Lysozyme and succinylated lysozyme as adsorbates and emulsifiers

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Lysozyme and succinylated lysozyme as adsorbates and emulsifiers

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Chapter 1

Introduction

1.1 Protein adsorption

When a solution containing proteins is exposed to another phase with which it forms an interface, more often than not the proteins adsorb at the interface. The behaviour of proteins at interfaces is important for a diverse range of applications. Especially, in biomedical science, biotechnology and in the food industry the possible uses of proteins are influenced by their surface behaviour. Proteins are, for example, often used in the stabilization of emulsions and foams in food stuffs. In medical science there is an interest in the adsorption of proteins on implant materials as used in, e.g., cardiovascular surgery and extracorporal assist devices like contact lenses and haemodialysis membranes. Another application of medical interest is the use of adsorption phenomena to design drug delivery systems. In biotechnology, enzymes are immobilized in bioreactors. To perform their function, these enzymes have to keep their biological activity and, hence, their structural integrity, after adsorption.
The major interactions determining protein adsorption are (a) electrostatic interactions between the protein molecules and the sorbent surface, (b) changes in the state of hydration of the sorbent surface and the protein molecule, (c) changes in the three-dimensional structure of the protein molecule and (d) Lifshitz-van der Waals interactions [1,2].

a) Electrostatic interaction between two charged compounds is determined by the sum of the interaction between separate charges. In aqueous environment proteins and most sorbent surfaces are electrically charged and they are surrounded by counter-ions and co-ions. The surface charge and the counter charge together form the electrical double layer and as a whole this is electroneutral. When the protein molecule approaches the sorbent surface the electrical double layers overlap, giving rise to charge regulation, i.e., a redistribution of the surface charges, the counter-ions and the co-ions. The electrostatic contribution to the adsorption affinity may be calculated by comparing the Gibbs energies of the charge distributions before and after adsorption, respectively.

b) Hydrophobic interaction refers to the tendency of apolar compounds to be rejected and, hence, to be driven together, by the surrounding water. The reason is that water adjacent to an apolar solute is in a low-entropy state because the water molecules are strongly oriented to form the maximum number of hydrogen bonds among each other as none can be formed with the apolar compound. Thus, dehydration of an apolar sorbent surface and of apolar patches of the protein molecule increases the entropy of the system and, therewith, is a strong driving force for adsorption.
c) The three-dimensional structure of a protein molecule is the net result of interactions within the molecule as well as between the protein and its environment. As the structure is usually marginally stable, a change of environment may induce a change in the protein structure. If adsorption-induced structural changes involve a loss of secondary and/or tertiary structure, the conformational entropy of the protein increases which, in turn, contributes to the adsorption process.
d) Lifshitz-van der Waals interactions or dispersion interactions are induced dipole interactions and depend on the polarisability of the interacting molecules. For proteins interacting with a surface across an aqueous medium these interactions are usually small and attractive.

1.2 Proteins at solid/liquid and liquid/liquid interfaces

This thesis deals with protein adsorption on solid/liquid and on liquid/liquid interfaces. The focus is on oil-in-water emulsions. Measurement of adsorbed amounts at the oil/water interface of emulsions is not unambiguous and, due to light scattering, spectroscopic investigation of the structure of the adsorbed proteins is virtually impossible. As a first approximation we therefore studied the adsorption of proteins at solid/liquid interfaces. To value the relevance of the experimental results obtained with these systems for the properties of oil-in-water emulsions we have to account for the different properties of solid and liquid interfaces, respectively, and the ensuring influence on protein adsorption behavior at the two types of surfaces. The main differences are [3]:

- Liquid/liquid interfaces, more so than solid/liquid interfaces, have the possibility to change shape (i.e., stretch of contract) on the timescale of the experiment, influencing the degree of spreading of the protein molecules at the interface.
- Liquid/liquid interfaces are smooth due to the contractile action of the surface tension. The surface tension keeps the interfacial area at a minimum and changes in the shape are counteracted by the surface tension. For solid/liquid interfaces, it is necessary to consider the surface roughness, surface heterogeneities or porosities.
- When liquid/liquid interfaces are charged, this is due to specifically adsorbing ions or by the adsorbing protein. Any influence of pH or ionic strength is caused by changes in the adsorbed ions or proteins. Solid surfaces are most often charged themselves and this charge is influenced by pH and ionic strength.
- A liquid/liquid interface requires two immiscible liquids. Most systems consist of water and an apolar liquid and the interface will thus often be hydrophobic. Solid/liquid interfaces can be either hydrophobic or hydrophilic.
- At the liquid/liquid interface, apolar parts of the protein may enter the apolar phase, whereas the polar parts prefer to stay in the polar phase (water). Protein penetration into a solid phase is not possible. Hence, it is to be expected that the adsorbed protein molecules unfold more extensively at a liquid/liquid interface than at a solid/liquid interface.
- Macroscopic liquid/liquid interfaces present only a very small surface-volume ratio. It is, therefore, difficult to determine the adsorbed mass by depletion measurements. The adsorbed amount may be derived from the change in interfacial pressure, but a straightforward and general relationship between the interfacial pressure and the adsorbed amount is not available. Various techniques to study protein adsorption like reflectometry, ellipsometry, neutron reflectometry, TIRF and FRAP are suitable on solid/liquid interfaces, but on liquid/liquid interfaces these techniques often suffer from the fact that the contrast between the refractive index of the protein and that of the oil, is small. Moreover, the incoming beam has to pass the oil/air interface before it reaches the oil/water interface. Passage of the air/oil interface causes scattering of the laserbeam, which perturbs the measurement.

1.3 Emulsions

An emulsion is a system of two immiscible liquids of which the one is finely dispersed in the other. Examples of emulsions are milk, mayonnaise, desserts, toppings and cream liquor. Man-made emulsions are made by vigorously stirring or otherwise homogenizing oil, water, and a surfactant. The homogenisation process provides the energy required to overcome the excess pressure (Laplace pressure) in the liquid droplets and to increase the interfacial energy involved in the enlargement of the oil/water interface [4]. Because of the large interfacial area and the proportionally high interfacial energy, emulsions are thermodynamically unstable and tend to coalesce [5]. Covering the emulsion droplets with protein will protect them against coalescence. Therefore, adsorption of proteins at the oil/water interface is an important step in the formation of an emulsion [6].

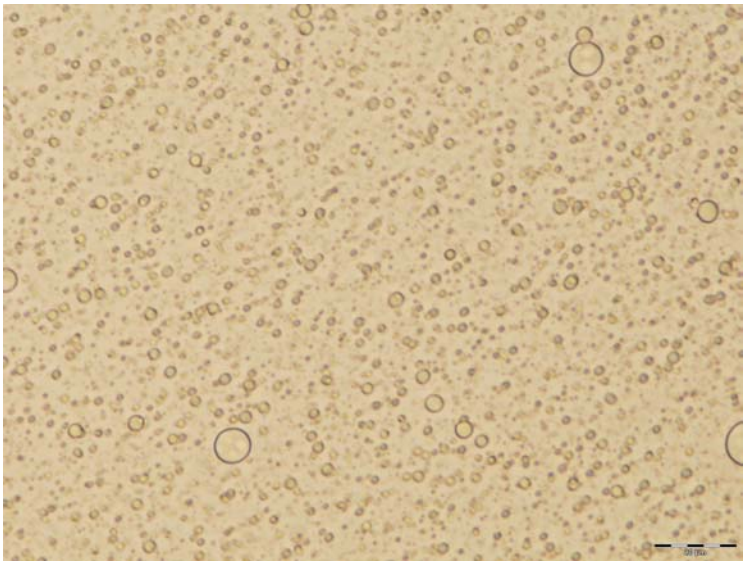

Figure 1.1 microscope picture of an emulsion stabilized by lysozyme

Proteins are highly surface active and at low concentrations all protein molecules adsorb. During the formation of an emulsion, the adsorption rate of the proteins is very high, since the protein is not transported by diffusion but by convection [4]. The interface is repeatedly expanded and compressed and these interfacial deformations are fast. The proteins can denature and coagulate on the surface when undergoing dilatational changes. Because of its dynamic behavior the rheological properties of the adsorbed protein layer is of great importance for the properties of the emulsion. A

satisfactory model for the rheological behavior of adsorbed protein layers is not available yet [7].

1.4 Outline of this thesis

The aim of this thesis is three-fold. The first one is to study the adsorption mechanism of two well-defined similar proteins at solid/liquid interfaces. Special attention is paid to the roles of hydrophobic and electrostatic interactions. To that end, both hydrophobic and hydrophilic surfaces were taken and the protein, lysozyme, is chemically modified by adding succinyl groups. The succinyl groups react with lysine and possibly other cationic groups, which are converted into anionic groups. Upon succinylation with an excess of succinyl, ten succinyl groups are linked to a lysozyme molecule. As a consequence, the isoelectric point of lysozyme shifts from pH 11 to pH 4.5. Besides affecting the charge of the protein, its structural stability changes. The influence of succinylation on the electrostatic properties and on the structure and structure stability of lysozyme is discussed in chapter 2.

Chapter 3 deals with adsorption characteristics of native and succinylated lysozyme at the hydrophobic and hydrophilic surface. In chapter 4, the kinetics of protein adsorption at solid hydrophilic and hydrophobic surfaces is studied. Here, we used lysozyme and α-lactalbumin. These proteins have a comparable size and shape but differ markedly in structural stability and isoelectric point.

The adsorption of lysozyme and succinylated lysozyme at a liquid/liquid interface is studied against the background of adsorption at the solid/liquid interface: the second aim of this thesis. The third aim is to investigate rheological properties of monolayers of the two proteins at the liquid/liquid interface and to relate these to the stability against coalescence of emulsions prepared in the presence of either one of the two proteins. Furthermore, emulsion aggregation phenomena were studied after mixing a positively charged emulsion (stabilized by lysozyme) with a negatively charged emulsion (stabilized by succinylated lysozyme) in varying proportions. The interfacial rheology measurements and the emulsion studies are included in chapter 5.

1.5 References

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Chapter 2

Effects of succinylation on the structure and thermostability of lysozyme[1]

Abstract

The influence of succinylation on lysozyme is studied using circular dichroism, fluorescence spectroscopy, and differential scanning calorimetry. The spectroscopic data reveal that at room temperature the structures of succinylated lysozyme and native lysozyme are similar. However, the calorimetric results show that the thermal stability of succinylated lysozyme is lower than that of native lysozyme. For succinylated lysozyme, the denaturation temperature (T_d) varies in the range of 325-333 K (52-60°C) and the associated denaturation enthalpy ($\Delta_{den}H$) between 225 and 410 kJ/mol. For lysozyme, T_d is 342-349 K (69-76 °C) and $\Delta_{den}H$ is 440-500 kJ/mol. From these data, the change in the heat capacity ($\Delta_{den}C_p$) upon thermal denaturation is derived. For lysozyme, $\Delta_{den}C_p$ is 7.5 kJ/mol/K and for succinylated lysozyme, it is 16.7 kJ/mol/K. The value of $\Delta_{den}C_p$ for lysozyme is comparable to previously reported values. The high value of $\Delta_{den}C_p$ for succinylated lysozyme is explained in terms of an extended degree of unfolding of the secondary structure and exposure of the apolar parts of the succinyl groups. Furthermore, the Gibbs energy of denaturation, as a function of temperature, derived from the thermodynamic analysis of the calorimetric data indicates a cold-denaturated state of succinylated lysozyme below 20°C. However, because a denatured state at low temperatures could not be detected by CD or fluorescence measurements, the native state may considered to be metastable at those conditions.

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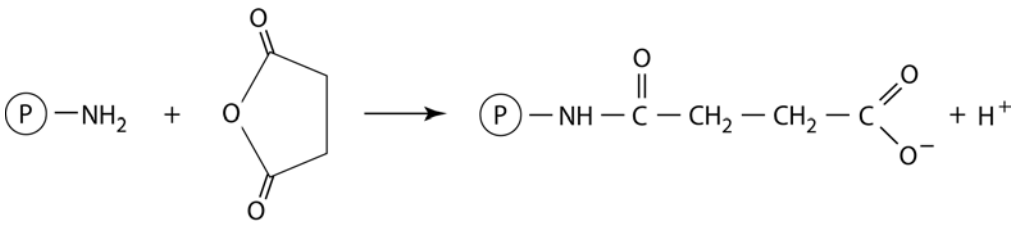
2.1 Introduction

The structure and structural stability of proteins play important roles in the processing and the sensorial perception of food, especially in emulsions and foams, where the formation and stabilization are only possible by adsorbing a surface active agent at the interface between the dispersed and continuous phases. Because of the GRAS status of many proteins, they are often applied in food stuffs. In this process, the dispersion forming and -stabilizing functionalities of a protein are strongly related to physical and chemical properties, in particular its structure and structural stability. With the aim to improve the protein's performance, adsorption studies are performed using modified proteins [1-3]. By comparing the adsorption behavior of a modified and a native protein, one may be able to identify the influence of different types of interactions on the adsorption process. Furthermore, phenomena like the formation of protein aggregates or the formation of a protein network at the interface play a role in the stability of emulsions or foams.
In chapter 3 [4], we report that succinylation of lysozyme strongly influences its adsorption behavior at silica surfaces. The influence was explained in terms of electrostatic interactions of the protein with the surface. Succinylation converts primary amino groups into carboxyl groups and thereby changes the protein charge, according to the following reaction mechanism:



Upon succinylation with an excess of succinic anhydride, the isoelectric point of lysozyme shifted from 11.0 to 4.5.
Lysozyme is a structurally stable protein and at room temperature it is expected to undergo little, if any, conformational alteration by mild modifications. This is observed more often when small modifications or mutations are introduced in proteins. Tian et al. [5] found identical secondary structures for several mutants of bacteriophage T4 lysozyme. Kosters et

al. [6] reported similar CD spectra for ovalbumin that was chemically modified with different methods. In contrast with these results, Lakkis et al. [7] observed shifts in the CD and fluorescence spectra when modifying casein, BSA and whey protein isolate with alkyl, acetyl and succinyl groups, except for the alkylation of BSA.

Here, we present a detailed analysis of the influence of succinylation on the structure and the structure stability of lysozyme. The conformations, i.e., the secondary and tertiary structures, of both lysozyme and succinylated lysozyme were probed with circular dichroism (CD) and fluorescence spectroscopy. The amount of the secondary structure was assessed by far-UV CD and the tertiary structure was probed by near-UV CD and fluorescence. The thermostability of the structure of lysozyme and succinylated lysozyme was determined with differential scanning calorimetry (DSC) and the experimental data allow for a thermodynamic analysis of protein denaturation as a function of temperature [8].

2.2 Materials and Methods

Materials

Hen egg white lysozyme was purchased from Sigma (L-6876) and used without further purification. All other chemicals were of analytical grade.

Modification of the protein

Lysozyme was dissolved in 20 mM phosphate buffer of pH 8. A large excess (more than 10 times the molar ratio) of succinic anhydride was added in small portions; the pH was kept between 8 and 9 by adding 0.1 M NaOH. Following this, the protein solution was dialyzed against water for four days at 4ºC and then freeze-dried. The mass of the protein after modification was measured by Maldi-tof. There were three fractions of modified lysozyme, succinylated at 8, 9 or 10 sites respectively. The isoelectric point of the modified protein was 4.5 as determined by gel electrophoresis [4].

Fluorescence spectroscopy

Fluorescence was measured with a Varian Cary Eclipse Fluorimeter equipped with a Peltier element for temperature control. The excitation wavelength was 280 nm and the fluorescence emission was measured between 300 and 400 nm. The excitation and emission slits were set at 2.5 nm. The measurements at constant temperature were made at 20 ºC. For the

temperature scans the temperature was changed at 0.1 ºC/minute. We did not use any external fluorescent probe.

Circular Dichroism

Circular Dichroism (CD) was measured using a Jasco J-715 spectropolarimeter. The far-UV CD measurements were performed in a quartz cuvette of 1 mm with a protein concentration around 3 μM in 10 mM phosphate buffer. The sample was scanned from 260 to 190 nm. The near-UV CD measurements were performed in a 1 cm quartz cuvette with a protein concentration around 30 μM. The sample was scanned over a wavelength range from 310 to 260 nm. For both methods, the spectra presented were an average of eight scans. A data pitch of 0.1 nm was used, and the scan rate was 20 nm/min. The bandwidth was 1.0 nm. The cell was thermostated with a Peltier element at 20 ºC unless specified otherwise. The concentration of the proteins was determined with UV absorption at 280 nm.

Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) of the protein solutions was performed at a scan rate of 1 ºC/min on a MicroCal VP-DSC with fixed cells of 0.5 ml. The proteins were dissolved in buffers containing 10^{-3} M phosphate for the high pH values and 10^{-3} M acetate for the low pH values. The ionic strength was adjusted to a value of 10^{-2} M using NaCl. The protein concentration was 5 g/l. The solution was degassed because air bubbles may alter the pressure during heating. Buffer against buffer measurements were performed and used for baseline correction.

2.3 Results and discussion

Structure of lysozyme and succinylated lysozyme

The fluorescence and circular dichroism (CD) spectra of lysozyme and succinylated lysozyme were measured and compared. For the fluorescence measurements, the six tryptophan residues present in lysozyme were used as intrinsic fluorophores. A wavelength of 280 nm was used to excite the tryptophans, and the emission of light was measured between 300 and 400 nm. In figure 2.1, the fluorescence spectra of lysozyme and succinylated lysozyme are shown. At room temperature, the position of the maxima of the emission spectra of the two proteins coincide, indicating that the tryptophans are in a similar environment. The intensity of the fluorescence is somewhat higher for native lysozyme. The

reason for this difference in intensity cannot unambiguously be explained. On the one hand, the intensity of the fluorescence of the tryptophans depends on the polarity of the moieties surrounding this residue. On the other hand, if there is a change in the surrounding polarity, the peak maximum is expected to shift as well, which is not observed. Possibly, the decrease in intensity is caused by a reduced contribution from the tyrosine residues that have reacted with succinyl. Anyway, the fact that the positions of the peak maxima of lysozyme and succinylated lysozyme coincide suggests that the tertiary structure of lysozyme is not significantly affected by succinylation.

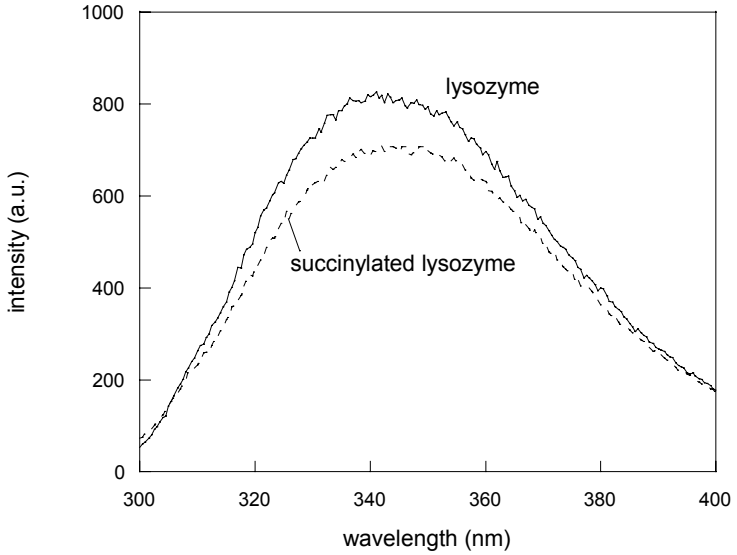


Figure 2.1 Fluorescence emission spectrum of lysozyme and succinylated lysozyme.

The tertiary structure is further probed by near-UV CD spectroscopy. In the near-UV region the CD spectrum of proteins is determined by aromatic side chains and disulfide bonds. The environment of aromatic compounds influences the CD spectrum, and the CD spectrum is therefore characteristic for the tertiary structure of the protein [9]. The near-UV CD spectra of the native and the modified protein are shown in figure 2.2. The largest parts of the spectra overlap; only around 280 nm is the ellipticity of succinylated lysozyme less than that of the native protein. Around 280 nm, tyrosine gives a peak in near-UV CD spectra [9]. This is a clear indication that tyrosine residues are succinylated.

