AN ABSTRACT OF THE THESIS OF

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Title: Molecular Properties Influencing the Surface Activity of α-Lactalbumin, β-Lactoglobulin and Bovine Serum Albumin

Abstract approved: / Dr. Joseph McGuire

The thermal stability of α-lactalbumin (α-lac), β-lactoglobulin (β-lg), and bovine serum albumin (BSA) was evaluated with differential scanning calorimetry (DSC), and their surface activity was studied at air-water, solid-water, and oil-water interfaces with ring tensiometry, ellipsometry, and emulsion conductivity measurements, respectively. The concentration dependencies of equilibrium surface pressure (Π) for single component and mixed solutions of α-lac, β-lg, and BSA were fit to a simple two parameter model. Application of the Gibbs adsorption equation to the Π vs. concentration relationships enabled qualitative prediction of adsorbed amount at hydrophobic solid surfaces. Surface tension kinetics were measured as well, and interpreted in terms of first-order rate constants defining adsorption and interfacial rearrangement. The effects of temperature and protein concentration on adsorbed mass recorded at
hydrophobic and hydrophilic solid surfaces were addressed and interpreted with reference to thermal stability as well as molecular size and flexibility. The oil-in-water emulsifying activity and emulsion stability exhibited by α-lac, β-lg, and BSA were evaluated at solution concentrations ranging from 0.100 to 20.0 mg/ml, by quantitative interpretation of changes in emulsion conductivity recorded during and shortly after homogenization. Differences observed in these emulsifying properties among the three proteins were found to be small. However, they were explainable with reference to important differences in their molecular properties revealed by earlier study of their behavior at air-water and solid-water interfaces.

Although molecularly dissimilar in several ways, the results indicated that the surface activity of the three proteins at each interface could be explained with reference to molecular size, flexibility and stability. Proteins small in size, with low stability and/or high molecular flexibility are more surface active than larger proteins, of higher stability and/or lower flexibility. The surface activity of BSA was treated as being largely governed by the behavior of one of its three domains in formulating this interpretation.
Molecular Properties Influencing the Surface Activity of $\alpha$-Lactalbumin, $\beta$-Lactoglobulin and Bovine Serum Albumin

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Chapter 1

Introduction

Apart from the nutritional aspect of proteins, their surface activity, i.e., their behavior at interfaces, is also of critical interest. The interfacial behavior of proteins can play a significant role in both biological and technological areas, including biomedical material compatibility, food and pharmaceutical processing, chromatography, and membrane application.

Protein Behavior at Solid-Water Interfaces

Some proteins affect the biocompatibility of foreign materials or devices exposed to blood or living tissue (1). For example, when inserted in an organism, any foreign material will be rapidly covered with protein layers (2, 3). Baier (3) found that platelet adhesion to non-physiological material did not occur until the material surface was enriched with a certain amount of the blood protein fibrinogen. Surfaces preadsorbed with a protein layer have been known
to enhance cell growth. For example, Albrektsson et al. (4) pointed out that the initial adsorption of κ-casein on titanium surfaces was important for integration of a titanium bone implant. The presence of protein in solution was found to enhance adhesion of the marine *Chlorella vulgaris* to glass (5). Preadsorption of serum proteins also enhances microbial and spore adhesion as reported by Schakenraad et al. (6). Similar phenomena apparently form the basis of plaque formation on teeth and dental restoratives (7).

Protein behavior at solid surfaces also plays an important role in purification and separation processes, particularly in membrane and chromatographic fractionation techniques. For example, immobilization of enzymes on a solid matrix is increasingly applied in industrial and chemical processes. An enzyme is “immobilized” when its release into the solution and its surface mobility are restricted or constrained by some physical or chemical means. While covalent binding of enzymes to solid carriers is the most common immobilization method, physical enzyme adsorption to the solid surface often precedes the formation of the covalent bond (8). Varying affinities between different proteins for an interface is exploited in some biochemical purification procedures, as well as in treating HPLC columns with protein to prevent further protein adsorption while still retaining their capacity to separate low molecular weight materials (9).

In some cases, proteins at interfaces can cause problems. In industry, these phenomena are usually referred as “fouling” or “biofouling.” Fouling of heat exchange surfaces is enhanced due to the heat sensitivity and high content of
protein in some fluid foods. Biofouling is also found in processes as diverse as desalination of seawater and operation of artificial kidney devices. Extensive cleaning operations must be carried out in order to bring such equipment back to its original state (10).

Protein Behavior at Air-Water and Oil-Water Interfaces

The behavior of proteins at air-water and oil-water interfaces is extremely important both theoretically and practically, particularly in the area of food science and technology. Proteins tend to accumulate at both air-water and oil-water interfaces constituting an interfacial layer or film, thereby altering surface properties (11). Films of proteins at these interfaces are significant to the food industry since these films can stabilize colloidal food systems, foams and emulsions, by reducing both interfacial tension and rates of collapse and coalescence (12). Many food products are foams, emulsions, or both, and include many dairy products, baked foods, ice cream products, meringue, mayonnaise, and the “head” on poured beer. Properties related to dispersion are usually crucial in determining the acceptability of many products (11-13).

Proteins are one of the two classes of surface-active agents used in formulating and stabilizing industrial colloidal food systems (14). Non-proteinaceous macromolecules are used in producing colloidal foods as well, but only a few polysaccharides, such as modified cellulose derivatives (15) or
acetylated pectin (16) are considered sufficiently surface active for practical purposes.

**Molecular Influences on Protein Interfacial Behavior**

Because of their amphipathic nature, protein molecules are surface active. Many proteins adsorb at interfaces at relatively low solution concentrations (12). The adsorption process involves transfer of protein molecules from solution to the sorbent surface along with concomitant displacement of solvent molecules and other components from that surface. The migration of proteins from solution to the interface is thermodynamically favorable since the conformational and hydration energy of the protein is reduced at the interface (17). It is believed that hydrophobic interactions not only play an important role in stabilizing the three-dimensional structure of proteins, but also contribute to stabilization of the protein at an interface (18, 19).

In any event, proteins are highly dynamic structures which are constantly “sampling” various conformations due to local thermodynamic fluctuations. Some such transient conformations could be stabilized in novel micro-environments (20). At an interface, therefore, an alternative way of folding the molecule to minimize hydrophobic free energy becomes possible, *i.e.*, rather than folding to the interior of a globule in the aqueous phase, apolar side chains can be located toward air, oil, or hydrophobic solid surfaces (17, 21-23). In addition, Soderquist and Walton (24) as well as Chan and Brash (25) have
demonstrated using CD spectra that various plasma proteins show a loss of secondary structure (α-helix or β-sheet) after elution from adsorbed surfaces.

The distribution of segments of the proteins at the interface depends upon many factors, particularly the free energy of adsorption of the various segments and the flexibility of the polypeptide chain (17, 26). As the free energy decreases with unfolding, proteins tend to cover as much of the interface as possible. Thus, adsorbed proteins are usually denatured, and desorption is energetically very unfavorable. For rigid-structured proteins like certain types of globular proteins, conformational changes are relatively limited, i.e., complete unfolding does not seem to occur and desorption might occur (27).

Unfolding or surface denaturation of protein involves a “rapid” cooperative transition in the protein tertiary structure when protein molecules reach the interface, followed by a relatively slow molecular rearrangement. In addition to protein and surface properties, the latter is very much affected by compressibility of the film and the manner of surface packing (17).

It is increasingly apparent that protein interfacial behavior is strongly influenced by its structural or molecular properties (17, 28). For example, Phillips (21) showed that proteins with a highly ordered native structure, giving a strongly cohesive interfacial film, were more efficient in stabilizing air-water and oil-water systems even though they were less surface active than proteins with a less rigid conformation. Horbett (29) proposed that factors which influence the surface activity of proteins are size, charge, structure (stability,
unfolding rate, cross-linking, and subunits), and other chemical properties (amphipathicity, oiliness, and solubility).

Based on spatial organization, Norde (30) classified protein molecules into three groups: (1) molecules that are highly solvated and flexible, resulting in a randomly coiled structure; (2) molecules that have adopted a regular (α-helix and β-sheet) structure, i.e., the fibrillar proteins; and (3) compact molecules that may contain α-helix, β-sheet, and random chain parts, i.e., the globular proteins. Most proteins, normally involved in enzymatic, immunologic, and biological transport functions belong to the globular category. Globular proteins, for which the three-dimensional structure in solution is stabilized mainly by intramolecular hydrophobic bonding, are sensitive to structural changes upon adsorption (31).

The molecular stability of protein is considered to be one of the most important factors influencing protein surface activity (28, 29, 31). Kato and Yutani (32) found that some surface properties of wild type and six mutant tryptophan synthase α-subunits substituted at position 49 were linearly correlated with the free energy of denaturation of these proteins. One might expect that the change in stability due to amino acid substitution at a single site would have an important effect on surface properties, evidently related to the greater ease of unfolding at the interface in case of less stable mutants. Besides, there are data supporting the thought that molecular flexibility and amphipathicity, which facilitate molecular re-orientation of proteins at interfaces, particularly at oil-water interfaces are also critical in formation of a stable interfacial film (33-35).
General Features of α-Lactalbumin, β-Lactoglobulin and Bovine Serum Albumin

The proteins α-lactalbumin (α-lac), β-lactoglobulin (β-lg), and bovine serum albumin (BSA) are all major components in milk whey. Of these, β-lg and α-lac are present in the highest concentration, 54 and 21% of the total whey protein, respectively (17) and are of primary importance in the overall properties of whey (36). On the average, common bovine milk contains 3 mg/ml β-lg, 1 mg/ml α-lac, and 0.4 mg/ml BSA (37).

β-Lg (162 amino acid residues, MW 18,320 for the monomer) exists as a dimer in solution at normal pH because of electrostatic interactions between Asp$^{130}$ and Glu$^{134}$ of one monomer with a corresponding Lys residue of another monomer (38). Each monomer contains two disulfide bridges and one free thiol group (-SH). The thiol group is important since it appears to facilitate SH/S-S interchange reactions which allow the formation of new structures or intermolecular disulfide-bonded dimers and polymers upon heating (39). The β-lg conformation is pH and temperature sensitive, even at pH 6.5, it undergoes some internal reorganization (17).

