Modulating Surface Rheology by Electrostatic Protein/Polysaccharide Interactions

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There is a large interest in mixed protein/polysaccharide layers at air−water and oil−water interfaces because of their ability to stabilize foams and emulsions. Mixed protein/polysaccharide adsorbed layers at air−water interfaces can be prepared either by adsorption of soluble protein/polysaccharide complexes or by sequential adsorption of complexes or polysaccharides to a previously formed protein layer. Even though the final protein and polysaccharide bulk concentrations are the same, the behavior of the adsorbed layers can be very different, depending on the method of preparation. The surface shear modulus of a sequentially formed β-lactoglobulin/pectin layer can be up to a factor of 6 higher than that of a layer made by simultaneous adsorption. Furthermore, the surface dilatational modulus and surface shear modulus strongly (up to factors of 2 and 7, respectively) depend on the bulk β-lactoglobulin/pectin mixing ratio. On the basis of the surface rheological behavior, a mechanistic understanding of how the structure of the adsorbed layers depends on the protein/polysaccharide interaction in bulk solution, mixing ratio, ionic strength, and order of adsorption to the interface (simultaneous or sequential) is derived. Insight into the effect of protein/polysaccharide interactions on the properties of adsorbed layers provides a solid basis to modulate surface rheological behavior.

Introduction

A classical application of electrostatic protein/polysaccharide interaction in the food industry is the use of pectin to stabilize casein micelles in acidified milk drinks. Due to electrostatic interaction negatively charged pectin molecules adsorb to the positively charged casein micelles. As a consequence electrostatic and steric repulsion prevent the micelles from acid-induced aggregation.[1−3] Polysaccharides are also used in food emulsions to prevent aggregation and creaming of emulsion droplets.[4−7] By layer-by-layer deposition of oppositely charged proteins, polysaccharides, and surfactants on emulsion droplets,[8,9] one can control the net charge on the droplets and enhance emulsion stability by electrostatic repulsion. However, depending on the relative concentrations, polysaccharides can also decrease emulsion stability due to bridging flocculation.[10]

Besides establishing electrostatic and/or steric repulsion, adsorbed layers at the air−water or oil−water interface may also affect foam and emulsion stability by their elastic behavior.[11] In this context ample attention has been paid to surface rheological behavior of different proteins depending on the pH, ionic strength, and aging of the interface.[12−16] When dealing with complex systems (e.g., protein/polysaccharide mixtures), it is important to understand how the functional behavior of the individual components, such as surface activity, rheology, etc., is affected by interactions between the components. Furthermore, rheological measurements can provide insight into the buildup of mixed protein/polysaccharide layers at liquid interfaces.

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Electrostatically driven coadsorption of non-surface-active anionic polysaccharides with proteins can affect the surface rheological properties.[17−19] Ducel and co-workers report that the oil−water interfacial rheological properties of plant protein/arabic gum coacervates are related to the interfacial rheological properties of the protein used.[20] Schmitt et al. describe an effect of the aging time of β-lactoglobulin/acacia gum complexes on air−water surface rheology in terms of reorganization of the complexes at the interface.[21] However, it is not known how the surface properties are related to the structure of/and interactions within the complex surface layer.

When mixing an anionic polysaccharide such as pectin with a protein at low ionic strength and above the pK_a of the polysaccharide, one can form protein/polysaccharide complexes. Depending on the protein/polysaccharide mixing ratio and protein/polysaccharide binding affinity, these soluble complexes can either be soluble or aggregate and phase separate (as a liquid coacervate phase or precipitate).[22] From previous work it is known that soluble β-lactoglobulin/low methoxyl pectin complexes (at pH 4.5) adsorb at air−water interfaces and that protein/polysaccharide interactions can be used to control the adsorption kinetics at the air−water interface.[23]

This work provides a mechanistic understanding of how surface dilatation and the surface shear rheological behavior depends on the electrostatic protein/polysaccharide interaction in the bulk solution, mixing ratio, ionic strength, and order of adsorption to the air−water interface (simultaneous or sequential). On the basis of all the observations, a model is proposed on how the parameters mentioned above could be used to control the buildup of protein/polysaccharide layers and, as a result, tune the surface rheological behavior at the air−water interface. In a different report it is shown that the model is also valid for oil−water interfaces.[24]

Materials and Methods

Materials. Acetate buffers (pH 4.5, ionic strength 9 mM) were prepared from analytical grade chemicals and deionized water (Barnstead EASYpure UV). Please note that values for ionic strength mentioned in the text represent the ionic strength of the buffer. Bovine β-lactoglobulin (isoelectric point 5.1) was purified using a nondenaturing method as described previously.[25] Low methoxyl pectin was supplied by CP Kelco, Lille Skensved, Denmark. The degree of methylation is 30.4% (only the nonmethylated galacturonic acid subunits have a free carboxyl group, p$K_a \approx 4.5$), the uronic acid content is 78.5%,[26] the number-averaged molar mass (M_n) is 1.5×10^5 g/mol, and the polydispersity (M_w/M_n) is 2.4.[23] Pectin solutions were prepared by wetting the powder with ethanol and subsequent dispersion in deionized water, followed by heating at 70 °C for 30 min. After overnight storage, the samples were centrifuged at 6000*g* for 10 min and stored at 4 °C until further use. Protein stock solutions (for the ζ-potential, 20 g/L; for all other measurements, 1 or 2 g/L) were prepared freshly every day by dissolving the freeze-dried protein in deionized water, allowing at least 30 min for dissolving. The pH of the protein stock solutions was 6.8 ± 0.2. After dilution of the pectin stock solution with buffer, addition of protein stock solution to a protein concentration of 0.1 g/L, and mixing, the protein/polysaccharide samples were equilibrated for 30 min before use to allow formation of protein/polysaccharide complexes.