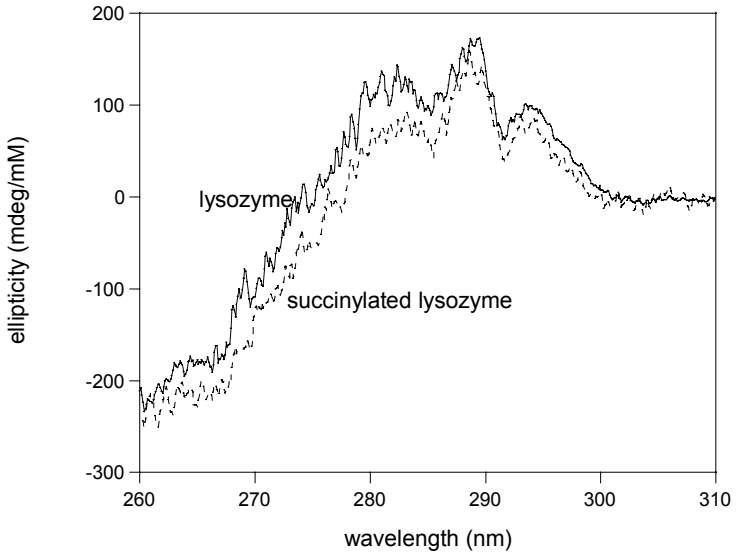


Figure 2.2 CD spectrum of lysozyme and succinylated lysozyme in the near UV region

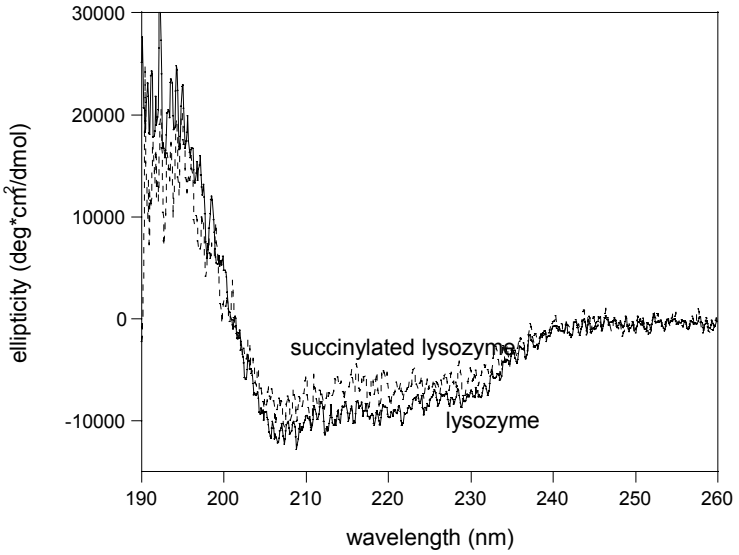


Figure 2.3 CD spectrum of lysozyme (thick line) and succinylated lysozyme (thin line) in the far UV region

Secondary structure elements like α-helices and β-sheets have dichroic activity in the wavelength range from 190 to 260 nm [9]. In figure 2.3, the far-UV CD spectra of lysozyme and succinylated lysozyme are given. The spectra are very similar for both proteins, indicating only a slight difference, if any, in secondary structure upon succinylation. This is observed more often when small modifications or mutations are introduced in proteins [5,6].

In conclusion, because the fluorescence and CD spectra of lysozyme and succinylated lysozyme are hardly different, the secondary and tertiary structures of the proteins at 20 ºC are considered to be similar, if not identical.

Thermal stability of lysozyme and succinylated lysozyme

Although their three-dimensional structures are essentially equal at 20°C, the thermal stabilities of these structures may be different for the two proteins. We monitored temperature-induced structural changes by measuring the fluorescence while heating the protein sample. Upon denaturation, the protein (partly) unfolds and it is expected that the tryptophan residues become more exposed to an aqueous, polar environment. As a result, the peak maximum of the fluorescence shifts towards higher wavelength. In figure 2.4, the ratio of fluorescence emission intensities at 360 (I_{360}) and 320 (I_{320}) nm is plotted against the temperature. By taking the ratio I_{360}/I_{320}, the shift becomes more pronounced.

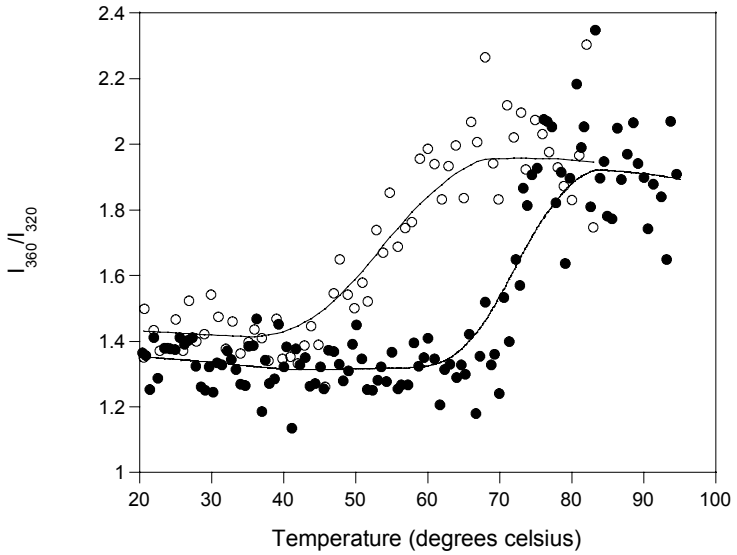


Figure 2.4 Fluorescence emission intensity at 360 nm divided by the fluorescence emission intensity at 320 nm on heating the samples for lysozyme (filled symbols) and succinylated lysozyme (open symbols).

A transition is observed in the temperature range 50-70ºC for succinylated lysozyme and between 70 and 80ºC for native lysozyme. For both proteins I_{360}/I_{320} is 1.4 for the native state and 2.0 for the denatured state and it implies that the tryptophan residues indeed become exposed to a more polar environment upon denaturation. The denaturation temperature decreases as a result of succinylation, meaning that the modified protein is less stable. Furthermore, the transition is more gradual for succinylated lysozyme, indicating either that

the transition is less cooperative or that the population of succinylated protein molecules is less homogeneous with respect to stability.
Another way to determine the structure stability of a globular protein is to measure the amount of energy required to unfold that structure. This energy may be measured by DSC. In a DSC apparatus, the protein is heated at the same rate as a blank and the measured differential heat is the heat required to denature the protein. In figure 2.5, the denaturation thermograms of lysozyme and succinylated lysozyme at pH 5 are shown. The peak area corresponds with the enthalpy of denaturation ($\Delta_{den}H$), and the temperature at the peak maximum is identified with the denaturation temperature (T_d). In this experiment, we find that succinylation lowers T_d by 20 °C and reduces $\Delta_{den}H$ by 200 kJ/mol.

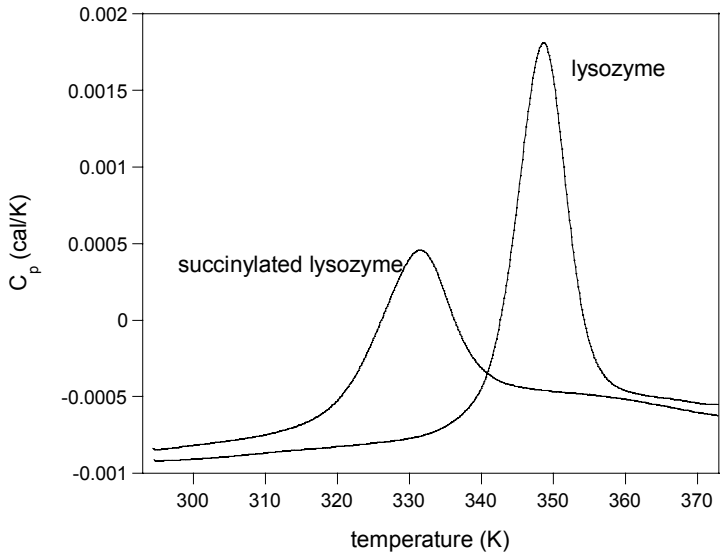


Figure 2.5 DSC thermogram of the denaturation of lysozyme and succinylated lysozyme at pH 5

To allow for a more complete thermodynamic analysis of the protein structure stability, DSC thermograms are taken at various pH values, for both lysozyme and succinylated lysozyme. Variation of pH causes variation in the protein structure stability and, hence, in the values for T_d and $\Delta_{den}H$. In our measurements, T_d of lysozyme varied between 342 and 349 K (69-76 °C) and $\Delta_{den}H$ between 440 and 500 kJ/mol. For succinylated lysozyme, the T_d varied from 325 to 333 K (52-60 °C) and $\Delta_{den}H$ between 225 and 410 kJ/mol. The complete set of data is given in table 2.1. The denaturation temperatures as measured with DSC compare well with the transition temperatures as observed with fluorescence (figure 2.4). Furthermore, the denaturation peaks of succinylated lysozyme are broader and reflect a more gradual thermal

denaturation transition of the succinylated protein sample, thus corroborating our observations with fluorescence.

pH	T_d(K)	$\Delta_{den}H$ (kJ/mol)	$\Delta_{den}H^{vH}$ (kJ/mol)	$\Delta_{den}H/\Delta_{den}H^{vH}$
	succinylated lysozyme			
3.5	330.5	290	563	0.8
4	333.6	425	253	1.68
5	330.4	320	323	0.99
7	329.8	309	310	1.00
8	327.1	268	256	1.05
9	326.8	257	252	1.02
11	325.6	239	243	0.99
	lysozyme			
3.5	348.8	500	457	1.10
4	349.0	500	456	1.10
5	347.6	491	485	1.01
6	346.7	494	417	1.19
7	345.3	459	434	1.06
8	345.2	477	492	0.97
9	345.6	493	508	0.97
11	342.7	451	389	1.21

Table 2.1 Denaturation temperature and enthalpy of lysozyme and succinylated lysozyme at different pH values.

Heating the protein sample to a temperature well above the thermal denaturation temperature causes irreversible behavior. It has been proven [10] that the irreversibility is caused by intermolecular aggregation of unfolded lysozyme and that the unfolding as such, reflected by the peak in the DSC thermogram, proceeds reversibly. We checked the reversibility of the denaturation for succinylated lysozyme by heating the sample to the denaturation temperature and keeping it at that temperature for 20 minutes. After that the sample still had a denaturation peak at the denaturation temperature.

The calorimetric enthalpy may then be compared with the Van 't Hoff enthalpy, $\Delta_{den}H^{vH}$, that may be derived from the thermograms as well [11]. These data are included in table 2.1. Similar values for $\Delta_{den}H$ and $\Delta_{den}H^{vH}$ point to a two-state denaturation process with no thermodynamically stable intermediate states. Thus, it is concluded that except for pH 11 lysozyme essentially denatures by a two-state process ($\Delta_{den}H$ / $\Delta_{den}H^{vH}$ deviates no more than 10 % from unity). The same holds for succinylated lysozyme except, perhaps, at pH 4 and

pH 3.5. Then, assuming a reversible two-state process, the enthalpy of denaturation at any temperature can be calculated according to:

$$\Delta_{den}H(T) = \Delta_{den}H_{Td} + \int_{Td}^{T} \Delta_{den}C_p dT \qquad (1)$$

where $\Delta_{den}H_{Td}$ is the denaturation enthalpy at T_d and $\Delta_{den}C_p$ is the difference in the heat capacity of the protein solution before and after the denaturation transition. The entropy ($\Delta_{den}S$) and Gibbs energy ($\Delta_{den}G$) of denaturation are calculated from the following thermodynamic relations:

$$\Delta_{den}S(T) = \frac{\Delta_{den}H_{Td}}{T_d} + \int_{Td}^{T} \Delta_{den}C_p d\ln T \qquad (2)$$

and

$$\Delta_{den}G(T) = \Delta_{den}H(T) - T\Delta_{den}S(T) \qquad (3)$$

It is well documented that unfolding of proteins in an aqueous environment leads to an increase in the heat capacity ($\Delta_{den}C_p$) of the system [8]. Most DSC thermograms, like the ones shown in figure 2.5, indeed show positive values for $\Delta_{den}C_p$. However, these C_p shifts

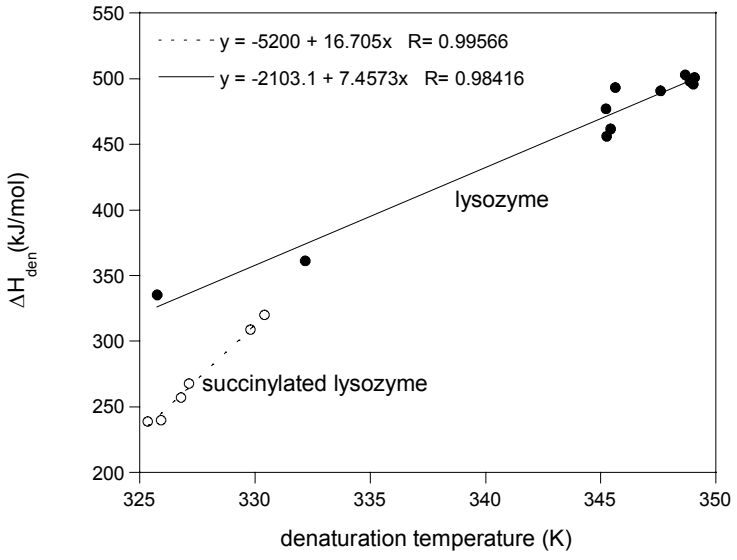


Figure 2.6 Denaturation enthalpy of lysozyme (closed symbols) and succinylated lysozyme (open symbols) plotted against the denaturation temperature.

are too small to be recorded with satisfactory reproducibility. A more accurate estimate of $\Delta_{den}C_p$ is obtained from the slope of a plot of $\Delta_{den}H$ as a function of T_d, shown in figure 2.6. For this plot, data points are used corresponding to the cases where $\Delta_{den}H$ / $\Delta_{den}H^{vH}$ deviates less than 10 % from unity. Larger deviations may be due to less reliable enthalpy data or may reflect that the transition does not proceed as a two-state process.

From the plots in figure 2.6, for lysozyme and succinylated lysozyme values of $\Delta_{den}C_p$ are obtained, which are 7.5 ± 0.2 kJ/mol/K and 16.7 ± 0.9 kJ/mol/K, respectively. The value for lysozyme compares well with literature data [8,12]. The value for succinylated lysozyme is much higher than usually found for globular proteins. It suggests a more progressed exposure of hydrophobic groups of the protein to water after unfolding. To test this suggestion, we measured the CD and fluorescence spectra of both proteins at a temperature above the denaturation temperature, i.e., 80°C. The near-UV CD measurement gave no signal, indicating that the tertiary structure of both proteins was denatured (data not shown). With fluorescence, for both proteins, I_{360}/I_{320} is 1.4 for the native state and 2.0 for the denatured state. The far-UV CD spectrum of lysozyme thermally denatured at 80°C was the same as that below the denaturation temperature, i.e., 20 °C (figure 2.3) but for succinylated lysozyme, a much smaller fraction of ordered secondary structure was found in the thermally denatured sample (figure 2.7). Therefore, the high value of $\Delta_{den}C_p$ for succinylated lysozyme

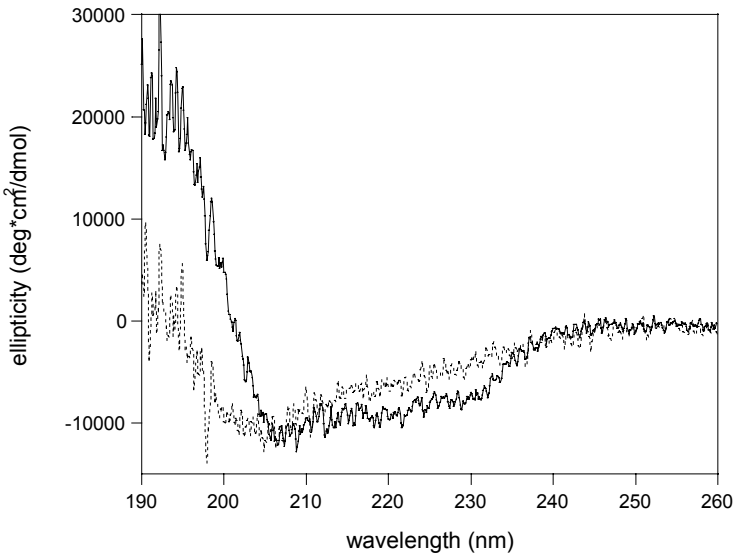


Figure 2.7 CD spectrum of succinylated lysozyme at 20°C (thin line) and 80°C (thick line).

is ascribed to a more extensive breakdown of its secondary structure. Further exposition to the aqueous environment of the apolar parts of the succinyl residues may also additionally contribute to the unusual high value of $\Delta_{den}C_p$.

Now, using the experimental data of $\Delta_{den}H_{Td}$, T_d and $\Delta_{den}C_p$, curves for $\Delta_{den}H$, $T\Delta_{den}S$ and $\Delta_{den}G$ are plotted as a function of temperature in figures 2.8 and 2.9. The entropy and enthalpy effects largely compensate each other so that the variations in the Gibbs energy are small and hardly visible in these figures. Therefore the Gibbs energy of denaturation is replotted on an enlarged scale in figure 2.10. It reveals that the Gibbs energy of denaturation for succinylated lysozyme is at all temperatures less positive (or more negative) than in the case of lysozyme. In other words, over the whole temperature range succinylation lowers the thermodynamic stability of the compact protein structure.

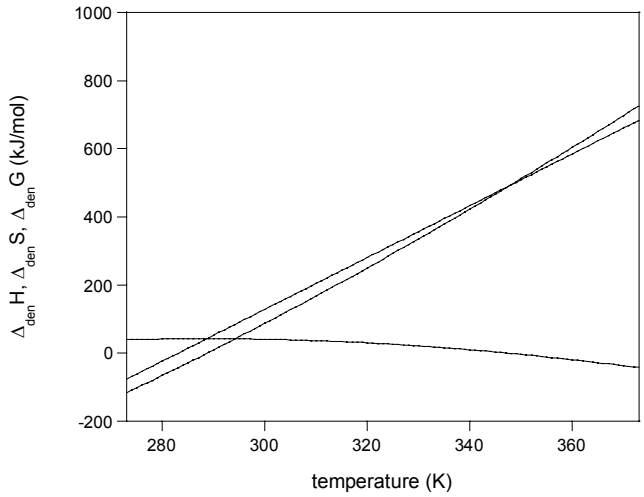


Figure 2.8 Enthalpy, Entropy and Gibbs energy of denaturation of lysozyme plotted against temperature

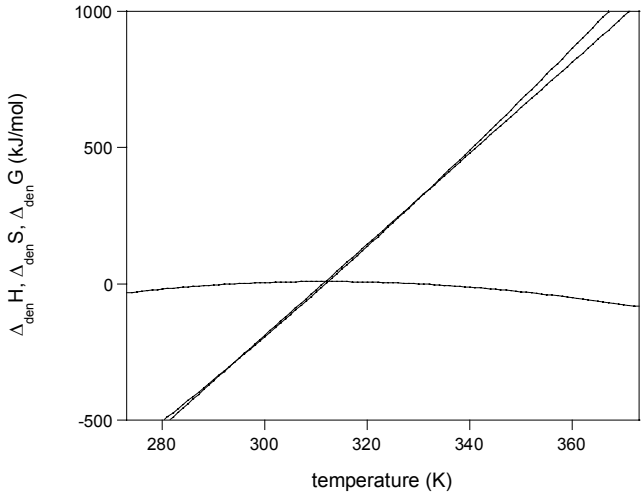


Figure 2.9 Enthalpy, Entropy and Gibbs energy of denaturation of succinylated lysozyme plotted against temperature

The temperatures where $\Delta_{den}G = 0$ are the denaturation temperatures. It appears that, the $\Delta_{den}G$ curve of succinylated lysozyme has two such temperatures. This would imply that in addition to a heat-induced denaturation, succinylated lysozyme should undergo a cold-induced denaturation as well. Cold denaturation of proteins has been reported more often in literature [8,13]. The cold denaturation of most proteins takes place at temperatures well below the freezing temperature of water. By increasing the pressure or by adding denaturants, like e.g. urea, the temperature of cold denaturation can be adjusted to above the freezing point [14].

