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Relation of Dynamic Changes in Interfacial Tension to Protein Destabilization upon Emulsification

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The objective of this study was to link conformational changes of proteins at a water/methylene chloride interface to their destabilization upon emulsification. When 4 aqueous protein solutions (bovine serum albumin, β-lactoglobulin, ovalbumin, or ribonuclease) were emulsified in methylene chloride, considerable proportions of all the proteins became water insoluble aggregates. There were also noticeable changes in the compositions of their water-soluble species. A series of water/methylene chloride interfacial reactions upon the proteins was considered a major cause of the phenomena observed. Based on this supposition, the interfacial tension was determined by a Krüss DVT-10 drop volume tensiometer under various experimental conditions. It substantiated that the interfacial tension was high enough to cause the adsorption of all the proteins. Under our experimental conditions, their presence in the aqueous phase resulted in reductions of the interfacial tension by the degrees of 8.5 - 17.1 mN m^{-1}. In addition, dynamic changes in the interfacial tension were monitored to compare relative rates at which the adsorbed proteins underwent conformational, structural rearrangements at the interface. Such information helped make a prediction about how easily proteins would denature and aggregate during emulsification. Our study indicated that emulsifying aqueous protein solutions in organic solvents should be handled with care, due to adverse interfacial effects.

Key words: Protein, Emulsification, Dynamic interfacial tension, Microspheres

INTRODUCTION

In recent years interests have increased in using emulsions and biodegradable poly-*d,l*-lactide-*co*-glycolide microspheres as parenteral protein and antigen delivery systems (Watts *et al.*, 1990; Vogel and Powell, 1995). The dosage forms provide their sustained release over a wide range of periods, making it possible to reduce the frequency of injections. This advantage contributes to improving patient compliance. In case where antigens are delivered in the dosage forms, their immunogenecity is often enhanced to a greater extent (O'Hagan *et al.*, 1991; Audran *et al.*, 1998). An early manufacturing process frequently used to load proteins or antigens into such delivery systems is to emulsify an aqueous protein solution in an organic solvent such as methylene chloride.

Emulsification involves the mixing of two immiscible liquid phases to generate an enormous interfacial area. A number of studies report on protein damage at the boundaries between aqueous and organic solvent phases, demonstrating that proteins adsorb to undergo structural changes at water/oil interfaces (Khmelnitsky *et al.*, 1984; Dickinson *et al.*, 1990; Walstra and Roos, 1993). It has also been reported that considerable amounts of proteins precipitate at water/oil interfaces when their aqueous solutions are emulsified in an oil phase (Sah, 1999). It is generally conceived that the surface activity of proteins, thanks to their amphipathicity and flexible conformation, permits them to adsorb at water/oil interfaces. Once adsorbed, they have a propensity for unfolding and spreading at the interface, thereby reducing the free energy of the system.

So far, little systematic work has relatively been carried out to address the issue of protein destabilization during emulsification. The aim of this study is to help better understand the protein instability observed when aqueous protein solutions are emulsified in methylene chloride. Efforts have been put into correlating the protein instability to adverse interfacial effects. In this study the precipitation

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of proteins at the water/methylene chloride (W/MC) interface and changes in the compositions of their water-soluble species are considered major indices for their instability toward emulsification. In particular, the adsorption and concurrent structural realignments of proteins at the interface have been demonstrated by a Krüss DVT-10 drop volume tensiometer: it has helped monitor dynamic changes in interfacial tension as a function of interface aging time, i.e., the duration of protein exposure to the interface. The model proteins used throughout this study include bovine serum albumin (BSA), β-lactoglobulin, ovalbumin, and ribonuclease A (RNase). Finally, methylene chloride has been chosen as a model organic solvent phase, since it finds wide applications in double-emulsion based microencapsulation processes (Watts *et al.*, 1990; Cho *et al.*, 2000).

MATERIALS AND METHODS

Materials: BSA (MW = 66,400), β-lactoglobulin (MW = 18,000), ovalbumin (MW = 44,300), and RNase (MW = 13,680) were purchased from Sigma-Aldrich Co. (St. Louis, MO). HPLC-grade methylene chloride was supplied from Fisher Scientific Company (Altanta, GA).

Emulsification: A protein (BSA, β-lactoglobulin, ovalbumin, or RNase) was dissolved in water to make a 0.5 mg/mL concentration. Three milliliters of the protein solution (total protein content = 1.5 mg) were emulsified in 12 ml of methylene chloride with the aid of a VirtiShear homogenizer equipped with a 10 mm shaft (The VirtisShear Co., Gardiner, NY). Emulsification was carried out at room temperature for 1 min at 16,000 rpm.