α-Lac (123 amino acid residues, MW 14,161) on the other hand, is a very compact, nearly spherical single chain globulin with four disulfide bonds but no thiol group. α-Lac has a biological function as well, that being to modulate the substrate specificity of galactosyltransferase in the lactose synthetase complex, which is responsible for the synthesis of lactose in lactating mammary tissue.
Calcium binding, which may stabilize the molecule against irreversible thermal denaturation, is another characteristic of α-lac (41).

BSA (MW 66,267) is a large globular protein in whey with a single polypeptide chain containing 582 amino acid residues with 17 intra chain disulfide bonds and one free thiol group. Synthesized in the liver tissue, BSA gains entrance to milk through the secretory cells (42). The BSA structure consists of three domains and nine subdomains (43). The multidomain structure of BSA is responsible for the anomalous behavior of the protein observed under denaturation conditions (44).

Research Goal

A great deal of effort has been devoted to study of different factors that influence the surface behavior of proteins. For example, the behavior of proteins at solid-water interfaces has been examined using a number of solid surfaces contacted with a number of different proteins (usually purified from blood plasma) in model systems. Much of that work has been dedicated to study of kinetic and equilibrium aspects of adsorption, and has greatly contributed to our present understanding of adsorption. The surface activity of some proteins, mostly food proteins, at air-water and oil-water interfaces has also been studied. However, most previous work has been performed with a particular protein used in order to explain a particular phenomenon. We presently know very little
about the molecular properties of proteins that influence their surface activity at air-water, oil-water, and solid-water interfaces.

Industrially relevant fluids can contain many different types of proteins. How different proteins in a mixture compete with each other to yield some overall surface functionality is not known in any quantitative manner. Study of the behavior of a set of well characterized food proteins at air-water, oil-water, and solid-water interfaces, and interpretation of observed behavior with reference to individual molecular properties should serve to improve present quantitative understanding.

It was the major purpose of this research to study the surface activity of globular food proteins at air-water, oil-water, and solid-water interfaces under controlled conditions in order to identify the protein molecular properties that consistently influence their surface activity. The milk proteins α-lac, β-lg, and BSA were selected as model globular food proteins. These major whey proteins are highly surface active, and among other things differ in structure, size, charge, hydrophobicity, and amino acid composition and sequence in known ways. Differential scanning calorimetry (DSC) was used in quantifying the molecular stability of the model proteins. Information regarding protein surface activity was gained using ring tensiometry, ellipsometry, and emulsion conductivity measurement.
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Chapter 2

The Surface Activity of $\alpha$-Lactalbumin, $\beta$-Lactoglobulin and Bovine Serum Albumin I. Surface Tension Measurements with Single Component and Mixed Solutions*

P. Suttiprasit, V. Krisdhasima, and J. McGuire

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Abstract

The concentration dependence of surface tension was evaluated with DuNoüy ring tensiometry for solutions of α-lactalbumin (α-lac), β-lactoglobulin (β-lg), and bovine serum albumin (BSA). Surface tension kinetics were measured as well, and interpreted in terms of first-order rate constants defining adsorption and interfacial rearrangement. Although molecularly dissimilar in several ways, the surface activity of each protein could be explained with reference to its relative flexibility and stability. Molecular size seemed to be an important factor in governing incorporation of protein into an interfacial layer. α-Lac, the smallest and least stable protein, was observed to be most surface active. BSA is the largest molecule, and consists of three large domains and nine subdomains. Its surface activity is consistent with the thought that it is largely governed by the behavior of one of these domains, and its activity after adsorption. β-Lg is roughly half the size of BSA, and apparently adsorbs faster than BSA, but exhibited a similar equilibrium Π behavior. This is attributed to its dimer structure and the presence of a free thiol in each monomeric subunit capable of facilitating thiol-disulfide interchange reactions, probably causing it to be less flexible than BSA.
Introduction

In a quantitative sense, we know very little about the molecular mechanisms influencing a protein's ability to compete with other proteins in a serum and situate itself, in some conformational state, at a surface. Surface activity is a cumulative property of a protein influenced by many factors: among these are its size, shape, charge, and thermal stability. Experimentally observed differences in surface activity among different protein molecules have been very difficult to quantify in terms of size, shape, charge and stability because proteins usually vary substantially from one another in each of these categories. Recent progress in recombinant DNA technology, particularly site-directed mutagenesis, has made it possible to modify protein structures almost at will (1). The use of synthetic protein mutants holds promise as a very attractive approach to gaining a quantitative understanding of protein behavior at solid surfaces, and researchers are now beginning to take advantage of this technology in order to relate chosen molecular properties to protein surface activity (2, 3).

The surface activity exhibited by the milk proteins α-lactalbumin (α-lac), β-lactoglobulin (β-lg) and bovine serum albumin (BSA) warrants continued study because of its immediate industrial significance. Problems associated with their role in fouling of membrane and heat exchange surfaces, and as possible mediators of microbial and spore adhesion have been reported (4, 5). When they accumulate at air-water or oil-water interfaces, the resulting interfacial layer can serve to stabilize a foam or emulsion by reducing interfacial tension
and rates of collapse and coalescence (6). Many food products are foams, emulsions, or both, and α-lac, β-lg and BSA can play an important role in stabilizing some of these systems.

Much is known about the chemistry of α-lac, β-lg and BSA; some selected properties of each molecule are listed in Table 2.1. It is thought that gross differences observed in the interfacial activity exhibited by these proteins might identify the molecular dissimilarities most important in governing such activity. There are a number of cases in which researchers have made reference to various chemical and structural characteristics of proteins that affect their interfacial properties. Shirahama et al. (14) measured sequential and competitive adsorption exhibited by different pair combinations of lysozyme, ribonuclease, and α-lac at hydrophilic- and hydrophobic, polystyrene-coated silica surfaces. These proteins are of similar size and shape; using a combination of reflectometry to measure adsorbed mass, and streaming potential measurements to gain information on adsorbed layer composition, they were able to discuss differences observed in interfacial activity with reference to their dissimilarities in charge and structural stability. The flexibility of a protein molecule has also been observed to be a very important factor influencing its interfacial behavior. Hunter et al. (15) reported that the flexibility of the β-casein molecule is more important than intermolecular interactions in directing the course of adsorption competition and exchange with lysozyme at the air-water interface.

Graham and Phillips (16) studied adsorption of β-casein, BSA and lysozyme at air-water and oil-water interfaces. For a single-component protein solution,
they suggested that the rate of change of spreading pressure can be defined with reference to two kinetic regions, each characterized by a first order rate constant. The first region is one in which both adsorbed mass and spreading pressure are observed to increase (the adsorption period); the other region is characterized by attainment of a plateau in adsorbed mass while spreading pressure continues to increase (the rearrangement period). They also reported that the primary monolayer dominates the surface pressure "isotherm": adsorbed mass can continue to increase after attainment of a plateau in spreading pressure, but it increases via multilayer formation where lower layers are reversibly adsorbed (17). Waniska and Kinsella (18) resolved spreading pressure vs. time data for β-lg in terms of these two types of first order rate constants. Their results could be explained largely in terms of electrostatic effects. In particular, they found that the pH dependence of the rearrangement rate constant was highest when the protein had the lowest net charge. They also reported that the rate of surface pressure development seemed to reflect the rate of rearrangement more than adsorption.

In this paper, we report on surface tension properties of α-lac, β-lg and BSA in single-component and mixed solutions at the air-water interface. Spreading pressures exhibited by the protein solutions were monitored as a function of concentration as well as time. Spreading pressure vs. concentration data were fit to a simple two-parameter model for each protein. Similar data recorded for proteins mixed in equal molar proportions were fit to the same model, and spreading pressure vs. time data recorded for each protein were
interpreted in terms of their relative rates of adsorption and rearrangement at the air-water interface.

**Materials and Methods**

**Proteins**

α-Lac (Type III, L-6010, Lot No. 89F8225), β-lg (L-0130, Lot No. 98F8030), and BSA (A-7906, Lot No. 15F0112) were obtained from Sigma Chemical Co. (St. Louis, MO). The three proteins were of the highest native pure grade prepared from bovine milk. The proteins were independently dissolved in 0.01 M phosphate buffer (pH 7.00) at a concentration of 2.0 - 2.5% and passed through filters of 0.22 μm pore width (Millipore Corp., Bedford, MA) to remove undissolved material and other impurities such as bacterial cells. The prepared solutions were stored at 5°C in a refrigerator and used in one day. The exact concentration of the proteins was determined by measuring absorbance with a Beckman UV-spectrophotometer (Model DU®-62, Beckman Instruments, Inc., Fullerton, CA). The following optical factors \( E_{1\% , 1cm} \) were used: α-lac, 20.6 at 280 nm (19); β-lg, 9.3 at 278 nm (20); and BSA, 6.67 at 279 nm (21).

The buffer used in this study consisted of analytical grade sodium phosphate monobasic, monohydrate, and analytical grade sodium phosphate dibasic, anhydrous (EM Industries, Inc., Gibbstown, NJ). The water was deionized and distilled in an all-glass still.
DuNoüy Ring Tensiometry

A DuNoüy tensiometer (Model No. 70535, CSC Scientific Co., Inc., Fairfax, VA) was used. The tensiometer employs the “ring method” of measurement, which is perhaps the most convenient method for the determination of surface tension and has been recommended as the only method yielding satisfactory results for colloidal suspensions (22, 23).

In the ring method, the force required to pull the ring from the surface of a liquid is determined. This force is equal to the weight of the ring plus the downward pull due to surface tension (22, 23). The value of surface tension given by the scale reading of a tensiometer is an “apparent surface tension”; to obtain the true surface tension, it is necessary to multiply the apparent surface tension by a correction factor to account for the force needed to support the weight of the liquid clinging to the ring at the break-point (22-24).