Figure 2.10 predicts cold denaturation of succinylated lysozyme at a temperature of 293 K (20°C). However, at this temperature, the protein presents itself in a globular, native-like state, suggesting that the onset of cold denaturation is (well) below 20°C. We tried to detect the cold denaturation transition of succinylated lysozyme by calorimetry. In a DSC experiment, the protein was cooled from 20°C to 5°C at a scan rate of 0.5 K/min [8,13]. The measurement did not produce a visible denaturation peak. We also tried to trace cold denatured protein by fluorescence and CD after keeping the protein overnight in a refrigerator. The fluorescence and the near-UV CD spectra of the cooled protein were measured at 5°C and the spectra were the same as that of the native protein. Hence, the expected denaturation could not be detected in the temperature range 5 - 20°C.

As the value of $\Delta_{den}C_p$ is subject to some statistical error, we checked whether this may affect our thermodynamic analysis and invalidate the conclusion that for succinylated lysozyme $\Delta_{den}G = 0$ somewhere between 5 - 20°C. This is not the case; the uncertainty in T_d is less then 2°C.

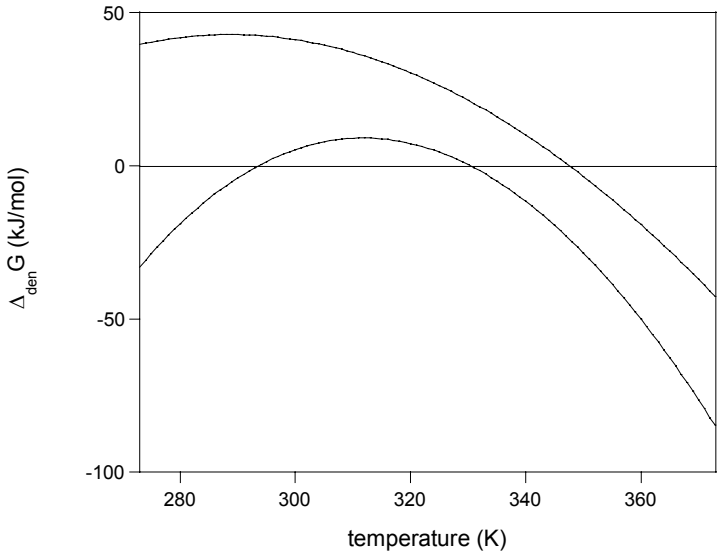


Figure 2.10 Gibbs energy of denaturation of lysozyme and succinylated lysozyme

Taking into account the standard deviation of the $\Delta_{den}C_p$ value did not significantly influence the curves in figures 2.8, 2.9 and 2.10. We therefore conclude that the compact structure of succinylated lysozyme is metastable at temperatures below 20°C: the compact structure is trapped in a local Gibbs energy minimum from which transition to the thermodynamically more favorable unfolded structure is (kinetically) hampered.

Conclusions

From CD and fluorescence measurements we conclude that lysozyme does not undergo major structural changes upon succinylation at room temperature. However, the stability of the native protein structure decreases as derived from a thermodynamic analysis based on differential scanning calorimetry. The influence of succinylation on the heat capacity change upon denaturation $\Delta_{den}C_p$ is remarkable. $\Delta_{den}C_p$ amounts to 7.5 kJ/mol/K for lysozyme and

16.7 kJ/mol/K for succinylated lysozyme. The much larger value for succinylated lysozyme is ascribed to a more extensive degree of unfolding, as indicated by circular dichroism spectroscopy, together with exposure of apolar parts of succinyl groups to the aqueous environment. The thermodynamic analysis of the DSC data predicts that succinylated lysozyme should denature below 20°C. As spectroscopic methods could not detect a denatured structure in the temperature range 5-20°C, it is inferred that below 20°C the protein is in a metastable state.

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Chapter 3

Electrostatic interactions in protein adsorption probed by comparing lysozyme and succinylated lysozyme[1]

Abstract

The influence of electrostatic interactions on protein adsorption was studied by comparing the adsorption of lysozyme and succinylated lysozyme at silica surfaces. The succinylation affects the charge of the protein, but also the stability. Although changes in stability can have an influence on adsorption, our data show that the primary effect can be entirely understood in terms of electrostatic interactions. The adsorbed amount as a function of pH has a maximum for both proteins. This maximum coincides with the isoelectric point for succinylated lysozyme, and is close to the isoelectric point for lysozyme. At pH values where the protein is electrostatically repelled by the sorbent, higher ionic strengths increase adsorption, and for electrostatic attraction higher ionic strengths decrease adsorption.

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3.1. Introduction

The behavior of proteins at surfaces is important for a diverse range of applications. For instance, in the food industry the possible uses of proteins are influenced by their surface behavior. Proteins are, for example, often used in the stabilization of emulsions and foams. In medical science there is an interest in the adsorption of proteins on implant materials as used in, e.g., cardiovascular surgery and extracorporal assist devices like contact lenses and hemodialysis membranes. Another application of medical interest is the use of adsorption phenomena to design drug delivery systems. In biotechnology, enzymes are immobilized in bioreactors. To perform their function, these enzymes have to keep their biological activity and, hence, their structural integrity.

Various interactions play a role in protein adsorption. Because most substrates and proteins are charged, electrostatic interactions are important. Moreover, because upon adsorption proteins often expose their hydrophobic core to the sorbent surface, changes in hydration of protein and sorbent play a role. Also, the structure stability and the flexibility of the protein are important, because the molecules often change shape upon adsorption. As all these effects are not independent of each other, it is difficult to estimate the contributions to the adsorption process made by each of them. Extensive discussions of the factors governing protein adsorption can be found in reviews by Haynes and Norde [1] and Malmsten [2].

The obvious way to study the effect of a single variable in the adsorption process is to use proteins that differ in only one property. This can be approached by using a pair of similar proteins or by comparing a modified protein with the corresponding native one. Pairs of structurally similar proteins are, e.g., lysozyme/α-lactalbumin and β-lactoglobulin A/β-lactoglobulin B. Elofsson et al. [3] compared the adsorption behavior of β-lactoglobulin A and B. The genetic variants A and B of this protein are very similar but differ in the dissociation constant of the dimeric form. It was shown that the adsorbed amount is proportional to the formation of dimers.

More recently, directed changes are introduced by genetic engineering [4-8] and by chemical modification [9]. Tian et al. [5] measured the CD spectrum of three stability mutants of bacteriophage T4 lysozyme adsorbed on silica. They measured a larger adsorption-induced change in the conformation of the protein when the structural stability in solution was lower. The data were fitted to a kinetic model assuming two states for the adsorbed protein. The protein with the lower stability adsorbed in the state that was more spread on the surface. Malmsten [6] investigated proteins in which specific peptides were inserted. The adsorption

on hydrophobic surfaces increased with the insertion of hydrophobic peptides in otherwise hydrophilic proteins. Comparison was made with predictions from a mean-field lattice model for block copolymers at interfaces, and a qualitative agreement between experimental observations and model predictions was obtained. In another article by Malmsten [7] charged amino acids were inserted together with the hydrophobic insertion. The adsorbed amount increased when the inserted amino acid had a favourable electrostatic interaction with the surface. Barroug et al. [9] studied lysozyme modified by attaching succinyl groups. The succinylated protein is more positively charged. The adsorption was measured on hydroxyapatite in the pH range of 6 to 9. It was found that the change in the protein charge causes a shift in the maximum adsorption as a function of pH; for lysozyme the maximum was at pH 9 and for succinylated lysozyme at pH 6.

Although many adsorption studies have been done with modified proteins, it is difficult to draw general conclusions about the influence of the modification on the adsorption process. More often than not, changes made in charge, hydrophobicity or size also affect the structure or structure stability of the protein. In this article we describe the adsorption of lysozyme and succinylated lysozyme. Lysozyme is a structurally stable protein and is expected to undergo little, if any, conformational alterations by modification. Succinylation converts primary amino groups into carboxyl groups and thereby changes the protein charge. We compare lysozyme with succinylated lysozyme with the aim to gain insight into the influence of electrostatic interactions on the adsorption of proteins.

3.2. Materials and methods

3.2.1 Materials

Hen egg-white lysozyme (L-6876) was purchased from Sigma and used without further purification. The proteins were dissolved in buffer or a salt solution (KNO_3 (pH 7), carbonate buffer (pH 10) or acetic acid buffer (pH 4)). Unless otherwise stated the adsorption experiments were carried out with 0.1 g/l protein solution at an ionic strength of 50 mM. Measurements at different ionic strengths were performed with the buffer concentration adjusted to that particular concentration. The substrates used in the adsorption experiments were strips of silicon with a silica layer of about 80 nm. These wafers were cleaned by sonication in alcohol followed by ozonization in a plasma cleaner. All measurements were performed at room temperature.

3.2.2 Modification of the protein

Protein was dissolved in 20 mM phosphate buffer of pH 8. A large excess (more than 10 times the molar ratio) of succinic anhydride was added in small portions; the pH was kept between 8 and 9 by adding 0.1 M NaOH. Following this, the protein solution was dialysed against water for four days and then freeze-dried. The degree of modification was checked by Maldi-TOF. The isoelectric point of the protein was determined by gel electrophoresis.

3.2.3 Mass Spectrometry

Mass spectra of lysozyme were acquired on the PerSeptive Biosystems Voyager DE RP MALDI-TOF MS. From a protein solution (1 mg/ml), 1 µl was diluted with 9 µl matrix. The matrix was freshly prepared and consisted of 10 mg sinapinic acid dissolved in 1 ml of 50% water, 50% acetonitril and 0.3% TFA. On a 100-welled goldplate, 1 µl of the protein/matrix solution was pipetted and crystals were formed at room temperature.

3.2.4 Gel electrophoresis

The isoelectric point of the modified protein was determined on a PhastSystem of Pharmacia Biotech. The protein was put on an IEF 3-9 phastgel and the measurement was done according to instructions supplied by the manufacturer. The protein was stained with Coomassie Blue.

3.2.5 Potentiometric titration

Potentiometric titrations of succinylated lysozyme were performed at three different salt concentrations of KNO_3; 10, 50 and 150 mM. For all three salt concentrations a blank titration was done. The slope of the Ag/AgCl-electrode was determined at –57.96 mV. The titration was performed with 0.1 M NaOH as base titrant and 0.1 M HCl as acid titrant.

3.2.6 Reflectometry

The adsorption of lysozyme on silica was measured by reflectometry. This technique is a simplified form of ellipsometry, in which the changes in intensity of a reflected polarised laser beam upon adsorption are measured. To this end a polarised laser beam is reflected on a surface at a fixed angle of incidence. After reflection from the bare surface the perpendicular (I_s) and the parallel intensities (I_p) of the light are measured. The output signal S is defined as the ratio I_p/I_s. Due to adsorption, the reflection changes from its value S_0 for the bare surface

to $S_0 + \Delta S$. The adsorbed amount per unit surface area, Γ, is calculated from the relative change in signal, as follows:

$\Gamma = \Delta S/S_0 * 1/A_s$

Here, A_s is a sensitivity factor that is determined by the optical properties of the surface. The value of A_s is calculated by an optical model in which every layer of the surface is assumed to be homogeneous and characterized by the thickness and the refractive index of that layer. We used a four-layer model: silicon, silica, protein and water. The thickness of the silica layer was measured with ellipsometry; the thickness of the protein layer was estimated. Refractive indices used are 3.85 for Si, 1.46 for SiO_2, 1.44 for protein and 1.33 for water. For the protein we used a refractive index increment of 0.185 ml/g [10]. A flow cell with stagnation point flow was used in this set-up. A measurement started by rinsing with buffer until a stable baseline was attained. Then a valve was switched and the buffer solution flow was replaced by a flow of protein solution. For a more extended treatment of reflectometry we refer to Dijt et al.[11].

3.3. Results and Discussion

3.3.1 Modification of lysozyme

Lysozyme was succinylated with an excess of succinic anhydride. We checked the degree of succinylation with Maldi-TOF. The mass spectrum is shown in figure 3.1. The mass of native lysozyme, as measured with Maldi-TOF, was 14.31 kDa. For succinylated lysozyme, three peaks were found with a mass of 15.11, 15.21 and 15.31 kDa. The mass of a succinyl group is 0.1 kDa, therefore, we can conclude that there are three fractions of modified lysozyme, succinylated respectively at 8, 9 or 10 sites.

Succinic anhydride is known to react with primary amino groups [12]. Lysozyme contains six lysine residues, which contain an ε-amino group, and one amino group at the N-terminal of the protein. This accounts for 7 reactive sites. It has been suggested that besides reacting with amino groups, the succinic anhydride may also react with tyrosine groups of which there are three in lysozyme. This would agree with a maximum number of 10 succinyl groups.

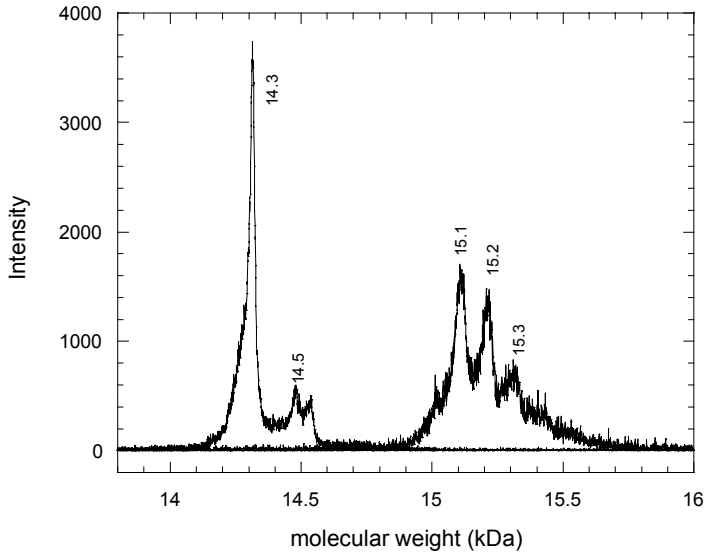

Figure 3.1 Maldi-TOF spectrum of lysozyme and succinylated lysozyme.

After modification, an amino or tyrosine group is converted into a succinyl group, which has a carboxylic acid as the endgroup. The intrinsic pK values of amino and tyrosine groups are around 10 and that of carboxylic at 4.8, so it is expected that after succinylation the isoelectric point of the protein has shifted to lower pH. We determined the isoelectric point of succinylated lysozyme with an isoelectric focussing gel (pH 3-9). Three bands were present at a pH of about 4.5 (figure 3.2). If every succinyl group adds to lowering the isoelectric point, this would be consistent with the three peaks in the Maldi-TOF.

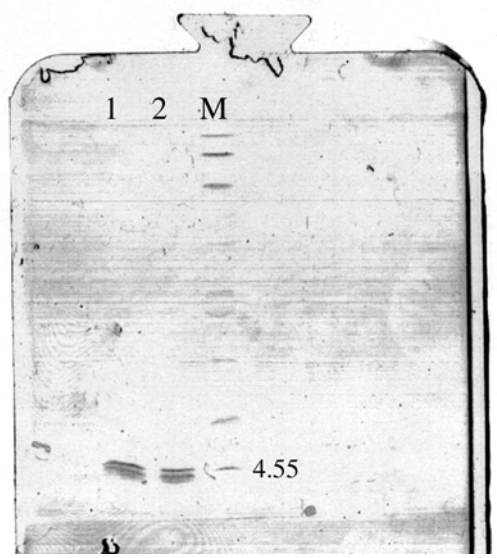

Figure 3.2 Iso electrofocussing gel of succinylated lysozyme (bands 1 and 2) and a marker (M). Marker bands are soybean trypsin inhibitor (4.55), β-lactoglobulin (5.2), bovine carbonic anhydrase B (5.85), human carbonic anhydrase B (6.55), horse myoglobin (6.85 and 7.35), lentil lectin (8.15, 8.45 and 8.65) and trypsinogen (9.30).

The thermal stability of lysozyme and succinylated lysozyme was measured with DSC and it was found that the thermal stability decreased upon modification, i.e. the denaturation temperature dropped from 75ºC to 60ºC and the denaturation enthalpy decreased from 500 kJ/mol to 410 kJ/mol. At room temperature, the structures of native and succinylated lysozyme were compared using CD and fluorescence. The structure of both proteins was very similar. There was a small difference in the near-UV CD spectrum and no difference in the far-UV CD spectrum. The peak maximum in the fluorescence spectrum was the same. These results are presented in chapter 2.

3.3.2 Potentiometric Titration

The potentiometric titration of succinylated lysozyme was performed at three ionic strengths. The point of zero charge is located at the common intersection point of the curves [13]. This is at pH 4.6, which is very close to the isoelectric point found with gel electrophoresis. The shift of the titration curve by varying the ionic strength is very small. By way of example, the titration curve of succinylated lysozyme at an ionic strength of 50 mM is shown in figure 3.3.

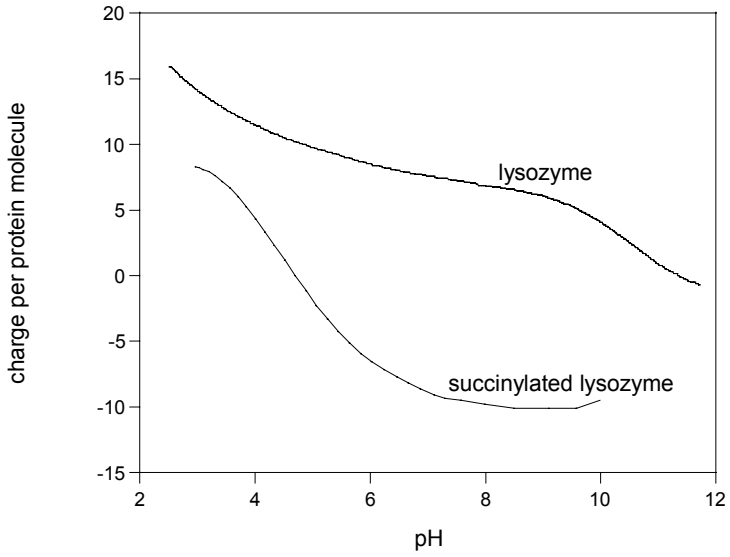

Figure 3.3 Titration curve of succinylated lysozyme at 50 mM ionic strength.

The titration curve of native lysozyme was taken from literature [14] and displayed in figure 3.3 as well. The two curves reveal that (1) succinylated lysozyme was more negatively charged then native lysozyme over the complete range of pH. (2) Succinylated lysozyme has the same number of titratable groups as native lysozyme and (3) the shape of the titration curves is different: the titration curve of native lysozyme has a steep area around pH 10,

(corresponding to the pK value for lysine and tyrosine) and for succinylated lysozyme the titration curve shows a steep part around pH 4.8 (close to the pK of the succinyl group).

3.3.3 Effect of succinylation on the adsorption of lysozyme

A typical result of the adsorption of lysozyme and succinylated lysozyme at silica at pH 7 is presented in figure 3.4. The adsorption was measured by reflectometry and the data were recorded as the adsorbed amount of protein per unit sorbent surface area in real time. At pH 7 silica is negatively charged. Succinylating the protein shifts the isoelectric point of lysozyme from 11 to 4.6. Hence, at pH 7 native lysozyme is positively charged whereas succinylated lysozyme is negatively charged. Figure 3.4 shows that the positively charged lysozyme adsorbs well: the adsorbed amount increases fast in the beginning and then levels off to an adsorbed amount of about 1.4 mg/m^2. This is comparable with the formation of a close-packed monolayer of side-on unperturbed molecules, which would yield 1.8 mg/m^2. Succinylated lysozyme does not adsorb under these conditions. The protein and the surface, that are both negatively charged, repel each other. Apparently, other interactions are not strong enough to compensate for the electrostatic repulsion.

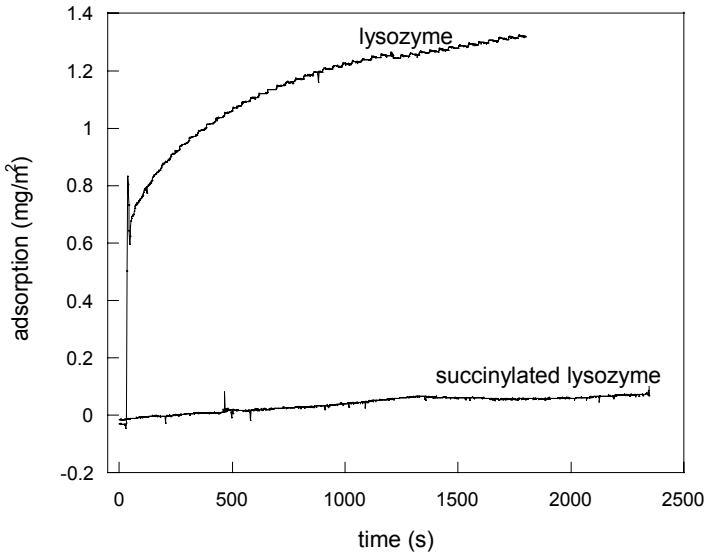

Figure 3.4 The adsorption of lysozyme and succinylated lysozyme at silica at pH 7.