Light Scattering. The second cumulant diffusion coefficients and scattered light intensities (at 90°) of the pectins and the β-lactoglobulin/pectin complexes were determined by light scattering[27] using an ALV 5000 light scattering instrument (Langen, Germany) equipped with a 400 mW argon laser tuned at a wavelength of 514.5 nm, as described before.[28] From dynamic light scattering hydrodynamic radii were calculated according to the Stokes−Einstein relation, assuming that the pectin molecules and the complexes are spherical. Averages and standard deviations were calculated from sets of 10 measurements. Temperature was controlled at 20 ± 0.1 °C. Stock protein (2 g/L), pectin (2 g/L), and buffer solutions (25 mM acetate buffer, ionic strength 9 mM, pH 4.5) were filtered through 0.45 μm filters (Acrodisc, Gelman Sciences, MI). For each mixing ratio a separate sample was prepared by subsequent addition of stock pectin and stock protein solution to the buffer solution. The protein concentration was 0.1 g/L for all samples, and the pectin concentrations varied from a protein/pectin mixing ratio of 1 w/w to a protein/pectin mixing ratio of 15 w/w. The 0 w/w sample is a 0.1 g/L pectin solution, without protein, in the same buffer. Protein/polysaccharide samples were equilibrated for 30 min before use to allow formation of protein/polysaccharide complexes.

Determination of the ζ-Potential. The electrophoretic mobility of soluble protein/polysaccharide complexes was measured using a Zetasizer 2000 HS (Malvern Instruments Ltd., U.K.) at 150 V applied voltage, using a He−Ne laser at 633 nm. The ζ-potential was calculated using the Helmholtz−Smoluchovski equation. The protein concentration could not be kept constant (but varied from 0.01 to 0.05 g/L) in these measurements because the scattered light intensity varies too much between the different mixing ratios to allow determination of the ζ-potential. Stock β-lactoglobulin and stock pectin solutions were mixed with buffer (ionic strength 9 mM, pH 4.5) to a final total concentration—depending on the mixing ratio—such that the signal retrieved was optimal and only a single narrow peak was observed in the scattered light intensity versus electrophoretic mobility curve. Average values and standard deviations were calculated over sets of 5−10 measurements. A duplicate set of samples was measured, and the differences were within 5%.

Drop Tensiometry/Surface Dilatational Rheology. Surface tension as a function of time was measured (for single-component and mixed solutions of protein and polysaccharide) using a profile analysis tensiometer (PAT1, SINTERFACE Technology, Berlin, Germany). Each experiment started with a clean interface of a freshly formed sample solution droplet (14 μL) hanging on the tip of a double concentrical needle connected to two separate syringes. Injection through the inner syringe, while liquid is withdrawn through the outer syringe such that the droplet surface area is kept constant, allows the solution inside the droplet to be exchanged while the previously formed adsorbed layer is kept intact. The rinsing velocity

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was 1 μL/s. The surface tension was determined by bubble shape analysis. All results are presented in terms of surface pressure $\Pi = \gamma_0 - \gamma$, where γ_0 is the surface tension of the solvent (72 mN/m) and γ is the measured surface tension.

Surface dilatational moduli (ϵ) were determined by subsequent expansion and compression of the interfacial area A (amplitude in area oscillation 6%, frequencies 0.01, 0.05, and 0.1 s^{-1}) and recording of the resulting change in surface tension: $\epsilon = d\gamma/(d \ln A) = -d\Pi/(d \ln A)$. Measurements of the dilatational modulus as a function of time were performed using a different drop tensiometer setup (ADT, ITCONCEPT, Longessaigne, France), where immediately before the start of the experiment an air bubble (7 μL) is formed on the tip of a needle in the sample solution as described before.[29] Periods of five oscillations (amplitude in area oscillation 4%, frequency 0.1 s^{-1}) were alternated with equally long resting periods. Average dilatational moduli, as well as the average surface tension during the oscillations, were determined using the last three oscillations of each oscillation set. All experiments were performed at least in duplicate; differences were within 5−10%. Temperature was controlled at 21 ± 1 °C. Experiments were performed at 2 mM ionic strength in addition to 9 mM, as indicated in the text, to enhance differences between the different samples.

Surface Shear Rheology. The surface shear rheological behavior of adsorbed protein/polysaccharide layers at air−water interfaces was investigated using a strain-controlled Couette-type interfacial shear rheometer, as described elsewhere.[30,31] Buffer solution was poured into the sample holder, and subsequently, at time zero, a 10 times concentrated protein or protein/polysaccharide solution was injected into the bulk solution at the bottom of the sample beaker to a final protein concentration of 0.1 mg/mL. Immediately after injection, a disk was suspended from a torsion wire until it just touched the interface. The sample was left to adsorb and equilibrate for 20 h at 21 ± 1 °C, during which the surface tension was monitored using a Wilhelmy plate. Deformation of the interface is achieved by turning the sample container at an angular velocity of 1.27 × 10^{-3} rad/s, and the resulting stress on the interfacial layer near the disk was calculated from the rotation of the disk. Stress−strain curves were calculated from these values. Since the relative deformation of the interface is not uniform due to the large gap width relative to the disk diameter, the ratio of stress and strain is to be considered as an apparent surface shear modulus. All experiments were performed in duplicate or triplicate.

