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Interfacial Tension Kinetics of Nisin and β-Casein at an Oil-Water Interface

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Abstract–The concentration- and time-dependence of interfacial pressure of nisin and β-casein at an *n*-hexadecane-water interface were evaluated by using DuNoüy tensiometry. The two emulsifiers attained interfacial saturation at a bulk concentration of about 0.1 mg/ml, the reduction of the interfacial tension by nisin at that concentration being about equivalent to that of β-casein. The time dependence of interfacial tension recorded for each protein was described by using two kinetic models. In the first, the reduction of interfacial tension with time was considered to be a result of molecular penetration into the interface followed by rearrangement. Nisin exhibited more rapid penetration and rearrangement at the interface than did β-casein. In the second, the model allowed for the parallel, irreversible adsorption of protein into each of two states from solution, where state 2 molecules occupy a greater interfacial area and are more tightly bound than state 1 molecules. The extent of adsorption in state 1 and state 2 was determined to be highly concentration dependent for each protein; adsorption occurs mostly in state 1 at high concentration and mostly in state 2 at low concentrations.

Key words: Adsorption, Nisin, β-casein, DuNoüy Tensiometry, Emulsifier, Kinetic Modeling

INTRODUCTION

Proteins adsorb at air-water and oil-water interfaces, decreasing interfacial energy. The rate and extent of this decrease depend on many factors, including the size, charge, and flexibility of the adsorbing protein [Graham and Philips, 1979]. Fluid interfaces differ from solid interfaces in allowing adsorbate molecules greater mobility at the interface and greater penetration into the non-aqueous phase [Dickinson and Matsumura, 1994]. Thus, studying protein adsorption at gas-liquid and liquid-liquid interfaces is important for proper understanding of the ability of proteins to stabilize emulsions and foams in a variety of applications [Dickinson and Matsumura, 1994; Ross and Morrison, 1988; Guzman et al., 1986; Cho et al., 1996; Farooq and Narsimhan, 1991; Hunter et al., 1991]. Graham and Philips [1979] suggested that native molecules must first penetrate the air-water or oil-water interfaces, then unfold and rearrange for optimal packing. They also stated that adsorption is controlled by diffusion, a function of the size of the molecule. Thus, at low surface coverage, every protein molecule that arrives at the interface adsorbs spontaneously. Eventually, a steady state is achieved when the interface is saturated, and all of the molecules have rearranged to their preferred orientation [Ross and Morrison, 1988].

The adsorption of proteins at air-water interface was described by a model that allows for tight adsorption of a first layer and loose packing of a second layer [Guzman et al., 1986]; in the first layer, protein adsorbs in different conformations with different occupied areas per adsorbed molecule, dependent upon surface concentration. Cho et al. [1996] reported that the native and alkylated derivatives of bovine serum albumin occupy greater area at the air-water interface than that corresponding to molecular dimensions. They also found that the rate of surface pressure increase for these proteins was higher for higher bulk concentration at low times. In the classical 2-state theory of the globular protein unfolding transition, there are two protein structures: native and highly disordered [Dickinson and Matsumura, 1994]. However, it has now been established that an intermediate conformation can be present, termed the "molten globule", defined as a protein with a native-like secondary structure but disordered (unfolded) tertiary structure. It was found that α-lactalbumin in the molten globule state (produced in the presence of EDTA) reduced the surface tension at the air-water and *n*-tetradecane-water interfaces more rapidly and to a lower level than the native protein. For globular proteins at the air-water interface, Farooq and Narsimhan [1991] proposed that adsorbed segments are present in the form of "trains". They concluded that the degree of unfolding of bovine serum albumin upon adsorption was greater than that of lysozyme, as they found that the number of segments per molecule increased linearly with the increase of surface concentration for bovine serum albumin, and was independent of surface concentration for lysozyme. Other studies suggested that protein molecules undergo conformational change during the adsorption process due to the interaction with the surface or during overcoming energy barrier to adsorption [Andrade et al., 1984; Song and Damodaran, 1991]. In particular, free energy change of a protein molecule for conformational change to a denatured form, 5 to 14 kcal/mol, is comparable to the free energy for adsorption, 5

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to 20 kcal/mol. Thus, the conformational change during adsorption is highly probable.