To make a better comparison between native and succinylated lysozyme, the adsorption was measured at pH values where both proteins are positively charged. For lysozyme this was at pH 7 and for succinylated lysozyme at pH 4.2. The results are shown in figures 3.5a and 3.5b. The adsorbed amounts of lysozyme and succinylated lysozyme are very similar at these

conditions. Anticipating the discussion on the influence of pH on adsorption saturation, it is fortuitous that the proteins adsorb to the same extent.

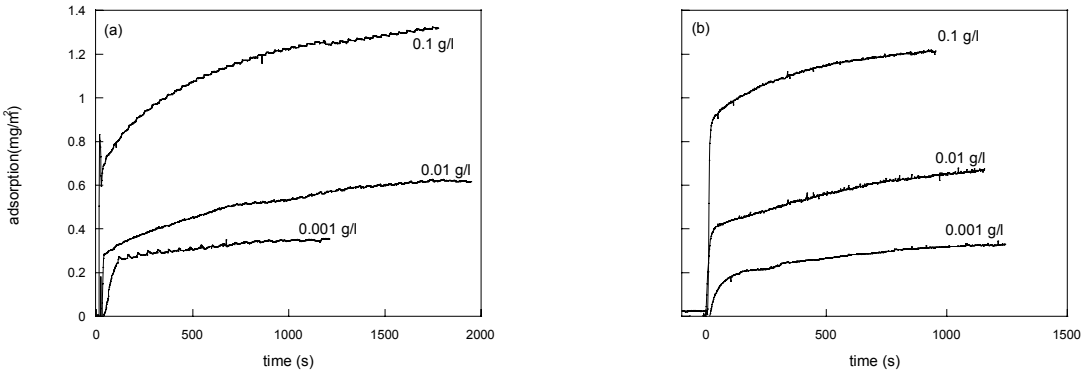

Figure 3.5 (a) The adsorption of lysozyme at pH 7, (b) The adsorption of succinylated lysozyme at pH 4.2.

The adsorption was monitored for three different protein concentrations in solution. Succinylation does not significantly influence the effect of protein concentration on the adsorption kinetics. The plateau value for the adsorbed amount decreased with decreasing the flux of protein. This is a supply rate effect: if the flux is lower the protein will have more time to spread on the surface, resulting in a lower amount of adsorbed protein per unit sorbent surface area [15,16].

3.3.4 Influence of pH

Figure 3.6 shows the adsorption of lysozyme at different pH values in the range of pH 9-10.3. It is difficult to measure at a pH beyond 10.5 because silica dissolves at such high pH. The amount of adsorbed protein is sensitive for the pH, especially between pH 10.0 and 10.2. The adsorption of succinylated lysozyme was also measured at different pH values, but in the range of pH 3-7 (data not shown). In order to derive more information out of this data, the adsorbed amount after a time of 1,000 seconds in the reflectometry experiment was taken and plotted against the pH. This is shown for lysozyme and succinylated lysozyme in figure 3.7. The chosen time of 1,000 seconds was a compromise between two considerations: it should not be too short so that the adsorbed amount is far from its saturation value, but if it is too long baseline drift of the reflectometer may cause erratic results.

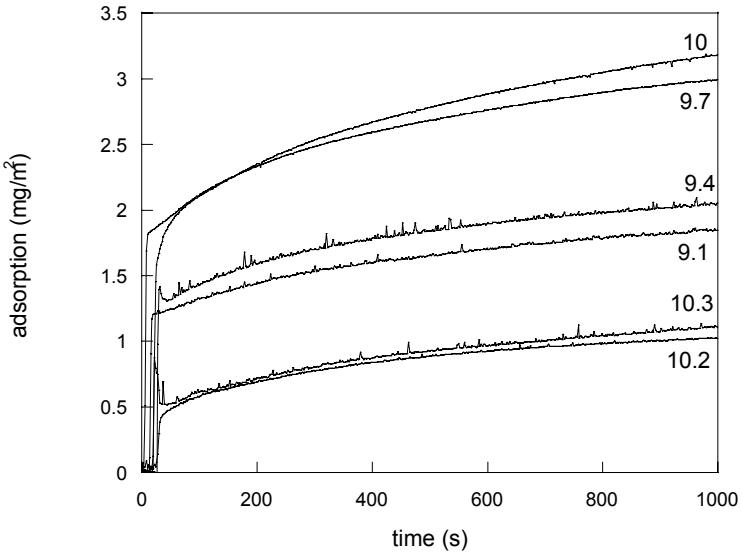

Figure 3.6 The adsorption of lysozyme at different pH values.

Figure 3.7 shows that the adsorbed amount as a function of pH passes through a maximum for both lysozyme and succinylated lysozyme. For succinylated lysozyme the maximum is at the isoelectric point of the protein. This is a rather general phenomenon in protein adsorption [1]. Proteins in the isoelectric point do not have a net charge, and hence, electrostatic repulsion between adsorbed molecules is at a minimum. The proteins can thus attain closer

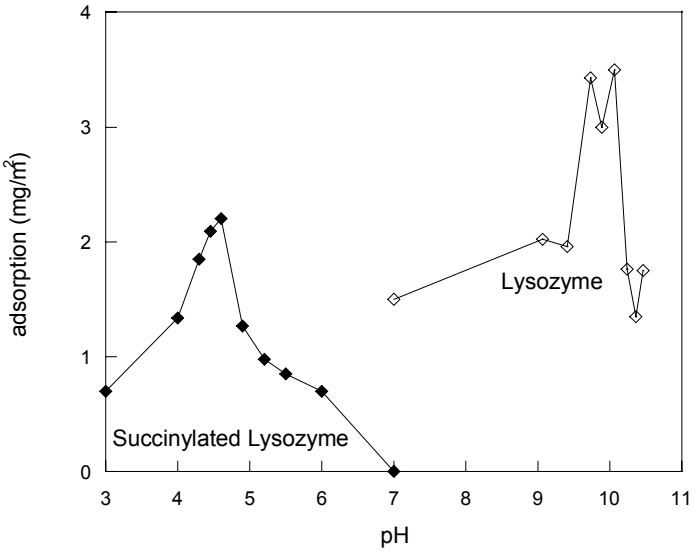

Figure 3.7 The adsorbed amount of lysozyme and succinylated lysozyme after 1,000 seconds plotted against the pH.

packing on the surface than proteins carrying a net charge. Another reason for the maximum might be that in the isoelectric region the protein has a higher structural stability and therefore a smaller tendency to spread at the interface. For lysozyme, the maximum adsorbed amount is at pH 9.8, which is about one pH unit below the isoelectric point of lysozyme. In this pH range the silica surface is heavily negatively charged. The maximum may thus well be at the isoelectric point of the protein-sorbent complex. Succinylated lysozyme adsorbs in smaller amounts than native lysozyme. The most plausible explanation is the lower structural stability, leading to a higher degree of spreading of the succinylated protein on the surface. In figure 3.8, we plotted the adsorbed amount of succinylated lysozyme against the absolute charge of the protein as determined by proton titration (figure 3.3). This gives a better insight in the role of protein charge in the adsorption process. From figure 3.7, it is concluded that the adsorbed amount of succinylated lysozyme decreases symmetrically, with respect to pH, at both sides of the isoelectric point. However, figure 3.8 reveals that this symmetry does not hold with respect to charge. At electrostatically attractive conditions the adsorption is higher than when protein and surface repel each other. This can be subscribed to a slower adsorption rate at repelling conditions allowing a protein to spread more extensively on the surface, before a neighbouring site becomes occupied by a subsequently adsorbing molecule. Also the lateral repulsion between neighbouring proteins is less effective if the sorbent-protein interaction is attractive.

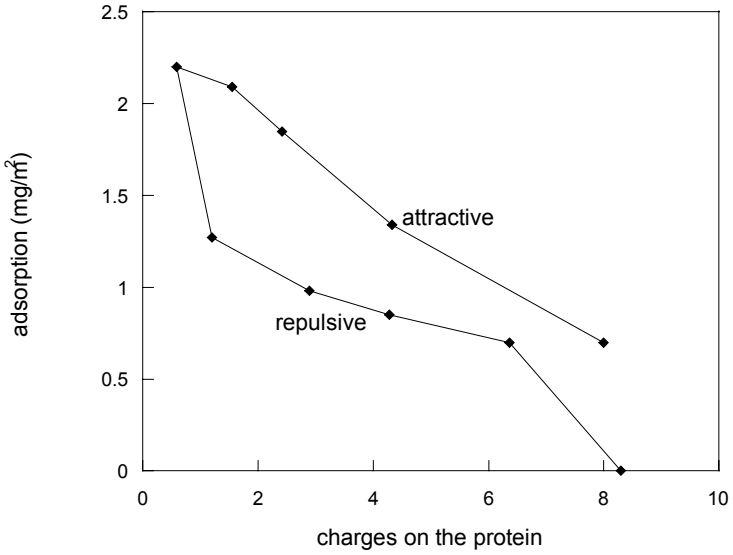

Figure 3.8 The adsorbed amount of succinylated lysozyme after 1,000 seconds plotted against the charge on the protein. The indication "attractive" refers to positively charged lysozyme and "repulsive" to negatively charged lysozyme, as the silica surface is negatively charged.

The adsorption of succinylated lysozyme has a different reproducibility above and below the isoelectric point. In figure 3.9, the adsorbed amount of succinylated lysozyme is depicted with the standard deviation indicated by error bars. The standard deviation was calculated over three adsorption measurements. The adsorption below and at the isoelectric point had a good reproducibility, but above the isoelectric point the reproducibility was less. Above the isoelectric point the protein, on approaching the surface, has to overcome an electrostatic barrier. Hence, a small difference in the conditions of the measurement may have a large influence on the adsorbed amount. We assume that the relatively poor reproducibility of the experimental data was caused by small differences in the silica surface.

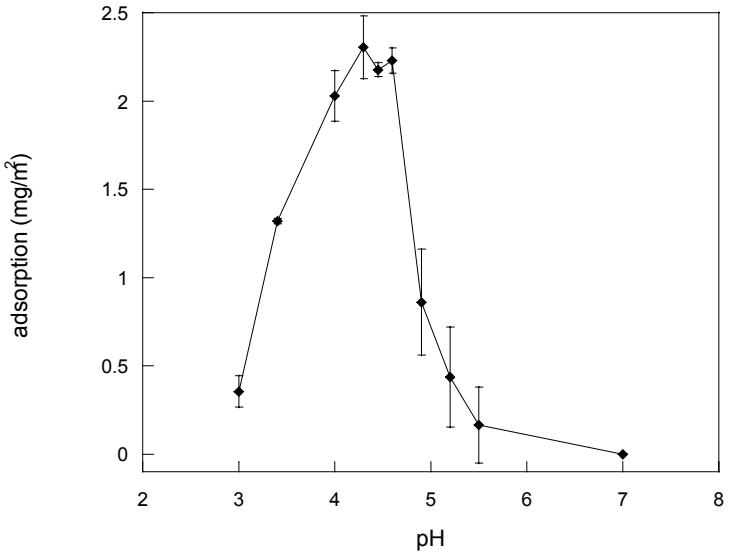

Figure 3.9 The adsorbed amount of succinylated lysozyme after 1,000 seconds plotted against the pH at 10 mM ionic strength with error bars.

Furthermore, the influence of pH on the desorption behavior is of interest. Figure 3.10 shows that, after about 1000 s of adsorption, rinsing with the solvent leads to partial desorption. The extent of desorption, expressed as a fraction of the pre-adsorbed amount, is maximal at the isoelectric point. Again, this may be explained by the smaller number of protein-sorbent contacts when the adsorbed protein molecule has undergone less spreading. Above the isoelectric point, desorption is stimulated by electrostatic repulsion, whereas electrostatic attraction at pH values below the isoelectric point fully suppresses desorption.

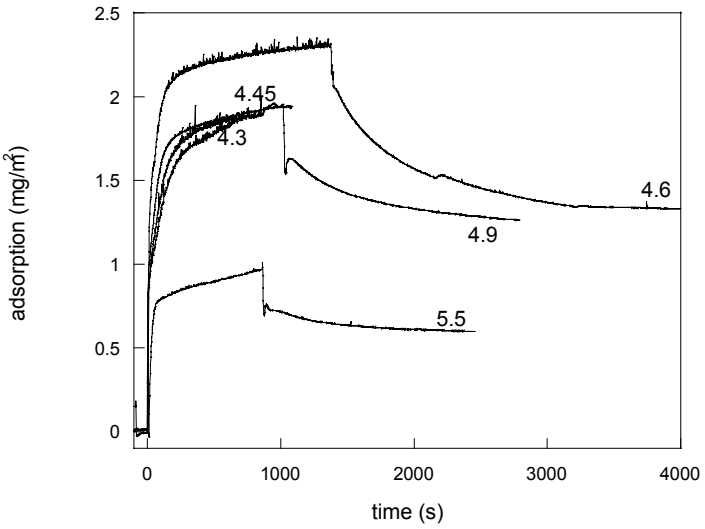

Figure 3.10 The adsorbed amount of succinylated lysozyme in time. The pH values of the measurements are given in the graph. After some time the surface is rinsed with buffer (see text).

3.3.5 Effect of ionic strength on the adsorption

The pH dependency of the adsorption of both lysozyme and succinylated lysozyme indicates that there is a strong contribution of electrostatic interactions to the adsorption process. Ions shield electrostatic interactions and it is therefore expected that the adsorbed amount of the proteins is affected by the ionic strength. The expectation is that at higher ionic strength protein adsorption is enhanced at electrostatically repulsive conditions (pH > i.e.p.) and depressed at electrostatically attractive conditions (pH < i.e.p.).

The adsorption of lysozyme and succinylated lysozyme was measured at three ionic strengths, i.e., 10mM, 50 mM and 150 mM. The results are shown in figures 3.11 and 3.12, where the adsorbed amounts of respectively lysozyme and succinylated lysozyme are plotted against pH.

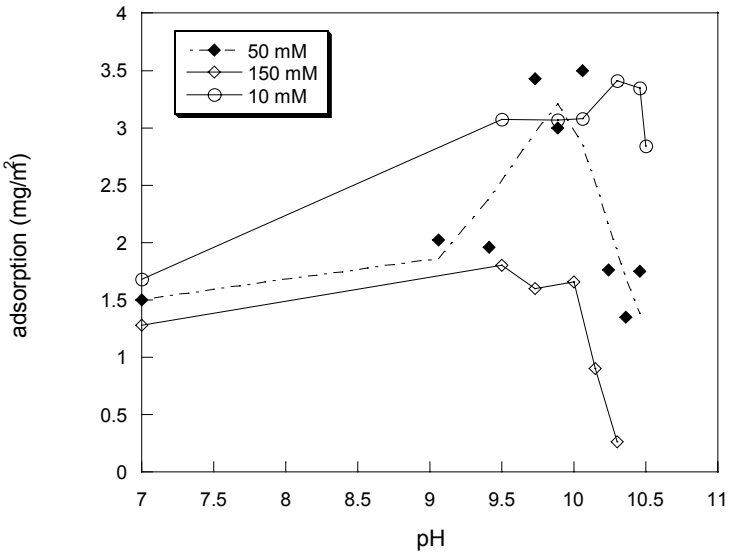

Figure 3.11 The adsorbed amount of lysozyme after 1,000 seconds plotted against the pH at three ionic strengths.

For lysozyme a rather complicated pattern is found. The adsorbed amount at both sides of the isoelectric point is reduced when increasing the ionic strength. In the isoelectric region the adsorbed amount is the same for 10 mM and 50 mM ionic strength but at 150 mM the maximum adsorption of lysozyme is strongly reduced. For succinylated lysozyme the shift in

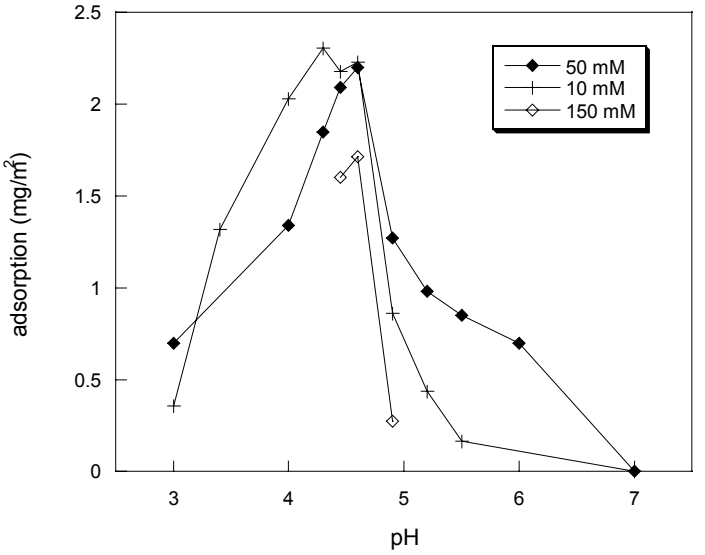

Figure 3.12 The adsorbed amount of succinylated lysozyme after 1,000 seconds plotted against the pH at three ionic strengths.

the adsorption profile when changing the ionic strength from 10 mM to 50 mM is more or less as expected. The curve for 150 mM is lower at both sides of the isoelectric point, as is the case with native lysozyme. This may well be due to adsorption competition between protein molecules and salt ions. It is known that specifically adsorbing (counter) ions can displace polyelectrolytes if their concentration is high enough [17].

3.4. Conclusion

It is difficult to modify a protein, by chemical modification or by genetic engineering, without causing further changes in the protein molecule. In this study we aimed at modifying the charge of lysozyme by chemical modification and use this as a method to obtain more insight into protein adsorption, especially in the influence of electrostatic interactions in this process. We found that the adsorption behavior of lysozyme and succinylated lysozyme is similar, but is shifted with respect to pH. For both proteins the maximum adsorbed amount is at the isoelectric point or in the isoelectric point of the protein-sorbent complex.
Lysozyme is a 'hard' protein. This means that it is a structurally stable protein and does not adsorb at hydrophilic surfaces under electrostatically repulsive conditions. Succinylated lysozyme has a lower stability as found with DSC. Directly above the isoelectric point, where the electrostatic interactions are repulsive, the adsorption of succinylated lysozyme is not zero. But on increasing the pH, the adsorption decreases steeply. Although the stability of lysozyme after succinylation is lower, it still behaves as a hard protein.
The desorption of succinylated lysozyme at the isoelectric point may lead to the conclusion that the protein does not spread under these conditions. This can also be the reason why the adsorption has a maximum at the isoelectric point.

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Chapter 4

Spreading of proteins and its effect on adsorption and desorption kinetics[1]

Abstract

The kinetics of adsorption of lysozyme and α-lactalbumin from aqueous solution on silica and hydrophobized silica is studied. The initial rate of adsorption of lysozyme at the hydrophilic surface is comparable with the limiting flux. For lysozyme at the hydrophobic surface and α-lactalbumin on both surfaces, the rate of adsorption is lower than the limiting flux, but the adsorption proceeds cooperatively, as manifested by an increase in the adsorption rate after the first protein molecules are adsorbed. Adsorption saturation, reflected in a steady-state value of the adsorbed amount, is invariant with the rate at which this value is reached. For both proteins, the saturated adsorption on hydrophilic silica is strongly dependent on the protein concentration in solution but on the hydrophobic interface this dependence is absent. Besides adsorption, desorption is studied. For lysozyme, it is found that the desorbable fraction decreases after longer residence time of the protein at the interface.

[1] submitted to Colloids and Surfaces B: Biointerfaces

4.1 Introduction

When a protein attaches to an interface, it may undergo shape changes to an extent depending on the forces exerted by the surface. Moreover, the shape change may in turn affect the interaction between adsorbed molecules, and lead to development of intermolecular association. All these processes may occur on widely varying time scales, so that it should come as no surprise that the kinetics of protein adsorption is very complicated indeed. One important aspect is the time dependence of the protein 'footprint'. The footprint is the surface area that a protein occupies on adsorbing. In many cases, the footprint increases with the residence time on the surface; this may be called spreading.