Measurement of surface tension

Both the surface tension-time dependency and equilibrium surface tension were determined at a controlled room temperature (26 to 28°C). Triplicate tests were performed for each measurement. First the protein stock solutions were diluted to the desired concentration with the phosphate buffer. The mixture was gently stirred for 45 s and a volume of 15 ml was then place in a Rodac plate cover (Becton, Dickinson & Co., Oxnard, CA). A DuNoüy ring was carefully cleaned between each measurement by rinsing twice with deionized and distilled water followed by flame treatment in the oxidizing portion of the flame of an
alcohol burner until the ring was "red" hot. The ring was then hung from the load cell and lowered about 5 mm below the surface of the liquid. The ring was pulled from the surface and the apparent surface tension recorded. Care was taken to assure consistency in pulling the ring from the liquid for each measurement. In all cases, a first measurement could be completed within about one minute after charging the plate cover with 15 ml of protein solution.

Single component solutions of α-lac, β-lg, and BSA were prepared separately at concentrations ranging from 0.05 to 20.00 mg/ml. Mixed solutions of these proteins were also prepared in equal molar ratios in the following combinations: (i) α-lac + β-lg, (ii) α-lac + BSA, and (iii) α-lac + β-lg + BSA. Mixtures were prepared such that total protein concentrations corresponded to those used in the single component tests. Equilibrium surface tensions of all protein solutions were measured after allowing 7 h for equilibration.

Surface pressure

The surface pressure, $\Pi$ (mN/m), of a protein solution corresponds to the reduction of the surface tension by a surfactant (25). In this study, $\Pi = \gamma_B - \gamma_P$ where $\gamma_B$ and $\gamma_P$ are the surface tension of the pure buffer (72.5 mN/m) and the protein solution, respectively.
Results and Discussion

Concentration Dependence of Surface Pressure for Single Component and Mixed Solutions

The concentration dependence of $\Pi$ for the single component and mixed solutions is shown in Figure 2.1. It is apparent that $\Pi$ increased with increasing protein bulk concentration ($C_p$, mg/ml), with $\alpha$-lac yielding a value of $\Pi$ approximately twice that resulting from either $\beta$-lg or BSA: about 34 mN/m vs. about 16 ($\Pi_{\beta \text{-} lg}$) and 17 mN/m ($\Pi_{\text{BSA}}$). At low $C_p$ levels, the increase in $\Pi$ exhibited by $\alpha$-lac was faster than that exhibited by either $\beta$-lg or BSA. At higher $C_p$ levels, $\Pi_{\beta \text{-} lg}$ and $\Pi_{\text{BSA}}$ attained a steady state value beyond 3.0 mg/ml. However, in the case of $\alpha$-lac, $\Pi$ continue to increase, albeit at a slow rate.

These results can be interpreted in terms of different molecular characteristics among the proteins that affect the nature of their adsorbed films. $\alpha$-Lac, $\beta$-lg and BSA are all globular proteins where, at pH 7.00, $\alpha$-lac and BSA are in a monomeric form while $\beta$-lg exists as a dimer (26). As a consequence of this, the size of BSA is roughly five times that of $\alpha$-lac and two times that of $\beta$-lg. The relatively small, compact size of $\alpha$-lac probably contributed greatly to its $\Pi$ values being higher than those of the other two proteins. In general, a more compact protein molecule can be more easily accommodated into an interfacial film (18). Moreover, analysis of each protein with differential scanning calorimetry (described in detail in Chapter 3) indicated that $\alpha$-lac first undergoes a thermally induced transition at an onset temperature of about
32.2°C. Transitions are not observed for β-lg and BSA until onset temperature of about 64.0 and 54.4°C are reached, respectively. This, in addition to the ability of α-lac to renature after denaturation should serve to aid its ability to adopt some energetically favorable stable, even in a crowded interface.

The concentration dependence of $\Pi_{\beta-lg}$ and $\Pi_{BSA}$ is similar. Both β-lg and BSA contain one free thiol group which is inaccessible to solvent at or below neutral pH (26, 27). However, although the free thiol of β-lg is normally "hidden" in the protein dimer, it displays increased reactivity at pH values above 6.5 and appears to facilitate thiol-disulfide interchange reactions allowing the formation of new structures upon heating (26). BSA consists of three large domains and nine subdomains (10). The multidomain structure of BSA is in part responsible for its anomalous behavior under denaturation conditions (28) as well as its interfacial activity (12). BSA contains 17 disulfide bridges whereas β-lg and α-lac contain two and four, respectively. Although BSA is relatively large and consists of a high number of disulfide bridges, its multidomain and high helix content (10, 13) render it somewhat flexible. The dimer structure and thiol - disulfide interchange reactions afforded by β-lg probably cause it to be less flexible than BSA. β-lg is nearly two times smaller than BSA, however, and this apparently compensates for its inflexibility. There is evidence supporting the thought that some protein molecules exist in an extended configuration at the air-water interface with hydrophobic side chains oriented toward the nonaqueous phase, and hydrophilic side chains directed toward the aqueous phase (29). Other work suggests that secondary structure is retained at the interface (30, 31).
BSA has a high α-helix content, and its helices are known to exhibit some amphiphilicity. There is good correlation between helix amphiphilicity and surface activity and presumably, BSA adsorption at the air-water interface may be largely influenced by adsorption of such helices (12).

Molecular charge and hydrophobicity also play a role in protein adsorption. Variation in hydrophobicity among these three proteins does not seem to be critical (see Table 2.1), whereas variation in charge does appear to be significant. A protein molecule with some net electrical charge must overcome an additional barrier to adsorption owing to the electrical potential set up at the interface by previously adsorbed protein (29). This barrier should be decreased for a protein of lower charge because of decreased electrostatic repulsion at the interface (18). At pH 7.00 the net charge of α-lac is only -2, and this low charge would be consistent with its Π vs. Cp behavior relative to that of β-lg and BSA. It is important to note, however, that electrostatic interactions probably play a very minor role in adsorption at the air-water interface (15). BSA adsorption at air-water and oil-water interfaces was shown to be governed largely by hydrophobic interactions (31), and among others, we have shown β-lg adsorption at a hydrophobic solid-water interface to be largely governed by nonelectrostatic interactions as well (32).

Figure 2.1 indicates that α-lac played a dominant role in determining the surface activity of the three protein mixtures. Comparing the amount of α-lac in each mixed solution at each Cp level, it is present in the greatest amount in the mixture α-lac + β-lg and the least amount in the mixture α-lac + β-lg + BSA. A
greater abundance of α-lac in a mixture resulted in higher values of \( \Pi \) at all \( C_p \) levels. Results are consistent with molecular flexibility considerations as well, \textit{i.e.}, once it enters the interface, BSA should be expected to compete more effectively with α-lac for surface sites than should the less flexible β-lg molecule. In fact, the mixture α-lac + β-lg more closely approximated the behavior of α-lac in single-component solution than did the mixtures containing BSA.

Equilibrium spreading pressures have been reported in the literature for a number of proteins under various solution conditions. The agreement between concentration-dependent values of spreading pressure used to construct Figure 2.1 and comparable data in the literature is reasonable. For example, data from Graham and Phillips (31) indicate a value of about 21 mN/m for \( \Pi_{\alpha\text{-lac}} \) at a concentration of 0.01 mg/ml; we measured \( \Pi_{\alpha\text{-lac}} \) to be about 24 mN/m at 0.05 mg/ml. Other data from Graham and Phillips (17) indicate a value of about 17 mN/m for \( \Pi_{\text{BSA}} \) at a concentration of 1.0 mg/ml; we measured \( \Pi_{\text{BSA}} \) to be about 15 mN/m at 1.0 mg/ml.

\( \Pi \) vs. \( C_p \) data were fit to a two-parameter model for each protein and mixture. Within the experimental concentration range, \( \Pi \) can be given by:

\[
\Pi = A_1 \ln (1 + A_2 C_p) \tag{2.1}
\]

where \( A_1 \) (mN/m) and \( A_2 \) (ml/mg) are function parameters. Values of \( A_1 \) and \( A_2 \) estimated for each protein along with the coefficient of determination \( (R^2) \) calculated for each fit to equation [2.1] are listed in Table 2.2.

It is of some qualitative interest to apply the simple form of the Gibbs
adsorption equation to gain an expression for the surface excess concentration of protein (\( \Gamma \), mol/m²). That is, assuming adsorption equilibrium and that the activity of protein at the interface equals its concentration in the bulk, the equation can be written (33):

\[
\Gamma = \frac{C_p}{RT} \left( \frac{d\Pi}{dC_p} \right)
\]  

[2.2]

where \( R \) and \( T \) are the universal gas constant and absolute temperature, respectively. Substitution of equation [2.1] into equation [2.2] yields:

\[
\Gamma = \left( \frac{A_1}{RT} \right) \left( \frac{A_2C_p}{(1+A_2C_p)} \right)
\]  

[2.3]

equation [2.3] indicates that relative plateau values of \( \Gamma \) among the proteins would be largely predictable with knowledge of \( A_1 \). That thought is consistent with results of other work from our laboratory in which we constructed the adsorption isotherm for these three proteins on a hydrophobic silicon surface (see Chapter 3). Considering the air-water interface as exposing protein to a model hydrophobic surface (15, 34), adsorption isotherms constructed at 27°C and shown in Figure 2.2 indicate that, in the region of the plateau, the number of moles of adsorbed \( \alpha \)-lac was about twice that of either \( \beta \)-lg or BSA. If the magnitude of \( \Pi \) is taken to correspond to the number of noncovalent contacts made at the interface, these data suggest that the number of contacts made by one \( \alpha \)-lac molecule roughly equals the number made by one \( \beta \)-lg or one BSA molecule.

\( \alpha \)-Lac is reported here as exhibiting a relatively high surface activity. It is important to recognize that \( \alpha \)-lac binds calcium (1 mol \( \alpha \)-lac : 2 mol Ca²⁺), serving to stabilize its structure (35). The calcium-free state yields the more
hydrophobic form of the molecule, and probably the more surface active form as well. The \( \alpha \)-lac used in this work contained less than 0.3 mol \( \text{Ca}^{2+} \) per mol \( \alpha \)-lac. It is therefore fair to interpret these results as reporting a higher surface activity for \( \alpha \)-lac than what might be expected of the molecule as it exists in milk. The results are further complicated by the fact that in addition to other metal ions, \( \text{Na}^+ \) can bind to the \( \text{Ca}^{2+} \) binding sites of \( \alpha \)-lac (35). This effect could presumably compensate somewhat for the \( \alpha \)-lac destabilization accompanying the lack of sufficient \( \text{Ca}^{2+} \) in these solutions.

