th>$\Delta E_{\text{barrier}}$/kT [-]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>1·10$^{-13}$</td>
<td>4</td>
</tr>
<tr>
<td>Monomeric</td>
<td>6·10$^{-11}$</td>
<td>0</td>
</tr>
<tr>
<td>Aggregated</td>
<td>1·10$^{-11}$</td>
<td>1</td>
</tr>
</tbody>
</table>

If the surface load is then plotted against the square root of time, the (apparent) diffusion constant of the protein can be calculated. When this is done for the results for non-modified ovalbumin (shown in figure 3.4), $D = 1·10^{-13}$ m$^2$/s is found, while the actual diffusion constant of ovalbumin in the bulk solution is $7·10^{-10}$ m$^2$/s. In other words, the adsorption of ovalbumin proceeds at a much lower rate than would be expected based on the diffusion rate of the protein. This indicates at the existence of an energy barrier for adsorption even in the early stages of the adsorption process, contrary to the consensus of barrier free adsorption of proteins.\textsuperscript{4-6} However, Damodaran \textit{et al.}\textsuperscript{9} reported a similar effect for native BSA. They found that partial unfolding of the protein increased the apparent diffusion constant, indicating at sub-optimal adsorption of the native protein, but they could not identify the underlying mechanism. To account for the energy barrier to adsorption, Ravera \textit{et al.}\textsuperscript{36} proposed a model where the diffusion constant in (1) is replaced with a renormalized diffusion constant $D^*$ which is defined as:

$$D^* = D_b e^{-\Delta E_{\text{barrier}}/kT} \quad (2.2)$$

where $\Delta E_{\text{barrier}}$ is the activation energy barrier for adsorption, $k$ the Boltzmann constant and $T$ the absolute temperature. Using this equation the energy barrier for the adsorption of native and caprylated ovalbumin to the air-water interface was calculated, the results are presented in table 2.2. For non-modified ovalbumin an activation energy for adsorption of 4 kT (or 10 kJ/mole) is found. A possible explanation is that non-modified ovalbumin adsorbs in its folded state, which has such a low exposed hydrophobicity that the kinetic energy of the protein drives the molecule back into the bulk solution. An increase in exposed hydrophobicity would then lead to a large gain in free energy upon adsorption, preventing back diffusion to the bulk phase. Indeed a negligible value of the activation energy was found for caprylated ovalbumin (see table 2.2). The apparent diffusion constant of the monomeric caprylated material was calculated with a correction for the 100 s time period between the
cleaning of the interface and the first ellipsometric data-point. For the aggregated species the bulk diffusion constant was calculated based on the assumption of a spherical shape of the aggregate. Using this value ($0.36 \cdot 10^{-11} \text{ m}^2/\text{s}$) the activation energy of $1kT$ was calculated. Deviations from the spherical shape will further decrease the estimate of the activation energy.

**Conclusions**

Protein exposed hydrophobicity has often been related to the kinetics of adsorption to the air-water interface. Since it is the commonly accepted view that no energy barrier for protein adsorption to “empty” surfaces can exist, hydrophobicity was said to play a role only in the latter part of adsorption, i.e. after filling of the monolayer and the subsequent development of an energy barrier. Using chemical modification a monomeric variant of ovalbumin was obtained with an increased exposed hydrophobicity, without changes in secondary or tertiary structure. The results presented in this work suggest that the assumption of barrier-free or purely diffusion limited adsorption of proteins (at low surface load) needs to be reconsidered. Our hypothesis is that there is an energy barrier for initial adsorption of any protein. The height of this barrier depends on the degree of hydrophobicity at the surface of the protein.

These observations might provide new opportunities e.g. to control foamability of food systems. Preliminary experiments demonstrated that for non-modified ovalbumin a concentration of around 10 mg/mL is necessary to form foam, while a concentration of only 0.1 mg/mL is needed if β-lg is used. Upon modification of ovalbumin with 3-4 capryl chains, the concentration needed to form foam could be reduced to 0.1 mg/mL (results not shown). Alternatively, introduction of groups that could shield the hydrophobic exposure of ingredients might prevent foam formation.

**References**


Adsorption Kinetics of Ovalbumin


Chapter 3

Quantitative description of the relation between protein net charge and protein adsorption to air-water interfaces

Abstract

In this study a set of chemically engineered variants of ovalbumin was produced to study the effects of electrostatic charge on the adsorption kinetics and resulting surface pressure at the air-water interface. The modification itself was based on the coupling of succinic anhydride to lysine residues on the protein surface. After purification of the modified proteins, five homogeneous batches were obtained with increasing degrees of modification, and zeta-potentials ranging from -19 to –26 mV (-17 mV for non-modified ovalbumin). These batches showed no changes in secondary, tertiary or quaternary structure compared to the non-modified protein. However, the rate of adsorption as measured with ellipsometry was found to decrease with increasing net charge, even at the initial stages of adsorption. This indicates an energy barrier to adsorption. With the use of a model based on the random sequential adsorption model, the energy barrier for adsorption was calculated and found to increase from 4.7 kT to 6.1 kT when the protein net charge was increased from –12 to –26. A second effect was that the increased electrostatic repulsion resulted in a larger apparent size of the adsorbed proteins, which went from 19 nm² to 31 nm² (non-modified and highest modification respectively), corresponding to similar interaction energies between adsorbed proteins at saturation. The interaction energy was found to determine the saturation surface load, but also the surface pressure as a function of the surface load. Further, it is shown that the build-up of protein surface layers can be described by the Coulombic interactions, exposed protein hydrophobicity and size. In this way proteins at interfaces can be described as hard colloidal particles.
Introduction

The interfacial layer between two bulk phases has chemical, physical and mechanical properties that are different from those of the bulk phases and these properties can change as a result of the adsorption of molecules from the bulk solution. Proteins form a major group of bio-macromolecules and are known to affect interfacial properties in many systems, resulting in much attention from a wide range of research fields (for reviews see refs. 1-3). A striking point in these reviews is the demand for a quantitative description of the relation between molecular parameters of the protein and changes in the mesoscopic system parameters such as the adsorbed amount and surface pressure.\textsuperscript{1-6} To satisfy this demand, a systematic approach to the problem is necessary.

In chapter 2 the role of exposed hydrophobicity was identified using chemical modification to selectively alter the exposed hydrophobicity of ovalbumin. Using the same approach in the present work, we aim at providing a quantitative description of the relation between electrostatic net charge of proteins and adsorption at the air-water interface. In this description we include the increase of adsorbed amount in time and the effect that the adsorbed proteins have on the surface pressure.

Effects of electrostatic interactions on adsorption behaviour have been studied at both liquid and solid interfaces (for studies on adsorption at solid interfaces see refs. 2,7-9). In this chapter the focus is on the liquid-gas interface, which has the advantage that it contains no ionisable groups and that it is homogenous, in contrast to solid interfaces. The first qualitative observations on the effects of net charge were based on the measurement of surface pressure in time for protein solutions at different pH values. Later, researchers used radioactive labelling or ellipsometry; both techniques have the benefit of measuring the adsorbed amount of protein directly. In these studies it was observed that the rate of adsorption and the total amount of adsorbed protein is highest at pH values close to the iso-electric point (IEP or pI) where the protein carries no net charge.\textsuperscript{10-13}

However, changing the pH not only changes the charge, but can also lead to changes in globularity of the protein. Many proteins adopt a molten globule form at acidic pH (pH<4.5),\textsuperscript{14} a state where the protein has lost its tertiary conformation while retaining secondary structure. This limits the range at which the pH can be chosen to specifically study
charge effects. Further, only few charged groups titrate in the pH range in which proteins are used in applications (pH 5-8). An alternative approach is then to screen the electrostatic charges by increasing the ionic strength.\textsuperscript{10,15-17} In this case the net charge on the protein is constant, while the interaction energy is decreased, effectively reducing the contribution of electrostatics to the observed phenomena. Decreasing electrostatic interactions were found to result in an increase in the kinetics of adsorption, the end-value of the adsorbed amount and the end-value of surface pressure. However, no quantitative description of the observations is offered in the mentioned articles.

Only few authors have tried to combine experimental results and theory to propose general equations describing the adsorption process. In the field of protein adsorption many models are based on the Ward and Tordai adsorption equation,\textsuperscript{18} which describes diffusion-controlled adsorption. A modification of this equation was proposed by MacRitchie and Alexander\textsuperscript{19} to account for charge effects. This modification describes the build-up of a wall potential at the interface as a result of the adsorption of charged molecules. More recently, it has been postulated that there is already an energy barrier for adsorption at low surface coverage. This energy barrier was found to limit the adsorption of phosvitin (measured directly using ellipsometry) to the air-water interface\textsuperscript{20} and has also been mentioned by others.\textsuperscript{17,21,22} It has been suggested that the clean air-water interface is not neutral, but displays a wall potential due to preferential orientation of the water molecules. A thorough review on this topic is given by Parfenyuk.\textsuperscript{23} Literature values for the potential of the air-water interface range from \(-1.1\) to \(+0.5\) mV, Parfenyuk concludes that it should be \(+0.10\) mV. This contribution seems to be negligible, compared to the much higher potentials found for the proteins. The major contribution to the energy barrier is the asymmetry of the diffuse double layer surrounding a charged protein near the interface. There are two effects responsible for this asymmetry. Firstly, the difference in the dielectric constant of the aqueous and the air phase. Secondly, there are no counter-ions in the air phase; this latter fact has probably a more pronounced effect. Effectively this results in an electrostatic repulsion that increases as the protein approaches the interface, as if an apparent image charge approaches the same interface from the low-dielectric gas phase. This phenomenon is known as the ‘image charge effect’.\textsuperscript{24}

It has been a matter of debate whether such a theoretical description can still be used to describe protein adsorption. The problem is that proteins are often not spherical and they have heterogeneous charge distribution, while theoretical models often assume that they can
be represented as such. Corrections have been made to include non-spherical shapes, but the effects of non-homogenous charge distributions are more complex to include. However, Roth et al. performed calculations for the interaction energy of lysozyme with a mica surface. They concluded that that the calculated interaction energy is not significantly different when the protein is modeled as a sphere rather than modeled using its 3D structure.

To obtain data that can be used for a quantitative description of the effects of electrostatic charge, ovalbumin was chemically modified using succinic anhydride. This modification is based on the reaction of succinic anhydride with the lysine residues of the protein, changing positively charged amino groups into negatively charged carboxylic groups. This approach has already been used to produce ‘charge ladders’ of modified proteins, where the effective charge of mildly modified proteins shows linear correlation with the degree of modification. In the presented work, five protein variants with increasing net charge were obtained. The kinetics of adsorption and the properties of the adsorbed protein layer were studied using an ellipsometer that was mounted on a Langmuir trough in combination with a Wilhelmy plate surface tensiometer. The obtained results are analysed using a theoretical model to obtain a validated quantitative analysis of the influence of increased protein net charge on the adsorption behaviour of the proteins.

**Materials and Methods**

Ovalbumin (Mₐ = 44 kDa) was isolated as described previously with the only adaptation being that ovalbumin was eluted from the ion-exchange material at 0.15M NaCl. Succinic anhydride, o-phthalaldehyde (OPA) and 2-ethyl-5-phenylisoxazolium-3’-sulfonate (Woodward’s reagent K) were from Sigma-Aldrich (St. Louis, Missouri, USA). All chemicals used were of analytical grade.

**Preparation of succinylated ovalbumin**

_Succinylation of ovalbumin._ Via the reaction of succinic anhydride with lysine residues of proteins, additional carboxylate groups are introduced, resulting in more negative charges on the protein (at pH 7.0). Using the method and conditions described by Kosters et al., three
batches of succinylated ovalbumin were prepared with succinic anhydride : lysine ratios of 0.38, 0.76 and 1.9 (mole/mole). These ratios were chosen to modify 10, 20 and 50% of the available lysine residues (or: to introduce 2, 4 and 10 succinate groups per protein) respectively.

Purification of succinylated ovalbumin. The procedure as described above results in heterogeneous modification of the protein. The three batches were fractionated using ion-exchange chromatography to obtain more homogeneous fractions with increasing degrees of modification. The batches (10 mg/ml, 10 mM phosphate buffer pH 7.0 + 0.1 M NaCl) were applied on an anion-exchange column (Source-Q, 280 ml) connected to an Äkta-explorer (Pharmacia Biotech, Sweden). The column was equilibrated in 10 mM phosphate buffer pH 7.0 containing 0.1 M NaCl (Buffer A). The gradient was increased from 0 to 70% buffer B (10 mM phosphate buffer pH 7.0, 0.5 M NaCl) over 20 column volumes with a flow rate of 60 ml/min. From all three batches, fractions were collected and pooled. These fractions were then dialyzed twice against distilled water, once against buffer A and subsequently re-applied to the column using the same conditions. Final fractions were collected from 11-20%, 22-27%, 28-43%, 45-53% and 57-67 % buffer B (further referred to as samples suc1, suc2, suc3, suc4 or suc5). The elution profiles of native or unmodified ovalbumin (further referred to as suc0) and suc1-suc5, on an analytical column under identical conditions, are shown in figure 3.1. It can be seen that the peak width for all fractions is comparable to unmodified ovalbumin (suc0) illustrating that all fractions are homogenous in the degree of modification (DM). After collection the fractions were dialyzed extensively against distilled water, lyophilized and stored at –20 °C.

![Figure 3.1. Ion-exchange elution profiles for all ovalbumin variants (lines 0-5 represent results of Suc0 –Suc5, respectively, pH 7.0, anionexchange)](image)
Chemical characterization

Detection of primary amino groups. The number of primary amino groups in ovalbumin variants was determined using o-phthalaldehyde (OPA) as described by Church et al.\textsuperscript{32} The OPA reagent was freshly prepared by dissolving 40 mg OPA in 1 mL methanol, followed by the addition of 25 mL 0.1 M sodium borate, 200 mg 2-(dimethylamino)-ethanethiol hydrochloride (DMA) and 5 mL 10% SDS. The total volume was adjusted to 50 mL with H\textsubscript{2}O. Samples were prepared (in triplicate) by adding 65 \(\mu\)L of a 0.1 mM protein solution to 3 mL of the reagent solution. The concentration in the original protein solution was determined from the OD at 280 nm (\(\varepsilon_{\text{ova}} = 29300 \text{ cm}^{-1} \text{ M}^{-1}\)).\textsuperscript{33} After addition of the reagent solution the samples were left to equilibrate for 2 minutes. The presence of alkyl-iso-indole derivatives formed after reaction of OPA with free amino groups, was measured by the absorbance at 340 nm. To calculate the number of primary amino groups per protein molecule a calibration curve was measured using leucine as a reference.

Detection of carboxylic groups. The number of carboxylic groups in ovalbumin variants was measured using the Woodward reagent K assay as described by Kosters et al.\textsuperscript{34} This method is based on the increase of the absorbance at 269 nm after reaction of the Woodward reagent K to the free carboxylic groups of the protein. All measurements were performed in duplicate.

Determination of \(\zeta\)-potential. The \(\zeta\)-potential of the proteins was determined on a Zetasizer 2000 (Malvern instruments Ltd., U.K.) with 10 mg/mL protein solutions (10 mM phosphate pH 7.0, 20°C). Electrophoretic mobility was monitored at 150 V applied voltage, using a He-Ne laser at 632 nm. The Smoluchowski equation was used to calculate the \(\zeta\)-potential from the measured electrophoretic mobility. The apparatus was calibrated according to supplier’s instructions. Samples were analysed in triplicate and the calculated \(\zeta\)-potential varied less then 10% between sample preparations.

Determination of secondary structure. Samples were dissolved (0.1 mg/mL) in a 10 mM phosphate buffer at pH 7.0. Far-UV CD spectra (190-260 nm) were recorded 16-fold and averaged on a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) using a 1 mm quartz cuvet. Spectra were measured both at 20 °C and at 90 °C, with a scan speed of 100 nm/min, a spectral resolution of 0.2 nm, a bandwidth of 1.0 nm and a response time of 0.125 s. All spectra were corrected for the corresponding protein-free sample and analyzed for the
secondy structure estimates using a non-linear least squares fitting procedure with reference spectra as described by de Jongh et al.\textsuperscript{35}

**Evaluation of tertiary structure.** The tertiary fold of ovalbumin variants was monitored with near-UV CD. Samples were dissolved (1.0 mg/mL) in a 10 mM phosphate buffer (pH 7.0) and near-UV CD spectra (250-350 nm) were recorded 16-fold on a Jasco J-715 spectropolarimeter (Jasco Corp., Japan) and averaged, using a 1 cm quartz cuvette. Further settings of the machine were identical to those for the far-UV CD experiments. Recorded spectra were corrected by subtracting the spectrum of a protein-free sample.

The intrinsic fluorescence of the tryptophan and tyrosine residues of 0.1 mg/mL protein solutions in 10 mM phosphate buffer (pH 7.0) was measured on a Perkin Elmer Luminescence Spectrometer LS 50 B. The excitation and emission slits were set at 5 nm. The excitation wavelength was 295 nm or 274, the excitation maxima of tryptophan and tyrosine respectively. The emission spectra were recorded from 300-450 nm with a scan speed of 120 nm/min. Each spectrum was the average of two scans and corrected for a protein-free sample.

**Characterization of interfacial properties**

**Adsorption kinetics measurements.** Adsorption to air-water interfaces of ovalbumin variants was monitored using a Multiskop ellipsometer (Optrell, Germany) combined with a Langmuir trough (Riegler and Kirstein, Germany) and Wilhelmy plate tensiometry. A good explanation of the theoretical background of ellipsometry has been given by Russev et al.\textsuperscript{36} With the use of the combination of ellipsometry and surface tensiometry, both the increase of surface load ($\Gamma$) and surface pressure ($\Pi$) in time could be measured. For all samples the rate of adsorption from 0.25 mg/mL solutions (in 10 mM phosphate, pH 7.0, 20 °C) was measured at least in duplicate. All experiments were started by removing the interfacial layer with a custom made suction device after which the clean interface was rapidly expanded to the maximum area (from 30 to 190 cm$^2$; the first data points are typically collected 100s after cleaning the interface). In this way, the initial conditions for each experiment approximated $\Gamma=0$ mg/m$^2$ at $t=0$ s. The values for the ellipsometric angles $\Delta$ and $\psi$ were used to calculate the adsorbed amount, using software from the supplier (Optrell). To do this, the refractive index and thickness of the adsorbed protein layer are fitted in a model based on two bulk phases (air and...
water) and one adsorbed layer, with parameters: $n_{\text{air}} = 1.000$, $n_{\text{protein solution}} = 1.3327$, $dn/dc = 0.18$; the angle of incidence was $50^\circ$. Control experiments with distilled water in between measurements confirmed that the cleaning method used (rinsing with ethanol and water) was sufficient to avoid contamination of the trough, i.e. surface pressure of a clean surface remained 0 mN/m during compression.

Surface rheology. Protein solutions (0.1 mg/mL in a 10 mM phosphate buffer at pH 7.0) were prepared 1 h before measurement. The surface tension was measured as a function of time (0-5000 s) on an Automated Drop Tensiometer (ADT; I.T. Concept.), a technique described by Benjamins et al.\(^{38}\) Basically, an air bubble is formed at the tip of a syringe needle placed in a cuvette containing the protein solution. Both the cuvette and the syringe are temperature controlled (20 ±0.1 °C). In these experiments, the bubble volume was kept constant at 4 µL, using the computer-controlled syringe plunger to compensate for gas diffusion from the bubble. The surface elastic modulus was measured by inducing sinusoidal changes in the interfacial area with a period of 10 s and amplitude of 10 %. The modulus was calculated from the measured changes in surface tension and surface area averaged over a sequence of five sinuses, every 500 s such a sequence was performed. These measurements of the modulus did not affect the development of surface pressure in time. All samples were measured in duplicate on two separate occasions.

Results and Discussion

Preparation of ovalbumin variants

Succinylation of ovalbumin was performed at three different succinic anhydride to lysine ratios. Ion-exchange chromatography was applied to obtain homogeneous fractions with increasing degrees of modification (DM), as described in the experimental section and shown in figure 3.1. The DM of the fractions was determined using the OPA assay; results are shown in table 3.1. An alternative method, the Woodward assay that detects the number of carboxylic groups, showed comparable results (data not shown). The DM was found to increase from 0 to 67 ±5% with regular intervals for the different variants, which yields theoretical net charges ranging from –12 to –26 (±1). The isoelectric points of the variants were also determined experimentally using iso-electric focussing. However, this approach
could only be used qualitatively, since the iso-electric point of the protein is too close to the pKₐ of the carboxylic groups.

Table 3.1. Chemical characterization of the degree of modification for succinylated ovalbumin

<table>
<thead>
<tr>
<th>Suc₀</th>
<th>-12</th>
<th>-17</th>
<th>0.21 - 0.25</th>
<th>0</th>
<th>5.19</th>
<th>5.19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suc₁</td>
<td>-18</td>
<td>-19</td>
<td>0.27 - 0.32</td>
<td>3</td>
<td>4.86</td>
<td>5.09</td>
</tr>
<tr>
<td>Suc₂</td>
<td>-20</td>
<td>-20</td>
<td>0.29 - 0.35</td>
<td>7</td>
<td>4.57</td>
<td>5.01</td>
</tr>
<tr>
<td>Suc₃</td>
<td>-22</td>
<td>-22</td>
<td>0.34 - 0.40</td>
<td>10</td>
<td>4.39</td>
<td>4.96</td>
</tr>
<tr>
<td>Suc₄</td>
<td>-24</td>
<td>-24</td>
<td>0.37 - 0.43</td>
<td>12</td>
<td>4.29</td>
<td>4.89</td>
</tr>
<tr>
<td>Suc₅</td>
<td>-26</td>
<td>-26</td>
<td>0.42 - 0.47</td>
<td>14</td>
<td>4.19</td>
<td>4.83</td>
</tr>
</tbody>
</table>

Chemical characterization

An extensive characterization of the chemical properties of the fractionated variants was performed to determine whether the chemical modification had induced any changes other than the increase in net charge. With the use of size-exclusion chromatography it was shown that all samples had identical elution patterns, confirming that all fractions consisted of monomeric protein (data not shown).

Figure 3.2. Far-UV CD spectra of Suc₀ (continuous) and Suc₅ (dashed line) ovalbumin, inset shows the near-UV CD spectra of Suc₀ and Suc₃ (20°C, 10mM phosphate buffer pH 7.0)

As illustrated by the far- and near-UV CD spectra for Suc₀ and Suc₅ (the most extremely modified sample) shown in figure 3.2, no significant differences in the globular folding state of the protein variants were found. Secondary structure estimates derived from spectral analysis indicated at a content of about 35 % α-helix, 48 % β-structure (β-helix and β-turn) and 16 % random coil for all variants; the differences in these values between samples was < 1 %. These values agree with other published results for far UV-CD of native ovalbumin.³⁹
This stability against modification agrees with findings by Bhaduri et al., but is in contrast with other literature where it has often been mentioned that chemical modification introduces large conformational changes in protein structure. Possibly the mild reaction conditions used during modification in this work and the limited number of modified groups is the explanation for this discrepancy.

The structural stability of the variants was tested using differential scanning calorimetry. In all cases denaturation temperatures of $78 \pm 2^\circ C$ were found (results not shown). The exposed hydrophobicity was monitored by hydrophobic interaction chromatography (conditions as described in chapter 7). All variants eluted at the same concentration of ammonium sulphate (0.7 M). Although no quantitative value can be obtained from this technique, it is sensitive to changes of the exposed hydrophobicity. The results clearly show that no differences result from the modification.

No discernable differences between the native and modified variants could be detected, and any differences in adsorption behavior of the ovalbumin variants can therefore be solely attributed to differences in net charge.

**Adsorption kinetics**

After characterizing the modified proteins, their adsorption behaviour was studied using ellipsometry. Both the adsorbed amount of protein (or surface load, $\Gamma$) and surface pressure ($\Pi$) were measured as a function of time during adsorption from a bulk solution (0.25 mg/mL) to an initially protein-free air-water interface.
Figure 3.3. The increase of surface load in time for all ovalbumin variants (0.25 mg/ml, 10mM phosphate buffer pH 7.0) for long time. Markers are shown from typical results (-Suc0, ×-Suc1, ○-Suc2, ÷-Suc3, △-Suc4 and ◊-Suc5). The lines are results from fitting the data with equation 3.2.