The spreading process is not only a function of intrinsic surface and protein properties, but must be influenced by the presence of neighboring molecules as soon as competition for available surface comes into play. E.g., the spreading may stop abruptly (jamming) or one molecule may (partly) displace a neighboring molecule.

An important consequence of this spreading process is the 'supply rate effect'.

As the protein concentration c becomes higher, the surface will be filled in a shorter time span. Hence, the time available for spreading will be shorter and if jamming stops the spreading, adsorbed proteins will achieve a smaller footprint at the surface. As a result the adsorbed amount Γ becomes larger. Thus, in many examples of protein adsorption, the spreading is less at a higher protein concentration [1-3]. Note that this is a kinetic rather than an equilibrium thermodynamic argument. The dependence of saturation coverage on the free solution concentration is often called 'adsorption isotherm', suggesting predominance of thermodynamic factors. However, the influence of wall shear rate on ultimate coverage is clear proof of kinetic control [4]. We will therefore not use the term 'isotherm' when we report $\Gamma(c)$ data.

If the spreading rate is much faster than the supply rate, the footprint of the protein molecules may reach its equilibrium value. The saturated adsorbed amount will be that of a layer of completely spread protein molecules. This adsorbed amount is independent of the supply rate. So, the final adsorbed amount is determined by a balance of the spreading rate and the rate of attachment [5,6].

What makes the matter so complex is that both the spreading rate and the rate of attachment may be influenced by many parameters, such as the surface chemistry

(charge, hydrophobicity) and protein characteristics like the structural stability and size. Wertz et al [4] measured the adsorption of albumin and fibrinogen on three different surfaces. They found that the initial adsorption rate was the same for the different surfaces, but the final footprint differed for the three interfaces, indicating a dependence of the interfacial spreading rate on the surface chemistry.

Once proteins are spread on the interface, they will not desorb easily. Wertz [4,7] studied the time dependence of the exchangeability of albumin by fibrinogen and found that albumin was less susceptible to exchange after a longer residence time of the protein on the surface. Vasina et al. [8] studied the exchange of α-chymotrypsin with radiolabeled α-chymotrypsin. These authors measured two exchange reactions in one experiment: first, labeled protein is exchanged by unlabeled protein and then this unlabeled protein is exchanged by labeled protein. In the work of Giacomelli et al. [9] homomolecular exchange is used to see the perturbations induced by the interface in stability and structure of BSA after exchange. They found that the structural characteristics of BSA are not affected after being exchanged from hydrophilic silica. Release from hydrophobic polystyrene yields BSA molecules of which the structural properties are altered.

Although much research has been done on this subject, a lot is still unclear with respect to the dynamics of protein adsorption. The objective of the present study was to investigate the influence of several variables (pH, surface wettability, protein characteristics) on adsorption kinetics, with a focus on factors affecting footprint and spreading behavior. We did this by directly measuring $\Gamma(t)$ under conditions of controlled diffusion layer thickness. This allowed us to compare the initial adsorption rate with the limiting flux. We could also determine the plateau adsorbed amount Γ_∞ at different protein concentrations in solution and we carried out desorption measurements while varying adsorbate residence time. The experiments were performed with two proteins, i.e., lysozyme and α-lactalbumin, which were supplied to two types of interfaces, i.e., silica and hydrophobized silica.

4.2 Materials and Methods

4.2.1 Materials

Hen egg-white lysozyme (L-6876) and α-lactalbumin (L-5385) were purchased from Sigma and used without further purification. The proteins were dissolved in acetate buffer (pH 4) or KNO_3 solution (pH 7). Unless otherwise stated, the adsorption experiments were carried out with 0.1 g/l protein solutions at an ionic strength of 50 mM.

The hydrophilic surfaces used were silica wafers with an oxide layer of about 80 nm. The wafers were cleaned by sonication in alcohol followed by ozonization in a plasma cleaner. To get a hydrophobic interface, the wafer was treated with dichlorodimethylsilane (DDS). The silane layer was attached to the silica wafer by dipping the wafer in a solution of 500 µl DDS in 30 ml trichloroethane for half an hour. The wafer was then rinsed with ethanol and water. The hydrophobicity was probed by measuring the contact angle with water and the wafers were considered well prepared if the contact angle was between 95° and 100°. Hydrophobized wafers were used without further cleaning.

The streaming potential of the wafers was measured from which the ζ-potential was calculated by standard procedures [10]. The ζ-potential of silica is - 45 mV in 5 mM KNO_3 and –27 mV in 50 mM KNO_3. At hydrophobic silica the ζ-potential is –32 mV in 5 mM KNO_3 and –25 mV in 50 mM KNO_3.

4.2.2 Reflectometry

The adsorbed mass of the proteins was measured as a function of time by reflectometry. This technique is a simplified form of ellipsometry, in which the changes in intensity of a reflected polarized laser beam upon adsorption are measured. For a more extended treatment of reflectometry we refer to Dijt et al. [11]. Briefly, a polarized laser beam is reflected on a surface at a fixed angle of incidence. After reflection from the bare surface the perpendicular (I_s) and the parallel (I_p) intensities of the light are measured. The output signal S is defined as the ratio I_p/I_s. Due to adsorption, the reflection changes from its value S_0 for the bare surface to $S_0 + \Delta S$. The adsorbed amount per unit surface area, Γ, is calculated from the relative change in signal, as follows:

$$\Gamma = \Delta S/S_0 \times 1/A_s \quad (1)$$

Here, A_s is a sensitivity factor that is determined by the optical properties of the surface. The value of A_s is calculated by an optical model in which every layer of the surface is assumed to be homogeneous and characterized by the thickness and the refractive index of that layer. We used a four-layer model: silicon, silica, protein and water. The thickness of the silica layer was measured by ellipsometry; the thickness of the protein layer was estimated. Refractive indices used are 3.85 for Si, 1.46 for SiO_2, 1.44 for protein and 1.33 for water. For the protein we used a refractive index increment of 0.185 ml/g [12]. A flow cell with stagnation point flow was used in this set-up. A measurement started by rinsing with buffer until a stable baseline was attained. Then the buffer solution flow was replaced by a flow of protein solution. The initial adsorption rate was compared with the limiting flux of the protein towards the surface as calculated by the expression:

$$\left(\frac{d\Gamma}{dt}\right)_{t=0} = kD^{2/3}c = k_{tr}c \quad (2)$$

Here k is a rate constant for a given hydrodynamic condition, c the concentration and D the diffusion coefficient of the polymer in the supplied solution. The value of k was determined by measuring the adsorption of polyethylene oxide (PEO) with a known diffusion coefficient on silica, assuming that the initial adsorption rate of PEO is equal to the limiting flux [11]. To derive the protein flux from the PEO result, corrections were made based on the concentration and the diffusion coefficient of the protein in the solution.

4.3 Results and Discussion

Effect of protein concentration

In figure 4.1, adsorption curves, $\Gamma(t)$ are given for α-lactalbumin at pH 4, adsorbing on a hydrophilic surface. First, we pay attention to the supply rate effect. The supply rate, varying proportionally with the protein concentration, influences the initial adsorption rate. If the initial adsorption rate is high, the filling time of the surface is short and the protein molecules have little time to spread. Consequently, more protein

molecules can be accommodated on the same surface area and adsorption saturation increases with increasing concentration of protein in solution.
In these data, the supply rate is varied over two decades. A very clear supply rate effect can be seen: at 0.001 g/l, Γ_∞ is no more than 0.5 mg/m^2, whereas at 0.1 g/l it reaches 1.7 mg/m^2, a more than threefold increase. Based on at the filling times τ_f observed in figure 4.1, we estimate that $\tau_f \approx 750$, 75 and 7.5 seconds for 0.001, 0.01 and 0.1 mg/ml, respectively. Assuming that Γ_∞ roughly spans the entire range of possible values (a dense monolayer of α-lactalbumine would correspond to ~ 2 mg/m^2), this would mean that the spreading rate τ_s is of order 100 s.

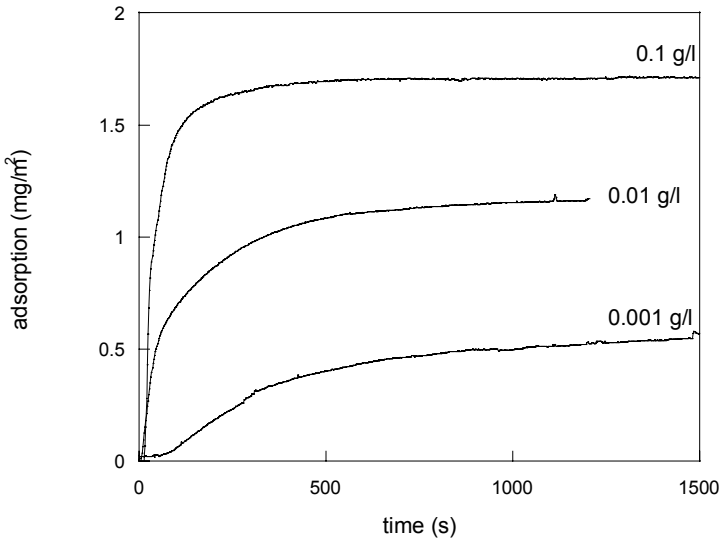

figure 4.1 Adsorption of α-lactalbumin on silica at different protein concentrations

In figure 4.2, the adsorption of α-lactalbumin on a hydrophobic surface at pH 4 is shown. Here, the situation is totally different. The initial adsorption rate spans a comparable range of values, but the plateau adsorbed amount is invariant with changes in concentration. Despite the variation in initial adsorption rate the adsorption levels off at the same plateau adsorbed amount.
Lysozyme shows similar behavior as α-lactalbumin: the initial adsorption rate increases with increasing supply rate and on the hydrophobic surface the same plateau value is reached for different protein concentrations, whereas at the hydrophilic

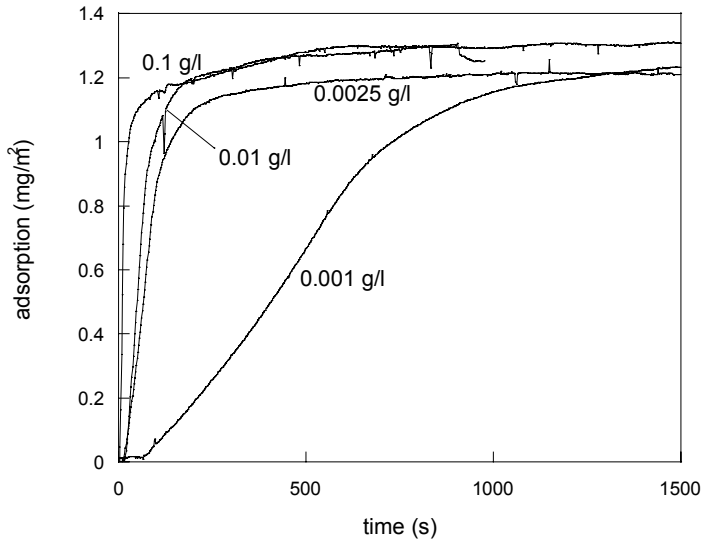

Figure 4.2 Adsorption of α-lactalbumin on silanized silica at different protein concentrations

surface the plateau value increases four-fold with increasing protein concentration. In figure 4.3, the adsorption plateaus $\Gamma_\infty(c)$, derived from the steady-state values of the $\Gamma(t)$ curves, are given for lysozyme and α-lactalbumin.

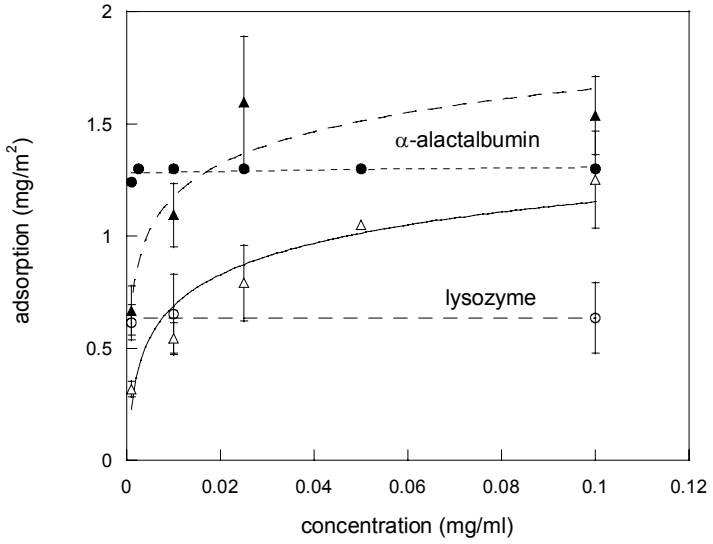

Figure 4.3 Adsorption of lysozyme and α-lactalbumin. open circles: lysozyme on hydrophobic surface; open triangles: lysozyme on hydrophilic surface; closed circles: α-lactalbumin on hydrophobic surface; closed triangles: α-lactalbumin on hydrophilic surface

Why is the behavior at hydrophilic and hydrophobic surfaces so different? If the supply rate and the spreading rate are of the same order of magnitude, the adsorbed amount must be affected by the supply rate. The protein molecules spread on the surface to an extent that is determined by the rate neighboring sites become occupied. The protein does not get enough time for complete relaxation. This is what happens on the hydrophilic interface over the concentration range presented in figure 4.1.
If the spreading rate τ_s is far greater or far smaller than the supply rate τ_f, the adsorbed amount will be independent of the supply rate. This is what happens on the hydrophobic surface. From the fact that the plateau adsorbed amount on the hydrophobic surface is lower than the saturation adsorption at the hydrophilic surface we conclude that spreading has occurred, hence $\tau_s << \tau_f$. On the hydrophobic interface, adsorbing protein molecules have sufficient time to spread, i.e. availability of sorbent surface area is not a limiting factor in the spreading process. As the supply rates to the hydrophilic and the hydrophobic surface are the same, it is concluded that spreading occurs faster at the hydrophobic surface.
Further comparison between adsorption saturation of α-lactalbumin at the two surfaces is presented in figure 4.4. At a high protein concentration (0.1 g/l) steady-state adsorption is higher on the hydrophilic interface, whereas at low protein

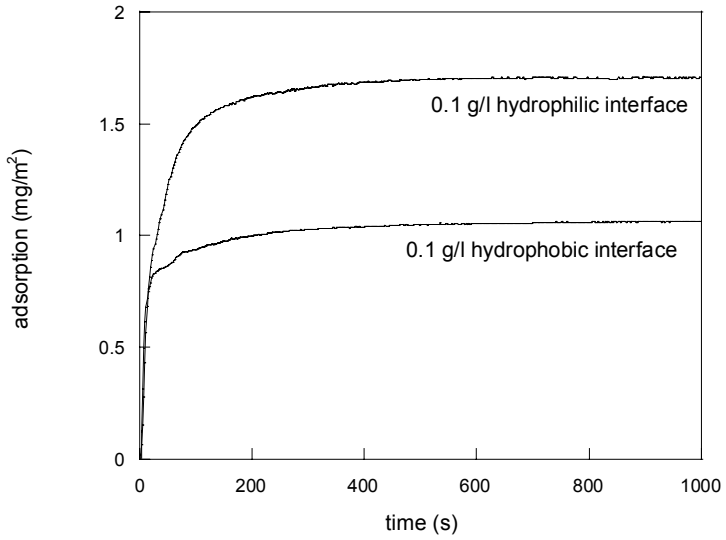

Figure 4.4a Adsorption of 0.1 g/l α-lactalbumin on both hydrophilic and hydrophobic interfaces.

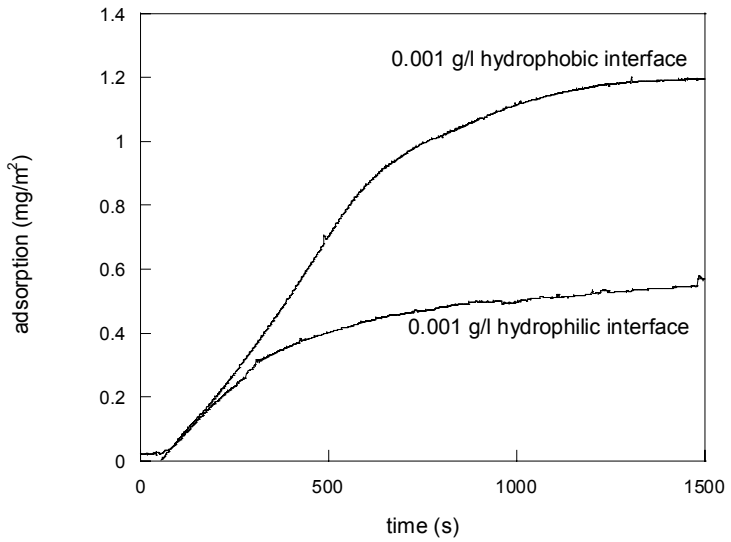

Figure 4.4b Adsorption of 0.001 g/l α-lactalbumin on both hydrophilic and hydrophobic interfaces.

concentration it is lower on the hydrophilic interface. Thus, although the spreading is faster on the hydrophobic interface, it spreads to a lesser extent. Apparently, the high affinity of adsorption at the hydrophobic surface leads to a less unfolded surface conformation of the adsorbed molecules. Remarkably, this means that α-lactalbumin spreads more extensively on the hydrophilic interface.

Effect of pH

The adsorption $\Gamma(t)$ of lysozyme on hydrophilic silica at different pH values is shown in figure 4.5. From pH = 9.1 to 10.1, the initial adsorption rate does not significantly vary with pH. This implies that the variation in the steady-state adsorption as a function of pH is not caused by the initial rate of adsorption, but by pH-dependent protein-sorbent interactions and/or interactions between the adsorbed protein molecules.

The adsorption kinetics of both lysozyme and α-lactalbumin on the hydrophobic surface are invariant with pH (data not shown). Apparently, variations in net charge do not significantly influence adsorption on the hydrophobic interface. This is in line with hydrophobic interaction being the major driving force for protein adsorption at

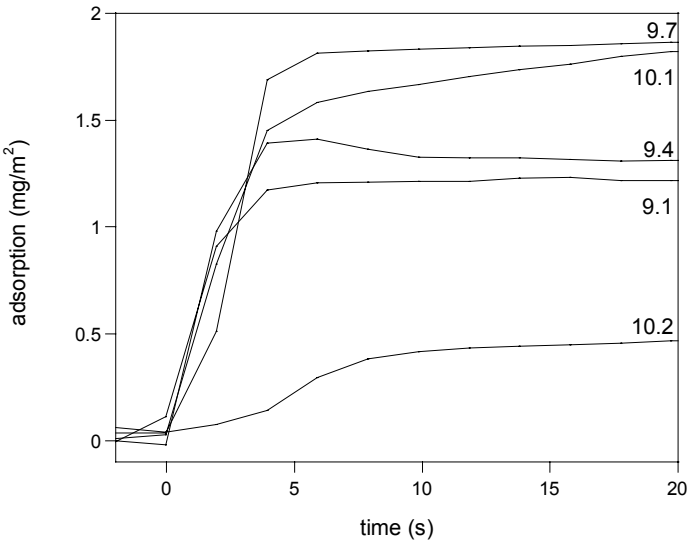

Figure 4.5 Adsorption of lysozyme at different pH values

hydrophobic interfaces [13]. Furthermore, zeta-potentials were derived from streaming potential measurements. We find that the hydrophobic silica has a lower zeta-potential than hydrophilic silica; this supports the suggestion that electrostatic interaction plays a minor role at the hydrophobic surface.

Initial adsorption rate

The data of figures 4.1 and 4.2 may be compared to the limiting flux. To this end, they are replotted in figure 4.6 as a function of *ct*. In this manner the data are normalized with respect to concentration, such that, if no other variables are involved, the curves would coincide. In addition, the protein arriving at the surface by the limiting flux (mg m^{-2} s^{-1}), as a function of the protein concentration in solution (mg m^{-3}), is plotted in the figure. If all the protein available near the surface adsorbs, the adsorption rate would equal the limiting flux. From the figure, it is clear that the rates of adsorption are lower than the limiting flux by about a factor of two. Apparently, the α-lactalbumin molecules arriving at both the hydrophobic and the hydrophilic interface have to pass a barrier before they can attach at the surface. The reason for the barrier is not clear. It is probably not of electrostatic nature since the protein and the interface have opposite charge signs. However, it cannot be excluded that an uneven distribution of the overall charge endows the protein molecule with a

positively and a negatively charged side. Then, only protein molecules that arrive at the surface in the right orientation may adsorb. For the highest protein concentration (0.1 g/l), the lower (normalized) rate is probably due to the enlarged scale of the graph. Due to cooperative binding of protein molecules at the sorbent surface the adsorption of the highest concentration.