**Time Dependence of Surface Pressure for Single Component Solutions**

The surface tension of polymer and protein solutions decreases with time, and approaches equilibrium after many hours (18, 34, 36). The \( \Pi \) vs. time relationship determined for each protein at different bulk concentrations are shown in Figure 2.3. The curves for each protein are plotted in order of concentration starting from 0.05 mg/ml (the lowest curve) to 20.00 mg/ml (the highest curve). Before and after reaching equilibrium, \( \Pi \) values for all solutions are dependent on concentration. At low concentrations, \( \Pi \) increased rapidly, with the rate of approach to equilibrium proceeding in the order \( \Pi_{\alpha \text{-lac}} > \Pi_{\beta \text{-lg}} > \Pi_{\text{BSA}} \). At sufficiently high concentrations an increase in \( \Pi \) was not immediately apparent. \( \Pi \) values reach a steady state due to the limitation in the number of noncovalent contacts that can be made at the interface. The number of contacts
is a function of the number of proteins, or available protein segments, that can occupy the interface (from 0.1 to 0.8 μg protein/cm²) (29).

These three proteins exhibit a more or less "typical" globular tertiary structure, and are suggested to be only partially unfolded upon adsorption (31, 37). As proposed for the globular proteins BSA and lysozyme (16, 37), these three protein molecules probably experience a concentration-driven reorientation after adsorption. At high concentrations, one or more additional, random and unsaturated layers probably adsorb to the first protein layer.

**Molecular influences on rates of adsorption and conformational rearrangement at the interface.** In order to evaluate surface tension kinetics, the following first-order rate equation has been used by a number of researchers (16, 18, 38):

\[
\ln \left( \frac{\Pi_\infty - \Pi_t}{\Pi_\infty - \Pi_0} \right) = -Kt
\]

[2.4]

where \( \Pi_\infty, \Pi_t \) and \( \Pi_0 \) are the surface pressure values at the steady or equilibrium state, at any time \( t \), and at \( t = 0 \), respectively. \( K \) is the first-order rate constant.

If equation [2.4] is to be used to monitor penetration into the surface and configurational rearrangements of adsorbed protein molecules, it is important to apply it only in the period beyond that affected by diffusion. Graham and Phillips (16) reported that at a bulk concentration of 0.001 mg/ml, changes in \( \Pi \) for BSA were diffusion controlled for about one hour. After this period, the surface coverage was such that an energy barrier to adsorption made the rate of adsorption lower than that predicted by the rate of diffusion. When the rate of adsorption equals the rate of diffusion, the total surface concentration of
molecules can be estimated by \( 2 C_p \left( \frac{Dt}{\pi} \right)^{1/2} \), where \( D \) is the diffusion coefficient. At the lowest concentration used in this study (0.05 mg/ml), if \( D \) is taken as concentration independent, adsorption in the present experiments should be diffusion controlled for only a matter of seconds. In any event, we applied equation [2.4] for the time period beginning with the first surface tension measurement, i.e., at about 30 s.

In practice, a plot of equation [2.4] usually yields two or more linear regions. The initial slope is taken to correspond to a first-order rate constant of adsorption (\( K_1 \)), while the second slope is taken to correspond to a first-order rate constant of rearrangement (\( K_2 \)), with rearrangement, by that time, occurring among a more or less constant number of adsorbed molecules (16, 17, 18). The slope describing an additional linear region (\( K_3 \)) would therefore be taken as qualitatively related to a rearrangement process as well. MacRitchie (39) has recently taken exception to this type of interpretation, particularly with reference to some workers' use of plots of \( \ln \left( \frac{d\Pi}{dt} \right) \) vs. \( \Pi \). These plots are usually characterized by a transition pressure above which the slope of the linear plot increases in magnitude, with the initial region interpreted as corresponding to protein penetration, and the increase in pressure in the second region attributed to molecular configurational changes in the absence of further adsorption. In particular, by comparing adsorption rates of \( \beta \)-lg recorded at constant area to those recorded at constant surface pressure, he could conclude that adsorption occurred at all surface pressures up to the maximum value measured. The general pattern of a \( \Pi \) vs. time plot was taken as the result of
initial adsorption, followed by a period of a slower net rate of adsorption (due to the onset of desorption), with attainment of a steady value in $\Pi$ being a result of equal rates of adsorption and desorption.

In any event, the surface tensions of each protein solution at a concentration of 0.05 mg/ml were separately measured as a function of time up to 240 min; a plot of $\ln \left( \Pi_\infty - \Pi_t \right)/(\Pi_\infty - \Pi_0)$ vs. time was constructed for each protein solution and is shown in Figure 2.4. The first-order rate constants estimated in each region, along with the coefficient of determination for each line, are shown in Table 2.3.

The importance of structural stability in dictating protein interfacial activity is reasonably well established (2, 38). The relative values of $K_1$ shown in Table 2.3 are consistent with the structural instability of $\alpha$-lac, and in qualitative agreement with work of Wei et al. (38) in which, at low concentrations (0.01 mg/ml), $K_1$ was observed to generally increase with decreasing stability for five model proteins. Naturally, the gross dissimilarity in size among the molecules makes it impossible to attribute differences in $K_1$ to stability alone, and it is important to indicate that the relative values of $K_1$ are consistent with expectations based on size as well. Indeed, in the case of BSA, size appears to be a dominant factor, since it is both more flexible and less stable (with reference to the onset temperature of its first thermal transition) than $\beta$-lg.

The rearrangement rate constants, $K_2$ and $K_3$ for $\alpha$-lac and BSA, and $K_2$ for $\beta$-lg exhibit less variation among molecules. Although $\alpha$-lac is flexible relative to $\beta$-lg, unlike $\beta$-lg it has the ability to fully renature after thermal
denaturation (40). Under these ambient conditions, it is conceivable that after rapid adsorption, a partial denaturation-renaturation phenomenon occurring in the crowded interface results in a slow net change in the number of noncovalent bonds made between protein and surface.

It is instructive to interpret data for BSA with reference to application of the "domain approach" to adsorption, as described by Andrade et al. (12). BSA can be resolved into three domains, the general shape of the whole molecule being viewed as three tennis balls in a cylinder (the prolate ellipsoid). Each domain contains disulfide bonded, α-helical subdomains. The domains are referred to as I, II, and III in going from the N- to the C-terminal. Whereas domains I and II are highly charged, domain III is uncharged. Presumably, upon entering the air-water interface BSA could initially adsorb "end on" with domains I and II extended toward the bulk. The amphiphilic nature of the subdomain helices might allow for disruption of their normal hydrophobic associations upon reaching an air-water interface. Based on this kind of thinking, they hypothesized that a slow, time-dependent denaturation of BSA (domain III) should be expected at a hydrophobic surface. In fact, that hypothesis is consistent with results for BSA shown in Table 2.3. At least, whereas adsorption apparently plays the major role in the rate of change of $\Pi_{\alpha\text{-lac}}$, rearrangement apparently plays a very important role in the time dependence of $\Pi_{\text{BSA}}$. 
Summary

α-Lac, β-lg and BSA are almost always present in the same sera, and their continued comparative study is important. Although molecularly dissimilar in several ways, their surface activity can be explained with reference to molecular size and issues related to flexibility and stability. Considering the surface activity of BSA to be largely governed by the behavior of one of its three domains was helpful in this regard. Further study with particular attention directed to the sequential and competitive adsorptive behavior of these proteins would serve to clarify these issues, but until individual molecular influences on surface activity are quantified in some kind of absolute sense interpretation of such data might be necessarily superficial.

Acknowledgment

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Table 2.1  Some physical properties of the three proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th># aa</th>
<th>MW (kD)</th>
<th>Shape &amp; dimension (nm)</th>
<th>Charge in neutral sol [3]</th>
<th>% α-Helix:β-sheet unordered (13)</th>
<th>Hydrophobicity (kcal/residue)</th>
<th>pI (per molecule)</th>
<th># S-S (per molecule)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-lac</td>
<td>123</td>
<td>14,161[^1^]</td>
<td>oblate ellipsoid (8) (2.3x3.7x3.2)</td>
<td>-2.0[^1^],[^3^]</td>
<td>45:45:10</td>
<td>1,019[^1^]</td>
<td>4.2 - 4.5</td>
<td>4</td>
</tr>
<tr>
<td>BSA</td>
<td>582</td>
<td>66,267</td>
<td>prolate ellipsoid with 3 domains (10) (4.2x14.1)</td>
<td>-18.0[^4^] (12) (-9, -8, -1)</td>
<td>-55:16:-29</td>
<td>995</td>
<td>4.7 - 4.9</td>
<td>17 + 1 SH</td>
</tr>
</tbody>
</table>

Notes:  
[^1^]. averaged between genetic variants A and B;  
[^2^]. dimer;  
[^3^]. based on Ref. (11);  
[^4^]. values in parentheses represent charge of each domain.
Table 2.2 Estimated model parameter values of single-component and mixed protein solutions.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$A_1$ (mN/m)</th>
<th>$A_2$ (ml/mg) x $10^{-6}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Lac</td>
<td>1.80</td>
<td>14.82</td>
<td>0.999</td>
</tr>
<tr>
<td>$\beta$-Lg</td>
<td>0.90</td>
<td>6.95</td>
<td>0.996</td>
</tr>
<tr>
<td>BSA</td>
<td>0.94</td>
<td>8.06</td>
<td>0.995</td>
</tr>
</tbody>
</table>