Results

To investigate how protein/polysaccharide interactions affect the properties of mixed protein/polysaccharide adsorbed layers, we first consider surface pressures. Figure 1a shows a typical surface pressure versus time curve for a pure β-lactoglobulin solution (0.1 g/L, pH 4.5, I = 2 mM, dotted line) and for a mixture of β-lactoglobulin and pectin (protein/polysaccharide mixing ratio 2 w/w, dashed line) measured with a pendant drop tensiometer. Pectin alone does not increase the surface pressure by more than 0.5 mN/m in 30000 s at the concentrations used (not shown). The double-syringe setup enables one to replace the protein solution inside the droplet with buffer solution. When this option is employed, one observes an instantaneous inhibition of surface pressure development (Figure 1a, solid curve). It appears that the increase in surface pressure due to protein adsorption can be stopped at any value by rinsing with buffer, after which the surface pressure remains constant in time (the fluctuations in surface pressure are due to vibrations caused by the rinsing and disappear when the rinsing stops). Because in the case of simultaneous adsorption the major part of the β-lactoglobulin is bound to pectin, the adsorption kinetics to the air−water interface in the presence of pectin are different from those of the pure protein case.[23] In particular, the exact amount of protein in the mixed layers is unknown and the role of pectin is unclear. To elucidate this, it is important to separate protein and polysaccharide effects. Using the rinsing method, one can compare interfaces with and without pectin starting from the same β-lactoglobulin layer. Rinsing a droplet—after formation of a protein layer—with a pectin solution (0.05 g/L, pH 4.5, I = 2 mM) instead of with buffer results in a 3 mN/m increase in

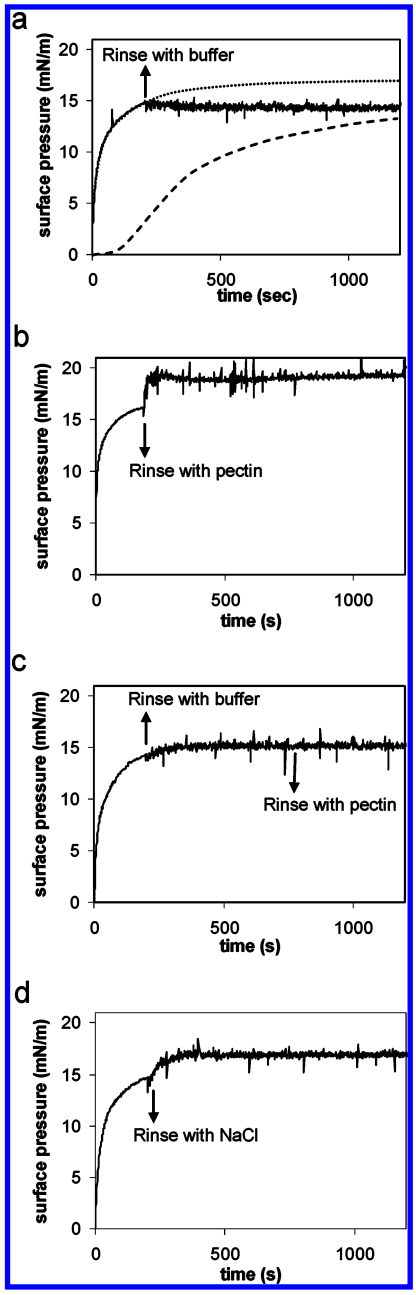

Figure 1. Surface pressure (at the air−water interface) versus time curves for a 0.1 g/L β-lactoglobulin solution, pH 4.5, I = 2 mM, flushed (from 200 s) with (a) buffer (the dotted line represents β-lactoglobulin without rinsing; the dashed line represents β-lactoglobulin/pectin in the case where β-lactoglobulin and pectin were mixed before the experiment started, also without rinsing), (b) pectin solution, (c) first buffer and then pectin (from 750 s), and (d) 50 mM NaCl solution.

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Table 1. Dilatational Moduli and Phase Angles of Different β-Lactoglobulin and β-Lactoglobulin/Pectin Layers Adsorbed to an Air−Water Interface[a]

	dilatational modulus (mN/m)			phase angle (deg)		
type of adsorbed layer	frequency 0.1 s^{-1}	frequency 0.05 s^{-1}	frequency 0.01 s^{-1}	frequency 0.1 s^{-1}	frequency 0.05 s^{-1}	frequency 0.01 s^{-1}
protein rinsed with buffer	68	66	57	10	12	13
protein rinsed with 0.05 g/L pectin	108	103	86	12	13	18
protein rinsed with buffer, then with 0.05 g/L pectin	109	104	92	11	12	15
protein rinsed with 50 mM NaCl solution	79	76	62	10	11	17
simultaneously adsorbed 2 w/w protein/pectin	61	56	43	13	14	16

[a] The β-lactoglobulin bulk concentration was 0.1 g/L, pH 4.5, ionic strength 2 mM, experimental error ±5%.

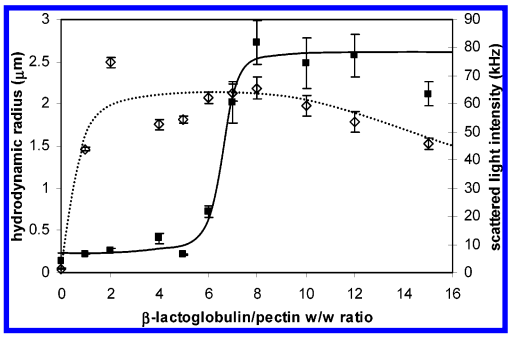

Figure 2. Dynamic light scattering of β-lactoglobulin/pectin mixtures at different w/w mixing ratios: (■) hydrodynamic radius, (◇) scattered light intensity. Lines serve to guide the eye. For all samples the protein concentration was 0.1 g/L, pH 4.5, $I = 9$ mM.