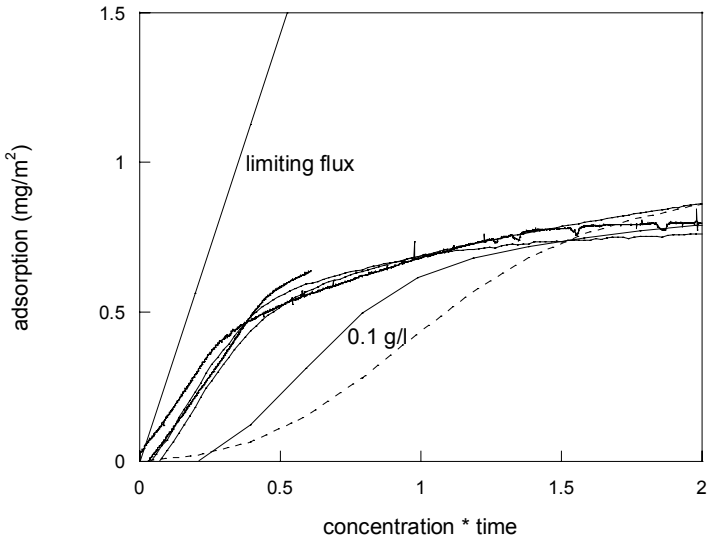

Figure 4.6 Comparison of the adsorption rate of α-lactalbumin with the limiting flux. Only for c=0.1 the rates differ between the two surfaces. Dashed line: hydrophilic interface; solid line: hydrophobic interface

In figure 4.7, the adsorption of lysozyme on a hydrophilic interface is plotted as a function of *ct*. Here, the initial rate of adsorption does not deviate much from the limiting flux, implying the absence of an adsorption barrier. Indeed, the protein and the surface attract each other electrostatically since at pH 7 lysozyme is positively and the silica negatively charged. At a certain critical surface coverage, the adsorption abruptly slows down for all three concentrations. This may correspond to the adsorbed amount where the surface charge of the silica is compensated by that of the adsorbed lysozyme molecules and, hence, to the point where electrostatically attractive (fast adsorption) conditions change into electrostatically repulsive (slow adsorption) conditions.

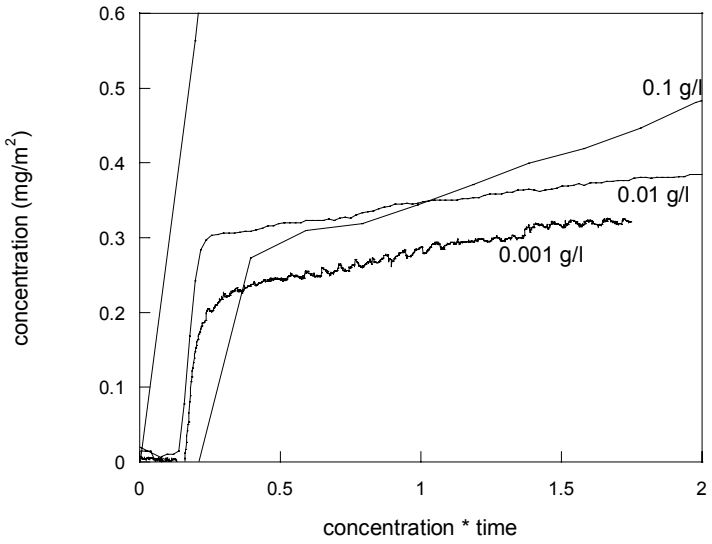

Figure 4.7 Comparison of the adsorption rate of lysozyme on hydrophilic surface with the limiting flux.

Cooperativity

The adsorption of lysozyme on silanized silica as a function of *ct*, is plotted in figure 4.8. Here, the initial adsorption rate at high concentration (0.1 g/l) is lower than the limiting flux and upon lowering the protein concentration it approaches the limiting

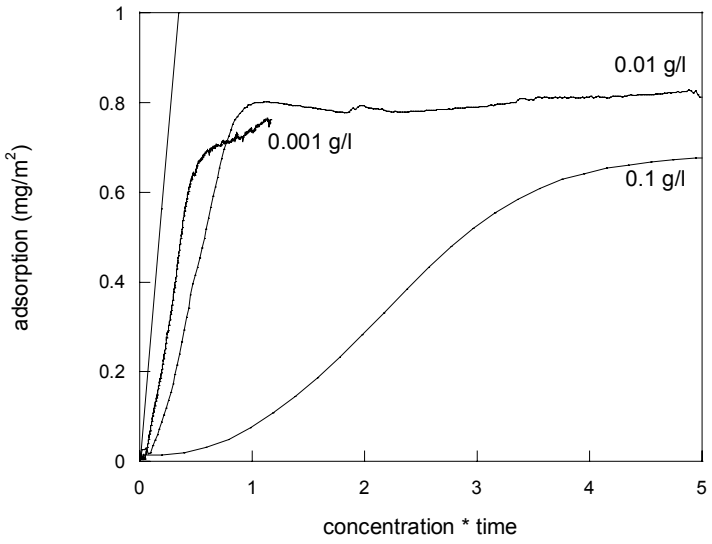

Figure 4.8 Comparison of the adsorption rate of lysozyme on hydrophobic surface with the limiting flux.

flux. The S-shape of the curves indicates that the first adsorbing molecules enhance adsorption of the subsequent ones, suggesting a cooperative adsorption mechanism. Cooperativity may be analyzed more quantitatively by calculating the adsorption rate constant k_a^{eff} as a function of time. According to [14]:

$$\frac{d\Gamma}{dt} = \frac{c_b - c_{eq}}{\frac{1}{k_{tr}} + \frac{1}{k_a^{eff}}} \tag{2}$$

in which k_{tr} is the rate constant for transport to the surface, c_b the concentration in bulk solution and c_{eq} the concentration in the solution near the surface. In the initial stage of the adsorption process $c_b >> c_{eq}$, and equation (2) may be written as:

$$k_a^{eff} = \frac{1}{\frac{c_b}{d\Gamma/dt} - \frac{1}{k_{tr}}} \tag{3}$$

To derive k_a^{eff}, $d\Gamma/dt$ is taken from the adsorption curves (figures 4.1 and 4.2) and k_{tr} is calculated from the flux (formula 1). For adsorption of lysozyme on hydrophobic silica, k_a^{eff} is plotted against time in figure 4.9. A sharp peak shows up for 0.1 g/l protein and a broader and higher peak for 0.01 g/l. In this plot cooperativity is now manifested by the initial increase in the rate constant for attachment, k_a^{eff}. The peak

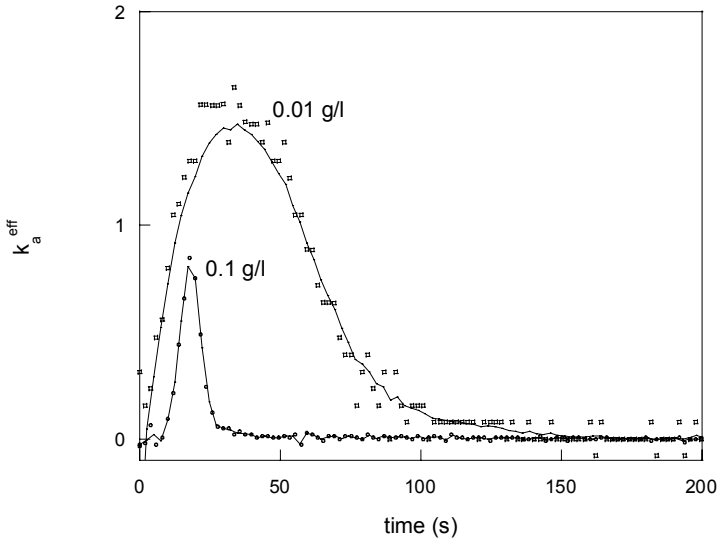

Figure 4.9 k_a^{eff} of lysozyme on hydrophobic silica

for 0.01 g/l is higher because the adsorption rate is closer to the limiting flux. The k_a^{eff} for 0.001 g/l is not plotted because the adsorption rate at this low concentration does not exceed the noise of the reflectometer output.

Adsorption of lysozyme on hydrophilic silica does not show cooperativity. The initial adsorption rate is very fast and levels off on filling the surface. α-Lactalbumin adsorbs cooperatively on both the hydrophilic and hydrophobic surface. The $k_a^{eff}(t)$ curves are very similar to the ones plotted in figure 4.9, except for the hydrophilic surface where the decline of k_a^{eff} is less pronounced.

Desorption

The influence of the kinetics of adsorption on the subsequent desorption upon dilution has been studied as well. In figure 4.10 the adsorption of lysozyme on the hydrophilic surface is monitored and after some time the surface is rinsed with buffer. The desorbed amount of lysozyme decreases with the residence time of the protein at the surface. After approximately 20 minutes the desorbable fraction becomes independent of the residence time. It is remarkable that even after this time a fraction of the protein still desorbs.

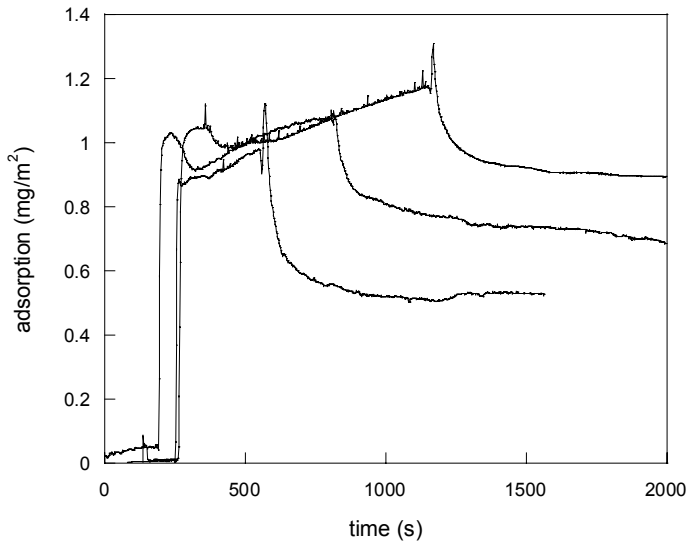

Figure 4.10 desorption of lysozyme from silica after different adsorption times

This feature is more clearly shown in figure 4.11, where the percentage of protein that remains adsorbed during rinsing is plotted as a function of the time the protein has

been in contact with the surface. The percentage increases more or less linearly until after 20 minutes the percentage of protein that sticks irreversibly on the surface is about 80 %. Hence, on the time scale of these measurements, 20 % of the protein remains loosely bound to the surface. If the probability of desorption is suppressed because lysozyme molecules have a larger footprint at the surface, one would be tempted to conclude from these data that the timescale of the spreading is about 20 minutes. However, this is not supported by the data concerning the supply rate effect. Hence, it seems that a second surface process occurs which converts loosely bound molecules into tightly bound ones. Furthermore, when this experiment is performed at higher protein concentrations (data not shown) the residence time needed for reaching the lowest desorbed fraction is shorter and this suggests that the irreversibility is due to protein aggregation on the surface.

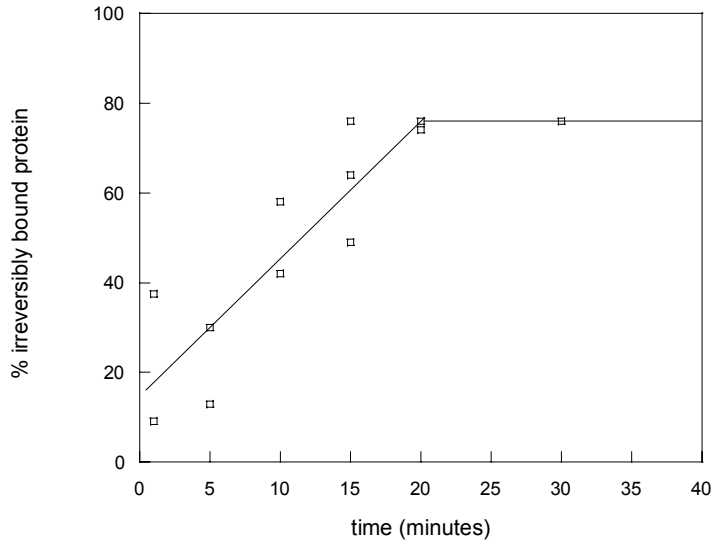

Figure 4.11 the desorption plotted as the percentage desorbed against the adsorption time

This experiment was also done with α-lactalbumin at pH 7. On rinsing the surface with buffer no significant desorption takes place (data not shown). At pH 7, where both the protein and the silica surface are negatively charged adsorption is electrostatically unfavorable. Therefore, the driving force for adsorption is non-electrostatic and dehydration of the hydrophilic surface would neither contribute to the adsorption process. Because α-lactalbumin is a soft protein, we expect that

adsorption-induced structural changes in the α-lactalbumin molecules increase protein adsorption affinity and therewith contribute to the irreversibility of the adsorption.

Conclusion

The kinetics of adsorption of lysozyme and α-lactalbumin from aqueous solution on silica and hydrophobized silica were studied. The conclusions from this work may be summarized as follows:

- For both proteins the saturated adsorption on hydrophilic silica is strongly dependent on the protein concentration in solution but on the hydrophobic interface this dependence is absent. It is concluded that on the hydrophobic interface the spreading rate is far greater than the supply rate, and on the hydrophilic interface the spreading rate is of order 100 s, which is of the same order as the supply rate.
- The initial rate of adsorption of lysozyme at the hydrophilic surface is comparable with the limiting flux. For lysozyme at the hydrophobic surface and α-lactalbumin on both surfaces, the rate of adsorption is lower than the limiting flux and the adsorption proceeds cooperatively, as manifested by an increase in the adsorption rate after the first protein molecules are adsorbed.
- For lysozyme it is found that the desorbable fraction decreases after longer residence time of the protein at the interface. This irreversibility might be due to surface aggregation of the protein, as suggested from the observation that diminishing the desorbable fraction occurs faster for higher protein concentration.

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Chapter 5

Lysozyme and succinylated lysozyme at the oil/water interface. Surface shear rheology and stabilization of oil-in-water emulsions

Abstract

The behavior of oil-in-water emulsions stabilized by lysozyme and succinylated lysozyme was compared with surface shear measurements on monolayers of the same proteins at an oil/water interface. Surface shear measurements were performed at different pH values. By succinylating the protein, the electrical charge of lysozyme was shifted to more negative values. Furthermore, succinylation lowers the thermal stability of the protein's globular structure. In the range measured, for both proteins the apparent surface shear viscosity was higher when the pH was closer to the isoelectric point. The monolayer of succinylated lysozyme was faster in forming a network, but that of lysozyme had the highest viscosity reached after 1400 minutes.

Oil-in-water emulsions were made and stabilized by lysozyme and succinylated lysozyme. Due to the adsorbed protein layer, these emulsions contain positively and negatively charged oil droplets, respectively. The emulsions were mixed and because of opposite charges on the emulsion droplets, they aggregate. The effect of mixing proportions was investigated and the largest aggregates were found when the mixing ratio deviated from unity.

After a prolonged period of time (more then 24 hours), the surface shear viscosity of lysozyme and succinylated lysozyme still increased, but no difference was found between mixing fresh or aged emulsions. It seems that the formation of a protein network at the interface is not decisive in the protection of the emulsion against coalescence.

5.1 Introduction

Many food products are emulsions, i.e., droplets of a liquid dispersed in another liquid. In processing such products, it is important to control the droplet size and the stability of the emulsion. For that purpose, often mixtures of surfactants and proteins are used. It is assumed that the low molecular weight surfactants stabilize the fluid interfaces by the Gibbs-Maragoni mechanism (which implies a certain degree of surface mobility), whereas the stabilizing role of the proteins is based on formation of a strong viscoelastic network in which the molecules are essentially immobile.

In this paper, we investigate the role of the viscoelastic network in emulsions by comparing surface shear measurements of proteins at the oil/water interface with the formation and stability of corresponding oil-in-water emulsions. Although proteins are frequently used as emulsifiers, relatively few fundamental studies have been done on proteins at oil/water interfaces, because experimental problems are often encountered when performing measurements at such an interface. For instance, reflectivity methods like IRRAS or ellipsometry suffer from the fact that the contrast between the refractive indices of the protein and the oil is small and, moreover, the interface of the overlaying oil layer with air scatters the incoming laser beam, which perturbs the measurement. Monolayers at the oil/water interface may be investigated by surface shear rheology. It allows determination of the apparent surface shear viscosity, which, in turn, gives information on the strength of a network formed between the molecules in the monolayer. However, this technique does not provide information on the amount of protein adsorbed at the interface. Literature reports on surface shear rheology of proteins at oil/water interfaces are scarce. A recent example is the work of Roth et al. [1] who investigated the pH dependence of surface shear characteristics for β-lactoglobulin in a water/dodecane interface. More data are available for air/water interfaces. For example, Martin et al. [2] investigated the surface shear of several proteins at the air/water interface and decided there was a correlation with surface pressure data. Krause et al. [3] studied the influence of succinylation on the interfacial behavior of legumin. They found a higher surface viscosity of the air/water interface upon succinylation. They also found that in an emulsion of decane in water, the droplet size decreased and the stability against coalescence increased with increasing degree of succinylation, and concluded that this is because the

modification causes the protein to dissociate into smaller subunits, therewith enhancing its molecular flexibility.

We studied the influence of succinylation on the interfacial behavior of lysozyme at an oil/water interface. Upon succinylation, the charge of lysozyme changes and hence succinylation is a good tool to study the influence of electrostatic interactions. Lysozyme is a relatively stable protein and although succinylation lowers the stability of the compact globular structure it does not completely destabilize the protein (this thesis, chapter 2). First, we investigated the surface shear rheology of lysozyme and succinylated lysozyme as a function of pH. Secondly, we studied emulsions made with lysozyme and succinylated lysozyme. These emulsions contain oppositely charged oil droplets and the effect of mixing was studied. By comparing the surface shear data with the emulsion experiments, we hope to find an indication for the role of network formation in emulsion stability.

5.2 Materials and Methods

5.2.1 Materials

Hen egg-white lysozyme was purchased from Sigma (L-6876). Succinylated lysozyme was prepared according to chapter 2. The oil used was tricaprylin from Sigma. Potassium acetate (pH 4) and potassium carbonate (pH 10) were used as buffer and potassium nitrate was used for the experiments at pH 7. All experiments were performed at an ionic strength of 10 mM.

5.2.2 Surface shear rheology

The instrument, shown in figure 5.1, has been described by Martin et al. [2]. Here, we use the apparatus with a thin layer of oil on top of the water phase. A stainless steel biconical disc with a radius of 30 mm is suspended from a torsion wire of 740 mm long and 0.2 mm thick, giving a torsion wire constant of 1.8×10^{-5} Nm/rad. The outer rim of the disc is exactly in the center of the investigated liquid/liquid interface. The dish containing the liquids has a diameter of 145 mm.