Table 2.3 First-order rate constant associated with adsorption and rearrangement for each protein.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_1$</th>
<th>$K_2$</th>
<th>$K_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^{-2}$min$^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>---------------------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>$\alpha$-Lac</td>
<td>9.937 (1.000)</td>
<td>1.336 (0.989)</td>
<td>0.330 (0.991)</td>
</tr>
<tr>
<td>$\beta$-Lg</td>
<td>3.864 (0.980)</td>
<td>1.522 (0.992)</td>
<td>-</td>
</tr>
<tr>
<td>BSA</td>
<td>2.739 (0.998)</td>
<td>1.755 (0.986)</td>
<td>0.897 (0.987)</td>
</tr>
</tbody>
</table>
Figure 2.1 Concentration dependence of surface pressure for single component and mixed protein solutions.
Figure 2.2 Adsorption isotherms constructed for each protein at a hydrophobic silicon surface at 27°C.
Figure 2.3 Time dependence of surface pressure for each protein as a function of concentration (a) α-lac, (b) β-lg, and (c) BSA. Concentrations used were 20.0, 10.0, 3.00, 2.00, 1.50, 1.00, 0.60, 0.30, 0.10, and 0.05 mg/ml.
Figure 2.4 Surface pressure data for each protein plotted according to equation [2.4].
References


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Chapter 3

The Surface Activity of α-Lactalbumin, β-Lactoglobulin and Bovine Serum Albumin II. Some Molecular Influences on Adsorption to Hydrophilic and Hydrophobic Silicon Surfaces*

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Abstract

The thermal transitions experienced by α-lactalbumin (α-lac), β-lactoglobulin (β-lg), and bovine serum albumin (BSA) during a temperature ramp from 15 to 160°C were evaluated with differential scanning calorimetry. Adsorption isotherms were then constructed for each protein on hydrophilic and hydrophobic silicon surfaces at 2, 27 and 52°C. α-Lac exhibited a more or less steady increase in adsorption with temperature for each surface, with the increase in adsorbed mass beyond 27°C being consistent with an α-lac transition occurring to facilitate favorable noncovalent contacts with the surface. Isotherms constructed at 27 and 52°C were similar for both β-lg and BSA, which do not undergo a thermally-induced transition until onset temperatures of about 64.0 and 54.4°C are reached, respectively. For each protein, adsorption was depressed at 2°C, consistent with decreasing stabilization by hydrophobic interactions and strengthening of hydrogen bonds occurring in the cold. Although adsorbed mass was observed to correlate reasonably well with molecular size at each temperature, the isotherm data are thought to provide only an indication of temperature and surface hydrophobic influences on relative amounts of single-component adsorption, and not relative adsorption affinities; i.e., molecular size effects are simply incidental, and proteins with low stability and/or high flexibility are preferentially adsorbed.
Introduction

In the preceding paper (Chapter 2), surface tension measurements with the milk proteins α-lactalbumin (α-lac), β-lactoglobulin (β-lg) and bovine serum albumin (BSA) in single-component and mixed solutions were analyzed as a function of concentration and time, and discussed with reference to size, flexibility and stability differences among the molecules. In general, industrially and medically relevant fluids can contain many different types of proteins. How different proteins in a mixture compete with each other to yield some overall surface functionality is not known in any quantitative manner. Study of the behavior of a set of well-characterized proteins at air-water, oil-water, and solid-water interfaces is one approach to gaining such an understanding. Graham and Phillips (1) measured adsorption isotherms for β-casein, BSA and lysozyme at air-water and oil-water interfaces. Those measurements constituted the first directly determined adsorption isotherms for proteins at two different interfaces. The general characteristics of adsorption of the three proteins were observed to be similar at the air-water and oil-water interfaces. The air-water interface presents a model hydrophobic surface to support protein adsorption, and one might expect to see some correlation between protein behavior at air-water interfaces and behavior at other hydrophobic interfaces for some proteins.

In this paper, we report on the adsorption of α-lac, β-lg and BSA from single-component solutions to hydrophilic and hydrophobic silicon surfaces. Arnebrant et al. (2) studied temperature effects associated with α-lac and β-lg
adsorption at a hydrophilic chromium surface. Adsorbed mass was monitored for one hour for each protein with \textit{in situ} ellipsometry. Adsorption was studied at several temperatures, including temperatures in the range of thermal denaturation. They were able to interpret results with reference to each protein's denaturation temperature, as well as its exhibited reversibility of denaturation. Differential scanning calorimetry (DSC) was used in the present work, as well as the surface tension measurements described in Chapter 2, to interpret adsorption isotherms constructed for \(\alpha\)-lac, \(\beta\)-lg and BSA on hydrophilic and hydrophobic silicon at 2, 27 and 52°C. Adsorbed mass was measured as a function of protein concentration with ellipsometry.

\textbf{Materials and Methods}

\textit{Proteins}

\(\alpha\)-Lac (Type III, L-6010, Lot No. 89F8115), \(\beta\)-lg (L-0130, Lot No. 98F8030), and BSA (A-7906, Lot No. 15F0112) were of the highest native pure grade available prepared from bovine milk and obtained from the same source as in Chapter 2. The proteins were independently dissolved in phosphate buffer (\(\text{NaH}_2\text{PO}_4\) and \(\text{Na}_2\text{HPO}_4\)) pH 7.00 and passed through filters of 0.45 \(\mu\)m pore width (Millipore Corp., Bedford, MA) to remove undissolved material and other impurities such as bacterial cells. Protein concentrations of 10 to 14\% in 0.5 M buffer were used in DSC experiments and concentrations ranging from 0.10 to 2.0 mg/ml in 0.01 M buffer were used in adsorption experiments. A high ionic
strength was required in the DSC tests to maintain a constant pH 7.00 in each concentrated protein solution prepared. The exact concentration of the proteins was determined by measuring uv absorbance as described in Chapter 2; the buffer and water used in this study were the same as in Chapter 2 as well.

**Hydrophobic and hydrophilic solid surfaces**

Polished silicon wafers (1-0-0 orientation, type N/PH, resistivity 0.1-0.16 ohm cm, Wacker Siltronic Corp., Portland, OR) were carefully cut into plates of 1.2 x 1.5 cm and then treated to exhibit hydrophilic or hydrophobic surfaces by methods adapted from Elwing *et al.* (3). The sample plates were rinsed with deionized-distilled water, washed in a solution of NH₄OH (29.2%) + H₂O₂ (30%) + H₂O (1:1:5 by volume) at 78°C for 5 min, rinsed in deionized-distilled water, washed in a solution of HCl (37.0%) + H₂O₂ (30%) + H₂O (1:1:5 by volume) at 78°C for 5 min, then thoroughly rinsed twice in deionized-distilled water. This treatment yields a hydrophilic surface with a contact angle for water less than 10°. The hydrophilic plates were stored in 50% ethanol until use.

Half of the hydrophilic surfaces were then treated to be hydrophobic. First, they were blown thoroughly with a nitrogen stream to expel water and then silanized by exposure to a 10% solution of dichlorodimethylsilane (Aldrich Chemical Co., Inc., Milwaukee, WI) in trichloroethylene (Mallinckrodt Inc., Paris, KY) for 10 min. Thereafter the surfaces were carefully rinsed in ethanol, followed by trichloroethylene, then again in ethanol. These hydrophobic surfaces showed a contact angle for water greater than 110°.
Hydrophobic plates were stored in a desiccator until use, but used within several hours after preparation.

**Adsorption**

Immediately prior to adsorption, hydrophobic and hydrophilic silicon plates were removed from storage and blown thoroughly with a nitrogen stream. The optical constants of each plate were measured by ellipsometry. Protein solutions were prepared at 3 mg/ml immediately prior to the adsorption test, then diluted to the desired concentration with 0.01 M phosphate buffer at pH 7.00. Both sample plates and protein solutions were separately equilibrated to 2, 27, or 52°C as desired preceding contact. The adsorption was carried out under static conditions by exposing one plate to 10 ml of protein solution for 4 hr in a temperature controlled refrigerator at 2 ± 0.5°C, in a controlled water bath constructed in our laboratory at 27 ± 0.5°C, or in a Lab-Line Orbit chamber (Model 3527, Lab-Line Instruments, Inc., Melrose, IL) at 52 ± 0.5°C. After adsorption, the silicon sample plates were carefully rinsed for 30 sec three times by dipping into three separate volumes of 600 ml of deionized-distilled water and thereafter were allowed to dry in a desiccator for 14 hr before ellipsometric analysis.

**Ellipsometry**

An automated Gaertner ellipsometer (Model L116B, Gaertner Scientific Corp., Chicago, IL) was used with angle of incidence 70° and wavelength 6328
The optical constants $\Delta$ and $\Psi$ for bare solid surfaces as well as adsorbed films were determined using the computer software “SubCA” supplied by Gaertner.

In ellipsometry, the change in the state of polarization of laser light upon reflection from the sample surface is measured. The state of polarization is defined by the phase and amplitude relationships between the two component plane waves into which the electric field oscillation is resolved. Ellipsometry is the measurement of the angles $\Delta$ and $\Psi$, where $\Delta$ is related to the phase change and $\Psi$ is related to the change in amplitude ratio. Changes in $\Delta$ and $\Psi$ resulting from protein adsorption onto the solid surface were resolved simultaneously into a film refractive index $n_f$, and a thickness $d$, using a computer program (4) written according to the calculation procedure described by McCrackin et al. (5). $n_f$ and $d$ were then resolved into values of adsorbed mass using the Lorentz-Lorenz relationship according to Cuypers et al. (6). To calculate the amount of mass adsorbed as a dry film, the ratio of the molecular weight to the molar refractivity ($M/A$) must be known for the adsorbing series; dried films were considered to be mixtures of protein in air ($n_{\text{air}} = 1.000$). Table 3.1 shows both the optical constants $\Delta$ and $\Psi$ obtained for the bare silicon surfaces used in this study, and the $M/A$ values calculated for the three proteins. The molar refractivity was calculated by summing the individual molar refractivities of all amino acid residues for each protein molecule. Individual molar refractivities for the 20 amino acid residues were taken from Pethig (8).

Completely randomized design with subsampling was used in adsorption
and ellipsometric experiments. Three replicates with five ellipsometric measurements of $\Delta$ and $\Psi$ were performed for each treatment.