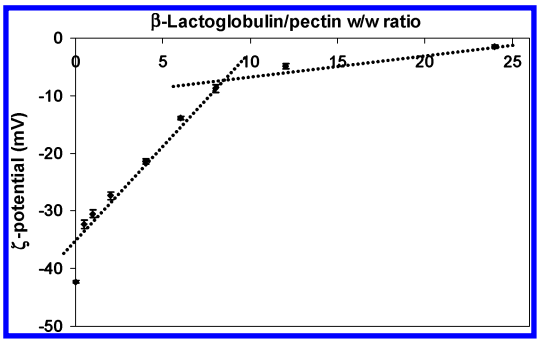

Figure 3. ζ-potential as a function of the β-lactoglobulin/pectin w/w ratio. Concentrations were adapted to the optimal signal, pH 4.5, $I = 9$ mM. Lines serve to guide the eye.

the surface pressure (Figure 1b). Interestingly, when the droplet was first rinsed with buffer to remove the remaining protein from the bulk solution and subsequently rinsed with the same pectin solution, the surface pressure remained constant (Figure 1c). Rinsing with a 50 mM NaCl solution (Figure 1d) resulted in a similar increase in surface pressure as observed after rinsing with pectin.

Besides the surface pressure, one can compare the surface rheological behavior of the different adsorbed layers using this setup. Dilatational moduli of all samples at three different frequencies were measured after the rinsing procedure, ∼30 min after the formation of the droplet (Table 1). Adsorption of pectin on a previously formed protein layer results in a 60% increase in modulus. In contrast, the presence of pectin in the protein solution before exposure to the interface leads to a 10% lower modulus than that of a pure protein layer. With decreasing oscillation frequency all layers exhibit a decrease in dilatational modulus, which coincides with an increase in phase angle. This behavior indicates that when there is more time available, the molecules can relax to a larger extent.

***β*-Lactoglobulin/Pectin Complexes in Solution.** Before we attempt to understand the behavior of the mixed adsorbed layers from simultaneous protein/polysaccharide adsorption, it is useful to have information about the mixing behavior of the components in bulk. Figure 2 shows the results of light scattering measurements, scattered light intensity and hydrodynamic radius as a function of the protein/polysaccharide mixing ratio at pH 4.5 and 9 mM ionic strength (ratio 0 is pure pectin; for all other mixing ratios, the protein concentration was 0.1 g/L). The scattered light intensity in the presence of protein, from ratio 1 w/w upward, is much higher than at ratio 0 w/w, in the absence of protein (Figure 2). The presence of protein does not influence the hydrodynamic radius until a mixing ratio of 6 w/w. Furthermore, from a previously measured decrease in adsorption kinetics it is known that at least 90% of the protein is bound up to a ratio of 6 w/w.[23] On the basis of these observations, it is concluded that soluble β-lactoglobulin/pectin complexes are formed. The soluble complexes aggregate and become insoluble (complex coacervation) at protein/polysaccharide mixing ratios from 7 w/w upward, as concluded from the steep increase in hydrodynamic radius and a strong increase in turbidity (as observed with the naked eye). The increased turbidity, which prevents light from going through the sample, could account for the decrease in intensity observed at 90° from ratio 8 w/w upward. Furthermore, the amount of phase-separated material decreases with the decrease in pectin concentration on increasing the mixing ratio. Comparing the number of charges on β-lactoglobulin and pectin on the basis of their proton titration curves (data not shown) suggests that if all molecules take part in the soluble complex formation, the total positive net charge on the protein molecules just compensates the total negative charge on the polysaccharides at the transition at ratio 7 w/w. Apparently the complexes aggregate and become insoluble when their net charge approaches neutrality, and an excess charge at lower mixing ratio prevents this. Small fractions of insoluble aggregated complexes below mixing ratio 7, due to heterogeneity in the stoichiometry, could heavily increase the average hydrodynamic radius and can count for the variation in hydrodynamic radius in the soluble complex regime.

The ζ-potential of the protein/polysaccharide complexes was measured to determine whether the net charge of the protein/polysaccharide complexes indeed decreases with increasing mixing ratio (Figure 3). On addition of protein to a pectin solution (at pH 4.5 and $I = 9$ mM), the ζ-potential, which is −40 mV for pure pectin, increases. First a linear increase in ζ-potential is observed until a value of −8 mV is reached for a protein/polysaccharide mixing ratio of 8 w/w. Upon a further increase of the mixing ratio the ζ-potential only slightly increases. As appears from Figures 2 and 3, a ζ-potential as small as −8 mV is not capable of preventing the complexes from aggregating and becoming insoluble. Although β-lactoglobulin is slightly positively charged at pH 4.5, the ζ-potential measured for the protein is 0 ± 1 mV.

Surface Dilatational Rheology. As mentioned before the rate of surface pressure development at air−water interfaces can be