β-casein is a single chained, fibrous protein of molecular weight 24,000 that has no disulfide bonds [Hunter et al., 1991]. The N-terminal 21-amino acid sequence of β-casein contains one-third of the charged residues at pH 7, and this portion of the protein is highly solvated and flexible. The remainder of the molecule is nonpolar and very hydrophobic, making β-casein distinctly amphiphilic. Hunter et al. [1991] modeled the adsorption of β-casein at the air-water interface using Langmuirian kinetics, defining an adsorption activation energy that depends on surface coverage. They found the isotherm exhibited two plateaus in surface coverage. The isotherm was interpreted as indicating adsorption of a saturated layer of β-casein at low concentration followed by molecular reorientation and continued adsorption until the surface is once again saturated.

Nisin is a polypeptide (3510 Da) consisting of 34 amino acid residues [van de Ven et al., 1991]. As far as the amino acid sequence is concerned, nisin possesses an amphiphilic character, with a cluster of bulky hydrophobic residues at the N-terminus and hydrophilic residues at the C-terminus. Nisin can withstand activity loss during thermal processing and exposure to acidic environments and pressure extremes [Bower, 1994]. These characteristics and others, such as non-toxicity, high surface activity and antimicrobial activity [Hurst, 1981], make it an attractive candidate for use as an emulsifier in food and pharmaceutical emulsions. The efficacy of nisin in emulsifying an oil in water was evaluated [Bani-Jaber et al., 1998] and found to be significant compared to β-casein and Tween® 80. The interfacial behavior of nisin and β-casein at hydrophilic and hydrophobic solid surfaces has been investigated by using ellipsometry [Krisdhasima et al., 1993; Lakamraju et al., 1996]. Each of the proteins, when dissolved in single-protein solution, more favorably adsorbed at hydrophilic than at hydrophobic surfaces.

In this work, we evaluated the interfacial tension kinetics of nisin and β-casein at *n*-hexadecane-water interface by using a DuNoüy tensiometer. In order to account for the effect of conformational change during adsorption, the interfacial tension kinetic data of each protein were analyzed with reference to a two state mechanism that allows for protein to adsorb in structurally dissimilar forms.

MATERIALS AND METHODS

Pure nisin (about 5.0×10^7 IU/g) was obtained from Aplin and Barrett Ltd. (Dorset, U. K.). *n*-Hexadecane (Lot No. 105H3530) and β-casein (Lot No. 25H9550) were purchased from Sigma Chemical Co. (St Louis, MO). Monobasic sodium phosphate monohydrate (Lot No. 77892KLJP), dibasic sodium phosphate heptahydrate (Lot No. 7914KJKA) and citric acid monohydrate (Lot No. 062777KMBX) were obtained from Mallinckrodt Specialty Chemical Co. (Paris, Kentucky). Sodium citrate (Lot No. 402346) was from J. T. T. Baker (Philipsburg, NJ).

1. Solution Preparation

Nisin was dissolved in 0.01 M sodium phosphate monobasic (pH 4.5), to ensure complete solubilization. A suitable volume of sodium phosphate dibasic (pH 9.1, 0.01 M) was added to the solubilized nisin to bring the pH 7.4. The same buffer was used to prepare β-casein solutions.

2. Interfacial Tension Measurement

A DuNoüy ring tensiometer (Model No. 70535, CSC Scientific Co., Inc., Fairfax, VA) was used to measure interfacial tension at the *n*-hexadecane-water interface. Immediately after gentle stirring for 45 s, 20 ml of protein solution at a concentration in the range of 1×10^{-6} to 1 mg/ml was placed in a beaker (5 cm dia). The ring was immersed about 10 mm below the surface of the protein solution, and this was followed by the addition of 20 ml *n*-hexadecane to the surface of the solution. The position (height) of the beaker was adjusted until the ring was in the interface and the apparent interfacial tension was measured (CSC Scientific Co., Operating Instructions). To account for the force needed to support the weight of the liquid clinging to the ring at the break point, the apparent interfacial tension was multiplied by a correction factor to get the true interfacial tension. All measurements were performed at room temperature (23-25 °C). Before each measurement, the ring was cleaned by rinsing in benzene followed by rinsing in methylethylketone, and then heating in the oxidizing portion of the flame of an alcohol burner.

3. Interfacial Pressure

Interfacial pressure, Π (mN/m), is the reduction of interfacial tension by a surfactant. The average of three interfacial tension readings between phosphate buffer (pH 7.4) and *n*-hexadecane was 52.4 mN/m. The interfacial pressure was obtained by subtracting the interfacial tension in the presence of a surfactant from this value.

RESULTS AND DISCUSSION

1. Effect of Concentration on Steady-state Interfacial Pressure

The concentration dependence of Π for nisin and β-casein is shown in Fig. 1. Each protein showed an increase in Π with increasing bulk concentration. The maximum reduction in interfacial tension was 30.4 and 32.2 mN/m for nisin and β-casein, respectively.