Adsorbed amount. In figure 3.3 typical curves of the adsorbed amount as a function of adsorption time are shown for suc0-suc5. Data were collected until 20,000 s but since the surface layer was already almost saturated after 5000 s only these data are shown. It can be seen that increasing the net charge has two major effects. First, the rate of initial adsorption decreases with increasing net charge, which indicates the presence of an energetic barrier for adsorption due to electrostatic repulsion. Secondly, the adsorbed amount at which saturation is reached ($\Gamma_{\text{sat}}$) decreases from 1.6 to 0.8 ±0.1 mg/m$^2$ (this is equal to 44 and 89 nm$^2$/molecule) for suc0 and suc5 respectively.

To obtain a more quantitative description of the results, the experimental data were fitted with equations based on the model of “random sequential adsorption”.$^{43-45}$ The RSA model assumes irreversible adsorption and calculates the chance of adsorption ($P_{\text{adsorb}}$, given by equation 3.1) as a function of surface coverage ($\theta$) based on excluded volume effects.

$$P_{\text{adsorb}} = 1 - 4\theta + \frac{6\sqrt{3}}{\pi} \theta^2 + 1.4069\theta^3$$ (3.1)

Where $\theta$ is the surface coverage [-], calculated via $\theta = \Gamma \Omega$ from the surface load ($\Gamma$, [#/m$^2$]) and the surface area taken up by adsorbed protein molecules ($\Omega$, [nm$^2$/molecule]). The rate of adsorption is then calculated from equation 3.2:
\[
\frac{d\Gamma}{dt} = D^* C_b P_{\text{adsorb}}
\]  

(3.2)

In this formula \(C_b\) is the protein concentration [#/m\(^3\)], and \(D^*\) the apparent diffusion coefficient. The apparent diffusion coefficient depends on the bulk diffusion constant \(D_b\) [7·10\(^{-10}\) m\(^2\)/s] in the bulk phase and an adsorption constant via:

\[
D^* \propto D_b \cdot k_{\text{adsorb}}
\]  

(3.3)

From \(k_{\text{adsorb}}\) an energy barrier for adsorption (\(\Delta E_{\text{barrier}}\)) can be calculated via the Boltzmann equation, if a proportionality constant of unity [m] is assumed.

\[
k_{\text{adsorb}} = e^{-\frac{\Delta E_{\text{barrier}}}{kT}}
\]  

(3.4)

To fit the experimental data only \(\Omega\) and \(k_{\text{adsorb}}\) were used as fitting parameters.

![Figure 3.4. Fitted values for the radius (▲) and electrostatic barrier (■) as a function of the protein net charge.](image)

The proteins were assumed to behave as “hard” particles, i.e. \(\Omega\) remains constant with time and surface pressure. This assumption is validated by the observation that the equation of state of ovalbumin does not depend on the bulk concentration (see chapter 4). Further, it has been shown by infra-red reflection adsorption spectroscopy (IRRAS) that the secondary
structure of ovalbumin adsorbed at the air-water interface was independent of the bulk concentrations.  

Table 3.2. Calculated values for the effective adsorption rate and energy barrier for adsorption

<table>
<thead>
<tr>
<th>Estimated net charge</th>
<th>$D^*$ [$10^{-13}$ m$^3$ s$^{-1}$]</th>
<th>$\Delta E_{\text{barrier}}$ [kT]</th>
<th>$R_\text{fit}$ [nm]</th>
<th>$\Gamma_\Pi - \Phi$ [mg/m$^2$]</th>
<th>$\Gamma_{\text{sat}}$ [mg/m$^2$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suc0</td>
<td>-12</td>
<td>6.4</td>
<td>4.7</td>
<td>2.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Suc1</td>
<td>-18</td>
<td>4.5</td>
<td>5.1</td>
<td>2.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Suc2</td>
<td>-20</td>
<td>3.4</td>
<td>5.3</td>
<td>2.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Suc3</td>
<td>-22</td>
<td>2.7</td>
<td>5.6</td>
<td>3.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Suc4</td>
<td>-24</td>
<td>2.6</td>
<td>5.6</td>
<td>3.1</td>
<td>0.8</td>
</tr>
<tr>
<td>Suc5</td>
<td>-26</td>
<td>1.6</td>
<td>6.1</td>
<td>3.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Suc0 (300mM NaCl)</td>
<td>-12</td>
<td>9.5</td>
<td>4.3</td>
<td>2.25</td>
<td>1.36</td>
</tr>
<tr>
<td>Suc2 (300mM NaCl)</td>
<td>-20</td>
<td>9.6</td>
<td>4.3</td>
<td>2.35</td>
<td>1.34</td>
</tr>
</tbody>
</table>

The lines in figure 3.3 are the result of the fitting procedure and in table 3.2 the values for the parameters $D^*$, $\Delta E_{\text{barrier}}$ and $\Omega$ are given. The energy barrier for adsorption increases linearly with increasing charge from 4.7 kT to 6.1 kT for suc0 and suc5 respectively (or 0.14 kT per charge) as shown in figure 3.4 ($R^2=0.95$). The fitted effective radius ($R_\text{fit} = \sqrt{\Omega/\pi}$) of the adsorbed proteins increases linearly with charge from 2.5 to 3.2 nm for suc0 to suc5, also shown in figure 4 ($R^2=0.92$; 0.07 nm per unit charge). This effective radius is the nearest centre-to-centre distance between adsorbing particles. For hard-sphere particles this is equal to two times the radius of a particle. In the case of charged particles this nearest distance is determined by the balance between the kinetic energy of the adsorbing particle, the attraction between the adsorbing particle and the interface and the repulsive energy between the particles. The repulsive energy between two particles (at separation $d=2R_\text{fit}$) was calculated for all variants using equation 3.4, representing the characteristic particle-particle repulsion energy.

$$U(d) = \frac{Z^2e_c^2}{\varepsilon} \left[ \frac{e^{\kappa R_0}}{1 + \kappa R_0} \right]^2 e^{-\kappa d} \frac{1}{d} \quad (3.5)$$

Where $Z$ is the charge on the molecule, $e_c$ is the unit electron charge ($1.6 \times 10^{-19}$ C), $\varepsilon$ the dielectric permittivity of the medium ($80$ C$^2$ N$^{-1}$ m$^{-2}$ for water), $\kappa$ the reciprocal Debye screening length [m$^{-1}$], $R_0$ the radius of the protein [m] and $d$ the distance between particles [m]. From the calculations it was found that that the interaction energy at the minimal distance between an adsorbing and an adsorbed protein ($2R_\text{fit}$) is similar for all variants (around 0.03 kT).
When the experiments were performed in the presence of 0.3 M NaCl, the fitted radius decreased to 2.3 nm (see table 3.2), which is close to the smallest radius found for native ovalbumin (dimensions of ovalbumin are 7.0x4.5x5.0 nm). At this salt concentration the difference between the variants disappeared, as illustrated by the results for suc2. This further confirms that the increase in radius should be contributed to the high electrostatic repulsion between adsorbed proteins.

*Figure 3.5. The increase of surface pressure in time for Suc0 to Suc5 (0.25 mg/ml pH 7.0, markers same as in figure 3.3)*

**Equation of state.** The relation between surface pressure and surface load is known as the “equation of state” and depends on the intermolecular interactions between adsorbed protein molecules. In figure 3.5 typical results of the surface pressure are plotted against the time of adsorption. The surface pressure of suc0 increases after 500 s. After this lag time the surface pressure increases sharply until a semi-equilibrium value is reached. For suc0 a surface pressure of approximately 20 mN/m is reached after 6000 s. With increasing net charge the time needed to reach equilibrium surface pressure increases to 8000 s for suc5, while the surface pressure that is reached is decreased (8 mN/m for suc5 after 12000 s). In the initial stages of adsorption the average distance between adsorbed proteins is such that there are no effective interactions. This can be illustrated by comparing figure 3.3 and figure 3.5, where it can be seen that for all variants the surface pressure starts to increase after 500 s, while at this time the adsorbed amount is lower for the variants with high net charge. The Π-Γ curves compiled from tensiometry and ellipsometry data are shown in figure 3.6. At longer time-
scales the ellipsometric data show more scattering, and those data are not used for calculations. Further, repeat experiments have shown that the trends indicated by the dotted lines are valid. The adsorbed amount where the surface pressure starts to increase ($\Gamma_{\Pi-0}$) is around 1.1 mg/m$^2$ for suc$_0$, and decreases with increasing net charge to 0.7 mg/m$^2$ for suc$_5$ (table 3.2). Using the calculation for the characteristic particle-particle interaction given by Yuan et al.$^{47}$ it was found that for all samples the interaction energy between adsorbed proteins within the surface layer, was similar (around 0.3 kT) at the point where the surface pressure starts to increase.

![Graph showing surface pressure as a function of surface load](image)

*Figure 3.6. The surface pressure as a function of surface load (combination of the results shown in figure 3.3 and 3.4), dotted lines are shown to guide the eye.*

The effect of ionic strength. If the observed difference in adsorption behaviour between suc$_0$ and modified protein is primarily the result of electrostatic interactions, an increase in ionic strength of the bulk solution should negate the differences. Under standard conditions (10 mM phosphate buffer pH 7.0) the Debye length can be estimated to be 3.2 nm; at high ionic strength (buffer + 300 mM NaCl) the Debye length is decreased to 0.6 nm.
In figure 3.7 the surface pressure of suc₀ and suc₂ is plotted versus the surface load at low and high ionic strength. At high ionic strength $\Gamma_{\Pi-0}$ is increased for suc₀ and the difference between $\Gamma_{\Pi-0}$ for suc₀ and suc₂ has disappeared. Moreover, the maximal surface load and surface pressure that is reached (after 5000s) is comparable for both proteins. That the high ionic strength leads to identical adsorption kinetics (data not shown) and equation of state, further confirms that the modification only affected the electrostatic properties of the protein.

Surface rheology. From the above the picture emerges that the static surface pressure can be fully described in terms of a packed surface layer. The question arises if this description is also valid for the dilatational properties of the surface. The dilatational elastic modulus of all variants was measured during adsorption and the results are shown in figure 3.8A. For suc₀ the modulus increases to 90 mN/m in 1000 s and then it remains constant. With increasing net charge the time needed to reach equilibrium is increased and the equilibrium modulus is decreased (40 mN/m for suc₅). This is similar to the development of surface pressure. A correction for the rate of adsorption can be achieved by plotting the modulus against the surface pressure (figure 3.8B). Until a surface pressure of around 7 mN/m all samples follow the same line. At higher surface pressures the results diverge. The modulus of suc₀ continues to increase almost linearly, while suc₅ levels of. Some authors have proposed that a lower dilatational modulus is the result of desorption of molecules from the interface. From this perspective it is interesting to note that for the variants no desorption of proteins from the interface was observed in ellipsometric experiments (results not shown), so that the elastic
modulus should be attributed to interactions between adsorbed molecules. This means that the different shape of the curves in figure 3.8B reflect the shift in the $\Pi$-$\Gamma$ relationship found in figure 3.6.

![Graphs showing elastic modulus vs. time and surface pressure for different variants.](image)

**Figure 3.8.** Elastic modulus vs. time (A) or vs. surface pressure (B) for all variants (top to bottom Suc0-Suc5, markers are as in figure 3.5).

**Conclusion**

With the use of five well-defined variants of ovalbumin with increased net charge, the influences of charge on adsorption kinetics and surface pressure development could be studied under constant system conditions. From adsorption experiments it became clear that the electrostatic charge affects the initial adsorption to the interface. This effect is due to the image-charge potential, which is the result of the difference in dielectric permittivity of the aqueous and air phase and the absence of counter-ions in the air-phase.

The contribution of net-charge to the kinetic barrier to adsorption should be seen in relation to the gain in energy due to hydrophobic interaction with the interface, as described in previous work. As the adsorption continues the chance of the adsorbing particle to arrive close to an already adsorbed particle increases, leading to a second barrier to adsorption. This steric barrier is due to excluded volume effects and is described by the Random Sequential Adsorption model. This model was used to describe the adsorption of all variants, and resulted in good fits of the experimental data. The apparent size of the proteins as fitted by the model ($R_{fit}$) could be related to the charge of the protein variants. Calculations show that the interaction energy due to electrostatic repulsion at separation distance $d=2R_{fit}$ is constant for
all variants, which validates the assumption that the ‘soft’ interaction potential due to electrostatic interactions can be approximated by a hard-core potential with a suitably chosen effective diameter.43-45,47

The development of surface pressure with increasing surface load was also affected by protein net charge. At increasing net charge the adsorbed amount needed to reach a certain surface pressure is decreased, the decrease in $\Gamma$ seems to follow the increase in $R_{\text{fit}}$. By plotting $\Pi$ against $\theta$ this is confirmed, since the results for all variants follow the same curve (not shown).

From the obtained results it may be clear that electrostatic properties are the most important molecular characteristic of globular proteins in understanding the adsorption process. One of the interesting findings is that the surface layer is less densely packed when the proteins have a higher charge. Preliminary data from neutron reflection experiments performed at ISIS (Didcot, UK), confirmed this idea. The larger amount of water at the adsorbed layers formed with proteins with higher net charge might be important in processes like coalescence of bubbles or emulsion droplets, or displacement of adsorbed proteins by low-molecular surfactants.

References

Electrostatics of Proteins at Interfaces


The adsorption and unfolding kinetics determine the folding state of proteins at the air-water interface and thereby the equation of state.

Abstract

Unfolding of proteins has often been mentioned as an important factor both during the adsorption process at air-water interfaces and in the increase of surface pressure at later stages of the adsorption process. In this work we focus on the question whether the folding state of the adsorbed protein depends on the rate of adsorption to the interface. The adsorption rate can be controlled by bulk concentration and therefore the adsorption of proteins with varying structural stabilities at several protein concentrations was studied using ellipsometry and surface tensiometry. For β-lactoglobulin the adsorbed amount (Γ) needed to reach a certain surface-pressure (Π) decreased with decreasing bulk concentration. Ovalbumin showed no such dependence. To verify whether this difference in behavior is caused by the difference in structural stability, similar experiments were performed with cytochrome c and a destabilized variant of this protein. However, both proteins showed identical Π-Γ curves and did not show any dependence on bulk concentration. From this work it was concluded that unfolding will only take place if the kinetics of adsorption is similar or slower than the kinetics of unfolding. The kinetics of unfolding depends on the activation energy of unfolding (which is in the order of 100-300 kJ/mole), rather than the free energy of unfolding (typically in the order of 10-50 kJ/mole). It was further shown that any unfolding during initial stages of adsorption is not reversed at later times, showing that there is no equilibrium between adsorbed protein conformation and the surface pressure.
**Introduction**

To understand the differences in surface behavior observed between different proteins –or one protein in different system conditions– it is necessary to identify the different processes involved in adsorbed layer formation and their relative contribution to the interfacial properties. One of these processes is the possible unfolding of proteins at the interface.\textsuperscript{1-3} That proteins may unfold at interfaces was hypothesized based on the observation that enzymes may lose their activity upon adsorption at the air-water interface.\textsuperscript{4,5} A gain in free energy of the system would be obtained if the protein changes its conformation so that the polar residues are oriented towards the aqueous phase and the non-polar (or hydrophobic) residues to the air phase. Such a reorientation will lead to a ‘loop-train’ configuration, where loops are the parts of the polypeptide that protrude in the bulk solution and the trains are segments of mainly hydrophobic residues that are adsorbed at the interface.\textsuperscript{3,6,7} In what way the observed interfacial properties are affected by such unfolding is not well understood. Some authors suggest that only an unfolded protein will adsorb at the interface, thereby relating structural stability to the initial adsorption.\textsuperscript{1,8,9} Other authors claim that protein unfolding is required to increase the surface pressure.\textsuperscript{10-12} In these models the conformational state of the proteins is often assumed to change with the surface pressure. In the equation of state model of Fainerman et al.,\textsuperscript{13,14} the decrease in apparent size of the adsorbed protein with increasing surface pressure is described as desorption of segments of the protein chain. However, from studies performed with non-unfolding particles (such as Stöber silica particles,\textsuperscript{15} glass microspheres,\textsuperscript{16} gelled polymer microbeads,\textsuperscript{17} latex particles,\textsuperscript{18} and colloidal silver particles\textsuperscript{19}) surface pressure-surface area relations are found that are remarkably similar to those typical for proteins. Another observed phenomenon that has been related to protein unfolding after adsorption is the increase in surface pressure at longer timescales, while little or no increase in adsorbed amount is measured.\textsuperscript{2,7,20} The above-mentioned articles illustrate the different phenomena that have been related to interfacial unfolding of proteins.

In contrast to the relative high amount of theories based on protein unfolding at liquid interfaces, the direct evidence seems to be limited. Detailed information on the exact globular structure of adsorbed proteins can be obtained from only a few techniques. Most techniques that can readily be used in bulk are difficult to apply in a reflection mode due to drastic decreases in signal intensity and subsequently a decrease of the signal to noise ratio. Neutron
and X-ray reflectivity have been used to obtain information on the density profile and structure of interfacial molecular layers, but the results cannot be decisive about possible conformational changes of proteins at the interface. More specific information on the structural fold of proteins at the air/water interface can be obtained from infrared reflection absorption spectroscopy (IRRAS). With this method, the spectrum of an infrared (IR) beam is analyzed after specular reflection at the interface. The sensitivity of the amide I region to changes in the secondary structure can be used to gain insight in the protein conformation at a secondary folding level. More recently also external reflection circular dichroism has been used to assess this information. From a combination of these techniques, several authors conclude that only limited changes in the conformation of proteins occurs upon adsorption at the air-water interface. Maximum changes of up to 10 % are observed in the secondary structure, but the globular folding state of the protein is generally found to remain intact. Furthermore, no significant changes of the structure in time have been reported so far.

With respect to characterizing the surface functionality of proteins it is important to learn in what way protein unfolding at interfaces affects the measured interfacial properties such as surface pressure. Therefore the current work focused on changes in the adsorbed state of the protein as a result of adsorption at rates, rather than differences between the adsorbed state and the conformation in bulk. Since the rate of adsorption is directly proportional to the bulk concentration of the protein, the adsorption of proteins with different structural stabilities at different bulk concentrations was measured with the use of ellipsometry and surface tensiometry.

**Materials and Methods**

β-Lactoglobulin (β-Lg) was isolated and purified (>98% purity) from fresh cow milk (A:B ratio 40:60) using the protocol described by de Jongh et al. The material was freeze-dried and stored at −20 °C. Ovalbumin was isolated as described previously with the only adaptation that ovalbumin was eluted from the ion-exchange material at 0.15 M NaCl to improve purity. Horse heart cytochrome c was purchased from Sigma-Aldrich (C7752), and dialysed against demineralised water, freeze-dried and stored at −20 °C until use.
Destabilization of cytochrome c. A destabilised form of cytochrome c was produced by reductive alkylation of the Methionine-80 as described by de Jongh et al. In this method the methionine-80 is first dissociated from the heme-group and then acetylated by incubating the protein (4 mM) in the presence of 24 mM iodacetic acid, 50 mM sodium acetate (pH 1.5) and 0.5 M NaCl at 37 °C for 8 h. Far-UV CD and absorbence measurements (375-425 nm to measure the Soret band as an indication for the presence of the heme-group) confirmed that the modification was complete, and that the modified protein exhibited the same structure as the non-modified protein under ambient conditions (results not shown).

Change in free energy by urea titration. The structural stability of the proteins at 20 °C was determined by monitoring the intrinsic fluorescence of tryptophan and tyrosine residues at increasing urea concentrations as described by Broersen et al. A 10 M urea stock solution was prepared by dissolving 1.103 g of urea (Sigma) per mL of phosphate buffer (pH 7.0). The protein samples were prepared by diluting a 5 µL of a 10 mg/mL solution in 1 mL urea-buffer mixture. Protein solutions (0.1 mg/mL or 2.7 µM in 10 mM phosphate pH 7.0) at increasing concentrations urea (0-8 M at 0.2 M intervals) were prepared one day before measurement, to ensure complete equilibration of the samples. Spectra were measured using a Cary Med Eclipse (Varian) fluorimeter, with excitation and emission slit widths of 5 nm. The excitation wavelength was 295 nm and the emission spectra were recorded from 300-400 nm with a scan speed of 100 nm/min. Each spectrum was the average of two scans and corrected for a protein-free sample. The titration curves were plotted by taking the band intensity for a fixed emission wavelength (310 nm) as a function of denaturant concentration. The free energy of unfolding was calculated from an extrapolation of the free energy of unfolding at increasing urea concentrations –assuming two-state unfolding- as described elsewhere.

Adsorption behaviour. The adsorption of proteins to the air-water interface and subsequent development of surface pressure was measured using a Multiskop ellipsometer (Optrell, Germany) combined with a Langmuir trough (Riegler and Kirstein, Germany) and Wilhelmy plate tensiometry. If proteins adsorb to the interface the ellipsometric angles Δ and Ψ are increased depending on the concentration in and the thickness of the adsorbed layer. The values for the ellipsometric angles Δ and Ψ can be used to calculate the adsorbed amount. To do this, the refractive index and thickness of the adsorbed protein layer are fitted in a model that constitutes of two bulk phases (air and water) and one adsorbed layer, with parameters:
Kinetics of Adsorption and Unfolding determine Equation of State

\[ n_{\text{air}} = 1.000, \ n_{\text{protein solution}} = 1.3327, \ dn/dc = 0.18; \] the angle of incidence was 50°. Using the combination of ellipsometry and surface tensiometry, both the increase of surface load (\( \Gamma \)) and surface pressure (\( \Pi \)) in time could be determined. The rate of adsorption from protein solutions 0.005-0.5 mg/mL (10mM phosphate, pH 7.0, 20 °C) was measured in duplicate. All experiments were started by removing the interfacial layer with a custom made suction device after which the clean interface was rapidly expanded to the maximum area (from 30 to 190 cm\(^2\)). The first datapoints taken are typically 100 s after cleaning the interface. In this way, the initial conditions for each experiment approximated \( \Gamma = 0 \) mg/m\(^2\) at \( t=0 \) s. Control experiments with destilled water in between measurements confirmed that the cleaning method used (rinsing with ethanol and water) was sufficient to avoid contamination of the trough.

**Results and discussion**

*Adsorption behaviour.* To link the potential unfolding of proteins at the interface to the exerted surface pressure, the kinetics of surface pressure development was monitored as a function of the protein bulk-concentration for \( \beta \)-lactoglobulin (\( \beta \)-lg) and ovalbumin, which have different folding stabilities in the bulk. The adsorption of these proteins was studied for three bulk concentrations: 0.5, 0.1 and 0.05 mg/mL (for \( \beta \)-lg also 0.005 mg/mL is included). In figure 4.1 the surface pressure is plotted against the adsorption time. For \( \beta \)-lg a decrease in both the rate of surface pressure development and plateau value of the surface pressure (\( \Pi_{2h} \)) are observed with decreasing concentration (\( \Pi_{2h} \) decreases from 25 to 16 mN/m). However, from the figure it can be seen that at all concentrations the interfacial layer reaches saturation with the time-span of the experiment. For ovalbumin saturation is reached at the highest, but not at lower concentrations (figure 4.1B, \( \Pi_{2h} \) decreases from 23 to 7 mN/m). Furthermore, a lag-time is found for the lower concentrations; at 0.05 mg/mL it takes 2000 s before surface pressure starts to increase.
To test whether the difference between the development of surface pressure by β-lactoglobulin and ovalbumin is due to a change in the adsorption kinetics, the adsorbed amount (Γ) in time for the same experiments was monitored by ellipsometry and plotted in figure 4.2. Here also a decrease in the rate of adsorption is observed for both proteins at lower concentrations, but the largest difference between the two proteins is found in the plateau-value of adsorption. The maximal adsorbed amount of β-lactoglobulin decreases from 2 mg/m² at 0.5 and 0.1 mg/mL to 1.2 mg/m² at 0.005 mg/mL. This latter value is close to that of ovalbumin at the lowest concentration, while at the highest concentration the adsorbed amount of ovalbumin (1.6 mg/m²) is lower than of β-lactoglobulin.
Figure 4.2. Surface load as a function of time for β-lactoglobulin (A, 0.5 (▲), 0.1 (■) and 0.05 (◆) mg/mL) and ovalbumin (B, 0.5 (△), 0.1 (□) and 0.05 (◇) mg/mL)

A better view on the effect of concentration on the adsorption behavior is obtained by plotting the surface pressure against the adsorbed amount, now also including results obtained from experiments at intermediate concentrations (figure 4.3). For β-lg, a decrease in bulk concentration of the protein results in a shift in the Π-Γ curve to lower values of Γ (figure 4.3A). The surface pressure is related to the interaction energy between adsorbed proteins. Apparently the interaction energy between β-lg adsorbed from low bulk concentrations is higher at certain Γ than when the proteins are adsorbed from higher bulk concentrations. In contrast, no shift in the Π-Γ curve of ovalbumin is observed (figure 4.3B). The only effect of bulk concentration is that the adsorbed amount at the saturation is lower (as seen in figure 4.2B), and subsequently also the maximal surface pressure reached is lower. This is due to the
fact that the measurement time was restricted to 8000 s. Since the adsorption rate is also determined by the bulk concentration, a decrease in the concentration of a factor 10 would also mean an approximate increase in the time to reach adsorption of a factor 100, according to the Ward and Tordai equation.