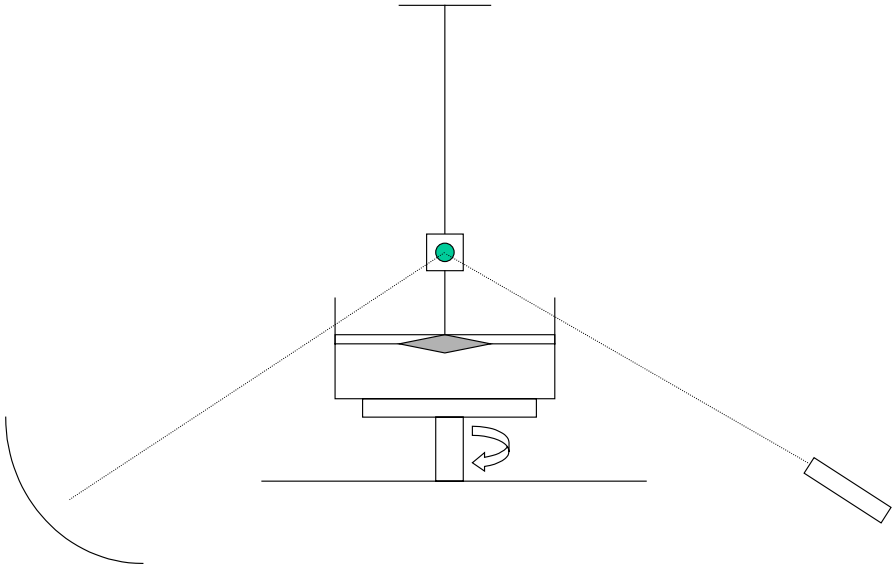

Figure 5.1 The set-up of the surface shear rheometer. Redrawn from Martin et al. [2]

Prior to the rheological measurement, the dish was filled with the protein solution (0.1 mg/ml), so that the disk just touched the surface. Next 50 ml of oil was carefully poured on top of the solution, so that formation of a protein monolayer at the oil/water interface could start. Directly after the oil was supplied the time was set at zero. After a certain time a constant rotation velocity of 1.27×10^{-3} rad/s was applied to the dish. The disc's final angle of rotation is used to calculate the apparent surface shear viscosity following the equation:

$$\eta_{app} = \frac{\tau}{4\pi\Omega}\left(\frac{1}{R_i{}^2} - \frac{1}{R_o{}^2}\right) \qquad (1)$$

Here, $\tau = K \times \Theta_i$ with K the torsion wire constant and Θ_i the angle of rotation of the disk; Ω is the angular velocity of the dish and R_i and R_o are the radius of the disk and the dish, respectively. We denote the viscosity as 'apparent' because it is not known how the strain in the film is distributed. The expression given assumes a homogeneous stress and no slip at the boundaries, but it has been repeatedly observed that in protein films the deformation may be very local along a fracture line [2]. According to Whorlow [4] the stress is defined as:

$$\sigma = \frac{\tau}{2\pi R_i^2} \qquad (2)$$

5.2.3 Emulsions

Oil-in-water emulsions were prepared by mixing 0.1 g/ml aqueous protein solutions of 10 mM KNO_3 with tricaprylin oil to yield a two phase system containing 20 (w/w) % oil. First, the solution was mixed for 1 minute using an ultraturrax (T25 basic, IKA, Staufen, Germany) equipped with an 18 mm dispersing element (S25KR-18 G, IKA). Then, the emulsion was homogenized for 10 passes at 50 bar (homogeniser unit HU-2.0, Delta Instruments, Drachten, The Netherlands). The final emulsion contained 0.8 w/w % protein. The droplet size of the particles was measured with a Malvern Mastersizer 2000 S. Creaming was checked with a turbiscan MA2000. Droplet aggregation was investigated under a light microscope. The zeta potential of the emulsion droplets was determined using a Zetasizer 2000 (Malvern). For the zetasizer measurements, the emulsion was diluted with 5 mM KNO_3 to obtain a solution with around 500 counts/min. Mixing of the emulsions was done by pipetting appropriate amounts of either kind of emulsion in eppendorf cuvettes that were subsequently shaken by hand.

5.3 Results and Discussion

5.3.1 Surface Shear Rheology

In figures 5.2 and 5.3 stress-strain curves of monolayers of lysozyme and succinylated lysozyme, at pH 9.5 and pH 4.6, respectively, are given. These pH-values are close to the isoelectric point of the two proteins. The surface shear experiment was done after different durations of resting time of the interface. It is observed that for both proteins the network strength increases in time: the stresses measured at a given strain increase strongly over timescales of hours. There are no major differences in the shape of the curves for lysozyme and succinylated lysozyme. All stress-strain curves exhibit a maximum in the stress. Martin et al. [2] ascribed this maximum to fracture of the protein film near the disk. The shape of the stress-strain curves indicates that the protein layers are visco-elastic. Upon continuous deformation, the protein network at the interface is, at least partly, destroyed.

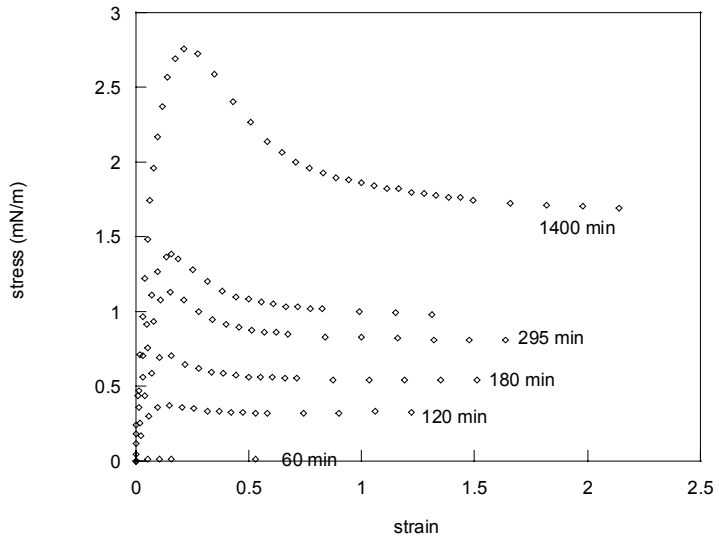

Figure 5.2 Stress-strain plot of lysozyme at pH 9.5 at different aging times

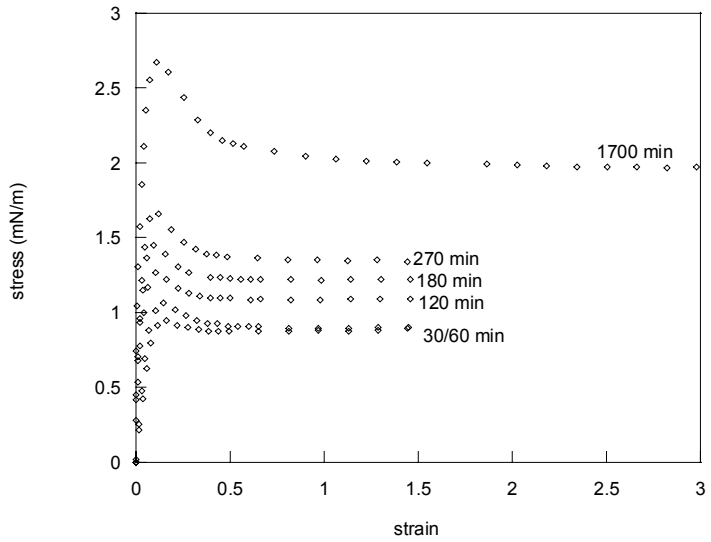

Figure 5.3 Stress-strain plot of succinylated lysozyme at pH 4.6 at different aging times

Beyond the maximum, the stress relaxes to a constant value, and one might say that the surface 'flows': it achieves a constant strain rate. The steady-state angle of rotation of the disk is used to calculate the apparent surface shear viscosity. In the figures 5.4 and 5.5 this viscosity is plotted against aging time of the film. For lysozyme, a lag time of one hour is

found before a measurable stress develops. For succinylated lysozyme, no lag time could be detected. The viscosity keeps increasing for both proteins at all pH values and for the whole duration of the experiment. The increase is steeper for lysozyme than for succinylated lysozyme. It was not possible to measure for longer times because microbial contamination occurred in the solutions, rendering the results unreliable.

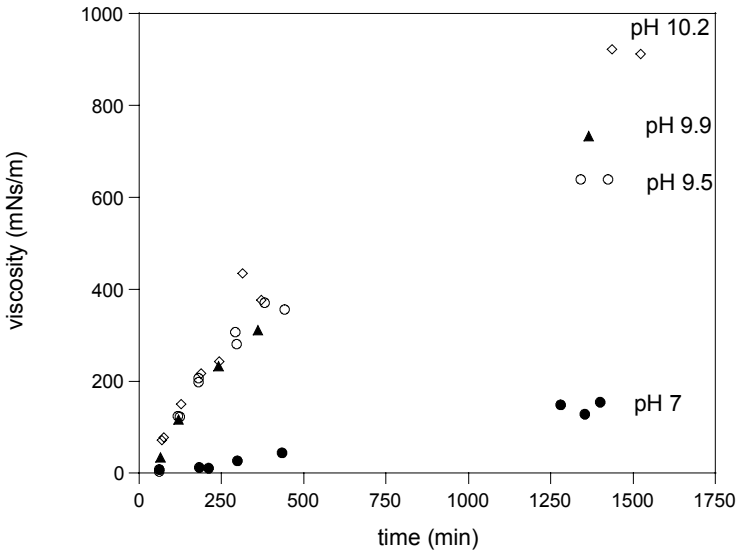

Figure 5.4 Apparent surface shear viscosity of lysozyme plotted against aging time for different pH values

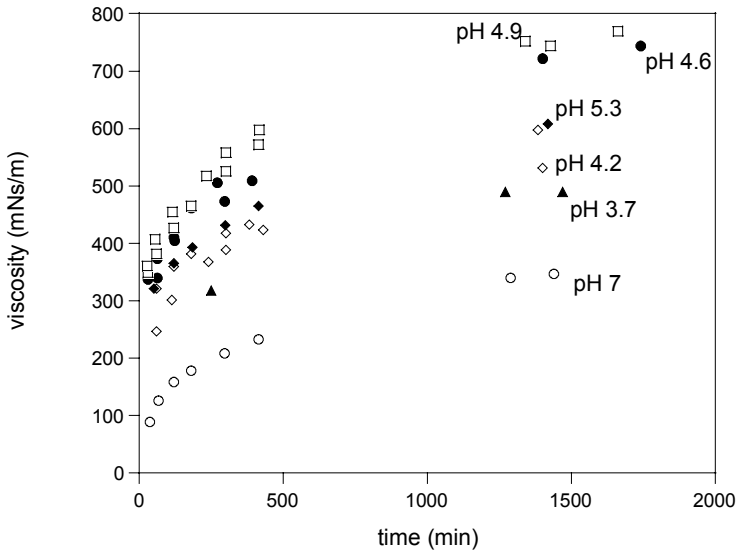

Figure 5.5 Apparent surface shear viscosity of succinylated lysozyme plotted against aging time for different pH values

The influence of pH is visible in figures 5.4 and 5.5. To get a better insight in the influence of the electrostatic interactions, the viscosity is given as a function of the charge on the protein in figures 5.6 and 5.7. The experiments were performed at different pH values and the charge was taken from proton titration curves of lysozyme and succinylated lysozyme (this thesis, chapter 3). Curves are given for a short (120 min) and a long (overnight) time of network formation. In both figures and for both proteins, it is clear that a stronger network is formed when the pH is closer to the isoelectric point of the protein. Furthermore, after 120 minutes of aging the viscosity for lysozyme is lower than for succinylated lysozyme, but after overnight aging the viscosity for lysozyme is higher than for succinylated lysozyme.

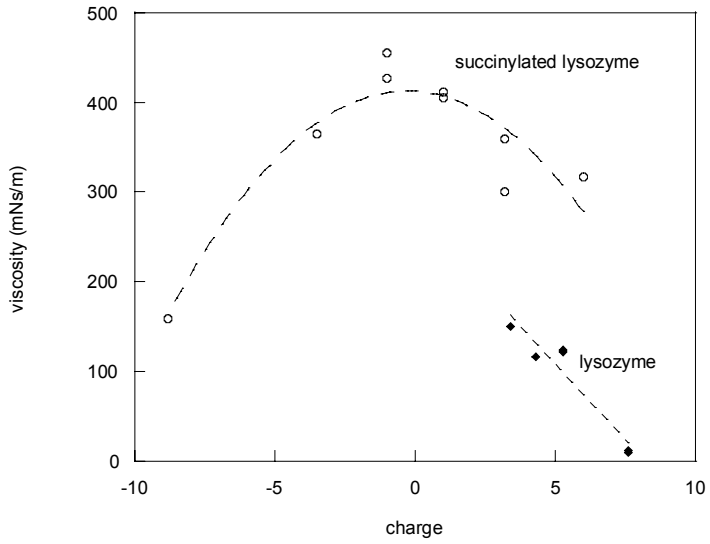

Figure 5.6 Apparent surface shear viscosity of lysozyme and succinylated lysozyme plotted against the protein charge after 120 minutes of aging time.

What is determining the surface shear viscosity of a protein layer? Martin [5] claims that the viscosity is related to the hardness i.e., the structural rigidity, of proteins. Using seven proteins of varying hardness (among which lysozyme) she found an exponential decrease of the steady-state stress with increasing normalized area (A_0/M) of the protein molecules at the interface. Besides that, it may be that the proteins unfold and make entanglements. In this work, we find that the surface shear viscosity is highest in the isoelectric point, that there is a large effect of resting time of the proteins at the interface, and that the less stable succinylated lysozyme is faster in forming a network.

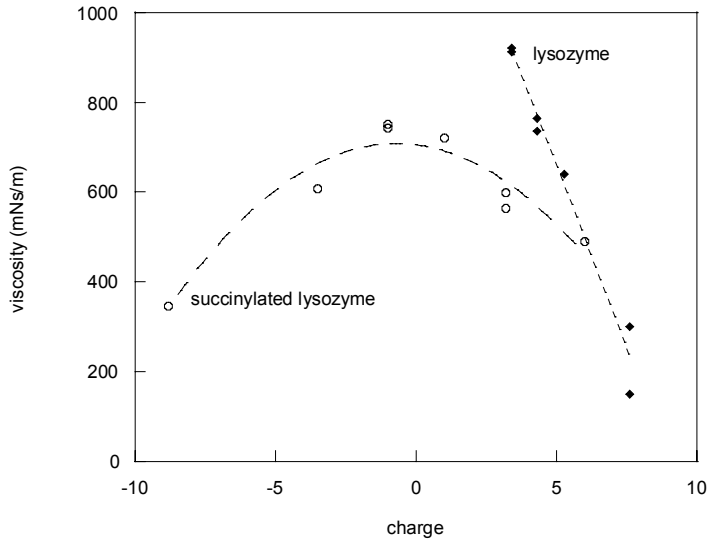

Figure 5.7 Apparent surface shear viscosity of lysozyme and succinylated lysozyme plotted against the protein charge after overnight aging.

For solid/liquid interfaces it is generally found that the adsorbed amount is largest in the isoelectric point [6] (this thesis, chapter 3). This may apply for the liquid interface as well. A larger adsorbed amount would contribute to a higher surface viscosity. Moreover, due to a lower electrostatic repulsion, neighboring protein molecules can contact each other more easily thus promoting network formation. Furthermore, it is thought that proteins are most stable in the isoelectric point. In this line of thought it is probable that the viscosity is largest here due to the hardness of the protein. Thus, the maximum value of the surface shear viscosity in the isoelectric point can support both interpretations, i.e. in terms of protein hardness and in terms of adsorbed amount.

The difference in time for the increase in the surface shear viscosity for lysozyme and succinylated lysozyme is related to the lower structural stability of the succinylated protein. This points to the formation of an interfacial protein network where (partial) unfolding of the protein plays a role. Due to its lower structural stability succinylated lysozyme unfolds faster than unmodified lysozyme, leading to faster network formation. Besides this, there is a large difference between the time scale of the adsorption process and the time scale of the increase in the surface shear viscosity. The adsorption process takes place within 1 hour [7,8] but the viscosity still increases after 24 hours. Apparently, the protein molecules in the monolayer continue to rearrange for a long time after adsorption. The influence of pH and the long time needed for network formation, together,

point to an influence of entanglement of unfolded protein molecules and network formation on the surface shear viscosity.

5.3.2 Mixed emulsions

Separate oil-in-water emulsions stabilized by lysozyme and succinylated lysozyme were prepared and then mixed in different proportions. The emulsions were unbuffered (aqueous phase contains 10 mM KNO_3) and the pH of the emulsions was about 6.5. At neutral pH, lysozyme is positively charged and succinylated lysozyme is negatively charged. Anticipating the discussion on zeta potential measurements, we note that the droplets in the two emulsions are oppositely charged as well.

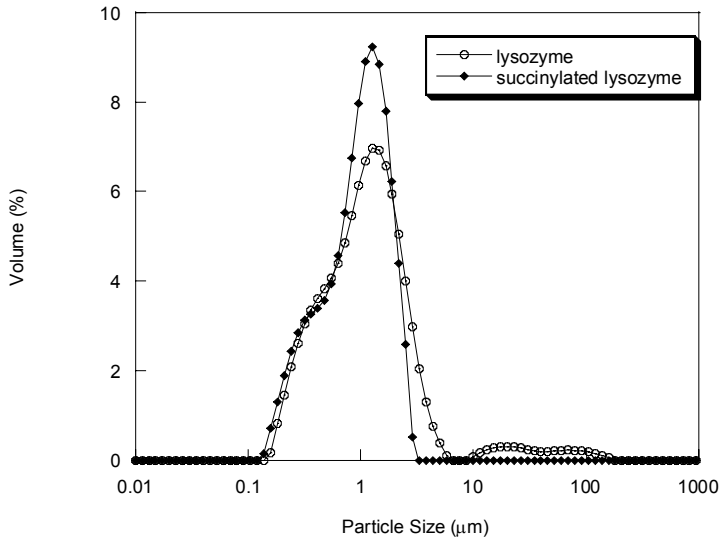

Figure 5.8 Size distribution of the single emulsions as measured with the mastersizer

The size of the emulsion droplets was measured with a mastersizer. The data for the separate emulsions are plotted in figure 5.8. The droplets in both emulsions were of similar size, i.e., about 1 μm in diameter. Figure 5.9 shows particle size distributions obtained when lysozyme-stabilized and succinylated lysozyme-stabilized emulsions are mixed in the proportions 80/20 v/v % and 20/80 v/v %. The particle sizes in these systems are about 10 μm. Hence, mixing the emulsions leads to aggregation and/or coalescence of the droplets.

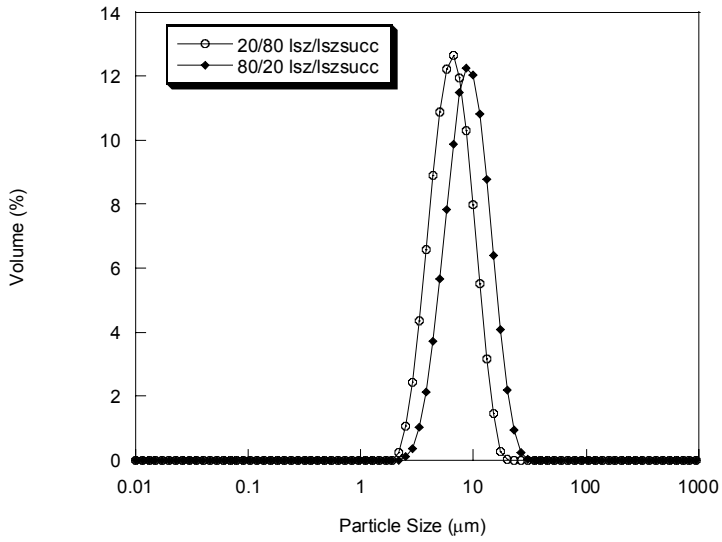

Figure 5.9 Size distribution of the mixed emulsions as measured with the mastersizer

Light microscope pictures of the single and the mixed emulsions are given in figures 5.10 and 5.11. The picture of the single emulsion displays single monomer droplets and in the mixed emulsion aggregates of individual droplets are visible. The size of these droplets is still 1 μm, implying the absence of coalescence.

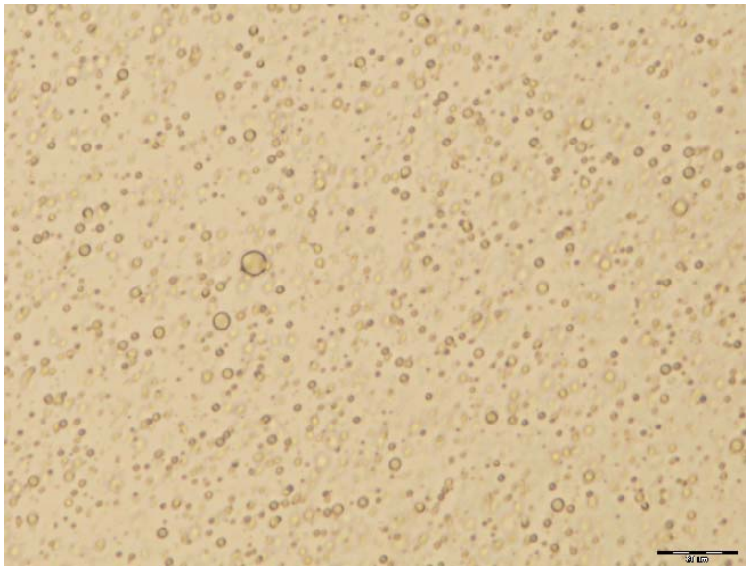

Figure 5.10 Microscope photograph of an emulsion made with succinylated lysozyme.