2. Time Dependence of Surface Pressure: Empirical Analysis

Fig. 2 shows the time dependence of Π for nisin and β-casein

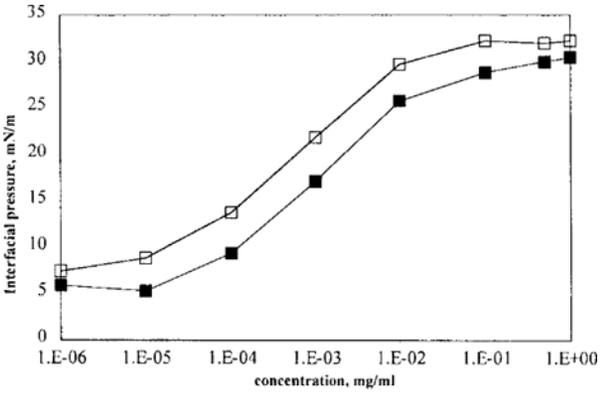

Fig. 1. Effect of concentration of nisin (filled squares) and β-casein (open squares) on interfacial pressure (concentration is plotted on semilog scale).

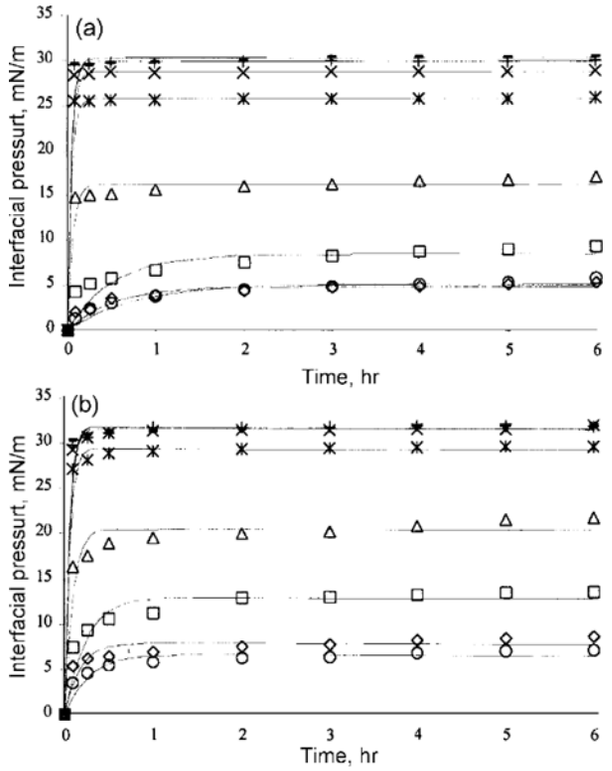

Fig. 2. Time dependence of interfacial pressure as a function of concentration for (a) nisin and (b) β-casein. The protein concentrations used were 0.000001 (circle), 0.00001 (diamond), 0.0001 (square), 0.001 (triangle), 0.01 (asterisk), 0.1 (cross), 0.5 (plus), 1 (minus), respectively.

(solid lines for the higher concentration data represent fits to a model that will be discussed in the next section). Both emulsifiers attained a steady state interfacial pressure, at all concentrations, after about 2 hours.

For single protein solutions of β-casein, BSA, and lysozyme, Graham and Philips [1979] suggested that the rate of change of interfacial pressure can be defined with reference to two kinetic regions, each characterized by a first-order rate constant. The first region, the adsorption period, is one in which both adsorbed mass and interfacial pressure are observed to increase. The other region, the rearrangement period, is characterized by attainment of a plateau in adsorbed mass while the interfacial pressure continues to increase. The rates of adsorption and conformational change can be represented by the first order equation:

$$\ln\frac{\Pi_\infty-\Pi_t}{\Pi_\infty-\Pi_0}=-kt, \tag{1}$$

where Π_∞, Π_t and Π_0 are the surface pressure values at steady state, at any time t, and at t=0, respectively, and k is a first-order rate constant.

Fitting the interfacial kinetic data for nisin and β-casein at 0.1 mg/ml according to Eq. (1) yielded two linear segments in each case. The slope of the first linear segment was considered as an adsorption rate constant, while the slope of the second was interpreted as a rearrangement rate constant. Compared to 0.183 and 0.003 for β-casein, nisin had an adsorption rate constant and a rearrangement rate constant of 0.736 and 0.012, respectively. The fast adsorption and rearrangement of nisin might be due to its small size and lack of tertiary structure.