In contrast to β-lg the interactions between adsorbed ovalbumin molecules are not affected by the bulk concentration. Since the possible contribution of electrostatic and hydrophobic interactions to the surface pressure is expected to be independent of the bulk concentration there must be another explanation for the shift in the Π-Γ curve with decreasing the bulk concentration, as observed for β-lg. Unfolding is a likely explanation, since it would result in a change in the apparent size of the adsorbed proteins. This would subsequently lead to increased surface pressure at lower values of Γ. Moreover, unfolding leads to the exposure of previously buried groups what may further affect the protein interaction potential.
Table 4.1. Molecular parameters for the proteins used in this study

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mw [kDa]</th>
<th>$\Delta G_{\text{unf}}$ [kJ/mole]</th>
<th>$\Delta F_{\text{act}}$ [kJ/mole]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>44</td>
<td>33</td>
<td>340 $^b$</td>
</tr>
<tr>
<td>β-Lg</td>
<td>18</td>
<td>27</td>
<td>400 $^c$</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>12</td>
<td>40</td>
<td>136 $^d$</td>
</tr>
<tr>
<td>Met-80 mod. Cyt-c</td>
<td>12</td>
<td>30</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

$^a$ Calculated from data in figure 4.4, $^b$ from Weijers & Broersen, $^c$ from Broersen, $^d$ from Mehta et al.

A measure of the tendency of a protein to unfold is the protein structural stability, typically described by the free energy of unfolding ($\Delta G_{\text{unf}}$). To test whether the concentration dependence of β-lactoglobulin could be the result of a lower structural stability, the intrinsic fluorescence at increasing urea concentrations was measured. The normalized fluorescence intensity, which is an measure of the fraction of unfolded protein, is plotted against the urea concentration (figure 4.4). The free energy of unfolding ($\Delta G_{\text{unf}}$) was calculated from these data assuming a two-state unfolding transition as described elsewhere, and the obtained values are given in table 4.1. For ovalbumin and β-lg these values were found to be 33 and 27 kJ/mole respectively. To evaluate whether the difference in structural stability is the explanation for the shift in the Π-Γ curve at different bulk concentrations, a comparison should be made with proteins that only differ in structural stability. Modification of cytochrome c can be used to obtain a protein with identical primary sequence and structural fold, but with decreased stability of the globular structure. This destabilized variant was produced by inhibiting the ligation of methionine-80 to the heme group as described in the method section. The titration curves for cytochrome c and the destabilised form are also given in figure 4.4, and the derived values for $\Delta G_{\text{unf}}$ in table 4.1. Cytochrome c is more stable than ovalbumin (40 kJ/mole), while the destabilised form showed a significantly decreased stability (30 kJ/mole), intermediate to that of β-lactoglobulin and ovalbumin.

$^a$ The absolute fluorescence intensity of Cytochrome c increases with increasing urea concentration, therefore we chose to show this data as 1 minus the normalized intensity.
Figure 4.4. Normalized fluorescence intensity as a function of the urea concentration for \( \beta \)-lactoglobulin (\( \triangle \)), ovalbumin (\( \square \)), cytochrome c (\( \odot \)) and Met-80 cytochrome c (\( \diamond \)).

The \( \Pi\)–\( \Gamma \) curves for both cytochrome c and the destabilized form (at 0.1 and 0.005 mg/ml) are given in figure 4.5. Surprisingly, all data are on the same curve. This means that for both cytochrome c and the destabilized variant no effect of bulk concentration is observed. While it was expected that the destabilized form of cytochrome c would show an enhanced ability to unfold at interfaces, this was not reflected in the concentration dependence of the \( \Pi\)–\( \Gamma \) curve.

Figure 4.5. Surface-pressure vs. surface load, for cytochrome c (\( \square \)) and Met-80 cytochrome c (\( \odot \)) at 0.005 and 0.1 mg/mL.
From the above, it must be concluded that the $\Delta G_{\text{unf}}$ is not the key parameter that describes the tendency of a protein to unfold at the air-water interface. To understand this better, we must first consider what the driving force for interfacial unfolding would be. In figure 4.6 a schematic representation is given of a protein at the interface. In this figure the arrows indicate the forces acting on the protein. The surface tension between air-water will act as a force towards expansion of the protein, while the surface tension between the protein and both bulk phases will result in a force towards retention of the globular shape.\textsuperscript{50} This situation is an analogy to the spreading of oil-droplets at the air-water interface. In this case, a simple calculation of the spreading coefficient\textsuperscript{51} will tell whether the oil droplet spreads or if it keeps its globular form.

\begin{center}
\textbf{Figure 4.6. Schematic representation of the forces acting on a protein at the air-water interface}
\end{center}

In the aqueous phase (bulk) the structure of globular proteins is defined, and the population of the unfolded state is very small for most globular proteins. To reach a (partially) unfolded state an activation energy needs to be overcome that is related to the breaking of for example ion-pairs on the protein surface and disruption of H-bonds and vanderWaals interaction between side-chain residues. In the case of $\beta$-lactoglobulin and ovalbumin the structure is further stabilised by covalent disulphide bridges. In aqueous solution a significant population of a more unfolded state can only be achieved by the presence of a denaturant or by varying a system parameter, like temperature; in both cases the Gibbs energy of the folded state increases, resulting in an increasing fraction of unfolded proteins. Similarly the spreading of proteins at the interface is constrained by the structural stability, leading to an (activation) energy barrier of unfolding. This barrier determines how fast proteins can unfold when the spreading coefficient is >0. While $\Delta G_{\text{unf}}$ reflects the change in free energy as a result of unfolding, this parameter is not directly related to the activation energy required to go from
the folded to the unfolded state. In other words, interfacial unfolding should be treated as a kinetic process, rather than a classical thermodynamic process.

![Graph showing kinetic unfolding](image)

**Figure 4.7.** Fraction unfolded protein as a function of time for β-lactoglobulin (□) and ovalbumin (△); 8M urea was added to the cuvette at t=0 s; inset shows the unfolding of the same samples at short times as measured by stopped-flow fluorescence (data from ref. 44)

A good way to evaluate the energy barrier to unfolding can be obtained by monitoring the kinetics of unfolding. Therefore, the intrinsic fluorescence of ovalbumin and β-lg was monitored as a function of time, after addition of 8M urea. The results are shown in figure 4.7. As can be seen in this figure, the unfolding of ovalbumin under the given conditions is only completed after 500 s, whereas for β-lactoglobulin the unfolding proceeds too fast to be accurately determined with this technique. To illustrate this, results from a stopped flow experiment performed by Broersen et al. under similar conditions are shown in the inset in figure 4.7. From the inset it becomes clear that β-lg unfolds almost completely at a time-scale of (sub-)seconds; while cytochrome c shows even faster (milliseconds) unfolding than β-lactoglobulin (results not shown). By measuring the kinetics of unfolding at different concentrations of urea and at different temperatures, the activation energy for unfolding (ΔE\text{act}^{\text{unf}}) can be established; the values reported in literature are given in table 4.1. Activation energies are in the order of 100-400 kJ/mole, while the difference in free energy between the folded and unfolded state is only in the order of 10-50 kJ/mole. In view of these activation energies it can be expected according to the Arrhenius equation that when unfolding of β-
lactoglobulin occurs at a time-scale of seconds, this will be at the 10-100 s timescale for ovalbumin (as indeed illustrated in figure 4.7) and at the microsecond timescale for cytochrome c. Destabilisation of cytochrome c will only cause this process to speed up (no literature value of $\Delta E^\text{act}_{\text{unf}}$ available). Assuming that for all proteins the spreading coefficient is not the limiting factor, then the time that the spreading force can act unperturbed on the protein will determine whether the protein will unfold. As can be seen from figure 4.2, the situation where no protein-protein interactions develop (i.e. where $\Pi<1 \text{ mN/m}$) is typically in the order of seconds for $\beta$-lg and cytochrome c. Obviously this time frame will allow most of the cytochrome c (modified or not) to unfold at the interface. The unfolding kinetics of ovalbumin is simply too slow and even though at very low bulk concentrations the surface pressure doesn’t increase until after 2000 s, no unfolding takes place. Apparently only for $\beta$-lactoglobulin both the unfolding kinetics and $\Delta E^\text{act}_{\text{unf}}$ are exactly in the critical range, where the folding state of the adsorbed protein depends on the rate of adsorption and thus on the bulk concentration.

**Conclusion**

The present work focused on the question whether the state of folding of adsorbed protein is affected by the bulk concentration. From the presented results, it is concluded that a change in the protein folded state can take place. However, this change does not depend on the stability of the protein as given by the change in free energy between the folded and the unfolded state. Rather, the process appears to be determined by the activation energy of unfolding, the spreading coefficient and the time available for unfolding. The unfolding was demonstrated by a shift in the $\Pi-\Gamma$ curve measured for $\beta$-lactoglobulin adsorbed from different bulk concentrations. For ovalbumin no evidence of additional unfolding was found, which is attributed to the fact that the unfolding of this protein is too slow. On the other hand, for cytochrome c also no changes in the $\Pi-\Gamma$ curve were found, but based on the very rapid unfolding of this protein it is suggested that in this case the proteins all reach the same unfolded state at the interface.
Chapter 4

The fact that the state of folding of adsorbed proteins can depend on the history of the adsorbed layer poses a serious challenge for the theoretical description of the adsorbed layer. However, only if the adsorption rate is close to the rate of unfolding, changes in bulk concentrations can lead to different adsorbed states. For practical applications, a rapid adsorption of proteins is necessary for foam formation. If the protein concentration is too low, or if the adsorption process is too slow, no foam is formed. This means that in such systems only proteins that have unfolding kinetics on the millisecond to second timescale will be sensitive for changes in adsorption kinetics.

Finally, the fact that the unfolding of adsorbed proteins can lead to a shift in the $\Pi-\Gamma$ curve, strongly suggests that this folding state is not purely determined by the surface pressure at the interface. It would even seem that once a protein has reached a certain state of unfolding at the interface, this (new) conformational state is retained even when the adsorption process continues. From these results it must be concluded that the role of unfolding in the description of adsorbed layers that has been the consensus in literature should be reconsidered.

Unfolding is neither necessary, nor a driving force for protein adsorption. Only after adsorption the protein can adopt to its new environment. This transition needs to occur at a time-scale that is shorter than the adsorption rate of neighboring proteins. From this it follows that the increase of surface pressure after saturation of the interface (constant $\Gamma$) cannot be attributed by unfolding processes.

References

Chapter 5

Importance of physical vs. chemical interactions in surface shear rheology


A densely packed adsorbed layer

A three-dimensional gelled system

From Dr. Chris Wijmans
Abstract

The stability of adsorbed protein layers against deformation has in literature been attributed to the formation of a continuous gel-like network. This hypothesis is mostly based on measurements of the increase of the surface shear elasticity with time. For several proteins this increase has been attributed to the formation of intermolecular disulfide bridges between adsorbed proteins. However, according to an alternative model the shear elasticity results from the low mobility of the densely packed proteins. To contribute to this discussion, the actual role of disulfide bridges in interfacial layers is studied. Ovalbumin was thiolated with S-acetylmercaptosuccinic anhydride (S-AMSA), followed by removal of the acetylblock on the sulphur atom, resulting in respectively blocked (SX) and deblocked (SH) ovalbumin variants. This allows comparison of proteins with identical amino acid sequence and similar globular packing and charge distribution, but different chemical reactivity. The presence and reactivity of the introduced, deblocked sulfhydryl groups was confirmed using the sulphydryl-disulfide exchange index (SEI). Despite the reactivity of the introduced sulfhydryl groups measured in solution, no increase in the surface shear elasticity could be detected with increasing reactivity. This indicates that physical rather than chemical interactions determine the surface shear behaviour. Further experiments were performed in bulk solution to study the conditions needed to induce covalent aggregate formation. From these studies it was found that mere concentration of proteins (to 200 mg/mL, equivalent to a surface concentration of around 2 mg/m²) is not sufficient to induce significant aggregation to form a continuous network. In view of these results, it was concluded that the adsorbed layer should not be considered a gelled network of aggregated material (in analogy with three-dimensional gels formed from heating protein solutions). Rather, it would appear that the adsorbed proteins form a highly packed system of proteins with net-repulsive interactions.
Introduction

Proteins and low-molecular weight (LMW) surfactants are both known for their ability to form interfacial layers that stabilise foams and emulsions. However, the mechanisms by which they stabilise the interface have been mentioned to be fundamentally different.\textsuperscript{2,3} The LMW surfactants are able to freely diffuse at the interface, thereby counteracting a surface pressure gradient across the interface. This is known as the Gibbs-Marangoni effect. For proteins the lateral surface diffusion in the saturated surface layer is found to be much smaller.\textsuperscript{4} In this case the stabilization proceeds via a different mechanism, which some authors have suggested to be related to the formation of a continuous network.\textsuperscript{5,6} This network would result in a mechanical force resisting deformation of the interface, the elastic modulus. The evidence for the existence of such a network has often been taken from surface shear rheological experiments. These experiments typically use a Couette type (or: concentric cylinders) geometry comparable to that used for the study of gelation in bulk systems. The technique was discussed thoroughly in 1979 by Izmailova\textsuperscript{7} and more recently by Bos et al.\textsuperscript{2} Although multiple articles have been published on the surface shear behaviour of protein layers\textsuperscript{8-17} a quantitative relationship of this behaviour to a fundamental parameter such as the adsorbed amount is still lacking.\textsuperscript{18} One reason for this might be that the interpretation of results from these measurements is not unambiguous. Several authors argue that the adsorbed protein layer is gelled, resulting in a network of highly interacting particles and that as such the rheological behaviour should be interpreted in analogy to the three dimensional equivalent (of heat set protein gels).\textsuperscript{1,8,13,19-21} This view is supported by observations of visibly coagulated layers of proteins as described by MacRitchie and Owens,\textsuperscript{22} even though these authors find that coagulation of a protein monolayer occurs only after the surface pressure is increased to higher values than those found for equilibrium spreading pressure of monomeric compounds. Furthermore, Izmailova\textsuperscript{7} mentions that formation of macroscopic films with folds and wrinkles is only described in cases of compressed layers, heated solutions and certain interfaces between water and hydrocarbons.

An alternative view, shared by several authors, states that the surface shear behaviour of adsorbed protein layers is the result of the dense packing of loose proteins.\textsuperscript{18,23,24} This
concept seems to be able to account for observations that the shear elasticity only increases once a certain concentration of adsorbed particles is reached,\textsuperscript{7,12} and that proteins are displaced from the interface relatively quickly by LMW surfactants.\textsuperscript{25-27}

If one wants to describe the interfacial behaviour of adsorbed proteins in all its complexity, it is necessary to distinguish between the two described conceptual models. One important difference between the two descriptions of the interfacial layer is the role of covalent interactions. In three-dimensional gels that are formed by heating protein solutions the formation of intermolecular disulfide bonds has been shown to increase the gel strength as measured by the elastic modulus.\textsuperscript{28} This observation has also been confirmed for gels formed by acidifying dispersions of pre-aggregated proteins.\textsuperscript{29-31} Several authors have suggested that intermolecular disulfide bonds will also be formed between adsorbed proteins if the adsorbed layer is in a gelled state.\textsuperscript{6,21,32,33} However, since the cysteine residues are typically located in the interior of the protein and are not readily accessible for the formation of intermolecular bonds, the proteins would need to partially unfold at the interface to expose these residues, as discussed by Damodaran and Anand.\textsuperscript{6} In the studies by Damodaran\textsuperscript{6} and Dickinson\textsuperscript{33} it was shown that after emulsification there was some polymerisation of \(\beta\)-lactoglobulin, but the surface shear elasticity was not determined. Other authors used chemical modification of proteins to introduce sulphydryl groups.\textsuperscript{34-36} Their results show that the introduction of sulphydryl groups itself did not improve the foam formation or stability.

To contribute to the above-mentioned discussion on the nature of adsorbed protein layers, the surface shear behaviour of chemically modified variants of ovalbumin is measured. Using the specific reactivity of S-acetylmercaptosuccinic anhydride (S-AMSA) towards the free amino groups of ovalbumin, groups with an acetylated sulphur atom can be introduced at the protein surface. Ovalbumin was chosen, since it was previously found that the protein structural integrity was not affected by mild modification of the lysine groups.\textsuperscript{37} The acetyl block can be removed by hydroxylamine, yielding free sulphydryl groups. Ovalbumin is a 42 kDa glycosylated and phosphorylated protein, containing one disulfide bridge and four free sulphydryl groups, but these are all located in the interior of the protein. After chemical modification and deblocking, reactive sulphydryl groups are present on the exterior of the protein, thus easily available for the formation of disulfide bridges. By combining the
investigation of the chemical properties of the modified proteins and the rheological properties of the adsorbed protein layers, we aim to provide a better understanding of the nature of the interfacial layer and the role of chemical interactions in this layer.

**Materials and methods**

**Chemicals** S-Acetylmercaptosuccinic anhydride (S-AMSA), Ortho-phthaldialdehyde (OPA), 2-Ethyl-5-phenylisoxazolium-3’-sulfonate (Woodward’s reagent K) and 2-(N-morpholino)-ethanesulfonic acid (MES) buffer were obtained from Sigma-Aldrich. N,N-dimethyl-2-mercaptopethyl-ammoniumchloride (DMA) and di-sodiumtetraborate decahydrate (Borax) were purchased from Merck. Sodiumdodecylsulfate (SDS) was from Serva. All chemicals used were of analytical grade.

**Introduction of sulphydryl groups into ovalbumin** Chicken egg ovalbumin was purified (>98%) as described previously. Primary amino groups in ovalbumin were thiolated essentially as described by Klotz. In this method S-acetylmercaptosuccinic anhydride (S-AMSA) is covalently linked to lysine residues on the protein surface. The modification was performed as follows: 200 mL of a 25 mg/mL ovalbumin (0.55 mM) solution in demineralized water was adjusted to pH 8.0 by the addition of 1 M NaOH using a pH-stat titration equipment (Metrohm) at room temperature. The reagent (S-AMSA) was added to the protein solution in small aliquots, while the pH was kept at 8.0 (± 0.1) by the addition of 1 M NaOH using the pH-stat apparatus. Two batches of modified ovalbumin were prepared by a total addition of 28.5 mg and 97.5 mg S-AMSA respectively to obtain a low and a medium degree of modification. After addition, the solution was stirred for another 30 min., followed by extensive dialysis against demineralized water at 4 °C. After dialysis each batch was separated into two fractions. To one fraction, 125 mL 0.01 M hydroxylamine (pH 7.3) was added, to remove the acetyl blocks. To the other fraction 125 mL distilled water was added to keep concentrations in both solutions equal. The deblocked fraction was then dialyzed against
distilled water; both samples were frozen, but not freeze-dried in order to avoid oxidation of the free sulfhydryl groups. The material was stored at -20 ºC until use.

Chemical characterization

Degree of modification. The total number of active sulfhydryl groups in non-modified, blocked and deblocked ovalbumin was determined using the Ellmann protocol. The reagent, 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB) reacts with free thiol groups of the protein. The reagent solution was prepared by dissolving 4.8 mg DTNB in 1.0 mL, 10mM Tris-HCl buffer (pH 8.0 and 2 % SDS) and subsequent addition of 2.5 mL Tris-HCl buffer (10mM, pH 8.0). 50 µL DTNB solution was added to 250 µL 5 mg/mL protein solution. The solution was mixed and incubated for 20 minutes at 25 ºC; then the absorbance was measured at 412 nm. The calibration curve, obtained by using various dilutions of a 0.50 mM cysteine solution, provided an extinction coefficient for the reduced conjugate of 13425 M⁻¹ cm⁻¹.

Reactivity of sulfhydryl groups. The chemical reactivity of the blocked and deblocked proteins was measured using the sulphhydryl-disulphide exchange index (SEI) as described by Owusu-Arptenen et al. The method determines both the number of available –SH groups and the reactivity of these groups. The method is based on the reaction between 2,2’-dipyridyldisulphide (PDS) and a free -SH group. The formation of the reaction product (2-thiopyridine; 2-TP) can be followed in time by measuring the absorbance at 343 nm. A stock solution of PDS was prepared by dissolving 40 mg PDS in 40 mL phosphate buffer (10 mM pH 7.0) and stirring for 3 hours at 20 ºC. Then the solution was filtered over a 0.45 µm filter and the concentration of PDS was determined at 281 nm, using a molar extinction coefficient of 9730 M⁻¹ cm⁻¹. For measurements the stock was diluted to 5·10⁻⁵ M; 2.7 mL of this solution was added to a cuvette and the absorbance at 343 was measured. Then 0.3 mL of sample solution was added and the adsorbance at 343 nm followed in time. Sample solutions used were 10 mg/mL protein (in 10 mM phosphate buffer, pH 7.0); Glutathion (0.1 mg/mL in 0.01 N HCl) was used for calibration. The total number of reactive –SH groups was calculated from the plateau value of the adsorbance (using a molar extinction coefficient of 7076 M⁻¹ cm⁻¹). The rate constant (k_SH) of this second-order reaction was calculated from the slope of the graph of the right-hand side of equation 5.1 against time.
When this rate constant is divided by the rate constant of glutathion, a standardized value called the SEI index is obtained; \( \text{SEI} = \left( \frac{k_{\text{SH}}}{k_{\text{glutathion}}} \right) \times 100\% \).

**Detection of quaternary structure.** Size exclusion chromatography (SEC) was used to study the aggregation state of the proteins. SEC was carried out on a Superdex S200 HR column (Pharmacia Biotech) with a bed volume of 24 mL. The column was equilibrated and run with 10 mM phosphate-buffer pH 7.0 and 50 mM NaCl at 20 °C. The sample (200 µL, 5 mg/mL in the same buffer) was applied to the column and the flow-rate was set to 0.4 mL/min; detection took place at 280 nm.

**Evaluation of secondary structure.** Far-UV circular dichroism (CD) spectra of 0.1 mg/mL protein solutions in 10 mM phosphate buffer (pH 7.0) were recorded at 20 °C in the range from 190 to 260 nm with a spectral resolution of 0.2 nm, on a Jasco J715-spectropolarimeter. The scan speed was 100 nm/min and the response time was 0.125 s with a bandwidth of 1 nm. Quartz cells with an optical path of 0.1 cm were used. Typically, 16 scans were accumulated and averaged. The spectra were corrected for the corresponding protein-free sample.

**Evaluation of tertiary structure.** The intrinsic fluorescence of the tryptophan and tyrosine residues of 0.1 mg/mL protein solutions in 10 mM phosphate buffer (pH 7.0) was measured on a Perkin Elmer Luminescence Spectrometer LS 50 B. The excitation and emission slits were set at 5 nm. The excitation wavelength was 295 or 274 nm, the excitation maxima of tryptophan and tyrosine respectively. The emission spectra were recorded from 300-450 nm with a scan speed of 120 nm/min. Each spectrum was the average of two scans and corrected for a protein-free sample.

**Iso-electric focusing.** The iso-electric points (IEP) of non-modified and modified ovalbumin variants were determined using the Phast System (Pharmacia). Ready to use PhastGel IEF 3-9 gels were used, which were stained with Coomassie brilliant blue. A calibration kit from Pharmacia was used with proteins that have iso-electric points ranging from 3.5 to 9.3.