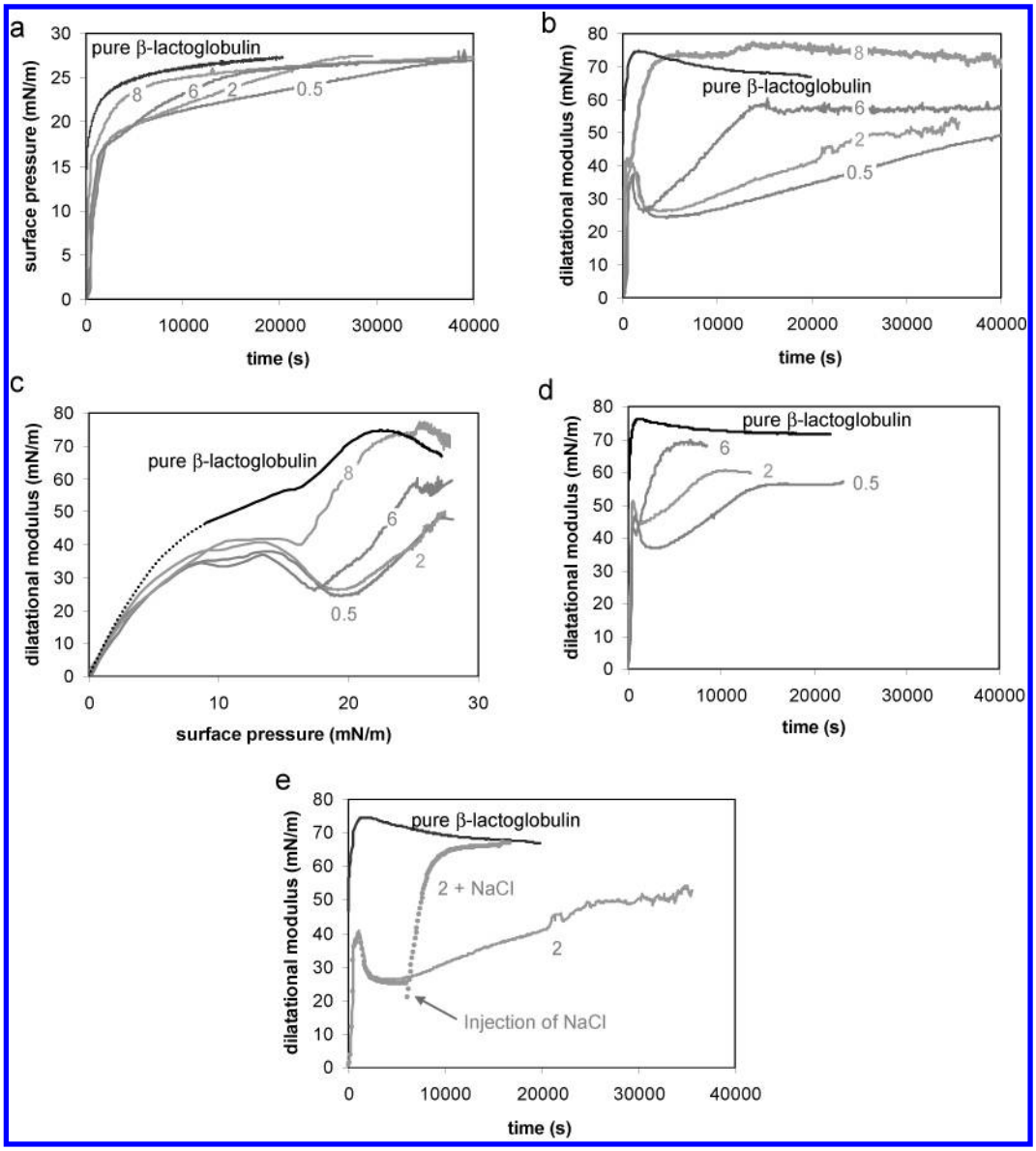

Figure 4. (a) Surface pressure (at the air−water interface) versus time curves of β-lactoglobulin and β-lactoglobulin/pectin mixtures at different ratios (as indicated in the graphs). For all samples the protein concentration was 0.1 g/L, pH 4.5, $I = 2$ mM. (b) Dilatational modulus versus time curves for the same samples, pH 4.5, $I = 2$ mM. (c) Dilatational modulus versus surface pressure curves. The dotted part of the β-lactoglobulin curve is estimated. (d) Dilatational modulus versus time curves at 9 mM ionic strength, pH 4.5. (e) Dilatational modulus of a 2 w/w β-lactoglobulin/pectin mixture as a function of time with and without NaCl injection up to 100 mM after 2 h.

affected by complexation of protein with polysaccharide in the bulk solution (Figure 1a). Furthermore, the presence of pectin in the interface appears to affect the rheological behavior of the adsorbed layer (Table 1). Because the net charge of the complexes depends on the mixing ratio and might affect the surface rheology, we measured the dilatational modulus (at 0.1 s^{-1}) of mixed β-lactoglobulin/pectin layers for different bulk mixing ratios (Figure 4). Figure 4a shows that the higher the protein/polysaccharide mixing ratio, the faster the surface pressure levels off to a constant value; at a mixing ratio of 8 w/w, the surface pressure versus time curve comes close to that of β-lactoglobulin alone (at this large time scale, the initial slopes are not resolved). This trend with mixing ratio is more pronounced for the development of the dilatational modulus. For the lower mixing ratios no steady-state value of the dilatational modulus is reached within 11 h (Figure 4b).

To compare the rheological properties of the interface in a way independent of adsorption kinetics, the modulus is plotted as a function of the surface pressure in Figure 4c. The shape of the curves, with a minimum in the dilatational modulus at a surface pressure between 15 and 20 mN/m, is not unique for the mixed systems, but is also observed for pure β-lactoglobulin layers (depending on the bulk concentration). The minimum in the dilatational modulus, coupled with a maximum in the phase angle (not shown), can presumably be attributed to conformational changes of the protein molecules at the interface. With increasing protein bulk concentration the minimum in the dilatational modulus becomes deeper (not shown), the maximum in the phase angle becomes higher, and the whole modulus versus surface pressure curve shifts to a slightly lower dilatational modulus. We tentatively interpret these differences in terms of layer structure. When protein adsorbs from low concentrations, it has the time to adopt a more favorable conformation at the interface before the layer is jammed, whereas on adsorption from higher concentrations the system is jammed before these conformational changes can occur. The latter layer structure is less ordered and less compact at $\Pi \approx 17$ mN/m, allowing for less pressure increase upon compression, i.e., a lower modulus. Possibly some (partial)