The empirical analysis of adsorption, while used frequently, can provide simple comparison but is not so meaningful in modeling. The empirical method for obtaining rate constants is good only if the system of interest obeys the mechanism underlying the empirical method. The system adopted in this study is more complex than a simple arrival and rearrangement sequence at the very least. In this system adsorption and rearrangement processes may take place simultaneously, not in a neat "step one-step two" sequence where no unfolding occurs until the surface is fully covered.

4. Analysis with Reference to a Kinetic Model

Wang and McGuire [1997] applied a kinetic model to describe the spreading pressure of T4 lysozyme solutions, allowing protein to be adsorbed in structurally and functionally dissimilar states. State 2 molecules are unfolded to some extent and more tightly bound to the surface (i.e., they reduce surface tension more per unit area than those in state 1); also the area occupied by a state 2 molecule (A_2) is larger than that occupied by a state 1 molecule (A_1). The same adsorption mechanism can be applied to protein adsorption at an oil-water interface. Let rate constants k_1 and k_2 describe adsorption into states 1 and 2, respectively. Equations describing the time-dependent fractional surface coverage of protein in each of the two states (θ_1 and θ_2) are

$$\theta_1=\frac{1}{1+ak_2/k_1}[1-\exp\{(-k_1C-ak_2C)t\}] \tag{2}$$

and

$$\theta_2=\frac{k_2/k_1}{1+ak_2/k_1}[1-\exp\{(-k_1C-ak_2C)t\}], \tag{3}$$

where a is A_2/A_1, and C (mg/ml) is the bulk protein concentration.

θ_1 and θ_2 were defined such that when the surface is covered,

$$\theta_1+a\theta_2=1. \tag{4}$$

If we assume that the interfacial tension reduction can be attributed to the amount adsorbed at the interface, then the interfacial pressure at any time is given by

$$\Pi=\Pi_{max}(\theta_1+\theta_2), \tag{5}$$

and

$$\Pi=\Pi_{max}\frac{1+k_2/k_1}{1+ak_2/k_1}[1-\exp\{(-k_1C-ak_2C)t\}], \tag{6}$$

In order to solve for k_1C and k_2C in Eq. (6), the values of a and Π_{max} should be known or approximated *in priori*. The maximum measured reductions in interfacial tension (32.2 for β-casein and 30.4 for nisin) provide a reasonable estimate for the theoretical Π_{max}. Based on molecular dimensions, 20 5 20 5 50 Å for nisin [van de Ven et al., 1991] and 14.6 5 14.6 5 175 Å for β-casein [Hunter et al., 1991], the parameters A_1 and A_2 can be estimated as the interfacial area occupied by adsorbed "end on" and "side on" molecules, respectively, such that a is 2.50 for nisin and 11.99 for β-casein. An experimentally determined "a" may allow for more accurate estimation of adsorption in states 1 and 2 by using Eq. (7) than what was estimated using the molecular dimensions. We assume that adsorption below these critical con-

centrations occurs in state 2 only, such that

$$\frac{d\theta_2}{dt}=k_2C(1-a\theta_2), \quad (7)$$

or

$$\Pi=\frac{\Pi_{max}}{a}\left[1-\exp\left(-\frac{k_2}{a}Ct\right)\right]. \quad (8)$$

Accordingly, a values obtained from Eq. (8) at the lower critical concentration (6.25 for β-casein and 4.79 for nisin) were used in estimating k_1C and k_2C for the two proteins according to Eq. (6) at concentrations higher than the lower critical concentration.

In general, we can see that the rate of adsorption in state 1 decreases with decreasing concentration, while the rate of adsorption in state 2 increases with decreasing concentration. At high bulk concentration, the ratio of the interfacial area to the total number of molecules available for adsorption is very small, and saturation of the interface is attained in a very short period of time. This suggests that the sub-layer would be crowded, with only little uncovered interface being available as adsorption progresses. Alternatively, the possibility for a protein molecule to extend its conformation and be adsorbed in state 2 would be maximized in a dilute solution. The isotherms of the steady-state interfacial pressure versus the bulk concentration shown on the Fig. 1 appear to be sigmoidal with upper and lower critical concentrations. The interfacial pressure does not change significantly below the lower critical concentration, probably because adsorption occurs only in state 2. The lower critical concentrations were selected as $1\text{-}5\times10^{-5}$ mg/ml for nisin and $1\text{-}5\times10^{-6}$ mg/ml for β-casein. The interfacial tension kinetic data, along with their fit to Eq. (6), are shown in Fig. 2. The values for k_1C and k_2C were estimated by using the MATLAB program. These estimates were plotted against the logarithm of concentration for both proteins shown in Fig. 3 and 4, respectively. Figs. 3 and 4 are also consistent with the thought that k_2C decreases with increasing concentration. The peaks observed in both figures could be mainly attributed to the experimental deviations. The abnormally high values of k_2C may only reflect a total adsorbed amount similar to that seen at high concentrations. Therefore, it was assumed that these peaks simply do not represent the overall tendency of k_2C with respect to