**Protein aggregation in solution.** The chemical reactivity of the modified ovalbumin variants in bulk solution was tested under similar conditions as experienced at the interface by

\[
k_{\text{SH}} \times t = \frac{1}{\left( [\text{PDS}]_0 - [\text{-SH}]_0 \right)\ln \left( \frac{[\text{PDS}]_0 - [2 \text{-TP}]_0}{[\text{-SH}]_0 - [2 \text{-TP}]_0 [\text{PDS}]_0} \right)}
\]
concentrating protein solutions to final concentrations of 100 or 200 mg/mL (using Centriprep centrifugal filtration units from Millipore). Further experiments were performed by heating 0.1 mg/mL protein solutions at 90 °C for 30 minutes, or by addition of ferricyanide. Any aggregation induced by these treatments was analysed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE 8-25 gradient gels (Pharmacia) were run on a Phast-System (Pharmacia). Samples were dissolved in sample buffer, containing 62.5 mM Tris-HCl (pH 6.8), 1.25 % SDS, 5 % glycerol, 0.00125 % bromophenol blue in the presence or absence of 1.25 % β-mercaptoethanol, and heated for 10 min at 100 °C before analysis. Gels were stained with Coomassie brilliant blue. A calibration kit with low molecular mass markers ranging from 14 to 94 kDa from Pharmacia was used.

**Surface shear rheology**

To study interfacial shear properties of adsorbed protein layers a Couette-type surface shear rheometer was used as described by Martin at al. The apparatus is schematically drawn in figure 5.1. First, 279 mL buffer (10 mM phosphate, pH 7.0, containing 300 mM NaCl) was filtered over a 0.2 μm filter and then placed in the trough. A concentrated protein solution (4 mL) was injected under the interface using a syringe, to obtain a final protein concentration of 0.1 mg/mL. A stainless steel biconical disk (diameter 30 mm) was suspended from a torsion wire of 0.15 mm and placed in such a way, that the disc edge was exactly at the air-water interface. The solution was left to adsorb and equilibrate for 22 hours while the surface tension was measured with a Wilhelmy plate (perimeter 18.1 mm) suspended from a NIMA tensiometer as illustrated in figure 5.1.
Figure 5.1. Schematic drawing of the surface shear setup: An electric motor (1) drives the stage (2), causing rotation of the dish containing the protein solution. The resulting rotation of the pendulum hanging from a torsion wire (3) is quantified by the reflection of a laser (4) from a mirror on the torsion wire on a scale (5). Simultaneously the surface pressure can be measured by a Wilhelmy plate connected to a NIMA tensiometer (6) mounted on the cover of the dish.

In replicate measurements with and without the Wilhelmy plate at the interface it was found that the presence of the plate did not affect the results from the surface shear measurements. Stress-strain curves were made as a function of time by rotating the sample dish at a fixed rate during 30 min. The stress acting on the inner disc ($\sigma$ [mN/m]) resulted in rotation of this disc that was quantified by reading the reflection of a laser beam from a mirror on top of the inner disk on a circular scale with a radius of 600 mm. Assuming homogenous deformation of the surface layer the strain ($\gamma$ [-]) applied can be calculated by equation 5.2:

$$\gamma = \frac{2R_o^2}{(R_o^2 - R_i^2)} \cdot \left( \Theta_o - \Theta_i \right)$$  \hspace{1cm} (5.2)

Where $R_i$ is the radius [m] and $\Theta_i$ the rotational displacement [rad] of the inner disc, and $R_o$ and $\Theta_o$ the radius and rotational displacement of the outer dish; $\Theta_o$ is given by the angular velocity of the trough and the time ($\omega^* t$) and $\Theta_i$ is measured from the displacement of the reflected laser light on the circular scale divided by two times the radius of the scale. The stress on the interface is calculated from equation 5.3:
The Interfacial layer: gelled or glas state?

\[
\sigma = \frac{\tau}{2\pi R_i^2}
\]  

(5.3)

with \(\tau = K\Theta\), the torque [Nm] exerted on the disc, and \(K\) the torsion wire constant. The torsion wire constant was determined by measuring the oscillation time with a calibration weight of 75 g, which is close to the weight of the pendulum disc. Values for the system parameters are: \(\omega = 1.27 \times 10^{-3} \text{ m}, \ R_i = 1.50 \times 10^{-2} \text{ m}, \ R_o = 7.25 \times 10^{-2} \text{ m}, \ K = 1.73 \times 10^{-5} \text{ N\cdot m\cdot rad}^{-1}\), the radius of the scale is 0.6 m. All experiments were performed at 20 °C. Duplicate measurements were performed after thorough cleaning of the trough by starting with new buffer and new injection of the protein solution.

**Results**

Chemical modification of ovalbumin with S-AMSA as described in the methods section yielded four variants; two blocked (SX) and two reactive (SH) variants with two degrees of modification (labelled 1 and 2). Size-exclusion chromatography results showed that all materials used consists of monomeric proteins (results not shown), illustrating that no auto-oxidation of the material had occurred during preparation, storage and handling. Far-UV CD and tryptophan fluorescence spectra from non-modified and the most extremely modified samples as shown in figure 5.2 and inset appear to be identical. This illustrates that no significant changes of the secondary and tertiary structure occurred as a result of the modification.

![Figure 5.2](image.png)

*Figure 5.2. Far-UV CD spectra of non-modified and modified ovalbumin (SX and SH) at 20 °C, 10 mM phosphate buffer pH 7.0, inset shows intrinsic Trp fluorescence of the same samples in the same buffer*
Chemical characterization. Table 5.1 shows the results of the chemical characterization of the modified ovalbumin variants. The presence of free –SH groups was determined using the Ellman determination in the absence and presence of SDS. Without SDS, no sulfhydryl groups were detected for non-modified and blocked proteins. For SH1 and SH2 proteins 1 and 2 groups per protein molecule were detected respectively. In the presence of SDS the proteins are structurally destabilised, and for non-modified ovalbumin all four free SH groups that are present in the primary sequence of ovalbumin are detected. For all modified samples the amount of detected sulfhydryl groups is also increased due to the exposure of the indigenous groups, in the presence of SDS. These results show that (1) the modification as such did not increase the exposure of the indigenous sulfhydryl groups, (2) the blocked variants were indeed blocked, and (3) unblocking was successful.

Table 5.1. Chemical presence and reactivity of sulphide groups after chemical modification

<table>
<thead>
<tr>
<th></th>
<th>Ellman (-SDS) ±1 [# SH/protein]</th>
<th>Ellman (+SDS) ±1 [# SH/protein]</th>
<th>SEI ±0.1 [# SH/protein]</th>
<th>K_{SEI} [M⁻¹ s⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-modified</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>SX1</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>1.8</td>
</tr>
<tr>
<td>SX2</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4.2</td>
</tr>
<tr>
<td>SH1</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>8.9</td>
</tr>
<tr>
<td>SH2</td>
<td>2</td>
<td>6</td>
<td>3</td>
<td>33</td>
</tr>
</tbody>
</table>

The kinetics of the reaction of sulfhydryl groups with the PDS reagent is shown in table 5.1 and in figure 5.3. The amount of SH groups per protein as measured by SEI is comparable to that found with Ellman (1 and 3 for SH1 and SH2 respectively). In figure 5.3 it is clearly visible that the deblocked variants have much more reactivity than either the non-modified or blocked variants. The high intensity and rapid increase of the absorbance at 343 nm found for SH2 in comparison with SH1 must be attributed to the fact that the protein was used in the same protein concentration, leading to higher molar concentrations of sulfhydryl groups. The reaction rate constant for SH2 is found to be three times higher than for SH1. Clearly the sulfhydryl groups that were introduced at the protein surface are reactive.
Surface shear rheology. After determination of the chemical properties of the blocked and deblocked variants of ovalbumin, the samples were used to study the surface shear rheology. Since Martin et al.\textsuperscript{41} and Dickinson et al.\textsuperscript{43} have shown that surface shear viscosities at air-water interfaces typically reach constant values after 22 hours, the measurements were started 22 h after injecting the protein solution in the shear dish. This time is thus expected to be sufficiently long for adsorption and any rearrangements or cross-linking processes to occur. During this equilibration period the surface pressure was monitored and found to be identical within the experimental error for all samples ($\Pi$ after 22 h was $20 \pm 1$ mN/m).

![Figure 5.3](image)

**Figure 5.3.** Fluorescence of 2-TP as a function of time for all variants, SX$_i$ (◇), SX$_j$ (△), SH$_1$ (●), SH$_2$ (▲).

![Figure 5.4](image)

**Figure 5.4.** Surface shear stress plotted against the applied strain for WT and modified variants, the line shown represents an interface with a constant shear Young modulus of mN/m, same symbols as in figure 5.3.
Chapter 5

The stress at the interface measured by the rotation of the inner disc as a function of the strain applied to the surface is plotted in figure 5.4, and values calculated from these data are given in table 5.2. The initial slope of stress versus strain represents the Young’s modulus for small deformations \( E_y = \sigma / \gamma \), data until a strain of 0.8) and varies only little between the samples. The deblocked variants have slightly lower values. At higher deformation the linear, elastic response of stress with strain changes to a more viscous response until at strain values higher than 2 a constant or steady-state stress is measured \( (\sigma_{ss}) \). Clearly, the presence of sulfhydryl groups on the surface of ovalbumin does not lead to significant increase of any of the parameters describing surface shear; in contrast, they appear to be lower. For reference values of the Young’s modulus and \( \sigma_{ss} \) (ranging from 0.0002 mN/m for \( \beta \)-casein to 3.2 mN/m for soy glycinin at pH 3) we refer to the work of Martin et al.\(^{21}\)

<table>
<thead>
<tr>
<th></th>
<th>( E_{\text{young}} (\pm 2) ) [mN/m]</th>
<th>( \sigma_{ss} (\pm 0.06) ) [mN/m]</th>
</tr>
</thead>
<tbody>
<tr>
<td>SX(_1)</td>
<td>9.3</td>
<td>1.4</td>
</tr>
<tr>
<td>SX(_2)</td>
<td>7.3</td>
<td>1.4</td>
</tr>
<tr>
<td>SH(_1)</td>
<td>6.6</td>
<td>1.0</td>
</tr>
<tr>
<td>SH(_2)</td>
<td>5.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Table 5.2. Surface rheological parameters calculated from the surface shear experiments**

*Covalent cross-linking in bulk* To test whether the introduced sulfhydryl groups are capable of covalent cross-links between proteins, solutions of each variant (in the same buffer as in the surface shear experiment) were concentrated to concentrations that are comparable to those encountered at the air-water interface:\(^{44}\) 100 and 200 mg/mL. Then the samples were left to equilibrate for 22 hours, identical to the surface shear experiments. All samples were analysed with SDS-page, with and without addition of \( \beta \)-mercaptoethanol to distinguish between covalently and non-covalently linked aggregates. The SDS-PAGE gels obtained are shown in figures 5.5A (reducing) and B (non-reducing). Under reducing conditions, only one single band (at 44 kDa) was visible for all samples, therefore only one example is shown in figure 5.5A. This demonstrates that under these conditions any aggregates formed were completely dissociated.
Under non-reducing conditions, some aggregation (<5 %) is observed for the SH variants, but not for the blocked variants (figure 5.5B-1). However, only dimers are formed, and no higher aggregates. From this the picture emerges that under the conditions used, there is little driving force for development of covalent interactions, even at these high protein concentrations. Extensive aggregation could only be obtained by heat treatment. In this case the unfolding of the proteins leads to increased aggregation, even at the low concentrations used. All variants showed similar electrophoresis patterns after heat treatment, of which one is shown in figure 5.5B-2. The increased exposure of the indigenous cysteine residues upon unfolding is sufficient to allow the formation of covalently linked aggregates even for non-modified protein; alternatively the addition of the oxidizing agent ferricyanide also induces the formation of covalently linked aggregates. An increase in the amount of aggregates is found for both SH1 and SH2, as compared to the amount formed in the first treatment (figure 5.5B-3). However, the blocked variants showed similar behaviour, indicating that the ferricyanide was also able to remove the blocking acetyl group from the introduced S-AMSA groups.

![Figure 5.5. SDS-PAGE results under reducing (A) and non-reducing conditions (B) with treatments: concentrated to 100 and 200 mg/mL (1), heated 90°C, 30 minutes (2) and treated with 5 mM K₃Fe(CN)₆ (3), band around 43 kDa indicates the monomeric form of ovalbumin](image-url)
Discussion

The role of disulfide bridge formation on surface shear behaviour of adsorbed protein layers was investigated using chemically modified ovalbumin variants. The degree of modification was controlled by varying the concentration of the reactant. After modification the introduced S-AMSA groups could be deblocked to yield a reactive sulfhydryl group, allowing a comparison of blocked and deblocked variants. In this way, side effects of the modification that have to be accounted for when comparing to non-modified protein are avoided. No significant changes in secondary, tertiary and quaternary structure resulted from the modification (figure 5.2). This is in line with the results obtained with other modifications of ovalbumin in earlier work, where typically little or no effects on the protein structure and structural stability are found for low degrees of modification of lysine residues of this protein. Whereas the introduced deblocked sulfhydryl groups were shown to be reactive with both Ellman and SEI (table 5.1), they did not result in an increase in surface shear viscosity (figure 5.4). Increasing the protein concentration in bulk solution, to reach essentially identical conditions as at the interface resulted in only little aggregation (<5 % was of the total protein present as dimer). Apparently, there is not enough attraction between protein molecules to provide temporary associations that allow the formation of short-range chemical reactions to occur.

It is important to note that since the modified groups are at the exterior of the protein, no unfolding at the interface is necessary to expose these groups, as would be the case for unmodified proteins. The small population of dimeric protein that might be formed at the interface (as indicated by the results in figure 5.5B-1) will not be sufficient to form a continuous network that would lead to an increase of surface shear elasticity. Rapid formation of higher aggregates could be induced, however, but only by thermal treatment. The thermal treatment results in an unfolding of the protein, increasing the hydrophobic exposure, which increases the tendency to aggregate.

In this respect, the work by Roth and coworkers provides interesting observations. In this work the surface shear viscosity of adsorbed β-lactoglobulin was measured during a 24 hour period. After this period the system was heated to 85°C or 90°C, cooled after a certain time and the surface shear viscosity ($\eta_s$) of the interface after heat treatment was measured.
The surface shear viscosity was found to increase from around 400 mN/m to 600-1000 mN/m upon heating. Combined with the observation that heating will cause extensive aggregation and even gelation (especially at the high concentrations at the interface) their results show that before the heat treatment the adsorbed proteins have not formed a continuous network. Faergmand and coworkers found a similar increase of $\eta_s$ after cross-linking adsorbed proteins with transglutaminase.$^{45-47}$ This confirms the notion that interfacial layers formed after protein adsorption should in general not be described in analogy to three-dimensional gelled systems, but in terms of a system with a dense packing of particles.

A good description of such a model based on packing density rather than covalent interactions can be found in the work of Cicuta et al.,$^{23,24}$ and Edwards.$^{48}$ In these models, the shear behaviour is the result of a decreased mobility of the particles due to the close packing. This description can be adjusted to allow for small clusters of aggregates. The occurrence of such small clusters of adsorbed particles was modelled by Ravichandran and Talbot$^{49}$ and their effect on shear viscosity has already been described by Eagland$^{50}$. However, even when small aggregates are accounted for, the dense packing description does not presume an order over higher length scales than that of the aggregate, due to formation of a continuous network. The data presented in the present study support this concept.

In conclusion, the inability of chemically reactive proteins at the interface to form covalent cross-links shows that the adsorbed protein layer obtained under relatively mild conditions is not in a ‘gelled’ state. This is an important observation to improve the understanding of results described in literature on this subject. However, as described by MacRitchie and Owen$^{51}$ and Izmailova,$^7$ coagulation of adsorbed proteins leading to the formation of a true gelled or continuous network, can and will occur under specific conditions where stress is applied to the system. Two different processes can be the cause of this transition. Firstly, the repulsion between adsorbed particles can be overcome by thermal treatment as described by Roth et al.$^{16}$ and Hellebust at al.$^{52}$ The latter authors found that the protein layer was mainly stabilized by non-covalent forces, although within the network some intermolecular disulfide bonds were detected. The second process is compression of the interface. In several studies it has been shown that if the desorption of particles during the compression is slow enough the particles will be pushed through the intermolecular repulsive
This will also result in a gelled state of the interfacial layer. During the formation of foam and emulsions, newly formed interfaces are subjected to large deformations. As a result, these interfaces might (partly) consist of a gelled network, which in turn may allow chemical cross-linking between proteins to occur, evidently leading to altered interfacial stabilisation properties.

References


Chapter 6

Intermezzo: Rendering a colloidal particle surface layer into a gelled protein system

H.H. J. de Jongh, P.A. Wierenga, submitted
Abstract

There is an on-going debate on whether a protein surface film at an air-water interface can be regarded as a gelled layer. There is literature reporting that such films show macroscopic fracture behaviour and a rheology comparable to three-dimensional bulk-networks. If this is the case, a complete description of the formation of adsorbed layers should include a transition from single, freely moving proteins, to a gelled layer. This report presents studies using spectroscopic techniques, like infrared, fluorescence and neutron spectroscopy, or ellipsometry, to derive molecular insight in situ to substantiate the intermolecular networking in surface films of chicken egg ovalbumin. It is concluded that protein films, generated by equilibrium adsorption from the bulk, behave as a densely packed colloidal repulsive particle system, where the proteins still have a significant rotational mobility, have a predominantly retained globular fold and show distinct (lateral) diffusion. Applied stresses on the surface film (by compressions of the interface) may result in protein denaturation and aggregation. This renders a surface film from a colloidal particle into that of a gelled system.
Introduction

That proteins can locally accumulate at interfaces is a vital functionality for some proteins in biological functioning. A prerequisite for such local accumulation is the presence of a gradient in dielectric constant, as is the case in for example a lipid structure, like membranes, or air-water interfaces. It is evident that a net energy gain is the most obvious reason for some proteins to locally cluster against entropic motions. The molecular functionalities that dictate such behaviour are, however, not understood. Not only in biological sciences, but also in many (bio)technological applications (f.e. nano-technology and chip development) film formation of proteins at interfaces is an essential functionality. The most widespread application of proteins films can be found in food technology where foams - gas cells stabilised by protein films - are an essential building block of many food structures. The ability of a specific protein to stabilize such interfaces has often been described and becomes nowadays better understood at a molecular level. These new insights relied heavily on the more recent developments of a number of spectroscopic tools that allow in situ detection of protein behaviour at air-water interfaces, like infrared reflection absorption spectroscopy, neutron reflection, and localized (time-resolved) fluorescence (correlation) spectroscopy. The aim of this report is to illustrate how these techniques allowed one to understand some of the molecular details of protein behaviour in relation to macroscopic formation and stabilization of air-water interfaces.

Materials and Methods

Ovalbumin was purified as described previously. The fluorescence correlation spectroscopic (FCS) and time-resolved fluorescence anisotropy (TRFA) measurements were carried out as described by Kudryashova et al. (refs. 3 and 4 respectively). Infrared reflection adsorption spectroscopy (IRRAS) has been described in detail by Meinders et al., while ellipsometry experiments were carried out as described by Wierenga et al. Neutron-reflection experiments were performed on the CRISP facility of ISIS (Didcot) using conditions as described elsewhere. All experiments were carried out at pH 7.0 (phosphate buffer) and 20 °C.
Results and Discussion

Adsorption kinetics

In order to stabilize a newly formed air-water interface the surface tension of the interface needs to be lowered. Adsorption of proteins results in such lowering by typically 15-25 mN/m. The adsorption process relies on the diffusion of proteins through the bulk phase to the interface, generally helped by convection streams in the solvent as well. However, it has been suggested that for many proteins diffusion is not the rate-limiting step in the adsorption process. In a number of studies we have recently been able to demonstrate the existence of a kinetic barrier of adsorption that reduces the chance to stick to the interface upon encounter. This kinetic barrier can be lowered by increasing the number of hydrophobic patches on the protein surface or decreasing the net charge (or zeta-potential) of the molecule.

Rheology of protein surface films

Previously it was shown that local protein concentrations at air-water interfaces can reach values up to 150-300 mg/ml (or 10-30 volume percent) and for some proteins it was suggested that these films could be multi-molecular in thickness. In view of these high local concentrations one would expect that these proteins could develop strong intermolecular interactions, yielding a protein network as observed in protein gels. This view was supported by studies where macroscopic fracturing of a surface film could be visualized upon rapid expansion of a formed surface film. Also, applying small deformations to a formed surface film provided responses in surface pressure that resembled typically the behaviour of proteins in a 3-dimensional gelled network. The question remains, however, whether the above-mentioned rheological properties of surface films can be substantiated by molecular insights at the air-water interface. To this end we used a variety of spectroscopic techniques to study protein surface films under static and dynamic conditions. In the work presented here we focus on some insights of chicken egg ovalbumin surface films.
Molecular details of protein surface films

Can proteins in surface films rearrange?

To get an idea on whether formed surface films of protein still allow molecular rearrangements, the effect of salt-addition on the surface film properties of ovalbumin was studied using neutron reflection. The results are shown in table 6.1. A reflectivity of $6 \times 10^{-6}$ is commonly found for proteins at air-water interfaces\(^8\) and a typical layer thickness of 10 nm was found (dimensions of the cigar-shaped protein are 4.5 by 7 nm). Interestingly, upon addition of salt the density of the layer becomes higher and the layer slightly thinner. When the same type of experiments was performed with a succinylated variant of ovalbumin (with a net charge of -26 instead of -12) a similar behaviour was observed (unpublished results). That a formed surface layer can still adapt to altered electrostatic screening, implies that the intermolecular interactions are such that rearrangements at the molecular level within an interfacial film can occur.

**Table 6.1.** Neutron reflection data on air-water surface films of ovalbumin in D$_2$O. The experiments were performed under equilibrium conditions and the salt was added after the surface film was formed.

<table>
<thead>
<tr>
<th></th>
<th>Reflectivity of adsorbed layer $[\times 10^{-6}]$</th>
<th>Thickness $[\text{nm}]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>$5.94 \pm 0.12$</td>
<td>$10.9 \pm 0.3$</td>
</tr>
<tr>
<td>Ovalbumin + 0.15 M NaCl</td>
<td>$6.42 \pm 0.18$</td>
<td>$9.3 \pm 0.2$</td>
</tr>
</tbody>
</table>

Do proteins develop an extensive network of interactions in surface films?

The most eminent test to see whether in surface films strong intermolecular interactions are developed comparable to those in bulk gels, is by evaluating their ability to form intermolecular covalent disulphide bonds within a surface film. To this end we developed a series of ovalbumin where on the protein surface an increasing number of blocked sulphydryl group were engineered (chemically). These blocked groups could be removed on command, yielding chemically reactive variants. Surface rheology measurements did, however, not provide any indication that a covalent-linked network was obtained at the interface.\(^{12}\) Moreover, concentrating these chemically reactive proteins in a solution up to comparable concentrations as in surface films, showed a dimerization for less than 5 % of all proteins. Providing a trigger for these proteins to start developing other intermolecular interactions by
denaturing the proteins using heat treatment, resulted in an immediate extensive multiple chemical cross-linking of all material.\textsuperscript{12} In conclusion, protein films that are generated by equilibrium adsorption from the bulk behave as a densely packed colloidal repulsive particle system.

This would imply then that, although hindered by the high protein concentration, proteins still exhibit lateral diffusion properties. Figure 6.1A shows fluorescence correlation spectroscopic measurements of ovalbumin in the bulk (curve 1) and residing at an air-water interface (curve 2). It can be observed that the translational diffusion time increases from 0.3 in the bulk to 9 ms at interfaces corresponding to a 25 times slower diffusion coefficient at the interface. Evidently, the protein at the interface is not as immobilized as in bulk ovalbumin-gels where no diffusion of proteins could be detected at all (unpublished results).
Does this ‘particle’-behaviour imply that proteins do not lose their globular structure?