Protein content in water and the W/MC interface : To determine the content of a protein in the aqueous phase, the water-in-oil emulsion was quickly centrifuged to facilitate phase separation. The content of the protein in the aqueous phase and the composition of its water-soluble species were determined by a native size exclusion chromatography (SEC)-HPLC assay described in the following section. The percent degree of protein recovery was calculated by comparing the aqueous protein concentration before emulsification with that of after emulsification, as follows: 100 × (the aqueous protein concentration determined after emulsification/the one before emulsification). This result led to the determination of the amount of the protein residing in the W/MC interface, which was equal to that of the protein disappearing from the aqueous phase. Each set of experiments was repeated at least triplicate, and results were presented as mean ± standard deviation in text.

Size exclusion chromatography-HPLC: Aliquots (40 μl) of protein sample solutions were injected into a Shimadzu HPLC system. They were eluted at a 0.8 mL/min flow rate from a TSK Gel G3000 SW analytical column by a mobile phase consisting of 10 mM phosphate buffer containing 0.1 M NaCl (pH 7.0). The elution of a protein was detected by a UV detector set at 280 nm. The protein concentration was determined by a calibration curve constructed by peak area integration of its standards of known concentrations.

Determination of dynamic interfacial tension: A Krüss DVT-10 drop volume tensiometer was used to measure the dynamic interfacial tension between methylene chloride and aqueous phases under various conditions. The detailed description on the tensiometer itself was described elsewhere (Campnaelli and Wang, 1997). Aqueous protein solutions at 0.5 - 3.0 mg/mL concentrations were delivered into methylene chloride via a 0.254-mm inner diameter bore capillary. A syringe pump (Model 44/Harvard Apparatus, South Natick, MA) was used to deliver the aqueous protein solution at a constant rate (1 to 0.02 mL/hr). As the protein solution was pumped into methylene chloride that was placed inside a glass tube, a drop grew and detached off the tip of the bore capillary. At this time, an infrared detector detected the drops as they detached, in order to measure the time that passed between subsequent drops. A change in the flow rate led to the manipulation of the exposure time of the protein upon the interface, which was referred to as interface aging time in text. Any variation in interfacial tension with flow rate was due to the effects of adsorption and reorientation of protein molecules present at the interface. Information on the flow rate and the time required to form each drop provided the determination of its volume. Interfacial tension, σ, was calculated by:

$$\sigma = \frac{V_{drop}(\rho_m - \rho_w)g}{\pi d} \quad (1)$$

where V_{drop} was the volume of an aqueous drop; g, the acceleration due to gravity; d, diameter of the bore capillary; ρ_m, the density of methylene chloride; and ρ_w, the density of the aqueous phase. For this experiment, the volumes of at least five drops were detected for each flow rate, in order to report the interfacial tension data as mean ± standard deviation. Interfacial pressure, π, was then given by:

$$\pi = \gamma_0 - \gamma \quad (2)$$

where γ_0 was the W/MC interfacial tension between

Table 1. Comparison of the compositions of water-soluble protein species before and emulsification[a]

	Water-soluble BSA species				Water-soluble obalbumin species		
	Monomer	Dimer	Oligomer	New aggregates	Monomer	Dimer	New aggregates
Before	82.6 ± 0.6	13.9 ± 0.2	3.4 ± 0.4	None	96.6 ± 0.3	3.1 ± 0.2	None
After	76.2 ± 1.6	17.3 ± 1.1	4.8 ± 0.1	3.5 ± 0.2	92.4 ± 2.1	5.3 ± 0.5	2.3 ± 0.8

[a]Before and after emulsification of 0.5 mg/ml aqueous protein solutions in methylene chloride, the contents of their water-soluble species were quantitated by the HPLC assay.

pure water and methylene chloride, and γ was the interfacial tension observed in the water phase containing a protein sample.

RESULTS

In the absence of methylene chloride, homogenization of 4 different aqueous protein solutions neither caused any considerable changes in their aqueous concentrations, nor did the compositions of their water-soluble species change. This substantiated that shear stress and/or the continual creation of a new air/water interface did not cause any physical and/or chemical aggregation under our experimental conditions.