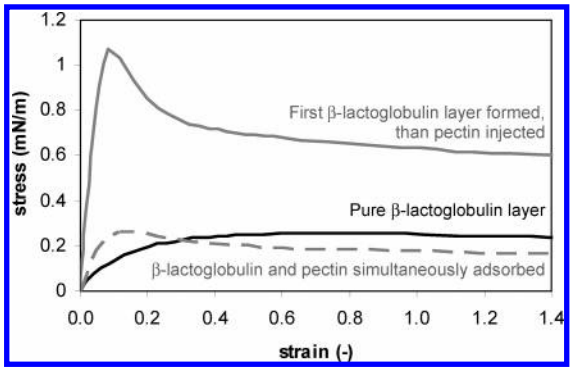

Figure 5. Typical examples of stress/strain curves of a pure β-lactoglobulin adsorbed layer at the air−water interface, a β-lactoglobulin/pectin layer simultaneously adsorbed from a 2 w/w mixture, and a mixed layer where β-lactoglobulin/pectin complexes adsorbed under a previously adsorbed protein layer (after ∼150 min), final overall mixing ratio 2 w/w. For all samples the protein concentration was 0.1 g/L, pH 4.5, $I = 9$ mM.

desorption occurs, too. At saturation, a similar steady-state value of the modulus for all concentrations is reached (∼70 mN/m) at steady-state surface pressure values increasing with concentration. The shift in surface pressure versus surface load curves to higher surface loads upon increasing the β-lactoglobulin bulk concentration, as observed by Wierenga et al., supports this hypothesis.[32] The time scale at which the dilatational modulus goes through this minimum is orders of magnitude larger for the protein/polysaccharide mixtures as compared to the pure protein case. For the protein/polysaccharide mixtures, the minimum in the dilatational modulus could possibly be interpreted as conformational changes of the protein molecules or as rearrangements of the protein/polysaccharide complexes or as a combination of both.

Comparing the mixed β-lactoglobulin/pectin layers in Figure 4c at a given value of surface pressure above 15 mN/m, one observes an increase of the dilatational modulus with increasing mixing ratio. At an ionic strength of 9 mM (instead of 2 mM), the difference between the different mixing ratios is diminished, and for all ratios a steady-state dilatational modulus is reached faster (Figure 4d). In Figure 4e, NaCl was injected into a 2 w/w β-lactoglobulin/pectin mixture up to an ionic strength of 100 mM 2 h after the start of the experiment. The dilatational modulus instantaneously increased approximately to the steady-state value of a pure protein layer, suggesting that protein liberated from its complex with pectin adsorbed rapidly.

Surface Shear Rheology. Surface shear deformation is different from surface dilatation in the sense that the area of the interface remains constant during shear deformation. This means there is no contribution of changing surface pressure during deformation, and therefore, the surface shear modulus is, compared to the dilatational modulus, more sensitive to elastic or viscous forces within the adsorbed layer. Surface shear stress was measured as a function of strain for the different samples (Figure 5).

A pure protein layer initially responds elastically; the stress increases with the strain. At higher strain, it becomes viscous; the stress levels off at a steady-state value of 0.24 mN/m. The steady-state shear stress for a mixture of 2 w/w β-lactoglobulin/pectin is not much different from that of a pure protein layer. However, the shape of the curves is different; the mixture exhibits a maximum stress, indicating yielding (or fracture) of the layer, which the pure protein layer does not. When a protein layer was adsorbed from a pure protein solution (for ∼150 min) prior to injection of pectin under the surface (into the bulk protein solution), much higher shear stresses were measured.

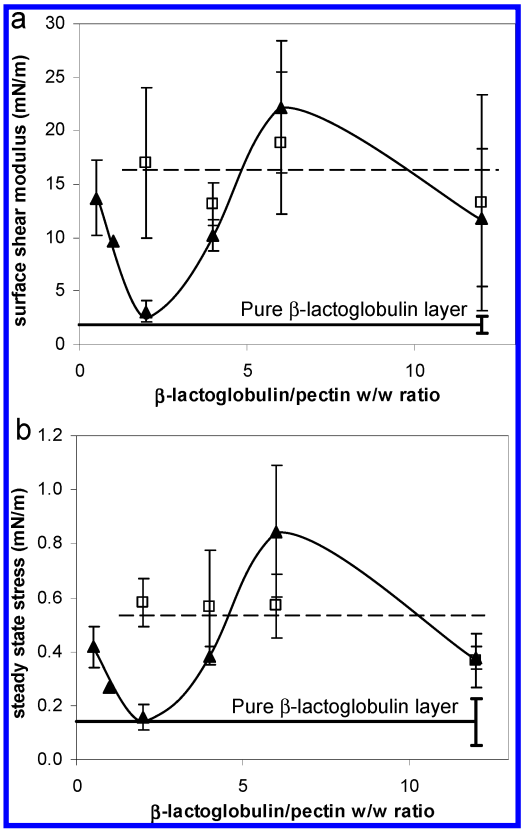

Figure 6. (a) Apparent surface shear modulus and (b) steady-state shear stress as a function of the β-lactoglobulin/pectin w/w mixing ratio in the bulk for (▲) simultaneous adsorption and sequential adsorption (□) at the air−water interface. For all samples the protein concentration was 0.1 g/L, pH 4.5, $I = 9$ mM. Lines serve to guide the eye.

The initial slope of a stress/strain curve represents an apparent surface shear modulus, also called an apparent Young's modulus. Figure 6a shows surface shear moduli for adsorbed layers of β-lactoglobulin/pectin mixtures at different bulk mixing ratios (solid triangles). Between ratios 2 and 6 the surface shear modulus strongly increased with increasing protein/polysaccharide mixing ratio. At a mixing ratio of 12 w/w, where the protein is in excess, the surface shear modulus may be decreased. If protein and polysaccharide do not simultaneously adsorb, but a protein layer has been formed before pectin is injected into the bulk, this dependence on mixing ratio is not seen (open squares). At all mixing ratios the surface shear modulus is higher than the surface shear modulus of a pure protein layer (dotted line), indicating the presence of pectin in the interface. The steady-state shear stress values at larger deformation show an effect of the mixing ratio in the case of simultaneous adsorption which is very similar to that for the (apparent) modulus. Again, this trend is absent in the case of sequential adsorption (Figure 6b).