To investigate whether proteins in a surface film lose their globular structure (partially) to allow non-polar residues to become exposed, a combination of IRRAS (Figure 6.2A), ellipsometry (6.2B) and time-resolved fluorescence anisotropy studies (6.2C) was applied to study these surface films. From IRRAS measurements it could be deduced from spectral simulation (figure 6.2A) that upon adsorption some small (in the order of 10% less β-strand) conformational changes occurred at a secondary structure level (see also ref. 4), independent of the bulk concentration (not shown). From the ellipsometry experiments (figure 6.2B), where the kinetics of adsorption (by means of the adsorbed amount \( \Gamma \)) was monitored while recording simultaneous the developed surface pressure \( (\Pi) \), it was shown that the protein bulk-concentration did not affect the \( \Pi-\Gamma \) plot (figure 6.2B). This suggests that major globular unfolding of ovalbumin did not occur (on the second timescale) since this would have resulted in a larger area occupied by a single protein and thereby in a shift of the \( \Pi-\Gamma \) plot. This latter behaviour was reported for example for β-lactoglobulin,\(^1\),\(^3\) for which it was also shown that the degree of unfolding related to the protein concentration in the sub-phase.\(^1\)\(^4\) The event of protein unfolding at interfaces was recently shown to be merely related to the kinetics of unfolding, ranging from microseconds to minutes, depending on the protein.\(^1\)\(^3\)

**Figure 6.2.** (A) IRRAS spectra recorded after 60 min for a 0.1 mg/ml bulk ovalbumin concentration. Curve “Exp” corresponds to experimental spectrum; curve “Sim” corresponds to simulated spectrum assuming a secondary structure as in the bulk; curve “Sim -10% βs” corresponds to simulated spectrum where 10 % of β-sheets is transformed into random coil. (B) Exerted surface-pressure as a function of the surface load as recorded by ellipsometry for ovalbumin (bulk concentrations range from 0.005 to 0.1 g/l). (C) Total fluorescence and fluorescence anisotropy decays for 0.1 mg/ml ovalbumin in bulk solution (top panels) and adsorbed at the air/water interface (bottom panels). In all panels the fitted and experimental curves (total fluorescence left, anisotropy right) are shown.
Finally, the time-resolved fluorescence anisotropy measurements (figure 6.2C) illustrate that proteins still have a significant rotational mobility at the interface, not strongly deviating from that in the bulk solution. From these data we tend to state that at air-water interfaces the globularity of ovalbumin is mainly intact.

Can compression of a surface film provoke protein aggregation within this film?

Although surface films are crowded areas, from the above we tend to conclude that extensive intermolecular interactions cannot be the source of the reported rheological and fracturing behaviour of the films.\textsuperscript{10,11} However, in both mentioned reports surface films were obtained using a procedure where the formed surface film was compressed significantly during handling. It was thus tested whether compression of formed ovalbumin surface films would be able to induce the molecules to develop intermolecular interactions. Figure 6.3A shows IRRAS spectra before and upon a more than two-fold compression of the available surface. The enhanced intensity at 1620 cm\textsuperscript{-1} can be assigned to the presence of anti-parallel $\beta$-sheet, indicative for protein-protein association. Moreover, from TRFA-measurements (figure 6.3B) it becomes evident that while at initial compression stages the molecules show a preferred rotation along the shorter axis, at compression over a factor 2 a strong immobilization of the molecules is observed. In the case of ovalbumin it could be shown using IRRAS that this aggregation was fully reversible, with relaxation rates on the hour-timescale (not shown).

\textbf{Figure 6.3.} (A) IRRAS spectra of an ovalbumin surface film before and after a reduction of the available surface by 50%. The dashed line indicates the position of anti-parallel $\beta$-sheets(B) Rotational correlation time from time-resolved fluorescence of ovalbumin in surface films as a function of compression of the available surface area.
This aggregation of proteins in surface films was also demonstrated by FCS (Figure 6.1B). Protein aggregates both in the bulk and at the interface have significant longer diffusion times (curve 1 and 3 respectively) compared to their monomeric counterparts. The reversibility of the aggregation is demonstrated by the presence of a contribution of faster diffusing molecules that corresponds to the monomers (see figure 6.1A, curve 2).

As stated in the introduction, there is an ongoing debate on the structure of interfacial protein layers. As shown in this report, a protein film is not by definition a gelled system. In contrast, adsorbed protein films will not gel, unless a certain stress is applied to the system. The origin of the in literature reported macroscopic fracture and other behaviour resembling 3D gelled systems must be found in the preparation of the interfacial layers. Many studies use compression of protein layers (either from initially bulk-adsorption or using a Trurnit-approach where a protein solution is spread along a wetted rod directly onto the interface) as a means to study the interfacial properties. This compression of the interface may lead to the transition of a surface film of packed colloidal particles to a gelled system. Obviously, such effects will result in changes of the macroscopic properties of the films. The importance of this finding is not only in evaluating the results already published in literature, but also in understanding for instance the difference in foaming-results between whipping or sparging techniques.

Acknowledgements

We gratefully acknowledge Elena Kudryashova for conducting some of the fluorescence and IRRAS measurements.

References


Chapter 7

Calculation of protein adsorption rates to air-water interfaces based on molecular parameters; a predictive tool for foaming capacity?

**Abstract**

The presented work is based on previous work in which the role of different protein molecular properties (such as net charge) on the surface functionality was studied. The adsorption was found to be limited by a kinetic barrier, the height of which is determined by the protein net charge density and hydrophobic exposure. The aim of this study is to show the extent to which the influence of these properties is reflected in the foam forming and stabilising properties of the protein. In this study, the molecular parameters of twenty different proteins were determined. Based on the hydrophobic exposure, net charge density and size of these proteins, a prediction was made of the adsorption kinetics of the proteins. To perform this calculation, a linear additivity between the electrostatic and hydrophobic contribution to the kinetic barrier was assumed. Experimentally, the actual kinetics of surface pressure development, the dilatational elastic modulus and the foam formation and stability was determined for all proteins.

For 7 of 20 proteins it was found that the initial protein concentration used in the foaming experiment (0.01 %) was not sufficient to allow foam formation. At higher concentration (0.05 %) these proteins did form foam, showing that the kinetics of adsorption was limiting the foam forming capacity. This limited foam forming capacity correlated with a threshold of the predicted adsorption kinetics. However, a quantitative relation could not be demonstrated. We feel that the assumption of additivity of hydrophobic and electrostatic leads to an overestimation of the hydrophobic contribution. Therefore, the nature of the balance between these two contributions needs to be reconsidered.

The foam forming capacity did seem to correlate with the rate of increase in surface pressure of the proteins. The foam stability, however, was not found to correlate with any of the measured parameters, not even with the dilatational elastic modulus. It seems therefore that other factors are (more) important in the stabilisation of foam, that are not reflected in either surface pressure or elastic modulus.
Introduction

Many food products are multi-phase systems such as foam (air-water) and emulsions (oil-water), where often protein acts as the interface stabilising agent. The quality of these dispersions can be described by textural or stability characteristics, and depends on three main factors: (1) the size and volume fraction of the dispersed particles, (2) the interaction between dispersed particles, and (3) the ‘stability’ of individual particles. These three factors can in turn be affected by the conditions used during formation and storage of the product. The effects of the applied conditions depend on the molecular properties of the protein (e.g. charge, size, etc.); so basically these properties determine the macroscopic characteristics of the system.

Fundamental research on the relation between molecular properties of proteins and the macroscopic characteristics of a multi-phase system such as foam is complicated. Therefore the advances in knowledge have to come from studies performed in model systems and subsequent extrapolation of the findings to the more complex system (foam). In the case of studies on proteins at air-water interfaces, the model system is often a planar interface, under static conditions. Using different techniques, the increase in adsorbed amount, surface pressure and elastic modulus of the interface can be followed in time. In this way, for set conditions, knowledge of adsorption behaviour at the interface can be extrapolated to the macroscopic properties of foam. However, for each change in system conditions (e.g. pH or ionic strength) new measurements are necessary, making such studies elaborate. This illustrates the need to predict the functionality of the protein under different conditions, based on parameters that can be more easily determined.

In previous work we have shown that the rate of adsorption of ovalbumin, even at the initial stages of adsorption ($\Gamma<0.5\, \text{mg/m}^2$), is lower than would be expected based on diffusional transport. This indicates the presence of an energy barrier for adsorption. Using chemically modified variants of ovalbumin it was found that the height of this energy barrier is determined by the exposed hydrophobicity and net charge.\(^1,2\) Both parameters were evaluated using hydrophobic interaction chromatography and zeta-potentiometry respectively.
The results provided the basis for a model presented in this work that attempts to quantitatively describe the adsorption of proteins to the air-water interface based on these two parameters, including of course also the protein diffusion to the bulk and thus the protein size. Other factors, such as the globular stability (related to potential unfolding processes, chapter 4), and the presence of chemically reactive sulphhydryl (chapter 5) were found not to be of significant importance for this process and are therefore not included in the model.

The presented work covers two areas of interest. Firstly, the net charge and hydrophobicity are assessed for a set of twenty different proteins, representing the typical variance of protein net charge density (at pH 7.0) and hydrophobicity. From the obtained data the adsorption rate constant of these proteins is predicted, based on the model mentioned above. This gives an indication of the expected variance in adsorption behaviour of these proteins. Secondly, the surface functional properties of these proteins are characterised by measurement of the development of surface pressure in time, the dilatational modulus and foam formation and stability. These results show the actual difference in surface functionality between the studied proteins. The obtained set of data (both from prediction and from experimental results) is than analyzed to obtain insight in the correlation between the molecular parameters, mesoscopic parameters (adsorption kinetics, surface pressure and dilatational modulus) and macroscopic properties (foam capacity, foam stability). The aim of such experiments is to ascertain whether certain molecular parameters can be identified as major predictors of the macroscopic properties.

**Materials and Methods**

*Chemicals* The proteins used in this study can be divided in 3 groups (1): Ovalbumin, β-Lactoglobulin, α-Lactalbumin, Lysozyme, BSA; (2): Bromelaine, Papaine, Cytochrome c, α-Amylase, Myoglobin, Phosvitin, Trypsin, Pepsin, Phospholipase A2; and (3): Patatin, Pea vicilin, Glycinin. The proteins from group one are the proteins most commonly used in the food industry as ‘pure’ protein preparations. The second group contains proteins that are not directly related to food applications, but that represent different structural families of proteins in order to obtain a range of protein properties that is as broad as possible. The last group represents food product related proteins, or rather protein isolates (potato, pea, soy bean),...
which are heterogeneous protein samples. The source and origin of the proteins used in this study is given in table 7.1. All chemicals used were of analytical grade and ordered from either Sigma-Aldrich or Merck.

Chemical characterization

Detection of protein size

The purity of proteins was checked using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were dissolved in sample buffer, containing 62.5 mM Tris-HCl (pH 6.8), 1.25 % SDS, 5 % glycerol, 0.00125 % bromophenol blue and 1.25 % β-mercaptoethanol, and heated for 10 min at 100 °C before analysis. Samples were applied on SDS-PAGE 8-25 gradient gels (Pharmacia) and run on a Phast-System (Pharmacia). Gels were stained with Coomassie brilliant blue. A kit with low molecular mass brilliant ranging from 14 to 94 kDa from Pharmacia was used for calibration.

Determination of ζ-potential

The ζ-potential of the proteins was determined on a Zetasizer 2000 (Malvern instruments Ltd., U.K.) with 10 mg/mL protein solutions (10 mM phosphate pH 7.0, 20 °C). Electrophoretic mobility was monitored at 150 V applied voltage, using a He-Ne laser at 632 nm. The apparatus was calibrated according to supplier’s instructions. Samples were analysed in triplicate and the measured ζ-potential, calculated from the mobility with the Smoluchowsky equation, varied less then 10 % between identical sample preparations.

Determination of exposed hydrophobicity.

The hydrophobic exposure of proteins is difficult to assess in an absolute manner. Hydrophobic chromatography was used to obtain a relative expression of hydrophobic exposure of the different proteins. A Phenyl FF column (high sub, 5 mL from Amersham, Biosciences) was used, with elution buffers A: 10 mM BisTris HCl, pH 7.0, B: buffer A + 0.85 M ammonium sulphate, and C: buffer A + 1 M ammonium sulfate. In those cases where a protein was found to be insoluble in 1 M (NH₄)₂SO₄ buffer B was used instead of buffer C. The column was equilibrated in buffer C and samples were prepared by dissolving 10 mg in 1 mL of buffer C and centrifuging for 5 min at 12000 rpm (eppendorf
centrifuge). A flow-rate of 5 mL/min was used and each experiment started by equilibration of the column with 5 column volumes (CV), followed by injection of 500 µL sample, followed by 2 CV of buffer C. Then the samples were eluted with a gradient from 100 % buffer C to 100 % buffer A, over a length of 20 CV, followed by 2 CV of 100 % buffer A. Detection took place at 280 nm and the exposed hydrophobicity is expressed as one minus the molarity of (NH₄)₂SO₄ where the highest absorbance is measured in the detector (this approach has also been used by Franco et al.).

**Surface functional properties**

*Automated drop tensiometry* The development of surface pressure and the surface dilatational elastic modulus were measured using an Automated Drop Tensiometer (ADT; I.T. Concept.), a technique described in detail by Benjamins et al. Basically, an air bubble is formed at the tip of a syringe needle placed in a cuvette containing the protein solution. Both the cuvette and the syringe are temperature controlled (20 ± 0.1 °C). In these experiments, the bubble volume was kept constant at 4 µL, using the computer-controlled syringe plunger to compensate for gas diffusion from the bubble. The surface elastic modulus (E) was measured by inducing sinusoidal changes in the interfacial area with a period of 10 s and amplitude of 10 %. The modulus was calculated from the measured changes in surface tension and surface area (E = dΠ / dlnA) averaged over a sequence of five sinuses; such a sequence was performed every 500 s. These measurements of the modulus did not affect the development of surface pressure in time. All protein solutions (0.1 mg/mL in a 10 mM phosphate buffer at pH 7.0) were prepared one hour before measurement and measured in duplicate.

*Ellipsometry* Adsorption to air-water interfaces of ovalbumin variants was monitored using a Multiskop ellipsometer (Optrell, Germany) combined with a Langmuir trough (Riegler and Kirstein, Germany) and Wilhelmy plate tensiometry. A good explanation of the theoretical background of ellipsometry has been given by Russev et al. Using the combination of ellipsometry and surface tensiometry, both the increase of surface load (Γ) and surface pressure (Π) in time were measured. For all samples the rate of adsorption from 0.1 mg/mL solutions (in 10 mM phosphate, pH 7.0, 20 °C) was measured at least in duplicate. All experiments were started by removing the interfacial layer with a custom made suction device.
after which the clean interface was rapidly expanded to the maximum area (from 30 to 190 cm²; the first data points are typically collected 100 s after cleaning the interface). In this way, the initial conditions for each experiment approximated $\Gamma = 0$ mg/m² at $t = 0$ s. The values for the ellipsometric angles $\Delta$ and $\psi$ were used to calculate the adsorbed amount, using software from the supplier (Optrell). To do this, the refractive index and thickness of the adsorbed protein layer are fitted in a model based on two bulk phases (air and water) and one adsorbed layer, with parameters: $n_{\text{air}} = 1.000$, $n_{\text{protein solution}} = 1.3327$, $dn/dc = 0.18$; the angle of incidence was 50°. Control experiments with distilled water in between measurements confirmed that the cleaning method used (rinsing with ethanol and water) was sufficient to avoid contamination of the trough, i.e. surface pressure of a clean surface remained 0 mN/m during compression.

** Foam formation and stability ** Foam was produced with the fan flutter technique in which foam is generated by high speed single rod whisking. The foam volume and bubble size distribution were determined as a function of time, using a photo camera in a special set-up (see figure 7.1A). The fan is made of stainless steel, with a width of 35 mm from spoke tip to opposite tip and had a thickness of 0.1 mm. The fan has 36 spokes. Mixing speed was 3500 rpm and mixing time 2 minutes. The typical sample volume was 60 mL and the protein concentration 0.1 mg/mL, if this concentration proved too low to obtain measurable foam volumes, a concentration of 0.5 mg/mL was used. Proteins were dissolved (one hour at room temperature 22 °C) in a sodium phosphate buffer pH 7.0 with an ionic strength of 10 mM. Foam was directly produced in a measuring cuvette (45 x 57 x 134 mm); teflon cubes were placed in the cuvette to elevate the foam to the height of the camera.

![Figure 7.8](image.png)

*Figure 7.8. (A) Set-up of the foam analyzer; (B) Example of an image obtained in reflection mode (left) and a processed image (right) where the analysed bubbles are colored.*
Foam was analysed in transmission and reflection mode. Images taken in transmission mode were used to determine the foam height; images taken in reflection mode were used for the calculation of bubble size distribution (see figure 1B). In the transmission mode light passed straight through the cuvette to a camera at the opposite side of the light source. For analysis in reflection mode a prism of 90° is placed on the 57 mm side of the cuvette. The camera is a Mega Pixel Progressive Scan Camera (1300 x 1030 pixels). After placing the cuvette in de Foam Analyser 50 images were taken at an interval of 30 s (total time 30 minutes) or less depending on foam capacity and stability, bubbles smaller than 5 pixels were discarded. The average bubble diameter and the cumulative number of bubbles as a function of bubble diameter are calculated.

Results and Discussion

Part I: Calculation of the adsorption rate constant

In previous work\textsuperscript{1,2} it has been shown that the adsorption of proteins to the air-water interface is limited by a kinetic energy barrier. The evidence for this was found from the adsorption behaviour of chemically modified variants of ovalbumin. In a first set of experiments, the hydrophobic exposure was increased by covalent coupling of capric acid onto lysine groups on the protein surface. Despite a slight increase in net charge, a drastic increase of the rate of adsorption was found.\textsuperscript{2} In another set of experiments, the net charge of the protein was increased by coupling of succinic anhydride to the protein lysine groups. In this way, the hydrophobic exposure remained unchanged, while a decrease in the rate of adsorption was observed with increasing net charge.\textsuperscript{1} It was further shown that the adsorption curves could be described by equation 7.1:

\[
\frac{d\theta}{dt} = D^* C_b P_{\text{adsorb}}
\]  

(7.1)

In this formula \(\theta\) is the surface coverage [-], calculated via \(\theta = \Gamma \Omega\) from the surface load (\(\Gamma\), [\#/m\(^2\)]) and the surface area taken up by adsorbed protein molecules (\(\Omega\), [nm\(^2\)/molecule]); \(k_{\text{adsorb}}\) is the adsorption constant, and \(C_b\) is the protein concentration [\#/m\(^3\)] in the bulk phase.
\( P_{\text{adsorb}} \) is the statistic chance [-] for an adsorbing protein to reach an available area at the interface, which is a function of \( \theta \) and calculated from the random sequential adsorption model.\(^7\)\(^-\)\(^9\) The apparent diffusion coefficient can be used to calculate the rate constant of adsorption (\( k_{\text{adsorb}} \)) as discussed in chapter 4. This constant is given by the probability that a protein adsorbs at the interface, when it arrives at an available location, and can be written as:

\[
k_{\text{adsorb}} = e^{-\frac{\Delta E_{\text{total}}}{kT}} \quad (7.2)
\]

It was suggested that the height of the kinetic energy barrier (\( \Delta E_{\text{total}} \)) mainly depends on the exposed hydrophobicity and net charge of the protein as discussed above. The net charge can be quantified by means of determining the \( \zeta \)-potential. An absolute method to determine the hydrophobicity of a protein is not available. Techniques that use the increase in fluorescence of fluorescent probes such as ANSA, PRODAN or cis-parinaric acid (CPA) in a hydrophobic environment have been used to assess hydrophobicity.\(^10\),\(^11\) However, the interaction depends not only on the protein hydrophobicity, but also on the size of the hydrophobic patches on the protein surface and the charge distribution.\(^10\),\(^11\) The technique does not provide an independent measure of exposed hydrophobicity, and is not useful for the comparison of proteins that can differ in electrostatic properties. Hydrophobic interaction chromatography offers a high throughput method with good reproducibility that is useful to determine the relative hydrophobicity of different proteins. In this study HIC was used as the main tool to assess exposed hydrophobicity, and PRODAN (a neutral fluorescent probe) as a control (for five proteins). The relative hydrophobicities calculated from both techniques were found to be similar for all five proteins (results not shown).

Using these tools the exposed hydrophobicity (HIC) and net charge (\( \zeta \)-potential) of all proteins used were assessed; the results are given in table 7.2. For both parameters the measured values are evenly distributed between the minimal and maximal value obtained, illustrating the heterogeneity of molecular properties represented by the proteins used in this study.
Additivity model

To relate the observed exposed hydrophobicity and \( \zeta \)-potential to energies contributing to \( \Delta E_{\text{total}} \), these contributions need to be converted into energy terms (kT). The height of the experimentally observed kinetic barrier is determined from the decrease in adsorption kinetics compared to pure diffusional transport of proteins to the interface. In figure 7.2 the value of \( \Delta E_{\text{total}} \) is shown as a function of net charge (panel A) and exposed hydrophobicity (panel B), based on the experimental data presented in chapter 2 and 3 using a single proteins with different degrees of modification. An assumption needs to be made on how the two contributions should be included in the calculation of \( \Delta E_{\text{total}} \). In a first approach, we propose the so-called “additivity model”. This model is based on the assumption that the energetic contributions of the electrostatic and hydrophobicity component to the total energy barrier are additive and independent. In this case the total adsorption barrier is calculated from:

\[
\Delta E_{\text{total}} = \Delta E_{\text{electrostatic}} + \Delta E_{\text{hydrophobic}}
\]

(7.3)

Figure 7.2A shows the measured \( \zeta \)-potential of succinylated ovalbumin variants as a function of the determined \( \Delta E_{\text{total}} \) (\( R^2=0.99 \)) as described in chapter 3. The relation appears to be linear, with an extrapolated barrier of 2.2 kT for ovalbumin if no net charge would be present at the protein. This remaining barrier suggests that the hydrophobic exposure of the protein should exceed a threshold value for efficient adsorption at the air-water interface.
Figure 7.2. ‘Calibration’ lines for the calculation of the adsorption barrier. (A) increased net charge leads to an increase of the barrier. (B) The hydrophobicity (if high enough) can lead to a decrease. These two panels are used as calibration curves for the linear additivity model.

Figure 7.2B shows the relation between the assessed hydrophobicity and hydrophobic part of the energy barrier,\(^2\) that was calculated by subtracting the electrostatic contribution from the total barrier for native and caprylated ovalbumin. This plot shows that with increasing hydrophobicity \(\Delta E_{\text{total}}\) diminishes, and that at higher values it takes on negative values. This means that the hydrophobic contribution not only decreases, but even counteracts the contribution of the electrostatic component. Support for this idea comes from experiments with \(\beta\)-lactoglobulin and with caprylated ovalbumin where the total energy barrier approaches zero, even though the net charge of these proteins is close to that of non-modified ovalbumin (around \(-14\)).\(^2\)

Using the two “calibration-plots” of figure 7.2 and equation 7.3, the energy barrier \(\Delta E_{\text{total}}\) for protein adsorption can be calculated for all proteins, based on their \(\zeta\)-potential and assessed hydrophobicity (third column in table 7.3). From this barrier the adsorption constant can be established using equation 7.2. Since the proteins used differ in size, this adsorption constant should be multiplied by the diffusion constant (calculated from the molecular weight of the proteins). In table 7.3 the adsorption rates are then given \((k_{\text{adsorb}}*D_b)\). The predicted values of \(k_{\text{adsorb}}\) vary from 0.4 to approximately 16800. This shows that the adsorption
behaviour between proteins can vary greatly. When comparing $k_{\text{adsorb}}$ with the energetic contributions, it becomes apparent that the hydrophobic contribution dominates the total energy barrier. Only for proteins with similar exposed hydrophobicity the electrostatic charge makes a small difference (as seen for example for β-lactoglobulin and α-lactalbumin). A second observation is that in some cases the adsorption process would be faster than diffusional transport, we will comment on this in the last part of this section.