All of the proteins emulsified in methylene chloride, however, displayed some degrees of instability (Fig. 1). Judged from the degree of protein recovery, ovalbumin suffered the highest degree of damage upon emulsification: its recovery was only 37.8 ± 0.8%. This meant that 930 ± 10 μg was the amount of water insoluble ovalbumin precipitates appearing at the W/MC interface (Before emulsification, the aqueous phase contained 1,500 μg of ovalbumin). A similar degree of interfacial precipitation was shown with β-lactoglobulin: its recovery after emulsification amounted to 41.0 ± 1.4%. Compared to ovalbumin and β-lactoglobulin, higher recovery yields

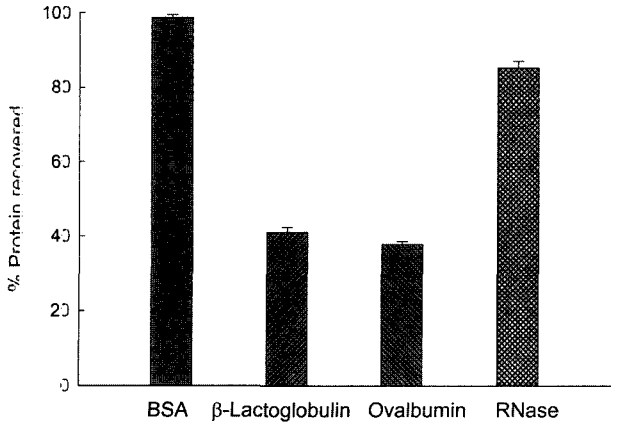

Fig. 1. The percentage protein recovery determined after emulsification. After 0.5 mg/mL protein solutions were emulsified in methylene chloride, its aqueous concentrations were determined by the SEC-HPLC assay.

Table II. The protein-free water/methylene chloride interfacial tension measured at 1 - 0.1 mL/hr flow rates

Flow rate (mL/hr)	Interfacial Tension (mN m^{-1})[a]
1.0	27.38 ± 0.42
0.5	27.12 ± 0.44
0.2	27.18 ± 0.41
0.1	27.40 ± 0.63

[a] Mean ± s.d. of 5 determinants.

were attained with BSA and RNase.

Even though BSA was almost completely recovered after emulsification, its underwent pronounced changes in the composition of its water soluble species. Before emulsification, the protein consisted of a monomer 82.6 ± 0.6, a dimer 13.9 ± 0.2, and a multimer 3.4 ± 0.4%. After emulsification, its monomer decreased to 76.2 ± 1.6%, whereas the dimer and multimer contents increased to 17.3 ± 1.1 and 4.8 ± 0.1%, respectively. In addition, a new water-soluble aggregate appeared in the aqueous phase emulsified. This indicated that emulsification led to conformational changes of BSA molecules, which facilitated the formation of intermolecular covalent linkages. A similar change in relation to the composition of water-soluble species was observed with ovalbumin (Table I). By contrast, only the same monomeric species appeared in the aqueous phase, when β-lactoglobulin or RNase was emulsified in methylene chloride.

Protein denaturation at the W/MC interface was attributed to a cause of the protein instability associated with emulsification. To buttress this supposition with details, the adsorption and conformational realignments of proteins at the W/MC interface were investigated by monitoring dynamic interfacial tension. Before this experiment, the interfacial tension between pure water and methylene chloride was first measured at different flow rates. Regardless of the flow rates used, the interfacial tension remained constant (Table II). The results made it clear that under our experimental conditions hydrodynamic effects associated with the process of droplet expansion did not influence the W/MC interfacial tension determined.

Interestingly, the presence of proteins in the aqueous

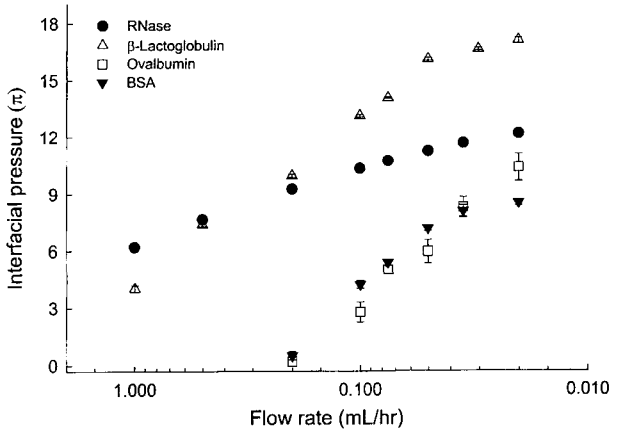

Fig. 2. Flow rate-dependent changes in the W/MC interfacial pressure π. The aqueous protein concentration was fixed at 0.5 mg/mL, and the pumping rate to deliver the solution into methylene chloride was changed from 1.0 to 0.02 mL/hr.