**Differential scanning calorimetry**

DSC is a technique in which the difference in energy input into a sample and a reference material is measured as a function of temperature, during which time the sample and the reference are subjected to a controlled temperature ramp. In this study, the Thermal Analyst 2000 system with a DuPont 910 DSC (DuPont Instruments, Wilmington, DE) was used. To eliminate moisture in and under the DSC cell, helium purging at the rate of 42.3 ml/min was applied. The system was calibrated for heat flow and two temperature points using DuPont calibration software. Indium and distilled water were used as calibration materials. Protein solutions were prepared immediately prior to DSC analysis; an exact volume of 12 $\mu$l was then placed in a coated aluminum pan (DuPont No. 900796 901) and sealed hermetically with a coated aluminum cover (DuPont No. 900790 901). The exact mass of the protein sample was calculated from the volume used and the uv spectroscopic data. The reference pan was the same as that of the sample, except that 0.5 M phosphate buffer pH 7.00 was used in absence of protein. For each protein, we performed six scans: an indium standard, a distilled water standard, a base line, and three replicate protein samples. A heating rate of 10°C/min was used in ramping temperature from 15 to 160°C. A sensitivity setting of 1x was selected for all scans. Duplicate analysis for peak temperatures (T$_{onset}$ and T$_{max}$) and heat of denaturation ($\Delta H$)
Results and Discussion

Protein Thermal Stability

The results of DSC experiments with the three proteins are represented in Figure 3.1 and Table 3.2. Within the experimental temperature range, two denaturation peaks were observed for β-lg and BSA, whereas only one peak was observed for α-lac. It is reasonably well-established that the first peaks are due to general denaturation or unfolding of the protein molecule (9-12). The second peak has been suggested to be caused by the unfolding of residual protein structure (12,13). This seems to be consistent with our results. Since the α-lac molecule is a rather small, "single domain" protein, unfolding probably occurs in one step. On the other hand, BSA consists of three domains and nine subdomains (14), and β-lg exists as a dimer under our experimental conditions. With increasing temperature, β-lg is thought to first dissociate into monomers that undergo partial unfolding, followed by polymerization of the monomers through sulhydryl oxidation and sulhydryl-disulfide exchange reactions (15).

Upon comparing data obtained from the denaturation peak of α-lac to that obtained from the first peaks of β-lg and BSA (Table 3.2), it is apparent that \( T_{onset} \) and \( T_{max} \) are highest for β-lg, and lowest for α-lac. This indicates that in solution at neutral pH, β-lg is the most stable and α-lac the least stable of the three proteins. However, α-lac has been observed to exhibit a very high degree
of "renaturation" ability (2,11). This phenomenon presumably accounts for the fact that α-lac is considered the most resilient of the whey proteins (16).

The denaturation peak for α-lac was somewhat broader than the first peaks of β-lg and BSA, which were similar to each other (as quantified by the difference between $T_{onset}$ and $T_{max}$). This is probably due to the renaturation ability of α-lac. In addition the second peak of BSA was about 1.7 times broader than that of β-lg. This is probably related to the fact that BSA is a large, multidomain molecule; moreover, BSA structural changes that occur upon heating to 65°C have been found to be partially reversible upon cooling to room temperature (17).

As discussed in Chapter 2, describing the behavior of α-lac is not completely straightforward. α-Lac binds calcium, serving to stabilize its structure. Due to the fact that protein preparations used in this work are lacking in Ca$^{2+}$, it is probably fair to interpret DSC results as reporting a lower thermal stability for α-lac than what might be expected of the molecule as it exists in milk. Again however, Na$^+$ can bind to the Ca$^{2+}$ binding sites of α-lac, perhaps compensating somewhat for the α-lac destabilization.

Comparing the enthalpy of the first thermal transition experienced by each protein, that of β-lg is the greatest, with those of α-lac and BSA being somewhat similar. ΔH of the second peak of β-lg is slightly more than twice that of BSA. These data are consistent with the conclusion that β-lg is the most stable, and α-lac the least stable protein. However, taken on a molar basis, these data would indicate that BSA is most stable, i.e., requiring the most energy to effect a
thermal transition, consistent with its high number of disulfide linkages. The point of interpreting molecular properties on a mass or molar basis with reference to their influence on surface activity is discussed in the last section.

**Temperature and Contact Surface Hydrophobic Effects on Adsorption**

Adsorption isotherms for each protein on hydrophobic and hydrophilic silicon surfaces were constructed at 2, 27, and 52°C, and are shown in Figure 3.2. For each protein, adsorbed mass was determined to be greater on the hydrophobic surface than on the hydrophilic surface at each temperature. This is in agreement with results of abundant previous work (3,18-21). The hydrophobic silicon surface consists of a thin layer of methyl groups covalently bound through silicon-oxygen bonds. These groups allow for hydrophobic interaction with adsorbing protein molecules. The hydrophilic surface consists of a thin layer of hydroxyl groups covalently bound to silicon atoms. These groups are amphoteric, being both proton acceptors and donors with a point of zero charge at about pH 4 (22). The hydrophilic silicon surface is negatively charged at neutral pH, with ability to bind positively-charged hydrophilic side chains of protein molecules. In any event, adsorbed mass was observed to be greater on hydrophobic surfaces at 27°C than on hydrophilic surfaces at 52°C for all three proteins.

Adsorption of proteins onto solid surfaces is thought to result in unfolding or partial denaturation, and irreversible adsorption (11,23,24). An increase in
temperature might be expected to enhance unfolding and result in an increase in adsorbed mass. However, only α-lac exhibited a more or less steady increase in adsorption with temperature for each surface. For α-lac, the increase in adsorbed mass beyond 27°C observed on each surface is consistent with the thought that the α-lac transition occurring between 27 and 52°C facilitates favorable noncovalent contacts with the surface. Arnebrant et al. (2) suggested that a heated solution of α-lac can contain molecules with different surface activities, due to the time-dependence exhibited by α-lac with respect to its reversibility of thermal denaturation. For α-lac, they stated that increasing the temperature increases the relative amount of denatured molecules in solution resulting in a greater adsorbed amount.

The isotherms constructed at 27 and 52°C appeared to be quite similar for both β-lg and BSA. The lack of difference in adsorptive behavior at 27 and 52°C for β-lg and BSA is consistent with thermal stability results obtained using DSC. β-Lg and BSA do not undergo a thermally-induced transition until onset temperatures of about 64.0 and 54.4°C are reached, respectively. Consequently, thermal stability influences do not appear to be affecting adsorption of β-lg and BSA up to 52°C. β-Lg and BSA adsorption is, however, depressed at 2°C, although the reason for this is not obvious. It is consistent with the belief that protein adsorption is entropically driven (25). Any hydrophobic interaction between the protein and silicon surface should be favored with increasing temperature; however this would be inconsistent with the similarity observed in the results obtained at 27 and 52°C. It is perhaps important to note that above
pH 6.5, the β-lg dimer is known to begin to dissociate, then to denature irreversibly while at first remaining in solution (26). This process is accelerated in the cold as compared to room temperature. Conceivably, the increased hydration of β-lg upon exposure to a cold (2°C) environment could cause adsorption of the molecule to be less energetically favorable. It is tempting to propose an analogous explanation for BSA, regarding its multi-domain structure. In any event, stabilization by hydrophobic interactions would be expected to decrease, with the formation and strengthening of hydrogen bonds favored, at temperatures near or below the freezing point of water (27).

**Molecular Effects**

Figure 3.3 shows the adsorption isotherms constructed for each of the three proteins, plotted together at constant temperature. At each temperature, adsorbed mass correlates reasonably well with molecular size, *i.e.*, the larger molecular size, the more mass adsorbed to a given surface. This is consistent with results of a good bit of previous work (28,29). In particular, a large molecular weight (large size) would suggest availability of a large number of potential noncovalent contacts with the surface. Besides molecular size, these proteins are dissimilar in flexibility and stability. As concluded in Chapter 2, molecular size and flexibility/stability properties strongly affect protein behavior at air-water interfaces. When isotherms of the three proteins were constructed in terms of adsorbed moles vs. concentration at 27°C, the relationship among the number of moles adsorbed to a hydrophobic surface agreed very well with the
relationship among the protein-specific parameters listed in Table 2.2 of Chapter 2. That is at first conflicting with the present observation. In particular, on a molar basis α-lac adsorbs most, and on a mass basis BSA adsorbs most. As plotted in Figures 3.2 and 3.3 (and Figure 2.2 of Chapter 2), these data provide only an indication of temperature and surface hydrophobic influences on relative amounts of single-component adsorption, and not relative adsorption affinities. If, as in blood serum, molecular weights among surface active proteins vary by one or two orders of magnitude, adsorption could be dominated by larger molecules in part simply because they are large. Indirect evidence provided in Chapter 2 suggested that both β-lg and BSA do not compete well with α-lac for surface sites. These data lead us to believe that in equilibrium studies, molecular size effects might simply be incidental. In any event, differences among the three proteins in stability and flexibility influence the adsorption at solid-water interfaces. Proteins are dynamic structures that apparently “sample” different conformations at an interface (23,30), and proteins with low stability and/or high flexibility will be preferentially adsorbed.

Acknowledgment

We are grateful to Dr. Edward Kolbe and Dr. DeQian Wang for assistance with the DSC. This material is based in part upon work supported by the National Science Foundation under Grant No. CTS-8910086. This is Technical Paper No. 9755 of the Oregon Agricultural Experiment Station.
Table 3.1  Optical constants of bare silicon surfaces and M/A ratio calculated for each protein.