**Part II: Characterization of surface functionality**

*Surface pressure development*

For each of the proteins listed in table 7.2 the development of surface pressure in time has been monitored. Measurements of the development of surface pressure in time of five proteins are shown in figure 7.3 as indication of the variation within all measured proteins. For proteins that adsorb fast (such as β-lg, line 1) a steady increase of $\Pi$ in time is measured. For the other proteins in figure 7.3, an initial lag time is observed before the surface pressure increases. This lag-time is indicative of slower adsorption kinetics, but is mainly due to the fact that surface pressure only starts to increase after a certain adsorbed amount is reached. Although the kinetics of surface pressure development varies greatly between proteins, only a small range is found for the surface pressure at saturation (16-29 mN/m).

![Figure 7.3. Surface pressure against time (logarithmic scale) 1- Myoglobin, 2- Cyt-C, 3- Ovalbumin, 4- β-lg, and 5- papain](image)
The surface pressure measurements only indicate the kinetics of adsorption under static conditions. To test the ability of the proteins to stabilise interfaces during foam formation, the foam forming properties were evaluated as well. The foaming capacity, or the initial amount of foam formed is given in table 7.2 (columns 10 and 11), the variation in foam volume between duplicate measurements was typically within 2 mL. The amount of foam formed at \( t=0 \), together with the amount of foam after 1 h is graphically shown in figure 7.4. In figure 7.4A the results are shown for those proteins that produced a measurable amount of foam at 0.1 mg/mL, while in figure 7.4B the results are shown of the proteins where a concentration of 0.5 mg/L was required.

\[ \begin{align*}
\text{BSA} & \quad 30 \\
\text{Bromelin} & \quad 15 \\
\text{Pepsin} & \quad 20 \\
\text{Phospholipase A2} & \quad 25 \\
\text{Trypsin} & \quad 10 \\
\beta\text{-Lactoglobulin} & \quad 30 \\
\alpha\text{-Lactalbumin} & \quad 20
\end{align*} \]

\[ \begin{align*}
\text{Phosvitin} & \quad 35 \\
\text{Glycinin} & \quad 30 \\
\text{Ovalbumin} & \quad 25 \\
\text{\( \alpha\) -Amylase} & \quad 20 \\
\text{Patatin} & \quad 15 \\
\text{Myoglobin} & \quad 10 \\
\text{Papain} & \quad 30 \\
\text{Lysosome} & \quad 25 \\
\text{Cytochrome C} & \quad 15
\end{align*} \]

Figure 7.4. Foam volume (A) 0.01% solutions at \( t=0 \) s (light bars) and \( t=7200 \) s (dark bars) and (B) 0.055% solutions at \( t=0 \) (light bars) and \( t=900 \) s (dark bars)

It is interesting to see that the distinction in higher and lower foaming capacity coincides with values of \( k_{adsorb} \) higher and lower than 0.1 respectively. The calculation of \( k_{adsorb} \) was based on adsorption studies under static conditions, where transport of proteins to
the interface is diffusion limited. During foam formation, diffusion can be neglected, since the mechanical stirring of the solution will result in convection that dominates transport of proteins. However, the adsorption constant \( k_{\text{adsorb}} \) is still important in this process. It can be concluded that the adsorption of proteins during foam formation is still limited by the kinetic barrier to such an extent that the adsorption is not fast enough to allow foam formation. The correlation between \( k_{\text{adsorb}} \) and \( V_{\text{foam}} \) is only qualitative, but the lack of quantitative correlation (shown in figure 7.5A, \( R^2 < 0.05 \)) might be due to the way in which \( k_{\text{adsorb}} \) was established using the ‘additivity’ model.

A second parameter related to foam formation is the initial increase of surface pressure in time. As shown in figure 7.5B, a clear positive relation is observed between \( d\Pi/dt \) and the initial foam volume \( (R^2 = 0.8) \). From this it must be concluded that apparently the increase in surface pressure is more directly related to foam formation than the adsorption of proteins at the interface. This means that for the prediction of protein functionality not only their adsorption behaviour, but also the relation between adsorbed amount and the surface pressure needs to be understood. The values for the surface pressure and the elastic modulus at the end of each experiment \( (t=2 \, \text{h}) \) are also given. Although both parameters show a broad range of values \( (\Pi \text{ from } 16-29 \, \text{mN/m and } E \text{ from } 22-121 \, \text{mN/m}) \) these parameters seem to have no predictive value for either the foam formation or stability \( (R^2 < 0.1) \).

![Figure 7.5](image-url)  
*Figure 7.5. Correlation between the amount of foam volume formed at \( t=0 \, \text{s} \) and (A) \( k_{\text{ads}} \) and (B) the initial rate of increase in surface pressure, lines shown are from linear regression, correlation coefficients are <0.1 and 0.8 for A and B respectively.*
Calculation of the adsorption rate constant

In the current study $k_{ads}$ was calculated using a linear additivity model. This model agrees with the observations that proteins with high enough hydrophobic exposure (e.g., β-lactoglobulin or caprylated ovalbumin) display adsorption kinetics that are faster than would be expected based on their net-charge. However, applying the additivity model means that $k_{ads}$ can reach values >1 if the hydrophobic contribution exceeds the electrostatic barrier. This in turn leads to the prediction of adsorption kinetics that is faster than pure diffusional transport, which is an unlikely situation (or, at least an unwanted situation in the general description of adsorption phenomena).

At this point it is important to note that it was recently discussed by Ladohkin et al., that electrostatic and hydrophobic contributions are not additive. In this work the partitioning of a 13 amino-acid cationic, hydrophobic polypeptide in anionic bilayer membranes was studied. Partitioning coefficients were measured for mutants of this peptide that differed in charge and/or hydrophobicity. The observed free energy of partitioning ($\Delta G_{obs}$) was found to depend linearly on both hydrophobicity and surface-potential. However, the slope of the plot of $\Delta G_{obs}$ decreased with increasing hydrophobicity of the polypeptide. Although they did not formulate an alternative to the additivity model, they did show that an increase in hydrophobic free energy of 3 kcal/mole resulted in a decrease of the electrostatic contribution by 20%.

Our experimental data (chapter 2 and 3) also suggest that increased hydrophobic exposure decreases the electrostatic barrier. In addition, it has been observed that adsorption of proteins proceeds much faster at the oil-water than at the air-water interface. However, the hydrophobic effect is commonly associated with the entropy change of water molecules when two hydrophobic molecules associate. If the hydrophobic attraction would only act at much smaller distances than the electrostatic repulsion, the latter interaction would dominate the process. Subsequently, the adsorption at oil-water and air-water interfaces should both be limited by the electrostatic effect and thus be equal. A possible explanation might be that the hydrophobic interactions act on length scales where electrostatic interactions are sensed. Although several authors have reported the long-range character of the hydrophobic
attraction\textsuperscript{13-16} this notion is still subject of both experimental and theoretical studies and no part of the consensus.

**Conclusions**

Based on the measured exposed hydrophobicity and $\zeta$-potential of twenty proteins predictions were made of their adsorption rates. This calculation should be improved with respect to the way in which both hydrophobic and electrostatic contributions are included in the calculation of the total energy barrier to adsorption. With only few exceptions, proteins with a calculated $k_{\text{adsorb}} \cdot D_b < 50$ showed no foam forming capacity at 0.01%, but only at 0.05%, while the other proteins had good foaming capacity already at 0.01%. This is an interesting observation, since $k_{\text{adsorb}}$ is calculated based on results from static adsorption experiments, while during foam formation more dynamic conditions are applied. Knowledge of the adsorption constant will thus enable a qualitative prediction of foam forming capacity. In equation 3.4 and 3.4.1 a proportionality-constant was needed to calculate $k_{\text{adsorb}}$ from the apparent diffusion constant found from $\Gamma$-t curves. The value was chosen as unity, but this does not affect the conclusions, since the same equations were used for calculation of the energy contributions from the apparent diffusion constant (calibrations) as for the calculation of the apparent diffusion constant ($k_{\text{adsorb}} \cdot D_b$) from the energy contributions.

The surface pressure development in time was also measured for all proteins. The initial rate of surface pressure increase showed a qualitative, but no quantitative agreement with $k_{\text{adsorb}}$. However, this parameter was found to correlate to the foam forming capacity (of the proteins that formed foam at 0.01%) with an $R^2$ value of 0.78.

With respect to the stability of the foam formed, no correlations were found with any of the measured parameters. Surprisingly, even the elastic modulus of the interface did not show any correlation with foam stability ($R^2$<0.1).

In conclusion, a number of interesting challenges are provided by the presented work. First among these is the calculation of the total energy barrier to adsorption, which determines the foam forming capacity of proteins. The most important issue to be addressed here is the
nature of the hydrophobic contribution to the adsorption constant. Further, a clear need is illustrated to find a parameter that can be used to predict the instability of the foam formed.

Acknowledgments

We would like to thank J. de Groot and H. Kosters from the Wageningen Centre for Food Science for their help with the characterization of the proteins, and M. Bos and H. Mocking-Bode from TNO Nutrition and Food Research Institute, the Netherlands for performing the foaming experiments.
Table 7.1. Protein parameters (origin and literature values), information on the primary sequence and modified residues were obtained from the Swiss-Prot and TrEMBL database (http://www.expasy.org/sprot)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>Origin</th>
<th>M&lt;sub&gt;w&lt;/sub&gt; [kDa]</th>
<th>Diffusion constant&lt;sup&gt;A&lt;/sup&gt; ([m^2/sec] \times 10^{-11})</th>
<th>Glycosylation sites</th>
<th>Phosphorylation sites</th>
<th>S-S bridges (free SH)</th>
<th>#COOH&lt;sup&gt;B&lt;/sup&gt;</th>
<th># NH&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;B&lt;/sup&gt;</th>
<th>(\sigma_w^c) [mC/m²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ovalbumin</td>
<td>From ref. 2</td>
<td>Henn egg white</td>
<td>44.5</td>
<td>9.1</td>
<td>Asn 292</td>
<td>Ser 68, 236, 240,344</td>
<td>1 (4)</td>
<td>47</td>
<td>35</td>
<td>-24.2</td>
</tr>
<tr>
<td>2 β-Lactoglobulin</td>
<td>From ref. 17</td>
<td>Cow milk</td>
<td>18.4</td>
<td>9.7</td>
<td></td>
<td></td>
<td>2 (1)</td>
<td>26</td>
<td>18</td>
<td>-21.9</td>
</tr>
<tr>
<td>3 α-Lactalbumin</td>
<td>Sigma L6010</td>
<td>Cow milk</td>
<td>14.2</td>
<td>13.3</td>
<td>Asn 45</td>
<td></td>
<td>4 (0)</td>
<td>20</td>
<td>13</td>
<td>-22.7</td>
</tr>
<tr>
<td>4 Lysozyme</td>
<td>Sigma L6876</td>
<td>Henn egg white</td>
<td>14.3</td>
<td>13.3</td>
<td></td>
<td></td>
<td>4 (0)</td>
<td>9</td>
<td>17</td>
<td>25.8</td>
</tr>
<tr>
<td>5 BSA</td>
<td>Sigma A4503</td>
<td>Cow milk</td>
<td>66.3</td>
<td>8.0</td>
<td></td>
<td></td>
<td>17 (1)</td>
<td>99</td>
<td>82</td>
<td>-19.7</td>
</tr>
<tr>
<td>6 Bromelaine</td>
<td>Sigma B4882</td>
<td>Pineapple Stem</td>
<td>22.8</td>
<td>11.4</td>
<td>Asn 117</td>
<td></td>
<td>3 (1)</td>
<td>17</td>
<td>21</td>
<td>9.5</td>
</tr>
<tr>
<td>7 Papaine</td>
<td>Merck 107144</td>
<td>Carica Papaya</td>
<td>23.4</td>
<td>11.3</td>
<td></td>
<td></td>
<td>0 (7)</td>
<td>16</td>
<td>22</td>
<td>13.9</td>
</tr>
<tr>
<td>8 Cytochrome-C</td>
<td>Sigma C7752</td>
<td>Horse heart</td>
<td>12.4</td>
<td>14.2</td>
<td></td>
<td></td>
<td>0 (2)</td>
<td>12</td>
<td>21</td>
<td>33.2</td>
</tr>
<tr>
<td>9 α-Amylase</td>
<td>Sigma 10065</td>
<td>Asp. Oryzae</td>
<td>12.3</td>
<td>8.6</td>
<td>Asn 297</td>
<td></td>
<td>4 (1)</td>
<td>54</td>
<td>30</td>
<td>-32.6</td>
</tr>
<tr>
<td>10 Myoglobin</td>
<td>Horse heart</td>
<td></td>
<td>17.0</td>
<td>12.5</td>
<td></td>
<td></td>
<td>0 (9)</td>
<td>21</td>
<td>21</td>
<td>0.0</td>
</tr>
<tr>
<td>11 Phosvitin</td>
<td>Sigma P1253</td>
<td>Henn egg white</td>
<td>34.0</td>
<td>10.8</td>
<td>Asn 67, 237</td>
<td></td>
<td>123 Ser</td>
<td>0 (0)</td>
<td>19</td>
<td>-139.3</td>
</tr>
<tr>
<td>12 Trypsin</td>
<td>Sigma 96310</td>
<td>Bovine pancreas</td>
<td>31.7</td>
<td>11.2</td>
<td></td>
<td></td>
<td>6 (0)</td>
<td>18</td>
<td>10</td>
<td>-18.4</td>
</tr>
<tr>
<td>13 Pepsin</td>
<td>Sigma 77152</td>
<td>Porcine Pancreas</td>
<td>23.3</td>
<td>9.9</td>
<td>Ser 68</td>
<td></td>
<td>3 (0)</td>
<td>42</td>
<td>3</td>
<td>-71.6</td>
</tr>
<tr>
<td>14 Phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Sigma 79483</td>
<td>Porcine Pancreas</td>
<td>14.0</td>
<td>13.4</td>
<td></td>
<td></td>
<td>6 (2)</td>
<td>15</td>
<td>13</td>
<td>-6.5</td>
</tr>
<tr>
<td>15 Patatin</td>
<td>From ref.</td>
<td>Potato</td>
<td>40.0</td>
<td>7.5</td>
<td>Asn 92, 301</td>
<td></td>
<td>0 (1)</td>
<td>43</td>
<td>32</td>
<td>-17.9</td>
</tr>
<tr>
<td>16 Pea vicilin</td>
<td>From ref. 18</td>
<td>Pea</td>
<td>81.0</td>
<td>6.3</td>
<td></td>
<td></td>
<td>0 (0)</td>
<td>69</td>
<td>56</td>
<td>-19.7</td>
</tr>
<tr>
<td>17 Glycinin A (B)</td>
<td>From ref. 19</td>
<td>Soy bean</td>
<td>32.6</td>
<td>5.6</td>
<td>Asn351</td>
<td></td>
<td>1 (5)/0 (0)</td>
<td>43 (15)</td>
<td>33(14)</td>
<td>-18.6 (-2.6)</td>
</tr>
</tbody>
</table>

<sup>A</sup> The approximate diffusion constant as calculated from the molecular weight using Stokes law; <sup>B</sup> #COOH and #NH₂ are the maximum number of negatively charged and positively charged groups in the primary sequence of the protein; <sup>C</sup> \(\sigma_w\) is the theoretical surface charge density on the protein.
The following table shows the derived data for delta E and K ads calculated according to the linear additivity model: ζ-potential [mV], ΔE_{el.st.} [kT], Hydrophobicity 1-[(NH₄)₂SO₄], ΔE_{hydr.} [kT], ΔE_{ads} [kT], K ads*D [m²/s]*10⁻¹², dΠ/dt (t=0), Π (t=2hrs), Π (t=2hrs), E_d [mN/m], Foam Volume (t=0; 0.01%), Foam volume (t=0; 0.05%).

<table>
<thead>
<tr>
<th>Protein</th>
<th>ζ-potential</th>
<th>ΔE_{el.st.}</th>
<th>Hydrophobicity</th>
<th>ΔE_{hydr.}</th>
<th>ΔE_{ads}</th>
<th>K ads*D</th>
<th>dΠ/dt (t=0)</th>
<th>Π (t=2hrs)</th>
<th>Π (t=2hrs)</th>
<th>E_d [mN/m]</th>
<th>Foam Volume (t=0; 0.01%)</th>
<th>Foam volume (t=0; 0.05%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosvitin</td>
<td>22.2</td>
<td>3.3</td>
<td>0.00</td>
<td>4.5</td>
<td>7.8</td>
<td>0.4</td>
<td>0.05</td>
<td>17</td>
<td>34</td>
<td>38.34</td>
<td>14.93</td>
<td>20.08</td>
</tr>
<tr>
<td>Glycinin</td>
<td>20.4</td>
<td>3.1</td>
<td>0.15</td>
<td>3.1</td>
<td>6.2</td>
<td>1.2</td>
<td>0.01</td>
<td>26</td>
<td>27</td>
<td>28.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipase A₂</td>
<td>8.4</td>
<td>1.3</td>
<td>0.00</td>
<td>4.5</td>
<td>5.8</td>
<td>4.0</td>
<td>0.62</td>
<td>26</td>
<td>121</td>
<td>38.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pea vicilin</td>
<td>17.7</td>
<td>2.7</td>
<td>0.15</td>
<td>3.1</td>
<td>5.8</td>
<td>2.0</td>
<td>0.06</td>
<td>24</td>
<td>67</td>
<td>20.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myoglobin</td>
<td>6.2</td>
<td>0.9</td>
<td>0.00</td>
<td>4.5</td>
<td>5.4</td>
<td>5.3</td>
<td>0.08</td>
<td>28</td>
<td>21</td>
<td>10.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome-C</td>
<td>3.7</td>
<td>0.6</td>
<td>0.00</td>
<td>4.5</td>
<td>5.1</td>
<td>8.7</td>
<td>0.03</td>
<td>25</td>
<td>25</td>
<td>11.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>17</td>
<td>2.6</td>
<td>0.25</td>
<td>2.2</td>
<td>4.8</td>
<td>8.3</td>
<td>0.01</td>
<td>22</td>
<td>77</td>
<td>5.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Amylase</td>
<td>14.2</td>
<td>2.1</td>
<td>0.28</td>
<td>1.9</td>
<td>4.0</td>
<td>15.9</td>
<td>0.01</td>
<td>16</td>
<td>33</td>
<td>4.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysozyme</td>
<td>3.8</td>
<td>0.6</td>
<td>0.21</td>
<td>2.5</td>
<td>3.1</td>
<td>59.6</td>
<td>0.01</td>
<td>29</td>
<td>22</td>
<td>5.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>14</td>
<td>2.1</td>
<td>0.38</td>
<td>0.9</td>
<td>3.0</td>
<td>48.0</td>
<td>0.66</td>
<td>21</td>
<td>76</td>
<td>39.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.9</td>
<td>0.1</td>
<td>0.31</td>
<td>1.6</td>
<td>1.7</td>
<td>202.2</td>
<td>0.46</td>
<td>25</td>
<td>45</td>
<td>31.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papain</td>
<td>1.3</td>
<td>0.2</td>
<td>0.36</td>
<td>1.1</td>
<td>1.3</td>
<td>309.2</td>
<td>0.01</td>
<td>16</td>
<td>52</td>
<td>18.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Lactalbumin</td>
<td>13.5</td>
<td>2.0</td>
<td>0.70</td>
<td>-2.2</td>
<td>-0.2</td>
<td>1518.4</td>
<td>0.15</td>
<td>20</td>
<td>46</td>
<td>29.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bromelaine</td>
<td>2.4</td>
<td>0.4</td>
<td>0.66</td>
<td>-1.8</td>
<td>-1.4</td>
<td>2160.0</td>
<td>0.05</td>
<td>22</td>
<td>51</td>
<td>26.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>14.1</td>
<td>2.1</td>
<td>0.80</td>
<td>-3.1</td>
<td>-1.0</td>
<td>4670.0</td>
<td>0.17</td>
<td>18</td>
<td>40</td>
<td>29.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patatin</td>
<td>18.7</td>
<td>2.8</td>
<td>0.99</td>
<td>-4.9</td>
<td>-2.1</td>
<td>6275.7</td>
<td>0.17</td>
<td>26</td>
<td>58</td>
<td>33.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pepsin</td>
<td>14</td>
<td>2.1</td>
<td>0.99</td>
<td>-4.9</td>
<td>-2.8</td>
<td>16792.4</td>
<td>0.25</td>
<td>20</td>
<td>42</td>
<td>34.72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
References


General Discussion

Does a colloidal approach suffice to describe protein surface layer formation at air-water interfaces?
In the general introduction to this thesis, the role of proteins in foam and emulsions was discussed. From this it was concluded that in order to understand this role, the contribution of protein molecular parameters to the interfacial functionality should be identified. Therefore, the basic aim of the presented work is to answer the question to what extent protein molecular functionalities determine the formation and functionality of adsorbed interfacial layers at the air-water interface. A secondary aim, with respect to the applicability of such knowledge, is of course to ascertain to what extent these properties are relevant in the formation and stabilization of a macroscopic system such as foam. In this chapter, we will present a discussion on these questions, including all findings presented in the preceding chapters together with some unpublished results and results obtained by other members of the group. We will start with the adsorption step where proteins adhere to the surface. Then the development of surface pressure as a result of interactions between adsorbed proteins, and rheological properties of the surface layer are discussed. Finally, these findings are put in perspective, based on a comparison of the foaming properties of twenty proteins, in relation to their molecular properties and measured surface behavior (surface pressure, etc).

8.1. Protein adsorption to air-water interfaces

8.1.1. Initial adsorption

The attachment of a freely diffusing protein from the bulk to the interface, thereby decreasing its diffusive mobility from 3 dimensions to only 2, is called adsorption. A thermodynamic interpretation of the process will conclude that the adsorption is driven by a gain in free energy of the system,\textsuperscript{1,2} basically relating the chemical potential of the protein in the bulk to that at the interface, and assuming equilibrium between these two potentials. However, both adsorption and desorption of proteins are found not to be simple equilibrium processes, but may be constrained by activation energy barriers, as will be demonstrated in this section.

In literature, the adsorption of a protein at an empty interface is assumed to occur instantaneously, without any limitations. However, the current work has shown that there is an energy barrier for protein adsorption, even at an empty interface. The presence of this barrier results in a lower effective adsorption rate of proteins. The height of this barrier
depends purely on the colloidal interaction between the protein, the interface and the two bulk phases (as discussed in chapter 2 and 3).

Increasing the net charge of the protein results in a higher repulsive interaction as the protein approaches the interface, thereby increasing the adsorption barrier. The major contribution to the repulsive interaction is deformation of the diffuse double layer, due to the fact that the air- or oil-phase have a low dielectric constant and contain no counter-ions (as discussed in chapter 3). Since oil and air have similar properties in this respect, the effect of the electrostatic barrier is expected to contribute to the barrier to adsorption at both types of interface.

The hydrophobic contribution is found to act in two ways. Firstly, if the hydrophobic exposure of a protein is too low, the difference in free energy between the adsorbed and dissolved state is not sufficient to keep the protein at the interface. Subsequently, the protein will diffuse back into the bulk resulting in a lower effective rate of adsorption. Secondly, high exposed hydrophobicity seems to counteract the electrostatic barrier. These effects are illustrated by the results shown in figure 8.1

![Figure 8.1](image-url)

**Figure 8.1.** Surface pressure as a function of time at air-water (A) and oil-water interface (B), for caprylated ovalbumin (Δ), non-modified ovalbumin (○) and succinylated ovalbumin (◇) at 0.1 mg/mL, 10mM phosphate buffer pH 7.0
In this figure, the development of surface pressure in time is given for three different proteins adsorbing to the air-water (panel A) or the oil-water interface (panel B). The proteins used are non-modified ovalbumin, caprylate d ovalbumin (having an increased exposed hydrophobicity) and succinylated ovalbumin (a variant with increased net charge).

In panel A the presence of a lag-time for non-modified ovalbumin is indicative of the decreased adsorption rate. The increased hydrophobic exposure of the caprylated variant results in an increase of the adsorption rate, visible in the faster increase of surface pressure. The succinylated ovalbumin shows an even slower development of $\Pi$, due to the increased net charge and subsequent increase of the adsorption barrier (as discussed in chapter 3).

However, at the oil-water interface (panel B), none of the curves show a lag-time. This means that the proteins adsorb so quickly that within seconds already sufficient protein has adsorbed to lead to an increase of $\Pi$. Other authors have also pointed at the difference in adsorption rate found at air-water and oil-water interfaces.\(^3\) The succinylated ovalbumin shows a slower increase of $\Pi$ and also a lower $\Pi$ at saturation of the layer. However, both effects might be due to the different relation between $\Pi$ and $\Gamma$ of non-modified and succinylated ovalbumin (this will be discussed later in this chapter).