phase led to drastic changes in the W/MC interfacial tension. Such changes were influenced to a great extent by the flow rate used and were protein specific (Fig. 2). At 1 - 0.2 mL/hr flow rates that permitted proteins to be briefly exposed to the interface, β-lactoglobulin reduced the W/MC interfacial tension to the greatest extent. If put it another way, there was the most noticeable evolution of interfacial pressure π. Under this experimental condition β-lactoglobulin was likely to be the most surface active of all the proteins tested in this study. RNase would be selected as the next protein, if their surface activity was ranked with regard to the magnitude of π change. In contrast, π was barely affected at 1 - 0.2 mL/hr flow rates, when BSA or ovalbumin was present in the aqueous phase. This

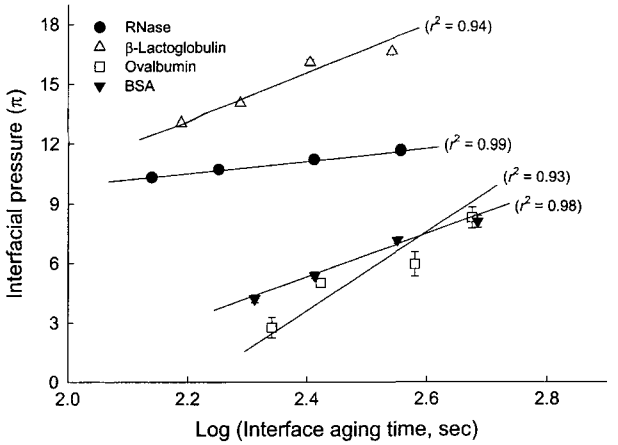

Fig. 3. Linear relationships between the W/MC interfacial pressure and log (interface aging time). Correlation coefficient was represented as r^2. The duration of exposure of proteins to the interface, referred to as interface aging time, was controlled by changing flow rates.

Table III. Comparison of π changes rates of 4 proteins, their degrees of recovery after emulsification, and changes in the composition of water-soluble species after emulsification

Protein	π change rate[a]	Recovery%	Changes in the composition
RNase	3.13	85.4 ± 1.6	×
β-Lactoglobulin	10.51	41.0 ± 1.4	×
BSA	10.60	98.7 ± 0.7	○
Ovalbumin	14.54	37.8 ± 0.8	○

[a]The slope of the linear lines shown in Fig. 3 (its unit is Nm m^{-1}/log sec).

meant that they did not reduce the W/MC interfacial tension at the flow rates used.

As the duration of protein exposure to the interface was prolonged, different results were observed. At 0.1 - 0.02 mL/hr flow rates, BSA and ovalbumin started to be effective in decreasing the W/MC interfacial tension. Furthermore, their π changes as a function of interface aging time were more pronounced, particularly compared with RNase. As shown in Fig. 3, it was of interest to notice that there were linear relationships between π and log (interface aging time). It was likely that the dynamic π changes represented the rates at which the adsorbed proteins underwent conformational rearrangements at the interface. The higher π change rate caused by a protein might indicate its greater conformational flexibility/instability at the interface. This speculation was in line with the experimental data. For example, RNase with a low π change rate displayed a high degree of recovery after emulsification (Table III). In contrast, a highest π change rate was obtained with ovalbumin that suffered the greatest damage upon emulsification. BSA, which underwent pronounced changes in the distribution of its water-soluble species, was also found to possess relatively a high π change rate.

It was questioned whether or not the W/MC interfacial area was fully covered when the initial aqueous protein concentration was 0.5 mg/mL. To address this issue, the aqueous BSA concentration was increased from 0.5 to 1, 2, and 3 mg/mL, and its concentration effect upon the interfacial tension was studied. Increases in its concentration led to further enhancement of π, especially when the flow rate was below 0.1 mL/hr (Fig. 4). A steeper concentration gradient helped more BSA molecules diffuse to and spread at the interface, thereby contributing to reducing the free energy of the system to a greater extent. Therefore, it would be probable to conclude that the entire interfacial area was not saturated by BSA molecules at the bulk concentration of 0.5 mg/mL.

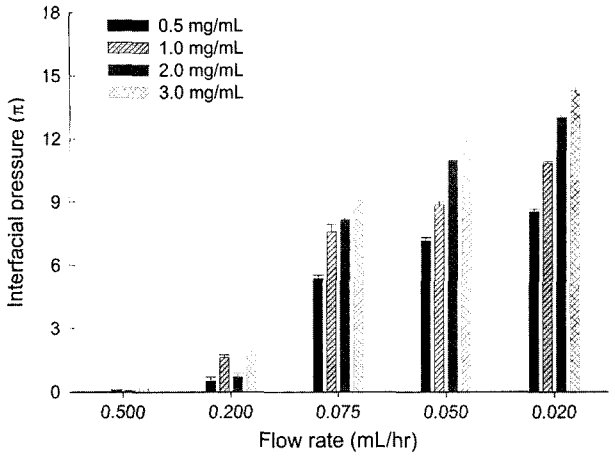

Fig. 4. The effect of BSA concentration upon the W/MC interfacial pressure π. The aqueous BSA concentration was changed from 0.5 to 1, 2, and 3 mg/mL, and π was determined over the flow rates of 0.50 - 0.02 mL/hr.