<table>
<thead>
<tr>
<th>Optical constant</th>
<th>Hydrophobic (standard deviation in parentheses)</th>
<th>Hydrophilic (standard deviation in parentheses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta$</td>
<td>172.23 (0.266)</td>
<td>174.84 (0.125)</td>
</tr>
<tr>
<td>$\Psi$</td>
<td>9.96 (0.022)</td>
<td>9.94 (0.016)</td>
</tr>
</tbody>
</table>

(b)  Protein M(7) A M/A (g/mol.cm$^3$)

<table>
<thead>
<tr>
<th>Protein</th>
<th>M(7)</th>
<th>A</th>
<th>M/A (g/mol.cm$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Lac</td>
<td>14,161*</td>
<td>3,710.6</td>
<td>3.816</td>
</tr>
<tr>
<td>$\beta$-Lg</td>
<td>18,320*</td>
<td>4,814.0</td>
<td>3.806</td>
</tr>
<tr>
<td>BSA</td>
<td>66,267</td>
<td>17,270.0</td>
<td>3.837</td>
</tr>
</tbody>
</table>

*averaged between genetic variants A and B

Table 3.2  Thermal transition properties obtained for each protein (standard deviations shown in parentheses).

<table>
<thead>
<tr>
<th>Protein</th>
<th>1st peak</th>
<th>2nd peak</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_{\text{onset}}$</td>
<td>$T_{\text{max}}$</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>$\alpha$-Lac</td>
<td>32.2 (0.085)</td>
<td>39.5 (0.025)</td>
</tr>
<tr>
<td>$\beta$-Lg</td>
<td>64.0 (0.052)</td>
<td>70.9 (0.038)</td>
</tr>
<tr>
<td>BSA</td>
<td>54.4 (0.085)</td>
<td>61.2 (0.008)</td>
</tr>
</tbody>
</table>
Figure 3.1 DSC scans recorded for each of the three proteins (a) α-lac, (b) β-lg, and (c) BSA.
Figure 3.2 Adsorption isotherms constructed for each protein at hydrophilic and hydrophobic silicon surfaces at 2, 27 and 52°C. Solid line correspond to hydrophilic ("L") surfaces, and dashed lines correspond to hydrophobic ("B") surfaces (a) α-lac, (b) β-lg, and (c) BSA.
Figure 3.3 Adsorption isotherms constructed for each protein at hydrophilic and hydrophobic silicon surfaces (a) 2°C, (b) 27°C, and (c) 52°C.
References


Chapter 4

On Evaluating the Emulsifying Properties of \( \alpha \)-Lactalbumin, \( \beta \)-Lactoglobulin and Bovine Serum Albumin

Using Conductivity Measurements*

P. Suttiprasit and J. McGuire

Abstract

Selected oil-in-water emulsifying properties of α-lactalbumin, β-lactoglobulin, and bovine serum albumin were evaluated at solution concentrations ranging from 0.100 to 20.0 mg/ml. Changes in emulsion conductivity were recorded during and shortly after homogenization, and quantitatively interpreted for each protein in terms of emulsifying activity and emulsion stability. Although differences observed in emulsifying properties among the three proteins were found to be small, they were explainable with reference to important differences in their molecular properties gained by study of their behavior at air-water and solid-water interfaces.
Introduction

The behavior of proteins at air-water and oil-water interfaces is important both theoretically and practically, particularly in the area of food science and technology. Proteins tend to accumulate at both air-water and oil-water interfaces constituting a layer or film, thereby altering surface properties (1). These films can stabilize colloidal food systems, foams and emulsions by reducing both interfacial tension and rates of collapse and coalescence (2). Many food products are foams, emulsions, or both, including many dairy products, baked foods, ice cream products, meringue, and mayonnaise. Properties related to dispersion are usually crucial in determining the acceptability of many products (1-3).

Proteins are one of the two classes of surface-active agents used in formulating and stabilizing industrial colloidal food systems (4). Non-proteinaceous macromolecules are used in producing colloidal foods as well, but only a few polysaccharides, such as modified cellulose derivatives (5) or acetylated pectin (6) are considered sufficiently surface active for practical purposes.

We recently analyzed the concentration- and time-dependence of surface tension for the milk proteins α-lactalbumin (α-lac), β-lactoglobulin (β-lg) and bovine serum albumin (BSA) in single-component and mixed solutions (7). We also reported on the adsorption of α-lac, β-lg and BSA from single-component solutions to hydrophilic and hydrophobic silicon surfaces between 2 and 52°C.
That work was motivated by existence of only little information about the molecular mechanisms influencing a protein's ability to compete with other proteins in a serum and situate itself at a surface. Much is known about the chemistry of $\alpha$-lac, $\beta$-lg and BSA, and we had hoped that differences observed in their interfacial activity might identify the molecular dissimilarities most important in governing such activity. Results of those studies could be explained with reference to size, flexibility and stability differences among the molecules. In particular we concluded that the molecular size effects on surface activity that we observed were simply incidental, and proteins with low structural stability and/or high flexibility would be most surface active.

The emulsifying properties of $\alpha$-lac, $\beta$-lg and BSA as well as other proteins have been studied before by Kato et al. (9) using a conductivity technique, and by Pearce and Kinsella (10) using a turbidimetric technique; in each case differences in the emulsifying properties among the three were found to be fairly small. In this paper, results from our earlier, more fundamental evaluations are applied to describe differences in the oil-in-water emulsifying properties we calculated for $\alpha$-lac, $\beta$-lg and BSA based on emulsion conductivity measurements.

**Materials and Methods**

**Proteins**

$\alpha$-Lac (Type III, L-6010, Lot No. 128F140), $\beta$-lg (L-0130, Lot No.
98F8030), and BSA (A-7906, Lot No. 15F0112) were of the highest native pure grade available prepared from bovine milk and obtained from Sigma Chemical Co. (St. Louis, MO). Corn oil (100% pure) was from Hunt-Wesson, Inc. (Fullerton, CA). The proteins were independently dissolved in 0.01 M phosphate buffer (NaH₂PO₄ and Na₂HPO₄, pH 7.00). Solutions were prepared at a concentration of 2.2-2.5% and passed through filters of 0.45 μm pore width (Millipore Corp., Bedford, MA) to remove undissolved material and other impurities such as bacterial cells. The prepared solutions were stored at 5°C and used within one day. The exact concentration of protein was determined by measuring UV absorbance as described earlier (7).

**Emulsion Conductivity Measurement**

Emulsion conductivity was measured continuously at a controlled room temperature (26-28°C) when 20.00 ml of protein solution and 6.67 ml of corn oil were combined and homogenized (Tissue-Tearor Homogenizer; Biospec Products, Bartlesville, OK) for 1.5 min (speed 18,600 rpm) in a glass column. The selected methods were adapted from Kato et al. (9). A special conductivity electrode (Orion Cat. No. 018014, Orion Research Inc., Boston, MA) for emulsion measurement with a cell constant of 1.0 was used. A schematic of the apparatus we used is shown in Figure 4.1. The electrode was connected to a conductivity meter (YSI Inc., Yellow Springs, OH). A strip chart recorder (Linear Instruments Corp., Reno, NV) was used to trace the electrical
conductivity (μmho/cm) of each emulsion for a period of 5 min following the onset of homogenization.

**Emulsifying Property Determination**

Stable emulsions were formed after homogenization for 1.5 min. Homogenization was therefore carried out for 1.5 min in all experiments. For each experiment, selected emulsion properties were calculated according to Kato et al. (9). The emulsion activity (EA) of each protein was determined by measuring the difference between conductivity of the protein solution and the minimum conductivity exhibited by the emulsion during homogenization for 1.5 min:

$$EA = C_p - C_e$$  \[4.1\]

where $C_p$ and $C_e$ are the conductivity of the protein solution and the observed minimum conductivity of the emulsion, respectively. Emulsion stability (ES) was estimated with the following expression:

$$ES = (C_p - C_e)\Delta t/\Delta C$$  \[4.2\]

where $\Delta t/\Delta C$ (min·cm·μmho⁻¹) is the reciprocal of the slope of the initial linear region of the conductivity curve.

**Results and Discussion**

**Emulsion Activity**

Figure 4.2 indicates that emulsion conductivity rapidly decreased with the
onset of homogenization, as nonconducting oil was mixed into the conducting protein solution. During emulsification, three processes take place: (1) droplet disruption; (2) adsorption of protein molecules on the newly created surfaces, and (3) coalescence. Coalescence during emulsification is more probable during vigorous agitation than during subsequent storage (1).

Table 4.1 shows the effect of protein concentration on EA. In general, EA values among the three proteins were found to be quite similar, particularly at 5.0 and 10.0 mg/ml. Kato et al. (9) measured the EA of ten proteins including α-lac, β-lg, and BSA at a concentration of 1.0 mg/ml. They found that EA values among the three milk proteins were quite similar as well, but different from the other proteins. On average, we measured EA of the three milk proteins at 1.0 mg/ml to be about 500 μmho/cm. This value is much lower than that recorded by Kato et al., i.e., about 1,350 μmho/cm. This difference is probably due to differences between our experimental conditions and those adopted by Kato et al., particularly regarding homogenizing power input and buffer salt concentration (0.01 M vs. 0.02 M used by Kato et al.).

In any event, we found that EA of α-lac, β-lg, and BSA each increased with protein concentration. Kato et al. (9) measured EA of κ-casein, 11s globulin, and ovalbumin at concentrations ranging from 1.0 to 10.0 mg/ml, and found that EA increased with concentration. They suggested that this phenomenon was compatible with the theory that the emulsifying activity of proteins is a function of protein concentration. This seems to be reasonable since proteins arrive at a newly formed oil-water interface (during homogenization) at a rate proportional
to their concentration (11). Higher concentrations provide more protein molecules to mediate the formation of emulsions, binding both water and oil molecules to form thick barriers that help prevent the oil particles from coalescing (12).