Since the protein/polysaccharide complexes are formed due to electrostatic interaction, the dependence of the surface shear modulus and steady-state shear stress on the ionic strength was studied (Figure 7). The β-lactoglobulin/pectin complex (w/w ratio 6) layer has a much higher surface shear modulus and steady-state surface shear stress than a pure protein layer under the same conditions at ionic strengths up to 300 mM. Pure pectin does not cause any surface shear elasticity at any ionic strength.

(32) Wierenga, P. A.; Egmond, M. R.; Voragen, A. G. J.; de Jongh, H. H. J. The adsorption and unfolding kinetics determines the folding state of proteins at the air−water interface and thereby the equation of state. *J. Colloid Interface Sci.* **2006**, *299* (2), 850−857.

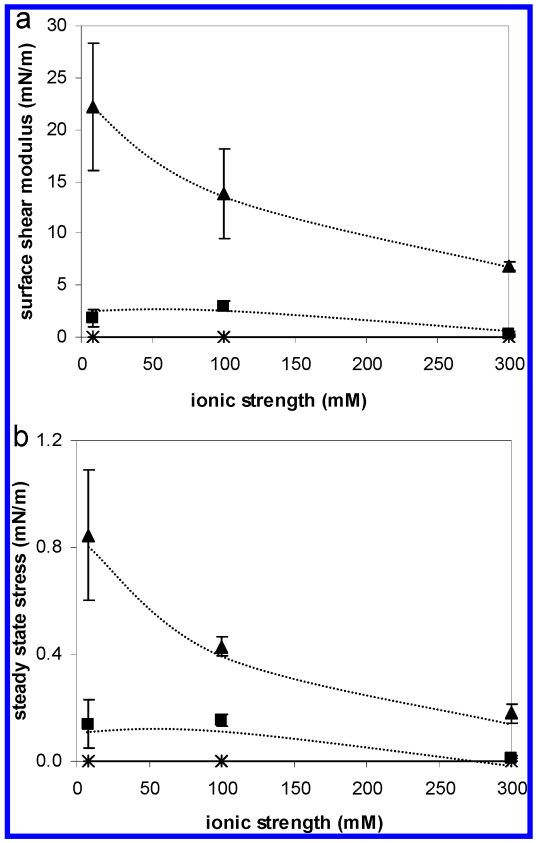

Figure 7. (a) Apparent surface shear modulus and (b) steady-state shear stress as a function of the ionic strength for 6 w/w ratio protein/polysaccharide complexes (▲), pure protein (■), and pure pectin (×) at the air−water interface. For all samples the protein concentration was 0.1 g/L, pH 4.5, $I = 9$ mM. Lines serve to guide the eye.

Discussion

With the rinsing experiments it was shown that pectin can adsorb to an adsorbed protein layer at the air−water interface and so affect the surface rheology. The fact that the surface pressure increased on rinsing with pectin, but did not increase when the bulk protein had been removed first by rinsing with buffer before the introduction of pectin (Figure 1), indicates that in the first case the increase in surface pressure is caused by additional protein adsorption; the injected pectin strongly attracts any protein it encounters in the droplet under these conditions, as demonstrated by dynamic light scattering and ζ-potential data. Similar to an increased ionic strength,[33,34] the presence of negatively charged pectin could reduce electrostatic repulsion between the positive protein molecules at the interface and in this way facilitate a denser protein packing. The higher dilatational modulus after rinsing with pectin, as compared to rinsing with NaCl (Table 1), indicates that the pectin—and not the extra adsorbed protein—is primarily responsible for the increased dilatational modulus.

We propose the layer compositions as depicted in Figure 8 to account for the data. In the absence of pectin, a pure protein layer is formed (Figure 8A). Using neutron reflection, the thickness of this layer was found to be approximately 4 nm, corresponding

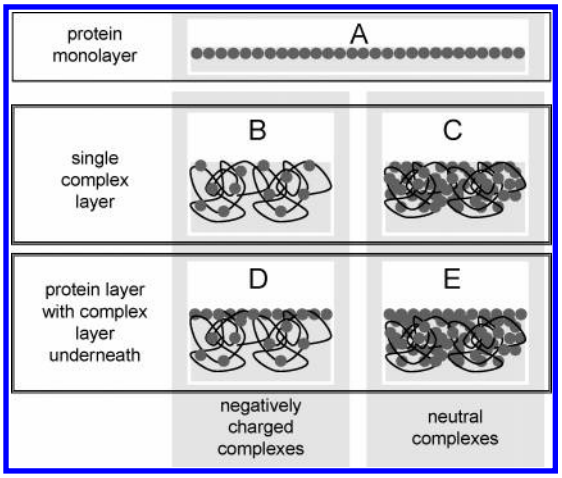

Figure 8. Schematic representation of different adsorbed layers at the air−water interface: (A) protein monolayer, (B, C) mixed layers from simultaneous protein/polysaccharide adsorption where (B) is from negatively charged complexes and (C) from net neutral complexes, (D, E) protein/polysaccharide complexes adsorbed at a previously formed protein layer where (D) concerns negatively charged complexes and (E) net neutral complexes.

to a monolayer of β-lactoglobulin dimers (data not shown). Comparing the values for the dilatational modulus obtained from sequential protein/polysaccharide adsorption with those obtained from simultaneous adsorption (Table 1) shows that, on one hand, adsorption of pectin (alone or in combination with extra protein) can reinforce an already existing protein layer at the interface, as in Figure 8D. On the other hand, when the pectin is present from the start, it seems to prevent the formation of a compact layer due to electrostatic repulsion between the net negatively charged protein/polysaccharide complexes, Figure 8B. In effect, the protein/polysaccharide complex constitutes a barrier against adsorption of protein molecules. The extent to which the latter effect occurs decreases with increasing protein/polysaccharide mixing ratio until the ζ-potential of the complexes is (close to) neutral. This is depicted in Figure 8 as going from panel B to panel C. Increasing ionic strength, and with that decreasing electrostatic repulsion between strongly negatively charged complexes (and finally breakup of the complexes), was demonstrated to counteract the effect of pectin.