DISCUSSION

The W/MC interfacial tension was great enough to cause protein unfolding that facilitated a series of interfacial reactions. Although the degree of damage was protein specific, emulsification brought serious problems to all of the proteins tested. The problem included the formation of water insoluble precipitates and/or changes in the compositions of their water-soluble species. Currently, many food emulsions use proteins to stabilize oil-in-water emulsions, based on the fact they form protective films at oil-in-water interfaces (Halling, 1981; Dickinson *et al.*, 1990). Proteins, such as ovalbumin, have also been used to stabilize a water/methylene chloride emulsion during the double emulsion-based microencapsulation process (Blanco-Prieto *et al.*, 1996; Raghuvanshi *et al.*, 1998). It can be inferred from our results that the proteins used might be subject to unfavorable physical and/or chemical changes at water/oil interfaces, losing their structural integrity as well as generating new precipitates. Therefore, their application in the stabilization of emulsions should be preceded with caution, especially when the emulsions are designed for a parenteral dosage form.

Efforts have been put to rationalize the surface activity of proteins with regard to their size, conformational stability, hydrophobicity, and the number of disulfide bonds (Nakai, 1983; Lanne *et al.*, 1987; Owusu and Cowan, 1989; Clarkson *et al.*, 1999). Our data indicate that the size does not seem to be a good index useful in predicting the strength of their surface activity. The magnitude of π changes reported in this study indicates that β-lactoglobulin and RNase with lower molecular weights are more surface active than BSA and ovalbumin with higher molecular weights. Previously, it was shown that a free thiol group and/or disulfide bond participated in the interfacially induced dimerization and oligomerization of BSA (Sah, 1999). It is likely that the W/MC interface triggers similar intermolecular thiol-disulfide exchange reactions to form the aggregates of β-lactoglobulin and ovalbumin. The characterization of the nature of such aggregates deserves further experimentation to explore the molecular aspects of interfacially induced reactions.

Interface aging time-dependent π changes substantiate that under our experimental conditions the W/MC interface does not reach an equilibrium state (Fig. 2 & 4). It reflects that the interface is perturbed continuously by the adsorption of proteins and the conformational realignments of the adsorbed protein molecules. As a consequence, the free energy of the system keeps declining, as the interface ages longer. This interpretation agrees well with the report that an enzyme exposed to a water/organic solvent phase continued to lose its activity, as its incubation time was prolonged (Cassells and Halling, 1990).

Interestingly, a time lag in the evolution of π was observed with BSA and ovalbumin, whereas the opposite was true for β-lactoglobulin and RNase (Fig. 2). The absence of a time lag for the latter proteins might indicate that the interface is occupied rapidly by sufficient amounts of β-lactoglobulin and RNase molecules, enough to decrease the interfacial tension. The subsequent π evolution furthermore suggests that the adsorbed protein molecules unfold to increase their contact area with the interface, contributing to a reduction in the W/MC interfacial tension. However, as shown in Table III, their propensity to spread and reorient at the interface differs from each other. This explains why there is a difference between them with regard to the degree of recovery after emulsification.

In comparison, the existence of an induction period for BSA and ovalbumin indicates that they behaved differently at the interface. Two theories are proposed to explain the presence of a time lag. The first idea is that the proteins in the aqueous phase require some time to migrate and adsorb at the interface. The data in Fig. 4, however, show that an induction period still is present, despite increases in aqueous BSA concentrations from 0.5 to 1, 2, and 3 mg/mL. Therefore, the first idea does not find a firm ground in logic, considering that increases in the bulk protein concentration should have helped more of its molecules diffuse to the interface. Another idea is that a certain time is needed for the adsorbed BSA and ovalbumin molecules to unfold and rearrange their structures at the interface. At the early stage of adsorption the proteins withstand interface-triggered adverse

reactions, but they are eventually subject to interfacial reactions with their exposure to interface increasing. This causes a subsequent increase in π values.

In summary, emulsification affects the structural integrity of proteins and renders them undergo a series of adverse interfacial reactions. The degree of their instability toward emulsification are different from one another. Information on dynamic π changes as a function of interface aging time is likely to be a good indicative of how easily proteins denature and aggregate upon emulsification.

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