If we compare the EA values among the three proteins at low and high concentrations, it is clear that at low concentration (0.1 mg/ml), \( EA_{\alpha\text{-lac}} \) and \( EA_{\text{BSA}} \) were not significantly different from each other, but higher than \( EA_{\beta\text{-lg}} \). At high concentration (20.0 mg/ml), \( EA_{\beta\text{-lg}} \) was the highest and \( EA_{\text{BSA}} \) the lowest. This might be related to important differences in molecular properties among the three proteins. At low bulk concentrations (0.01 mg/ml) Wei et al. (13) found a correlation between protein structural stability and adsorption kinetics at the air-water interface, i.e., less stable proteins adsorbed at a faster rate than proteins of greater stability. The \( \beta\text{-lg} \) molecule is the most thermally stable, while \( \alpha\text{-lac} \) is the least stable. Moreover, at low concentration, there is a greater surface area available per protein molecule, which would afford more unfolding at the interface. Both the \( \alpha\text{-lac} \) molecule and the particular domain of BSA thought to govern its behavior at apolar interfaces are quite flexible; that property is also consistent with the EA data recorded at low concentration. At high concentration, interpretation of the data is less straightforward. Wei et al. (13) found that protein "surface" hydrophobicity correlated well with adsorption rate at high bulk concentrations. However, \( \beta\text{-lg} \) is only slightly more hydrophobic than \( \alpha\text{-lac} \) and BSA (14). In any event, it is probably fair to note that concerning BSA, the ability of its domains to undergo conformational
change at an apolar interface contributes relatively much to its surface activity (7). The short protein-surface contact times associated with these experiments, along with reduced available surface area per molecule at high protein concentration, are consistent with its low value of EA.

Emulsion Stability

After homogenization, emulsion conductivity gradually increased as shown in Figure 4.2. This is consistent with the thought that dispersed oil droplets rise and coalesce to form a floating layer. Additionally, for all experiments an initial linear region of high slope is followed by a final linear region of low slope. This qualitative result is completely consistent with what Kato et al. (9) observed at 1.0 mg/ml. And in quantitative agreement with data of Kato et al., at termination of an experiment the emulsion conductivity was about 400 μmho/cm below the original (protein solution) conductivity. However, the pattern of the curves in our data is uniquely different from that of Kato et al. during the same time period following the emulsification. In particular, the slopes of the final linear region that we measured are not as high as those measured by Kato et al. Again, the conditions used in our experiments were somewhat different from those used by Kato et al. and one might suspect that the emulsions formed in our experiments are physically dissimilar to those of Kato et al. However, one should still expect to gain the same conceptual information regarding the emulsion stability associated with each protein.

To calculate the ES values shown in Table 4.2, we used the slope from the
initial linear region. It is important to note that in many cases the linear portion of the first region lasted less than 15 s (Kato et al. used the slope measured in the second region when the linear part of the first region lasted less than 15 s). In the case of Figure 4.2, however, estimation of ES based on the slope of the second region would yield a value of ES one or two orders of magnitude higher than those “typically” measured (9-10).

At 1.0 mg/ml, our ES values actually compare very well with those of Kato et al. (9) when differences in measured EA are taken into account. They found $ES_{\alpha-lac} \approx ES_{\beta-lg} = 9 \text{ min}$, and $ES_{BSA} \approx 7 \text{ min}$. Using a turbidimetric method, Pearce and Kinsella (10) found $ES_{\alpha-lac} = 21$, $ES_{\beta-lg} = 22$, and $ES_{BSA} = 12 \text{ min}$. However, our data (Table 4.2) show that $ES_{\beta-lg}$ and $ES_{BSA}$ are comparable while $ES_{\alpha-lac}$ is lower.

In general, the conductivity of $\beta$-lg and BSA emulsions immediately rise to their terminal value (i.e., the value of emulsion conductivity five minutes after the onset of homogenization) whereas $\alpha$-lac shows a similar initial rise, but followed by a slower increase to the terminal value (see Figure 4.2; slopes of the second linear region are tabulated in Table 4.2). This implies that although $ES_{\alpha-lac}$ was estimated to be lowest according to equation [4.2], it actually exhibited a higher stability in that its approach to the terminal value involves a fast step plus a slow step, as opposed to one fast step. At 0.1 mg/ml, each of the proteins exhibits a two step approach to the terminal value. At low concentration, there is more available area for each adsorbed protein molecule. Adsorbed protein molecules might unfold as much as their tertiary structure and
time allow. At higher concentrations, the surface layer becomes compressed, and there is less available area for molecular rearrangement. α-Lac, however, is the most flexible of the three proteins, and its resiliency to unfolding is well documented (15). We have found its kinetic behavior at the air-water interface to be consistent with the thought that α-lac would experience a rapid adsorption, during which period the number of noncovalent contacts made with the surface is already at or near its maximum value. That is, rapid, partial denaturation-renaturation events occurring in the crowded interface might allow α-lac to make more noncovalent contacts per area than the other proteins, at high concentrations.

In any event, overall, the emulsifying properties of these three proteins are similar. This finding is in qualitative agreement with Kato et al. (9) using a similar conductivity technique, and with Pearce and Kinsella (10) using a turbidimetric technique. However, it appears that if differences in emulsifying properties among apparently similar proteins are to be more clearly defined, and if the emulsion conductivity method is to be used to do it, the methodology must either be standardized or the entire conductivity vs. time plot must be quantitatively analyzed in some way to yield consistent results. We hope that this latter topic will contribute to the subject of future study.

Acknowledgment

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Center, Logan, Utah: Oregon State, Utah State, and Brigham Young Universities cooperating. This is Technical Paper No. 9955 of the Oregon Agricultural Experiment Station.
Table 4.1  The emulsion activity of each protein calculated according to equation [4.1].

<table>
<thead>
<tr>
<th>Protein Concentration (mg/ml)</th>
<th>Emulsion Activity (µmho/cm)</th>
<th>α-Lac</th>
<th>β-Lg</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.0</td>
<td>618</td>
<td>640</td>
<td>582</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>573</td>
<td>579</td>
<td>565</td>
<td></td>
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<tr>
<td>5.00</td>
<td>538</td>
<td>522</td>
<td>539</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>519</td>
<td>484</td>
<td>510</td>
<td></td>
</tr>
<tr>
<td>0.500</td>
<td>499</td>
<td>467</td>
<td>502</td>
<td></td>
</tr>
<tr>
<td>0.100</td>
<td>487</td>
<td>464</td>
<td>489</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.2  The emulsion stability calculated according to equation [4.2] and based on the slope of the initial linear region, and slope values evaluated in the second linear region for each protein.

<table>
<thead>
<tr>
<th>Protein Concentration (mg/ml)</th>
<th>Emulsion Stability (min)</th>
<th>Slope (µmho/min)</th>
<th>α-Lac</th>
<th>β-Lg</th>
<th>BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>α-Lac</td>
<td>β-Lg</td>
<td>BSA</td>
<td></td>
</tr>
<tr>
<td>20.0</td>
<td>4.27</td>
<td>4.48</td>
<td>5.82</td>
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<tr>
<td>10.0</td>
<td>3.17</td>
<td>3.80</td>
<td>4.43</td>
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<tr>
<td>5.00</td>
<td>2.82</td>
<td>3.47</td>
<td>3.54</td>
<td>12</td>
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<td>3.00</td>
<td>3.15</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
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<td>2.56</td>
<td>2.88</td>
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</tr>
<tr>
<td>0.100</td>
<td>2.40</td>
<td>2.85</td>
<td>3.02</td>
<td>23</td>
<td>6</td>
</tr>
</tbody>
</table>
Figure 4.1 Apparatus designed for measurement of emulsion conductivity.
Figure 4.2 Emulsion conductivity curves for (a) α-lac, (b) β-lg, and (c) BSA. Concentrations used were, from left for each protein, 20.0, 10.0, 5.00, 1.00, 0.500, and 0.100 mg/ml. The conductivity of each protein solution immediately preceding the onset of homogenization is indicated with an arrow on each plot.
References


Chapter 5

Conclusions

α-Lac, β-lg and BSA are almost always present in the same sera, and their continued comparative study is important. Although molecularly dissimilar in several ways, the surface activity of each protein could be explained with reference to their molecular size, flexibility and stability.

α-Lac, the smallest and least stable protein, exhibited the highest spreading pressure over the concentration range studied and the highest rate of arrival from bulk solution to the air-water interface. This indicates that α-lac is the most surface active and is consistent with the thought that small molecular size would facilitate incorporation of a molecule into an interfacial layer. At the oil-water interface, the ability of α-lac to stabilize an emulsion was calculated to be lower than that calculated for both β-lg and BSA, which were similar. However, α-lac may actually have exhibited a higher ability to stabilize an emulsion than calculated, based on quantitative analysis of the entire conductivity curve.

BSA is the largest molecule, and consists of three large domains and nine subdomains. The surface activity of BSA was found to be similar to that of β-lg, as indicated by its concentration-dependent behavior at air-water, solid-water and oil-water interfaces. β-Lg, in its dimer form, is roughly half the size of BSA. Surface tension kinetic tests indicated that β-lg adsorbs faster than BSA, but also that relative to β-lg, molecular rearrangement plays an important role in the
time-dependent behavior of BSA. This indicates that BSA surface activity is consistent with the thought that it is largely governed by the behavior of one of its three domains, and its activity after adsorption, *i.e.*, a slow disruption of its subdomain helices allowing for hydrophobic association. Moreover, the presence of a free thiol in each monomeric subunit of β-lg is capable of facilitating thiol-disulfide interchange reactions, probably causing it to be less flexible than BSA.
Chapter 6

Recommendations for Future Research

The surface activity exhibited by the food proteins α-lac, β-lg and BSA warrants continued study because of its immediate industrial significance. The results from this research indicated that the surface activity of the three proteins could be explained with reference to molecular size, stability, and flexibility.

It would be instructive to evaluate the adsorbed mass of each protein at air-water interfaces using appropriate techniques such as radiolabelling or in situ ellipsometry. The results could be used to verify the two parameter model proposed in chapter 2.

As suggested in chapter 4, the conductivity method for evaluating the emulsifying properties should be standardized. Otherwise the entire conductivity vs. time plot should be quantitatively analyzed in some way to yield consistent results. The results would enhance the practical use of conductivity methods.

One might try to apply other methods such as radiolabelling, fluorescence spectroscopy, infrared spectroscopy, or 3D molecular computer graphics to study the surface activity of the globular food proteins α-lac, β-lg and BSA.

A further step is the use of site-directed mutagenesis to produce protein mutants, acquisition of naturally occurring genetic variants, or even use of proteins with chemically modified side chains to gain a better quantitative understanding of molecular properties affecting their interfacial behavior.
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