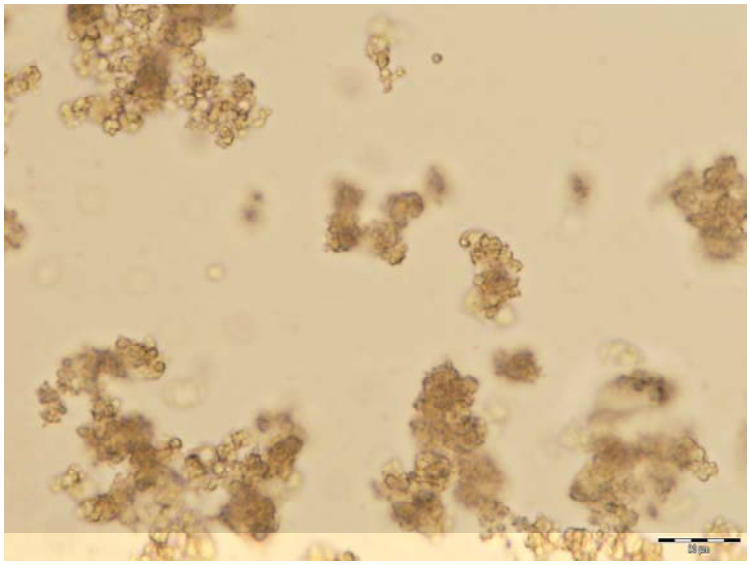

Figure 5.11 Microscope photograph of a mixture of 80 % lysozyme-stabilized emulsion and 20 % succinylated lysozyme-stabilized emulsion

Neither the single nor the mixed emulsions creamed in the turbiscan. The droplets in the separate emulsions are probably too small to cream during the observation time, i.e., 24 hours, as can be concluded from an estimate of the creaming velocity. For the aggregated emulsions, the particle size, as measured in figure 5.9, would certainly be expected to cream rapidly. However, creaming does not occur. The absence of creaming might perhaps be an indication that the aggregates are tenuously structured, keeping water in between the aggregated oil droplets. Aggregates with a compact structure would have a larger density difference with the surrounding water and this would make them more susceptible to creaming. However, it is more probable that on mixing the emulsions, a (weak) particle-gel is formed. If all the emulsion droplets participate in a network, they will not cream. The zeta potential of the single and the mixed emulsions is measured with a zetasizer. On the emulsion droplets of the separate emulsions the zeta potential is + 45 mV for lysozyme and –47 mV for succinylated lysozyme. For all mixing proportions, the aggregates in the emulsion mixtures are slightly positively charged. This points to a charge compensation mechanism in the aggregation process. One scenario would be that equal numbers of droplets from each charge sign are clustered together in one aggregate. If this happens, there should be left over monomer emulsion droplets in the uneven mixtures. In the mastersizer measurements as well as in the microscope pictures no monomers are visible. This leaves co-adsorption of ions or protein molecules in the aggregates as the most probable charge regulation mechanism. We note that the emulsions are prepared with an

excess amount of protein so that a significant amount of free protein in the water phase is available for incorporation in the aggregates.

The effect of mixing proportions is investigated by plotting the size measured in the mastersizer of the aggregates against the mixing proportion. In figure 5.12, the size of the aggregates D[3,2] is highest in the cases were the mixing is unequal. This is where the amount of protein complex is lowest. What is the meaning of the measured sizes? In the mastersizer, the aggregates are subjected to high shear rates. Besides being subjected to shear, the emulsions are diluted with water. In case of weak aggregation the aggregates would have fallen apart upon dilution. Because the mixed emulsions do not cream and are expected to form a gel, we think that this system falls apart in smaller structures during the mastersizer experiment.

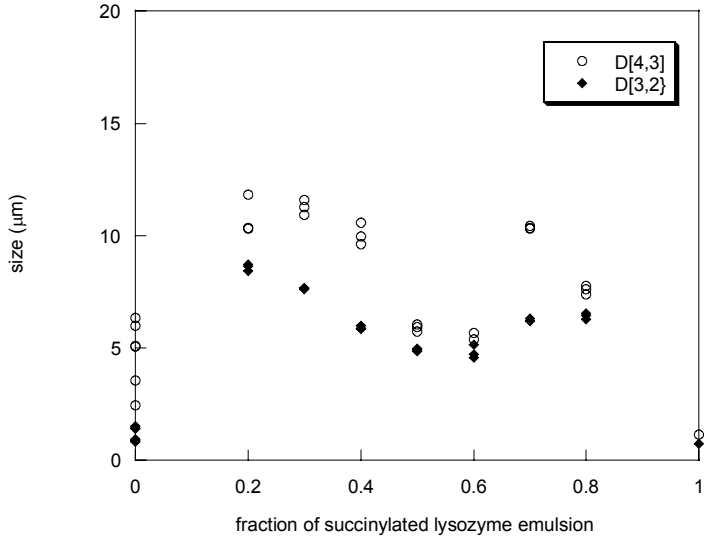

Figure 5.12. Size D[3,2] and D[4,3] of mixtures of emulsions plotted against the fraction of emulsion stabilized with succinylated lysozyme

The difference in size in the mastersizer experiments thus reflects a difference in the stability of the aggregates. In the picture that we have of these aggregates, excess protein molecules bridge the emulsion droplets to form an aggregate. In the work of Biesheuvel et al.[9] it is found that mixtures of lysozyme and succinylated lysozyme form complexes in solution above a certain (salt dependent) threshold protein concentration. With the presence of emulsion droplets, these complexes can accumulate at the surface of emulsion droplets. Due to equal amounts of both proteins, in the 50/50 mixture the amount of protein to form a complex available is at the maximum. In this mixture the protein layer in

between the emulsion droplets is thicker and thus easier to split up in the mastersizer. An experiment to verify this would be to measure the size of the aggregates by mixing washed emulsions in which the excess protein is removed. If the stability is influenced by excess protein bridging the emulsion droplets, the 50/50 mixture would be more stable with less excess protein and the size of the aggregates would be larger.

5.3.3 Comparing surface shear rheology with mixing of the emulsions

It has been suggested that the formation of a protein network in the oil/water interface is required in protecting emulsions against coalescence [10]. To compare the surface shear data with the behavior of the emulsions, the emulsions are mixed after aging for a day. In this way the protein network on the emulsion droplets has had time to form. We find that there is no obvious difference in mixing fresh or aged emulsions. Thus, we do not find a significant effect of time on the behavior of the mixed emulsion, whereas formation of a network, as measured in the increase in the surface shear viscosity, is strongly time-dependent. It seems that the formation of an adsorbed interfacial layer on the surface of the oil droplets is already protecting the emulsion against coalescence.

5.4 Conclusions

Surface shear measurements on monolayers of lysozyme and succinylated lysozyme in an oil/water interface were performed at different pH values. In the range measured, for both proteins the apparent surface shear viscosity was higher when the pH was closer to the isoelectric point. The monolayer of succinylated lysozyme was faster in forming a network, but that of lysozyme had the highest final viscosity reached after 1400 minutes. The influence of pH and the long time needed for network formation, together, point to an influence of entanglement of unfolded protein molecules and network formation on the surface shear viscosity.

Oil-in-water emulsions were made and stabilized by lysozyme and succinylated lysozyme. Due to the adsorbed protein layer, these emulsions contain positively and negatively charged oil droplets, respectively. The emulsions were mixed and because of opposite charges on the emulsion droplets, the droplets aggregate. The effect of mixing proportions was investigated and the largest aggregates were found when the mixing ratio deviated from unity. More experiments are needed to fully explain this phenomenon.

The mixing of fresh emulsions showed no difference in the aggregation behavior with the mixing of aged emulsions, despite continuous increasing of the surface shear viscosity of

lysozyme and succinylated lysozyme until 24 hours. It seems that the formation of a protein network at the interface is not decisive in the protection of the emulsion against coalescence.

5.5 References

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Summary

Many food products are emulsions, i.e., a system of two immiscible liquids of which the one is finely dispersed in the other. In many emulsions, a monolayer of protein molecules in the liquid/liquid interface prevents coalescence of the droplets. Therefore, in the formation of a protein-stabilized emulsions the adsorption of proteins at the oil/water interface is an important step. To obtain a better insight in the characteristics of the protein layer that are relevant for stabilizing emulsions we studied protein adsorption using model systems. The proteins we selected were lysozyme and α-lactalbumin. Their adsorption and characteristics in the adsorbed state were investigated at solid/liquid, i.e., silica/water, and liquid/liquid, i.e., oil/water, interfaces.

The major interactions determining protein adsorption are (a) electrostatic interaction between the protein molecules and the sorbent surface, (b) changes in the state of hydration of the sorbent surface and the protein molecule, and (c) changes in the three-dimensional structure of the protein molecule. In this thesis, there is an emphasis on electrostatic interactions. To study the influence of electrostatic interactions on protein adsorption, lysozyme is chemically modified by adding succinyl groups. The succinyl groups react with lysine and other cationic groups which are converted into anionic groups. Upon succinylation with an excess of succinyl, ten succinyl groups are linked to a lysozyme molecule. As a consequence, the isoelectric point of lysozyme shifts from 11 to 4.5. Besides affecting the charge of the protein, its structural stability decreases.

In chapter 2, the influence of succinylation on the structural properties of lysozyme is studied using circular dichroism, fluorescence spectroscopy, and differential scanning calorimetry. The spectroscopic data reveal that at room temperature the structures of succinylated lysozyme and native lysozyme are similar. However, the calorimetric results show that the thermal stability, as reflected in the denaturation temperature and the Gibbs energy of denaturation, of succinylated lysozyme is lower than that of native lysozyme. It is furthermore remarkable that the change in the heat capacity ($\Delta_{den}C_p$) upon thermal denaturation for succinylated lysozyme is much higher than for native lysozyme. This is explained in terms of an extended degree of unfolding of the secondary structure and full exposure to the aqueous environment of the apolar parts of the succinyl groups.

In chapter 3, the influence of electrostatic interactions on protein adsorption was studied on solid/liquid interfaces, by comparing the adsorption of lysozyme and succinylated lysozyme at silica surfaces. The succinylation not only affects the charge of the protein, but also its structure stability, as described in chapter 2. Although changes in stability may have an influence on adsorption, our data show that the primary effect of succinylation can be entirely understood in terms of electrostatic interactions. The saturated adsorbed amount as a function of pH has a maximum for both proteins. This maximum coincides with the isoelectric point for succinylated lysozyme and is close to the isoelectric point for lysozyme. At pH values where the protein is electrostatically repelled by the sorbent, higher ionic strengths increase adsorption, and for electrostatic attraction higher ionic strengths decrease adsorption.
In chapter 4, the kinetics of adsorption of lysozyme and α-lactalbumin on silica and hydrophobized silica is investigated. For lysozyme at the hydrophilic interface, the rate of adsorption increases proportionally with increasing protein concentration in solution. The adsorption rate is comparable with the supply rate. It implies that all of the positively charged lysozyme molecules attach at the negatively charged silica surface. For α-lactalbumin, which is also positively charged, the initial rate of adsorption is a fraction of the supply rate. For both proteins, adsorption saturation increases with increasing supply rate, indicating less spreading of the adsorbed protein molecules when the sorbent surface becomes more rapidly covered by the protein.
At the hydrophobic interface, the initial adsorption rate of both proteins is considerably lower than the supply rate, especially for the highest concentration measured. The final adsorbed amount of both proteins is invariant with the supply rate. It suggests that structural rearrangements in the adsorbed protein molecules occur at a shorter time scale than that of the supply.
It is furthermore remarkable that for lysozyme at the hydrophobic interface and for α-lactalbumin at both types of interfaces, the adsorption proceeds in a cooperative manner, as is manifested by an increase in the adsorption rate after the first molecules are adsorbed. Besides adsorption, desorption is studied. For lysozyme, it is found that the desorbed amount decreases after longer residence time of the protein at the interface.

In chapter 5, the behavior of oil-in-water emulsions stabilized by lysozyme and succinylated lysozyme was compared with surface shear measurements on monolayers of the same proteins at an oil/water interface. Surface shear measurements were performed at different pH values. In the range measured, for both proteins the apparent surface shear viscosity was higher at pH values closer to the isoelectric point. The monolayer of succinylated lysozyme was faster in forming a network, but that of lysozyme had the highest final viscosity reached after 1400 minutes.
Oil-in-water emulsions were made and stabilized by lysozyme and succinylated lysozyme. Due to the adsorbed protein layer, these emulsions contain positively and negatively charged oil droplets, respectively. The emulsions were mixed and because of opposite charges on the emulsion droplets, the droplets aggregate. The effect of mixing proportions was investigated and the largest aggregates were found when the mixing ratio deviated from unity.
After a long time (more then 24 hours), the surface shear viscosity of lysozyme and succinylated lysozyme still increased, but no difference was found between mixing fresh or aged emulsions. It seems that the formation of a protein network at the interface is not decisive in the protection of the emulsion against coalescence.

Samenvatting

Veel levensmiddelen, zoals melk, mayonaise, slagroom, sladressing en crème likeur, zijn emulsies. Een emulsie is een mengsel van olie (zoals bijvoorbeeld zonnebloemolie) en water, waarbij de ene vloeistof, als kleine druppeltjes, zweeft in de andere vloeistof. Deze druppeltjes zijn niet vanzelf stabiel en als er niet een extra component wordt toegevoegd zullen de twee vloeistoffen ontmengen. Deze extra component wordt emulgator en/of stabilisator genoemd.

In veel levensmiddelen worden eiwitten als emulgator toegevoegd. Een laagje eiwit gaat in de rand van de druppel zitten en vormt daar een laagje. Deze laag van eiwit in het grensvlak voorkomt dat de druppeltjes kunnen samenvloeien. Daarom is het van belang dat de eigenschappen van eiwitten aan oppervlakken bestudeerd worden. In dit proefschrift is vooral gekeken hoe het eiwit naar het oppervlak gaat en hoeveel er uiteindelijk op zit. Deze adsorptie is bestudeerd op vast/vloeistof en olie/water grensvlakken.

De eiwitten die wij bestudeerd hebben zijn lysozyme en α-lactalbumin. Lysozyme wordt gehaald uit het wit van kippeneieren en α-lactalbumin wordt gehaald uit melk. Beide eiwitten zijn globulair. Ze zijn van dezelfde grootte maar de stabiliteit van de globulaire structuur is erg verschillend. Ook de elektrische eigenschappen van de eiwitten verschillen sterk. Daarnaast is lysozyme chemisch gemodificeerd met succinyl groepen. Door de modificatie verschuift het iso electrisch punt van lysozyme van 11 naar 4.5. Hierdoor kan de invloed van elektrostatische interacties op de adsorptie van eiwit bestudeert worden.

Omdat er naast een effect op de elektrische lading van het eiwit ook een effect op de stabiliteit van het eiwit verwacht wordt, is in hoofdstuk 2 de invloed van succinyleren op de structuur en structuur stabiliteit van lysozyme onderzocht. De structuur is bestudeerd met circulair dichroisme en fluorescentie spectroscopie. De stabiliteit is gemeten met differentiële scanning calorimetrie. Uit de spectroscopische metingen blijkt dat de structuur van lysozyme niet significant verandert door de chemisch modificatie. Uit de calorimetriemetingen blijkt echter dat de stabiliteit van gesuccinyleerd lysozyme beduidend lager is dan dat van natief lysozyme.

De warmtecapaciteit van een eiwitoplossing in water neemt toe ten gevolge van denaturatie van dat eiwit. Deze verandering van de warmtecapaciteit ($\Delta_{den}C_p$) is bepaald voor het gesuccinyleerde en het ongemodificeerde eiwit. Het blijkt dat

succinylatie een sterke verhoging van $\Delta_{den}C_p$ veroorzaakt. Dit wordt verklaard doordat gesuccinyleerd lysozyme verder ontvouwt en doordat de apolaire delen van de succinylgroepen aan het oplosmiddel (water) zijn blootgesteld.
In hoofdstuk 3 is de invloed van elektrostatische interacties op eiwit adsorptie bestudeerd door een vergelijking te maken tussen de adsorptie van lysozyme en gesuccinyleerd lysozyme op silica. Zoals beschreven is in hoofdstuk 2 is gesuccinyleerd lysozyme anders geladen en minder stabiel dan lysozyme. Ondanks deze verandering in de stabiliteit, is de invloed van de elektrostatische interacties duidelijk te zien in de adsorptiemetingen. De geadsorbeerde hoeveelheid heeft een maximum als functie van de pH voor beide eiwitten. Dit maximum komt overeen met het iso electrisch punt voor gesuccinyleerd lysozyme en is dichtbij het isoelectrisch punt voor lysozyme. Ook de invloed van de zoutconcentratie is gemeten. Bij pH waarden waar het eiwit elektrostatisch wordt afgestoten door het oppervlak, is de adsortpie hoger bij een hogere zoutsterkte en bij elektrostatische aantrekking verlaagt een hogere zoutsterkte de adsorptie.
De snelheid waarmee het eiwit op het oppervlak gaat zitten (de kinetiek van de adsorptie) van lysozyme en α-lactalbumin op silica en hydrofoob silica is bestudeerd in hoofdstuk 4. De hoeveelheid die na een bepaalde tijd op het silica geadsorbeerd is, is voor beide eiwitten sterk afhankelijk van de concentratie van het eiwit. Dit in tegenstelling met het hydrofobe oppervlak waar de maximale geadsorbeerde hoeveelheid onafhankelijk is van de concentratie. Daarnaast is de snelheid van adsorptie vergeleken met de aanvoersnelheid. Voor lysozyme op silica is de adsorptiesnelheid vergelijkbaar met de aanvoersnelheid. De adsorptiesnelheid van lysozyme op het hydrofobe oppervlak en α-lactalbumine op beide oppervlakken is lager dan de aanvoersnelheid. Hierbij valt op dat in tijdens het ‘vollopen’ van het oppervlak de adsorptiesnelheid toeneemt en dat de adsorptie dus coöperatief is.
In een experiment waarbij na de adsorptie het oppervlak gespoeld wordt, is gevonden dat de hoeveelheid die van het oppervlak gespoeld wordt, afneemt als het eiwit langer op het oppervlak zit. Dit komt doordat het eiwit aggregeert op het oppervlak.
In hoofdstuk 5 is gekeken naar de rheologische eigenschappen van geadsorbeerde lagen van lysozyme en gesuccinyleerd lysozyme aan olie/water grensvlakken en naar de menging van emulsies gemaakt met lysozyme en gesuccinyleerd lysozyme. De oppervlakteviscositeit van de grenslaag is gemeten en dit is een maat voor de

netwerkvorming van de eiwitten. Deze oppervlakteviscositeit werd voor beide eiwitten hoger, dichter bij het iso electrisch punt . Een verschil was dat het netwerk van gesuccinyleerd lysozyme sneller gevormd werd, maar met lysozyme werd uiteindelijk de hoogste viscositeit bereikt.
Olie in water emulsies gestabiliseerd met lysozyme en gesuccinyleerd lysozyme bevatten respectievelijk positief en negatief geladen oliedruppeltjes. Deze emulsies zijn gemengd en door de tegengestelde ladingen op de druppeltjes trekken deze elkaar aan en klonteren ze samen (aggregeren). Het mengen is gedaan in verschillende verhoudingen en daarbij viel op dat de grootste aggregaten werden gevonden bij een ongelijke mengverhouding.

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Marijn

Levensloop

Marijn van der Veen werd op 21 oktober 1972 geboren te Heerenveen. Na het behalen van het VWO-diploma in 1991 aan de RSG te Heerenveen begon zij in hetzelfde jaar met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. In november 1998 werd deze studie afgesloten en per 1 januari 1999 begonnen met een promotie onderzoek bij de leerstoelgroep fysische chemie en kolloidkunde aan Wageningen Universiteit. De resultaten van dit promotie onderzoek staan beschreven in dit boekje.

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