The results of surface shear measurements support this mechanistic view on the structure and composition of mixed protein/polysaccharide adsorbed layers. The surface shear modulus of layers created by simultaneous protein/polysaccharide adsorption (Figure 6) strongly increases with the mixing ratio, between mixing ratios of 2 and 6. Above this range, the surface shear modulus does not further increase. Since above a mixing ratio of 6 the ζ-potential is close to zero, one can imagine that at this point more compact layers can be formed (Figure 8C), resulting in a higher surface shear modulus and a higher steady-state stress as compared to those at a ratio of 2, where complexes are strongly negatively charged (Figure 8B). Monteux et al.[35] observed a similar dependence of the surface shear and dilatation rheology on the mixing ratio of oppositely charged polyelectrolytes and surfactants. Since soluble complexes aggregated at a mixing ratio of 7 or higher, as shown by dynamic light scattering (Figure 2), presumably this can also occur in the interface, possibly resulting in a macroscopic network. It is possible that the interface is wetted by a liquid coacervate phase in situations C and E, but

(33) Cho, D.; Narsimhan, G.; Franses, E. I. Adsorption Dynamics of Native and Pentylated Bovine Serum Albumin at Air−Water Interfaces: Surface Concentration/ Surface Pressure Measurements. *Journal Colloid Interface Sci.* **1997**, *191* (2), 312.

(34) Wierenga, P. A.; Meinders, M. B. J.; Egmond, M. R.; Voragen, A. G. J.; de Jongh, H. H. J. Quantitative description of the relation between protein net charge and protein adsorption to air−water interfaces. *J. Phys. Chem. B* **2005**, *109* (35), 16946−16952.

(35) Monteux, C.; Fuller, G. G.; Bergeron, V. Shear and dilational surface rheology of oppositely charged polyelectrolyte/surfactant microgels adsorbed at the air−water interface. Influence on foam stability. *J. Phys. Chem. B* **2004**, *108* (42), 16473−16482.

the thermodynamic nonequilibrium character of protein adsorption might not allow the formation of a pure viscous layer. It is not clear yet why the surface shear modulus and steady-state shear stress were higher at w/w mixing ratios of 0.5 and 1, compared to a ratio of 2 (Figure 6); this trend is not observed with the dilatational modulus. Possibly at these low mixing ratios the layers are thicker because the complexes are swollen due to strong electrostatic repulsion.

A pure protein layer (Figure 8A) exhibits only a low surface shear modulus and no maximum in the stress/strain curve. Possibly, individual protein molecules can easily be sheared along each other. When pectin is injected under the surface, it will complex with the remaining protein in the bulk. When these complexes are negatively charged, they will most likely bind to the slightly positively charged protein layer at the interface. In this case a compact layer of protein molecules has already adsorbed before the polysaccharides have a chance to interfere. The existing protein layer can now be reinforced by adsorption of protein/polysaccharide complexes, resulting in a layer as in Figure 8D. One can imagine why in this case the shear elasticity of sequentially adsorbed layers does not depend on the mixing ratio (compare parts D and E of Figure 8) as was observed for the layers formed from simultaneous adsorption (Figure 8B,C); with sequential adsorption the mixing ratio primarily affects the second layers. Apparently as long as it is negative the absolute value of the net charge of the complexes in the second layer is not of primary importance. At a mixing ratio of 12 w/w, where protein is in excess and the complexes are net neutral (as shown by the ζ-potential experiments), the system becomes unstable (as appeared from light scattering); the soluble complexes aggregate and eventually phase separate. One might expect that in this case only free protein molecules would adsorb at the interface, but it appears that the formed layer still has a higher shear modulus than a pure protein layer, indicating the presence of complexes in the interface. Presumably after its injection into the bulk solution, pectin immediately forms complexes with protein in the bulk due to the high affinity. Eventually it could be more favorable for polysaccharides to complex with proteins at the interface, which have already lost much of their entropy, as compared to protein molecules that can freely move through the bulk solution. This hypothesis is supported by the observation that, in the presence of 300 mM NaCl, at a mixing ratio of 6, the shear modulus is still higher than that of a pure protein layer, while in previous work it was shown that the effect of the presence of pectin on the protein adsorption kinetics disappears at an ionic strength of 80 mM.[23] Apparently the electrostatic binding of pectin on an adsorbed protein layer at the interface can occur under circumstances where binding with protein molecules in bulk solution is no longer detectable. Presumably in this case binding is not so strong that the polysaccharide can prevent the formation of a densely packed protein layer at the interface as was observed at low ionic strength.

One might expect that in time a layer as depicted in Figure 8B transforms into a layer as in Figure 8D, because the overall bulk solution concentrations are the same in both cases; however, within the time scale of the experiments, up to 20 h, this does not occur, indicating a thermodynamic nonequilibrium situation.

Insight into the mechanism of mixed protein/polysaccharide adsorption provides an opportunity to modulate the surface rheological properties by manipulating the protein/polysaccharide interaction. The protein/polysaccharide mixing ratio and the order of adsorption at the interface are shown to be important parameters. A major advantage is that at the interface there is a net attraction between the protein and polysaccharide over a wider range of ionic strengths than in solution. Varying the protein/polysaccharide binding affinity, e.g., by varying the charge density and distribution on the protein or polysaccharide, is expected to provide a broad opportunity to modulate the surface rheological behavior.

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