The electrostatic contribution to the adsorption barrier depends on the diffuse double layer surrounding the adsorbing protein and is thus acts on a length scale in the order of 1-100 nanometer. Furthermore, this contribution is expected to be similar air- and oil-water interfaces. That the adsorption at the oil-water interface is found to be higher than at the air-water interface leads to the conclusion that the effect of the electrostatic barrier is affected by the hydrophobic properties of the interface. That the hydrophobic exposure of the protein itself is equally important is illustrated by the adsorption behaviour of caprylated ovalbumin at the air-water interface. A similar observation was made by by Damodaran et al.\(^4\) In this study, structural intermediates of bovine serum albumin were produced, that varied in exposed hydrophobicity. The adsorption rate was found to increase with hydrophobic exposure.

The intriguing part is that this hydrophobic contribution seems to compete with the electrostatic contribution, while the hydrophobic effect is commonly associated only with the gain in entropy when two hydrophobic compounds associate in an aqueous medium. This
means that the hydrophobic contribution would only be sensed at very short distances. At the moment there is little theory developed to describe the nature of the balance between hydrophobic and electrostatic interactions, and our experimental data proved to be too limited to provide a solid basis for a quantitative description of this effect.

Another important conclusion from this work is that apparently the colloidal interactions dominate the initial adsorption process. No evidence was found for a contribution to the adsorption that could be related to the unfolding of proteins, while such a contribution has been mentioned by several authors (as discussed in the introduction).

8.1.2. Adsorption at an occupied surface

As adsorption continues, the surface layer goes through several stages, which have been denoted as the gaseous, liquid and solid phase, depending on the amount of interactions between the adsorbed proteins and their lateral mobility.\(^3,^5\) In literature the description of the adsorption rate during the gaseous phase has often been assumed to be purely diffusion limited, as described by the Ward and Tordai function. To describe the rate of adsorption in the later stages, corrections to this model have been made to account for the ‘work against surface pressure’, or the ‘build up of surface wall potential’ due to the net charge of the adsorbing protein. Although these modifications have been applied by several authors, there are certain drawbacks, such as the presence of parameters that are difficult to confirm by other measurements. The apparent surface area of the adsorbing protein is such a fitting parameter, but calculations of this value using these models have commonly yielded values that are much smaller than the actual protein size.\(^4,^6-^8\)

A good alternative method to account for the filling of the adsorbed layer is given by the random-sequential adsorption (RSA) model. This model is based purely on a decrease of available (free) surface area due to the presence of previously adsorbed proteins. From the available surface area, a statistical chance can be calculated for an adsorbing particle to arrive at a location at the interface where enough surface area is available to allow adsorption.

The RSA model is based on hard particles, but as was shown in chapter 3, an increase in protein net charge resulted in an increase of the fitted apparent size of the adsorbed
molecules at the interface (see figure 3.4). The estimates of the apparent size gave values that agreed with the cross-sectional area of the proteins studied, and were found to increase with increasing net charge density of the protein. If this apparent size was used to calculate the interaction energy between two touching proteins, it was found that for all modifications a similar interaction energy was found. This shows that a ‘hard-particle’ approximation is valid for proteins with soft repulsive interactions. The importance of these repulsive interactions is illustrated by the fact that for proteins with increasing net charge the adsorption continued until the total interaction energy between adsorbed particles reached a typical value of 0.3 kT.

8.2. Reversibility of adsorption

While the adsorption is essential in the formation of the interfacial layer, the stability of the interface (under deformation) might be affected by desorption of the adsorbed proteins. Especially in a foam, drainage of liquid or shrinking of the bubble due to Oswald ripening could change the conditions of the interface in such a way that desorption might occur.

8.2.1. Desorption during static conditions

Desorption of adsorbed proteins has been studied by Fainerman et al.\textsuperscript{9} and Miller et al.\textsuperscript{10} They followed the surface pressure during adsorption for a certain amount of time after which the protein solution was replaced by clean buffer. Both articles show that at 20 °C no significant decrease in surface pressure is found when the bulk phase is thus depleted from protein. This illustrates that adsorption under static conditions is essentially irreversible. However, it was shown by Mackie et al.\textsuperscript{11,12} and Roth et al.\textsuperscript{13} that addition of surfactants to the bulk solution leads to formation of localized areas at the interface where only surfactants are present. Initially this leads to the condensation of the protein layer, followed by the formation of folds and wrinkles at the interface, rather than desorption of displaced protein molecules. Only at higher concentrations of the surfactant limited desorption was observed.
8.2.2. Desorption during compression of surface layers

As described above, desorption under static conditions is negligible. However, in foam the interface can be subjected to deformation. An example is the processes of Oswald ripening, one of the major processes that lead to instability of foam. During Oswald ripening, gas from an air bubble will go into solution in the surrounding liquid, causing a decrease of the interfacial area of the bubble. A theoretical study of the system has predicted that this process should be counteracted by a high elastic modulus of the surface layer.\(^{14}\) However, Dickinson et al. studied the dissolution of air bubbles stabilised by four different proteins and found that in all experiments the bubbles disappeared. This shows that the elastic modulus does not reach high enough values to counteract the shrinking process. They concluded that these findings should be attributed to desorption of the proteins as a result of the decreased interfacial area.

To study desorption under compression, several researchers have used surface pressure measurements during compression and expansion of adsorbed layers.\(^{9,15}\) The studies were able to show the existence of hysteresis in the response of surface pressure on compression expansion. However, the surface pressure does not give direct information on the adsorbed amount. Such information could, however, be obtained with a combination of Wilhelmy plate surface tension measurements and ellipsometry. We have performed such experiments with β-lactoglobulin, ovalbumin, succinylated ovalbumin and β-casein. Adsorbed layers were formed as described in the previous chapters, and proteins were allowed to adsorb during 2 hours. After this period the surface area was decreased from 192 to 40 cm\(^2\) (80% compression) by moving the barriers of the Langmuir trough at a speed of (0.2 cm\(^2\)/s. During the compression both the surface tension as the adsorbed amount of protein were measured at 10 s intervals. A theoretical adsorbed amount during compression (assuming that no desorption occurred) was calculated from the initial trough area and adsorbed amount before compression. The desorption behaviour of β-casein and succinylated ovalbumin is represented in figure 8.2.
In figure 8.2, markers are shown for the measured adsorbed amount, the lines indicate the theoretical increase in adsorbed amount (based on the surface area and the initial adsorbed amount. Clear differences in desorption behavior between the proteins is seen. During compression, the adsorbed amount of β-casein remains almost constant, indicating significant desorption. The total desorbed amount can be calculated from the theoretical and measured adsorbed amount after compression and is around 80 % for β-casein. For β-lactoglobulin and ovalbumin a different behavior is observed (results not shown). Although these latter proteins show some desorption, the total amount of desorbed protein at the end of the experiment is much less than for β-casein (20 and 10 % for and β-lactoglobulin and ovalbumin respectively).

In the field of adsorbed protein layers, desorption has often been related to protein size. From studies on the interfacial behavior of particles (e.g. glass), the formulas are given to calculated the energy needed for desorption. So, if the adsorbed protein is represented as a particle at the interface, the wetting angle ($\theta$) of the protein can be calculated from the surface tension between the three phases (air, water, protein) using Young’s equation:
A Colloidal Description of Adsorbed Protein Layers

\[ \cos(\theta) = \frac{\gamma_{\text{Protein-Air}} - \gamma_{\text{Protein-Water}}}{\gamma_{A-W}} \]  

(8.1)

Where the term \( \gamma_{\text{Protein-Air}} - \gamma_{\text{Protein-water}} \) can be interpreted as the transfer energy of the protein from air to water. This approach gives an interesting perspective, since this transfer energy may be related to the adsorption energy, or the energy gained upon dehydration of part of the protein surface upon adsorption. Inversely, the desorption energy would be related to the energy of (re-)hydration of the exposed part of the protein. The change in free energy depends on the area of contact between protein and water: \( A_{PW} = 2\pi R^2(1+\cos(\theta)) \), and the flat area of water missing: \( A_{MW} = \pi R^2(1-\cos^2(\theta)) \). Subsequently, the energy \( (U) \) for removing a particle from the interface to the air can be calculated as:

\[ U = 2\pi R^2(1+\cos(\theta))(\gamma_{PA} - \gamma_{PW}) + \pi R^2(1-\cos^2(\theta))\gamma_{AW} \]  

(8.2)

Which can be rewritten using equation 8.1 as:

\[ U = \pi R^2\gamma_{AW}(1+\cos^2(\theta)) \]  

(8.3)

As can be seen, the larger the protein, the more energy would be needed to remove the protein from the interface. However, the difference in protein size between β-lactoglobulin and β-casein is not sufficient to explain the difference in desorption behavior. Another factor that might be expected to promote desorption is an increased net charge and subsequent repulsion between adsorbed proteins. However, results obtained with succinylated ovalbumin showed that this protein was as tightly adsorbed as the non-modified ovalbumin (figure 8.2, 8 % desorption).

An alternative, plausible explanation for the high desorption found for β-casein, is based on the characteristic of β-casein that it can form micelles. This association behavior has been described in bulk solutions, but may also affect its interfacial properties. Apparently, as the interface is compressed, the protein is pushed into formation of micelles that can easily
desorb from the interface, similar to the behavior of low molecular weight surfactants. In this way the activation energy for desorption would be decreased.

Except for β-casein, upon compression the adsorbed amount reaches values higher than calculated for saturated monolayers. Using infrared reflection adsorption spectroscopy (IRRAS) it could be shown that for ovalbumin significant aggregation resulted from the compression (see figure 8.3).24 This interfacial aggregation is indicated by the formation of anti-parallel β-sheets, and has also been observed by refs. 25-27. Also in the case of compression of monolayers of particles it has been shown that these particles form aggregated systems upon compression.28,29

![Figure 8.3. Correlation between the degree of aggregation of ovalbumin at the air/water interface as deduced from IRRAS spectra (from Kudryashova et al.)30, determined by the fraction of anti-parallel β-sheet (sharp intensity at 1624 cm⁻¹ in IRRAS spectra) relative to that present in thermo-aggregated ovalbumin (squares) and the surface pressure as determined simultaneously using a Wilhelmy balance (dashed line). The ovalbumin bulk concentration is 0.1 mg/mL.](image)

Apparently, the compression pushes the adsorbed species through the repulsive barrier that prevents aggregation under static conditions. The resulting aggregation is not only in localized clusters, but involves all adsorbed particles. Simulations using Brownian and molecular dynamics show similar behavior.31,32 Upon expansion of these compressed layers, typically fracture behavior is observed. Such behavior was described by Hotrum et al. in a study of spreading of oil droplets at the air-water interface.33 In this study first a spread protein layer was prepared, which was subsequently compressed to reach a surface load of
approximately 6 mg/m$^2$. A small amount of emulsion droplets was then inserted under the interface, after which the interface was expanded. Upon expansion macroscopic fractures were observed, which were the locations where oil-droplets were found to adsorb and spread. Similar experiments on the compression and subsequent expansion of the interfacial layer were performed by Kudryashova et al.$^{24}$ Aggregation was observed by IRRAS in the compressed layer, but upon expansion the amount of aggregates slowly decreased in time (in a time-scale of hours), showing that the aggregation is reversible. We therefore suggest that fraction behavior is not a property inherent to adsorbed protein layers formed under quiescent conditions. Rather, the observation of such fracture behavior should be regarded as an indication that the observed interface has been subjected to compression.

8.3. *Interfacial unfolding*

In literature interfacial unfolding of proteins has been related to different stages of protein adsorption (as discussed in the introduction). However, in chapter 4 we have given an overview of literature that shows that for most globular proteins the adsorbed conformation is not much different from that in the bulk. Only by decreasing the rate of adsorption, for proteins with fast unfolding kinetics (in the order of milliseconds to seconds) the conformational state of adsorbed proteins was found to change, as illustrated by a shift in the Π-Γ curve. Even after saturation was reached (even after 2 hours), the difference in the Π-Γ relation remained. This indicates that the conformational transition that leads to the shift in the Π-Γ curve is not readily reversed.

8.4. *Surface functional properties*

In the previous section, the adsorption of proteins is discussed, since it is a first requirement for the formation of an interfacial layer that can help to form and stabilise foamed and emulsified systems. The second requirement is that the protein adsorption increases surface pressure. Furthermore, the way in which the surface pressure responds to deformation of the interface is important. The changes in these mesoscopic characteristics of the interface as a result of adsorption will be discussed in this section.
8.4.1. Development of surface pressure

In the introduction a short overview was given on the models that have been used to describe the relation between the adsorbed amount of protein and the surface pressure. In general two types of models can be distinguished. The models based on a polymeric description, assume that the protein chain is adsorbed via attachment of different segments. These segments can then be adsorbed or desorbed, depending on the interfacial pressure. However, as shown above, most adsorbed proteins will not resemble a flexible polymer chain. Therefore, it seems reasonable that the basis of the model should assume that the protein is a hard particle. Application of the theories based on this assumption yield results where the surface pressure increases too sharply and reaches too high values. So, an adjustment of these models is needed. A good indication of the parameters needed in the model can be obtained from chapter 3, where the Π-Γ relationship for ovalbumin variants with increasing net charge was studied. In figure 8.4 the results of these studies are plotted in two ways. The first approach is the basic plot of Π versus Γ, where it is observed that at higher net charge (e.g. line 5) the surface pressure at any surface load is higher than for proteins with less net charge (e.g. line 0). However, in this chapter it was also observed that an increase of the protein net charge also resulted in an increase of its apparent size (Ω). For all variants shown, the apparent size (calculations are discussed in chapter 3) was used to calculate the fractional surface coverage (θ=ΓΩ). The resulting plot of Π-Ω is shown in figure 8.4B.

![Figure 8.4](image)

**Figure 8.4.** Surface pressure as a function of the adsorbed amount (A) or the fractional surface coverage θ (B); lines 1-5 represent modified variants of ovalbumin (line 0) with increasing net charge (data from figure 3.6)

It is striking to observe how all curves from figure 8.4A fall on an identical curve in figure 8.4B. This shows that apparently the effective radius that is predicted from the RSA adsorption model for the variants with higher net charge can be used as the effective radius of a ‘hard particle’. However, this still doesn’t answer the question how the Π-θ relation should
be described. Our proposition is that the proteins should be considered as hard-particles with soft repulsive interactions. These repulsive interactions are the result of the net charge on the protein. The repulsive interaction between two particles can be easily calculated. However, as saturation is reached, the proteins will be so close together that multiple diffuse double layers will overlap. The interaction energy calculated between adsorbed proteins at saturation was found to be constant (0.3 kT) for all variants. We have not found analytical solutions for the calculation of the electrostatic repulsion covering the whole adsorption process.

8.4.2. Dilatational rheology

Typical experiments to measure the dilatational rheology of adsorbed layers use expansion/compression of maximally 10 % of the total interfacial area, within a time scale of seconds. This situation is not comparable to the compression of the interface discussed in section 8.2.2, since in this situation the amount of compression is much less and proceeds much faster. The desorption studies performed and described in section 8.2.2 showed only limited desorption, even after 80% compression. From this it may be concluded that during dilatational experiments no desorption of proteins will occur. Adsorption of proteins during the sinusoidal deformation in dilatational experiments will also not be negligible, based on two reasons. In the case of fast adsorption, the interface will quickly reach saturation, resulting in a small amount of available surface area, even after 10 % expansion. In the case of slow adsorption, the rate of expansion and expression will be faster than the adsorption. Therefore in both cases no significant adsorption during deformation is expected.

Another factor which has been commonly used in the interpretation of results from dilatational rheological experiments is the change of protein conformation (or adsorption and desorption of segments). The observations presented in chapter 4 have shown that the protein conformation is not reversible, so no significant changes in the tertiary structure are expected to occur during either expansion or compression.

Based on the above, we conclude that the dilatational data reflect the \( \Pi - \Gamma \) relation. Evidence in support of this statement is presented in figure 3.8. Further prove can be obtained if results from for example ellipsometric studies of the \( \Pi - \Gamma \) curve were used to calculate the dilatational modulus of the systems under study. In this way, the observed dilatational modulus can be compared with the calculated value. A deviation between these two values would indicate that there is a contribution to the surface pressure during the dilatational experiments, which is absent during measurement of the \( \Pi - \Gamma \) curve.
8.4.3. **Shear rheology**

The surface shear behaviour of adsorbed protein layers has long been interpreted in analogy to three-dimensional gelled systems. Such systems are formed by associative interactions between proteins that lead to aggregation. If the size and concentration of these aggregates is sufficient, the interactions between aggregates will lead to the formation of a continuous space-filling network. Typically, this network has also the ability to ‘support itself’. However, such a description does not fit our observations on adsorbed protein layers.

Proteins adsorb at the interface due to the affinity between the protein and the interface, rather than the affinity between proteins. This means that even though the concentration of proteins at the interface is high (100-200 mg/mL) the conditions are such that there is a net repulsive interaction between adsorbed proteins. This net repulsive interaction will prevent aggregation and subsequent formation of a continuous network. Further evidence for the free mobility of adsorbed proteins is found by fluorescence correlation spectroscopy (FCS). As discussed in chapter 6 the translational mobility is still measurable for adsorbed proteins, while such mobility is not found in bulk gels of ovalbumin. A second important observation is that even after introduction of reactive sulfhydryl groups no increase of the surface shear rheology was measured (chapter 5), while the presence of these groups is expected to strengthen any intermolecular bonds formed. Therefore, we propose that the surface shear rheology of adsorbed layers should be described as the behaviour of a densely packed system of freely movable proteins.

In contrast to adsorbed layers formed under static conditions, evidence has been found for the gelation of interfacial protein layers upon compression of the interface (chapter 5 and 6). This contrast may be important in relating the observations of shear rheology (under static conditions) to the characteristics of the interfacial layers in foam and emulsions. If whipping or homogenization is applied, typically large stresses are applied to the (newly formed) interfacial layers.

8.5. **Relating protein surface functionality to foaming behavior**

In the previous paragraphs we have described how the molecular properties affect the adsorption behavior and surface functional properties. The challenge of such research is of
course to see the extent to which such knowledge can be extrapolated in the description of macroscopic systems such as foam.

8.5.1 Foam formation

There are two basic techniques to produce foam: whipping and sparging. During whipping the air is incorporated into the aqueous phase at high speed, resulting in high stresses on the newly formed interface. In contrast, the sparging (which resembles the rise of gas bubbles in a beer) is a very mild process, where the air is introduced in the solution through a porous medium. It may be anticipated that the transport of proteins to the interface during sparing reflects the adsorption at a static air-water interface. The highly dynamic conditions during whipping do not reflect such conditions. However, the results presented in chapter 7 show that even during whipping there is a clear difference in the ability of proteins to adsorb at the newly interface in the time that the interface is formed. This difference is reflected in the minimum concentration needed to produce foam. Furthermore, it was shown that the kinetic barrier to adsorption that was determined under static conditions correlated (quantitatively) with the concentration at which foam was formed. In figure 8.5 this is illustrated by the foam formed after whipping non-modified ovalbumin (a kinetic barrier of around 4.7 kT) and caprylated ovalbumin (0.01 kT). It was found that a concentration of 10 mg/mL was needed under these conditions to produce stable foam with the non-modified ovalbumin, while the caprylated ovalbumin produced foam at 0.1 mg/mL.
These results show that the adsorption as studied under static conditions can be used to predict the foam forming capacity. However, as discussed in chapter 7, the relation between these two parameters is only qualitative at the moment. From the above, it may be clear that for sparging we also expect a major influence of the kinetic barrier to adsorption on the foaming behavior of proteins.
8.5.2 Foam stability

Foam stability is mainly determined by the processes of coalescence and Oswald ripening, since the other two instability processes (drainage and creaming) do not directly affect the amount, the size or size-distribution of gas bubbles. The Oswald ripening process results in shrinking of gas bubbles due to dissolution of gas, resulting in compression of the interfacial layer. In section 8.4.2 it was already discussed that such compression is expected to lead to an increase of the elastic modulus of the interface, which could eventually stabilise the interface against further shrinking. However, experiment by Dickinson et al.\textsuperscript{35} have shown that for four different proteins no such stabilisation was observed. Apparently, there is still desorption of proteins, even after the protein layer is compressed, which would result in at least in some aggregation (as shown in chapter 6). The question then arises, how and if Oswald ripening can be stopped, since even aggregation of the proteins is not sufficient to prevent desorption. A good indication of the answer is given by Hellebust.\textsuperscript{36} In this work, a description is given of the production of gas bubbles stabilised by human serum albumin. These gas bubbles are stable over time periods of several days and have found a wide spread application as an ultrasound contrast agent, that can be used in medical research (e.g. acoustic cardiography). Such bubbles are formed after sonification, and heat treatment. In other work it was shown that sonication leads to the formation of anti-parallel $\beta$-sheets, which are indicative of aggregation. These results can be taken as a strong indication that the compression under mild conditions (Oswald ripening) only leads to aggregation in what is called the ‘secondary’ minimum. These aggregates can then still dissociate in time (as demonstrated by Kudryashova et al.)\textsuperscript{24}. Stable aggregate formation at the interface can be obtained by heating the proteins, and apparently such interfacial layers resist shrinking.

In addition to dilational rheology, also shear rheological behavior has been mentioned as an important factor in the stabilization of foam. Interestingly, aggregation of proteins as a result of heat treatment has been shown to lead to dramatic increase of the shear elastic modulus of adsorbed layers.\textsuperscript{13} From this, the conclusion can be drawn that although proteins can already stabilize the interface under non-denaturing circumstances, subsequent heating of the adsorbed protein layer increases its stability.
**Putting the scientific results in an industrial perspective**

We feel that with the presented work, we have provided a coherent description of the different phenomena related to adsorbed protein layers, based on protein molecular properties. In general, this will enable the industry to understand results obtained either from published work, or from own experience. However, a specific discussion of the relevance is hindered by the diverse nature of industrial situations in which foam formation is important. For certain applications such as food, the foam formation is wanted. On the other hand, many different industrial processes are hindered by unwanted foam formation, for example wastewater treatment. In a recent paper by Pelton, the relevance of anti-foam agents in industry is discussed (as an indication, it is mentioned that a large kraft pulp mill will spend in excess of a million dollars per year on anti-foaming agents).  

With respect to applications in food industry, it is important to note that there are only two main sources of proteins that are used as additives to affect interfacial processes. These sources are egg white proteins and whey proteins. However, many different commercial preparations are available of these proteins. These preparations differ in the purity of the protein (both protein heterogeneity and presence of non-protein compounds) and in the way these preparations are processed (heating, spray drying, etc.). This means that in terms of protein molecular parameters there is not much difference between the preparations, but that the composition and treatment can be tuned to the application in which the preparation should be applied.

Based on the presented work, several principles of the role of proteins have been identified, that can be used as guidelines in controlling foam formation and stability. For example, the spray-drying of proteins can lead to denaturation, which increases the protein exposed hydrophobicity, which will result in increased adsorption kinetics. The pH and ionic strength of the solutions will be also be important in adsorption kinetics, but mainly in the surface pressure that results from adsorption, since these parameters determine the electrostatic interactions.
Conclusion

When all results are taken into account, the general picture emerges that to understand the role of proteins in interfacial phenomena, the protein may be considered as a colloidal particle. In contrast with existing literature, this would suggest that although the protein structure is complex, the details of this structure are not necessary for a satisfying description of its interfacial behavior. The only effect of intrinsic protein characteristics was found in the interfacial unfolding that may occur if the adsorption kinetics is slower than the kinetics of unfolding. Other phenomena, ranging from initial adsorption to the development of surface shear elasticity were found to be determined by the colloidal interactions between the proteins and the interface.

We feel that the presented work provides a coherent description of the interfacial properties of proteins. This description could be based on observed molecular parameters, of which the hydrophobicity and net charge density were the most important.