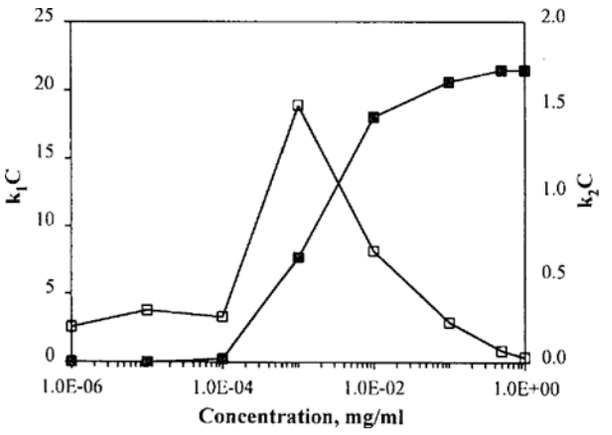

Fig. 3 Adsorption rates k_1C (filled square) and k_2C (open square) (ml/mg.min) for nisin as a function of concentration based on fitting interfacial pressure kinetic data to Eq. (6).

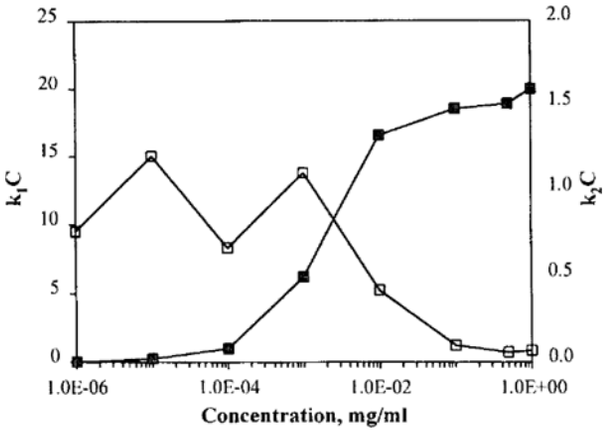

Fig. 4. Adsorption rates k_1C (filled square) and k_2C (open square) (ml/mg.min) for β-casein as a function of concentration based on fitting interfacial pressure kinetic data to Eq. (6).

bulk concentrations. The estimates of k_1C were higher at high concentrations and k_2C were higher at low concentrations. Furthermore, the ratio of k_2/k_1 increases as concentration decreases for all data presented, which has very useful meaning mechanistically. Guzman et al. [1986] modeled the dependence on surface concentration for adsorption rate constants in terms of activation energies for adsorption and desorption. Accordingly, adsorption rate constants, k_iC might be represented in the form $k_i=k_{i0}\exp(-E_{ai}/RT)$, where E_{ai}, the activation energy for adsorption, is considered to be surface-coverage dependent. On the other hand, Lee et al. [1999] used the wild type of bacteriophage T4 lysozyme and its synthetic mutants in order to develop a simulation model at solid-water interfaces. They proposed that adsorption rate constants as a function of contact time and attained prediction in a good agreement with experimental data. The results in our study suggest that the activation energy for protein adsorption increases with increasing surface concentration of nisin and β-casein; consequently, the ease with which a protein molecule adsorbs should decrease with increasing surface concentration. Hunter et al. [1991] found that for β-casein, at low concentrations ($<10^{-3}$ mg/ml), adsorption at the air-water interface was "cooperative", becoming easier as the surface coverage increased, while at higher concentrations ($>10^{-3}$ mg/ml), adsorption became more difficult as the surface coverage increased.

The areas of state 1 and 2 might not be constant with changing concentration. In addition, the two-state kinetic model might not work well at very low concentrations where adsorption from highly dilute solutions can be a transport-limited process; i.e., the rate of diffusion to the surface is slower than the rate of protein binding to the surface [Wang and McGuire, 1997]. Moreover, protein adsorption at such low concentrations might not result in complete coverage of the interface at steady state. Modeling of protein adsorption in two states can allow quantitative characterization of the adsorption process incorporating the conformational events taking place at interfaces.

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