References


Proteins readily adsorb from solution to many different types of interfaces. Of special importance is the adsorption at liquid (air-water or oil-water) interfaces, since this governs the formation and stabilisation of emulsions and foam. In these systems the function of proteins is to stabilize the interface, which can be described by parameters such as the surface pressure, surface rheology, etc. To predict the formation and stabilization of foam and emulsions, it is necessary to distinguish the contributions of system parameters (e.g. pH, T) from interfacial properties (e.g. solid, liquid, charged or not) and from protein functionalities (e.g. charge, folding stability).

In current literature, the processes and phenomena related to surface functionality are described with different concepts. The adsorption behavior of proteins is often interpreted as a process where the folding state changes from a globular (dissolved) to a ‘loop-train’ (adsorbed) conformation. For the description of the development of surface pressure some authors use this ‘loop-train’ model (soft-particle), while others use a hard particle model. And the surface shear rheology is often seen as the result of the formation of a two-dimensional gelled layer at the interface. The disadvantage of such different explanations for observed phenomena is that they are difficult to combine in one complete conceptual model. Therefore the research performed in this thesis was aimed at providing an understanding of the mesoscopic parameters used in the description of adsorbed layers (adsorption rate, surface pressure, etc.). We further aim to use measured chemical and physical properties of the proteins as the basis of this understanding. To this end, chemical modification has been used in this work to specifically alter one molecular parameter of a protein (such as exposed hydrophobicity, net charge or chemical reactivity). With this technique different sets of modified protein variants could be obtained, yielding proteins with identical secondary and tertiary structure, but varying values for the targeted molecular parameter (for instance net charge). The modified proteins were thoroughly characterized with respect to chemical and
physical properties, to assure that apart from the selected property the modified protein was similar to the unmodified protein. Subsequently, the surface functional characteristics of the modified proteins were determined, as will be described in the next sections.

**Exposed hydrophobicity**

The role of exposed hydrophobicity on the kinetics of protein adsorption to the air-water interface is studied in chapter 2. The exposed hydrophobicity of ovalbumin was increased by covalent coupling of capric acid to the lysine residues of the protein. No change in the molecular structure of ovalbumin was observed upon caprylation. The exposed hydrophobicity of a monomeric caprylated ovalbumin (with an average of three capryl groups per protein) and non-modified ovalbumin was measured using 8-anilino-1-naphtelenesulfonic acid (ANSA) fluorescence. The exposed hydrophobicity of the monomeric fraction was significantly higher than the non-modified protein.

Adsorption kinetics of both samples was studied by measuring the increase in surface load ($\Gamma$) and in surface pressure ($\Pi$) as a function of time ($t$) using an ellipsometer and a Wilhelmy plate respectively. For non-modified ovalbumin the increase of surface load in time (even at low surface coverage) is much lower than the value that was calculated from diffusional transport, showing that the process is limited by an energy barrier for adsorption. In contrast, no significant effect of an energy barrier was detected in the adsorption kinetics of caprylated protein. The surface pressure at a certain surface load ($\Pi-\Gamma$) was not affected by the modification, indicating that the effect of increased hydrophobicity is limited to the adsorption process.

**Net charge**

In chapter 3 a set of five chemically engineered variants of ovalbumin (with $\zeta$-potentials ranging from -19 to -26 mV) was produced using succinylation. These variants showed no changes in secondary, tertiary or quaternary structure compared to the non-modified protein. It was found that the rate of adsorption (measured with ellipsometry) decreased with increasing net charge, even at the initial stages of adsorption. Using a model
based on the random sequential adsorption model, it was calculated that the energy barrier to adsorption (already mentioned in chapter 3) increased from 4.7 kT to 6.1 kT when the protein net charge was increased from –12 to –26.

A second effect was that the increased electrostatic repulsion resulted in a larger apparent size of the adsorbed proteins, which went from 19 nm\(^2\) to 31 nm\(^2\) (non-modified and highest modification respectively). The larger apparent size of the proteins resulted in an increase of the surface pressure at certain \(\Gamma\), and thus a shift of the \(\Pi-\Gamma\) curve. It was further found that saturation of the monolayer was reached at lower values of \(\Gamma\). The distance between adsorbed proteins at saturation was found to correspond to similar interaction energies between adsorbed proteins.

Interfacial unfolding

Chapter 4 focuses on the question whether the folding state of adsorbed proteins depend on the rate of adsorption to the interface. By changing the bulk concentration, the adsorption rate can be controlled. The adsorption of proteins with varying structural stabilities at several protein concentrations was studied using ellipsometry and surface tensiometry. For \(\beta\)-lactoglobulin the adsorbed amount needed to reach a certain surface-pressure decreased with decreasing bulk concentration. Ovalbumin showed no such dependence. Further experiments with cytochrome \(c\) and a destabilized variant of this protein showed that both proteins showed identical \(\Pi-\Gamma\) curves and did not show any dependence on bulk concentration. Since the electrostatic contribution to surface pressure (as discussed in chapter 3) does not depend on the bulk concentration, the shift in the \(\Pi-\Gamma\) curve indicates an increase in the apparent size of \(\beta\)-lg as a result of (partial) unfolding of the protein. From this work it is concluded that unfolding will only take place if the kinetics of adsorption is similar or slower than the kinetics of unfolding. The kinetics of unfolding depends on the activation energy of unfolding (which is in the order of 100-300 kJ/mole), rather than the free energy of unfolding (typically in the order of 10-50 kJ/mole). It was further shown that any unfolding during initial stages of adsorption is not reversed at later times, showing that there is no equilibrium between adsorbed protein conformation and the surface pressure.
**Chemical reactivity**

To test whether the surface shear rheology of adsorbed layers is due to the formation of a continuous gel-like network, or the low mobility of the densely packed proteins, the role of disulfide bridges in interfacial layers is studied in chapter 5. Disulfide bridge formation increases the gel strength of bulk gels formed upon heat treatment of protein solutions, but will not occur if the proteins are not associated. Ovalbumin was thiolated with S-AMSA, followed by removal of the acetylblock on the sulphur atom, resulting in respectively blocked (SX) and deblocked (SH) ovalbumin variants. The presence and reactivity of the introduced, deblocked sulfhydryl groups was confirmed using the sulfhydryl-disulfide exchange index (SEI). Despite the reactivity of the introduced sulfhydryl groups measured in solution, no increase in the surface shear elasticity could be detected with increasing reactivity. This indicates that physical rather than chemical interactions determine the surface shear behaviour. In view of these results, it was concluded that the adsorbed layer should not be considered a gelled network of aggregated material (in analogy with three-dimensional gels formed from heating protein solutions). Rather, it would appear that the adsorbed proteins form a highly packed system of proteins with net-repulsive interactions.

In chapter 6, a short review is given on the transition of the state of the adsorbed layer from a densely packed system to a continuous network. This transition is found to occur as a result of compression of the interface and results in fracture behaviour of the interfacial layer.

**From model systems to foam**

In chapter 7, a set of experiments is presented, where the molecular parameters and surface functional properties (surface pressure and dilatational modulus) are determined for a series of proteins. Furthermore, foaming experiments were performed to assess the foam forming capacity and the foam stability of these proteins. First, a prediction was made of the adsorption constant, based on the hydrophobic exposure and net charge density. The contribution of these two parameters to the total adsorption barrier was calculated using data from chapter 2 and 3, under the assumption that both contributions were additive. The results shows that 7 out of 20 proteins did not form foam at a concentration of 0.01 %, but only at 0.05 %. This distinction was also reflected by the calculated adsorption rate ($k_{\text{adsorb}}*D_b$); for the poor foaming proteins this value was $< 50$, while for the other proteins this value was higher. The foam forming capacity was also found to correlate with the initial increase of surface pressure. This correlation was quantitative ($R^2=0.78$), while the correlation with $k_{\text{adsorb}}$
was poor ($R^2<0.1$). For foam stability, no correlation was found with any of the measured parameters ($R^2<0.1$), not even with the dilatational modulus.

This work illustrates that foam formation is limited by the same kinetic barrier as the adsorption at static planar air-water interfaces.
Eiwitten adsorberen vanuit oplossing aan verschillende typen oppervlakken. De adsorptie aan vloeibare (lucht-water of olie-water) grensvlakken is van bijzonder belang, omdat dit de vorming en stabilisatie van emulsies en schuim bepaalt. De functie van eiwitten in deze systemen is het stabiliseren van het grensvlak; deze stabilisatie kan worden beschreven met parameters als de oppervlakte druk, oppervlakte reology, etc. Om de vorming en stabilisatie van schuim en emulsies te kunnen voorspellen is het noodzakelijk om onderscheid te maken in de bijdragen van systeem parameters (zoals pH, T), grensvlak parameters (zoals vast, vloeibaar, geladen of niet) en eiwitfunctionaliteiten (zoals lading, structuurstabiliteit).

In de huidige literatuur worden de processen en verschijnselen met betrekking op oppervlakte functionaliteit beschreven met verschillende denkbeelden. Het adsorptie gedrag van eiwitten wordt vaak geïnterpreteerd als een proces waarbij de vouwings-staat van het eiwit overgaat van een globulaire (in oplossing) naar een ontvouwen of ‘loop-train’ (geadsorbeerde) conformatie. Voor de beschrijving van de ontwikkeling van oppervlakte druk gebruiken sommige auteurs dit ‘loop-train’ model (soft-particle), terwijl anderen een hard-particle model gebruiken. Verder wordt de oppervlakte shear reology vaak gezien als een resultaat van de vorming van een twee-dimensionaal gegeleerde laag aan het grensvlak. Het nadeel van zulke diverse verklaringen voor de waargenomen verschijnselen is dat ze moeilijk een compleet conceptueel model te verenigen zijn. Daarom is het onderzoek in dit proefschrift gericht op het vormen van begrip van de mesoscopische parameters die gebruikt worden in de beschrijving van geadsorbeerde lagen (adsorptie snelheid, oppervlakte druk, etc.). Daarnaast willen we dit begrip baseren op gemeten waarden van de chemische en fysische eigenschappen van de eiwitten. Om dit doel te bereiken zijn eiwitten chemisch gemodificeerd, om specifiek één moleculaire parameter van een eiwit te veranderen (zoals geëxposeerde hydrophobicititeit, netto lading of chemische reactiviteit). Met behulp van deze techniek zijn verschillende groepen van gemodificeerde eiwit varianten gemaakt, die bestaan
uit eiwitten met identieke secondaire en tertiaire structuur, maar verschillende waardes voor de geselecteerde moleculeaire parameter (bijvoorbeeld electrostatische lading). De chemische en physische eigenschappen van de gemodificeerde eiwitten werden uitgebreid onderzocht, om er zeker van te kunnen zijn dat behalve de geselecteerde eigenschap het gemodificeerde eiwit gelijk was aan het niet-gemodificeerde eiwit. Vervolgens zijn de oppervlakte functionele eigenschappen van de gemodificeerde eiwitten bepaald, zoals in de volgende secties beschreven wordt.

**Geëxposeerde hydrophobiciteit**

De rol van geëxposeerde hydrophobiciteit in de adsorptie kinetiek van eiwit adsorptie aan het lucht-water grensvlak is onderwerp van hoofdstuk 2. De geëxposeerde hydrophobiciteit van ovalbumine was vergroot door covalente binding van caprylzuur aan de lysine residuen van het eiwit. Er werd geen verschil in de globulaire structuur van ovalbumine waargenomen na caprylatie. De geëxposeerde hydrophobiciteit van een monomeer gecapryleerd ovalbumine (met gemiddeld drie capryl groepen per eiwit) en niet-gemodificeerd ovalbumine was gemeten met 8-anilino-1-naphtelenesulfonzuur (ANS) fluorescentie. De gemeten waarde voor was significant hoger voor het gomodificeerd dan het niet-gemodificeerde eiwit.

Van beide monsters was de adsorptie kinetiek bepaald door het meten van de toename in geadsorbeerde hoeveelheid ($\Gamma$) en in oppervlakte druk ($\Pi$) als een functie van de tijd (t) met behulp van respectievelijk een ellipsometer en een Wilhelmy plaat. Voor niet-gemodificeerd ovalbumine is de toename van de geadsorbeerde hoeveelheid in de tijd (zelf bij lage bezettingsgraad) veel lager dan de waarde die was berekend op basis van diffusie transport, wat laat zien dat dit proces beperkt wordt door een energie barrière voor adsorptie. Daarentegen werd geen significante bijdrage van een energie barrière bepaald voor de adsorptie kinetiek van gecapryleerd eiwit. De relatie tussen de oppervlakte spanning en de oppervlakte belading ($\Pi-\Gamma$) was niet beïnvloed door de modificatie, wat er op wijst dat het effect van de verhoogde geëxposeerde hydrophobiciteit beperkt is tot het adsorptie proces.
**Netto lading**

In hoofdstuk 3 is een verzameling van vijf chemisch gemodificeerde varianten van ovalbumine geproduceerd (met ζ-potentialen variëren varierend van -19 tot –26 mV) middels succinylatie. Voor deze varianten werden geen veranderingen in secundaire, tertiaire of quartenaire structuur waargenomen ten opzichte van het niet-gemodificeerde eiwit. Wel werd er gevonden dat de snelheid van adsorptie (zoals gemeten met ellipsometrie) daalde met verhoging van de netto lading, zelf in het initiële stadium van adsorptie. Met behulp van een model dat gebaseerd is op ‘random sequential adsorption’, werd geconcludeerd dat de energetische barrière voor adsorptie (al genoemd in hoofdstuk 3) steeg van 4.7 kT tot 6.1 kT als de netto lading op het eiwit werd verhoogd van –12 tot –26.

Een tweede effect, was dat de verhoogde electrostatische repulsion leidde tot een stijging van de effectieve (of: schijnbare) grootte van de geadsorbeerde eiwitten, de waarde steeg van 19 nm² tot 31 nm² (niet-gemodificeerd en de hoogste modificatie respectievelijk). Deze grotere schijnbare grootte van de eiwitten resulteerde in een verhoging van de oppervlakte druk bij bepaalde Γ, en daarmee een verschuiving van de Π-Γ curve. Verder werd er waargenomen dat de verzadiging van de monolaag werd bereikt bij lagere waardes voor Γ. De afstand tussen geadsorbeerde eiwitten bij verzadiging bleek overeen te komen met een gelijke interactie energie tussen geadsorbeerde eiwitten.

**Oppervlakte ontvouwing**

Hoofdstuk 4 is gericht op de vraag of de vouwings staat van geadsorbeerde eiwitten afhangt van de snelheid van adsorptie aan het grensvlak. Door de bulk concentratie te veranderen kan de snelheid van adsorptie worden gecontroleerd. De adsorptie van eiwitten met verschillende structuur stabiliteit bij verschillende eiwit concentraties ellipsometry werd bepaald middels oppervlakte spanningsmetingen en ellipsometrie. Voor β-lactoglobulin daalde de hoeveelheid geadsorbeerd materiaal die nodig was om een bepaalde oppervlakte druk te bereiken, met een daling van de bulk concentratie. Deze afhankelijkheid werd niet gemeten voor ovalbumine. Verdere experimenten met cytochrome c en een gedestabiliseerde variant van dit eiwit, lieten gelijke Π-Γ curves zien voor beide eiwitten, die ook niet afhankelijk was van de bulk concentratie. Aangezien de electrostatische bijdrage aan de oppervlakte druk (zoals besproken in hoofdstuk 3) niet afhangt van de bulk concentratie, moet de verschuiving van de Π-Γ curve
duiden op een verhoging van de effectieve grote van β-lg als gevolg van (gedeeltelijke) ontvouwing van het eiwit. Uit dit werk werd geconcludeerd dat ontvouwing slechts optreed als de kinetiek van adsorptie gelijk of langzamer is dan de kinetiek van adsorptie. De kinetiek van ontvouwing werd bepaald door de activatie energie voor ontvouwing (met een typische waarde van 100-300 kJ/mole), en niet zozeer door de vrije energie van ontvouwing (typisch in de orde van 10-50 kJ/mole). Verder werd duidelijk dat voor zover ontvouwing plaats heeft in de initiële stadia van adsorptie, deze ontvouwing niet ongedaan wordt gemaakt op latere tijdstippen, wat laat zien dat er geen evenwicht is tussen de conformatie van geadsorbeerde eiwitten en de oppervlakte druk.

Chemische reactiviteit

Om te testen of de oppervlakte shear reology van geadsorbeerde lagen het gevolg is van de vorming van een continu gel-type netwerk, of door de lage mobiliteit van een eiwitten in een dichte pakking, is in hoofdstuk 5 de rol van disulfide bruggen in geadsorbeerde lagen bestudeerd. In gelen die verkregen zijn door het verhitten van eiwit oplossingen is aangetoond dat disulfide bruggen de gelsterkte verhogen, maar disulfide bruggen worden niet gevormd als de eiwitten niet geassocieerd zijn. Ovalbumine was gethiooleerd met S-AMSA, gevolgd door verwijdering van de acetylblokkade op het zwavel atoom, was resulteerd in respectievelijk geblokkeerde (SX) en gedeblokkte (SH) ovalbumine varianten. De aanwezigheid en reactiviteit van de geïntroduceerde, gedeblokkeerde sulfhydryl groepen was bevestigd door meting van de sulfhydryl-disulfide uitwisselingsindex (SEI). Ondanks de reactiviteit van de geïntroduceerde sulfhydryl groepen, zoals gemeten in bulk oplossing, kon er geen verhoging van de surface shear elasticiteit gedetecteerd. Dit is een indicatie dat het surface shear gedrag eerder wordt bepaald door fysische dan door chemische interacties. In het licht van deze resultaten werd geconcludeerd dat de geadsorbeerde laag niet moet worden gezien als een gegeleerd netwerk van geaggregeerde eiwitten (in analogy aan drie dimensionale gels gevormd door verhitting van eiwit oplossingen). Het lijkt er eerder op dat de geadsorbeerde eiwitten verkeren in een glas-achtige toestand, waarbij een hoge pakkingsdichtheid wordt bereikt van deeltjes met onderlinge repulsie.

In hoofdstuk 6 wordt een kort overzicht gegeven van de overgang van de staat van de geadsorbeerde laag, van een dicht gepakt systeem naar een continu netwerk. Deze overgang
Van modelsystemen naar schuim

In hoofdstuk 7 worden de resultaten gepresenteerd van een verzameling experimenten, waarbij de moleculaire parameters en oppervlakte functionele eigenschappen (oppervlakte druk en dilatatie modulus) van een aantal eiwitten zijn bepaald. Aansluitend zijn schuimexperimenten uitgevoerd om de schuimvormende en schuimstabiliserende eigenschappen van deze eiwitten te beoordelen.

Als eerste werd op basis van de gemeten geëxposeerde hydrophobiciteit en de netto ladingsdichtheid een voorspelling gedaan van de adsorptie constante. De bijdrage van deze twee parameters aan de totale adsorptie barrière werd berekend met gebruikmaking van de data uit hoofdstukken 2 en 3, onder de aanname dat beide bijdragen additief zijn. De berekende waardes van de adsorptie snelheid ($k_{\text{adsorb}} \cdot D_h$) bleek een relatie te hebben met de schuimvormende eigenschappen van de eiwiten. De resultaten van de schuimproef laten zien dat 7 van de 20 eiwiten geen schuim vormden bij concentraties van 0.01%, maar alleen bij de hogere concentratie van 0.05%. Deze slecht schuimvormende eiwitten hadden een berekende adsorptie snelheid van $< 50$, terwijl deze waarde hoger was voor de andere eiwiten. De schuimvormende capaciteit bleek verder te correleren met de initiële toename van de oppervlakte druk. Deze correlatie was ook quantitatief ($R^2=0.78$), terwijl het verband tussen met $k_{\text{adsorb}} \cdot D$ slecht was ($R^2<0.1$). Voor schuimstabiliteit werd geen enkele correlatie gevonden met een van de gemeten parameters ($R^2<0.1$), zelfs niet met de dilatatie modulus.

Dit werk laat zien, dat schuimvorming wordt gelimiteerd door dezelfde kinetische barrière als de adsorptie aan een statisch, vlak lucht-water grensvlak.
Dankwoord

Zoals de meeste dingen, is het schrijven van een proefschrift iets dat wel door één persoon wordt gedaan, maar wat niet hetzelfde was geworden als er geen andere mensen bij betrokken waren geweest. Daarom wil ik mijn proefschrift graag beëindigen met een dankwoord voor al die mensen die binnen en buiten mijn werk betrokken zijn geraakt bij dit project.

Ik was in de gelukkige positie dat ik twee promotoren had, die elk vanuit zijn achtergrond mijn project heeft begeleid. Daarbij wil ik Fons bedanken, voor de betrokkenheid die je had en natuurlijk voor de leuke discussie over hoe wetenschappelijke kennis toegepast kan worden in de levensmiddelenindustrie. Maarten, jouw kennis was een waardevolle aanvulling en met je inzichten bracht je telkens een nieuw licht in de gesprekken die we voerden.

Daarnaast wil ik hierbij Harmen de Jongh, de kapitein van het B009 schip, danken voor de kans om bij hem in het project te werken. De combinatie van mensen uit verschillende achtergronden en met verschillende interesses heeft het misschien niet altijd gemakkelijk gemaakt om naar één doel te streven. Maar zoals bij een klok alle radaren op de juiste manier in elkaar grijpen is ook bij B009 uiteindelijk een eenheid ontstaan. Met name wil ik je bedanken voor de vrijheid die je me hebt gegeven en alle discussies, waarin ik leerde over mezelf, over het denken over resultaten en wetenschap. Natuurlijk gaat mijn dank ook uit naar alle andere leden van B009, Jolan de Groot en Hans Kosters, die mij veel geholpen hebben met diverse experimenten; Kerensa Broersen voor de onspanning die je me liet kennen buiten het werk om, Renate Ganzevles, voor alle creatieve uitspattingen en de gezelligheid als we de grensvlakspanning even kwijt willen raken. Met name ook dank voor Jan-Willem Simons, die het pad van eiwitmodificatie voor mij heeft gebaand, en Marcel Meinders, voor de vele zinvolle discussies en al het werk en de tijd die je zowel in de modellen als in de IRRAS hebt gestoken. Bij de IRRAS hoort ook Misses Irras, oftewel Anneke Martin, eindelijk kan ik je in dit proefschrift de uitkomst van mijn resultaten laten zien. Ik vondt het erg gezellig om met jou de ins en outs van de IRRAS te leren kennen en natuurlijk ook het plezier dat je kunt beleven aan shear rheologische metingen.
Dankwoord

Mijn collega’s van de Leerstoelgroep levensmiddelenchemie wil ik bedanken voor de vele leuke momenten zowel tijdens bijeenkomsten als bij de diverse labuitjes waren een zeer welkome onderbreking van de serieuzere kanten van het onderzoek. Met name de zeer geslaagde AIO reis naar Japan is een mooi voorbeeld van veel ontspanning in combinatie met een ontzettend leerzame en interessante ervaring binnen de academische en industriële wereld van Japan. Met het WCFS was er natuurlijk de AIO reis en de diverse activiteiten georganiseerd door het AIO platform.

Bijzondere dank voor Julia Diederen en Kerenza Broersen, die vanaf het eerste begin mijn kamergenoten zijn geweest. Met het gekakel op het kantoor zelf, zijn we zelfs adoptie-ouders geworden van onze eigen kip. De vele leuke, intensieve en onzinnige discussies en de creatieve uitspattingen op ons kantoor (en natuurlijk het reisje naar Engeland) zijn echt onvergetelijk.

Buiten de directe collega’s heb ik ook veel steun gehad van mensen uit andere groepen. Robert Dalgleish en Stephen Holt, thanks for the nice welcome at ISIS and the help you gave me before, during and after the experiments with neutron reflection. Uit de leerstoelgroep van Fysische en Colloïdkunde wil ik Willem Norde, Maarten Biesheuvel, Marcel Minor bedanken voor de gesprekken over metingen, resultaten en analyse. Olga Eliseeva I want to thank you for the nice cooperation on the ellipsometer, en natuurlijk Remco Fokkink voor de hulp bij het opzetten van metingen met de ellipsometer, die uiteindelijk een grote bijdrage aan mijn werk hebben geleverd.


Als laatste natuurlijk de mensen waar alles mee begon: mijn ouders, Bob en Rita, dank voor de steun die jullie altijd hebben gegeven, Hans-Robert en Maarten, en natuurlijk Laëtitia, Thomas en Emillie, bedankt voor alle leuke momenten en gezelligheid als ik bij jullie was.

Peter

168
List of Publications


