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Received for review May 23, 1989. Accepted November 9, 1989.

Effects of Controlled Sulfitolysis of Bovine Serum Albumin on Droplet Size and Surface Area of Emulsions

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Bovine serum albumin (BSA) was modified by oxidative sulfitolysis to cleave 10 and 17 disulfide bonds. The average droplet diameter and surface area of oil in water emulsions stabilized by native and modified BSA were determined with a computerized imaging system at three oil volume fractions (ϕ), namely, 0.22, 0.42, and 0.62. At $\phi = 0.22$, no difference in the emulsifying properties of the proteins was observed. At $\phi = 0.42$ and 0.62, the emulsions stabilized by modified BSA had smaller average diameters and greater surface areas than emulsions stabilized by native BSA. The emulsifying activity of BSA was enhanced by the increased flexibility and by exposure of hydrophobic segments of BSA resulting from the cleavage of disulfide bonds.

Proteins stabilize emulsions by mechanisms that involve reduction of interfacial tension, formation of an interfacial film around the droplets, and repulsion between droplets (Graham and Phillips, 1979; Dickinson and Stainsby, 1982). Different proteins possess a range of emulsifying properties reflecting their dynamic structure and the presence of hydrophobic and hydrophilic regions (Kinsella, 1982; Nakai, 1983). These properties, which vary with different proteins, make some proteins useful as emulsifying agents in formulated and fabricated food systems (Kinsella, 1984). The mechanisms accounting for the emulsifying activity of proteins have been the subject of many studies (Graham and Phillips, 1979; Nakai et al., 1980; Waniska et al., 1981; Shimizu et al., 1986; Kato et al., 1986; Das and Kinsella, 1989), but an understanding of the exact role of proteins in the formation and stabilization of emulsions has not been fully elucidated.

Emulsions are formed by the input of energy to increase the interfacial area and by including a surfactant to stabilize the dispersed droplets. As surfactants, proteins migrate to and adsorb at the interface, orient polar groups to the aqueous phase and nonpolar groups to the apolar phase, and lower the interfacial tension (Davis and Rideal, 1963; Graham and Phillips, 1979). The solubility, hydrophobic/hydrophilic balance, size, and net charge of a protein determine how rapidly it reaches the interface and thereby partially determine the initial emulsion characteristics. Once adsorbed to the interface, the protein may partially unfold, spread, and interact to form a continuous cohesive film (Graham and Phillips, 1979; Kinsella, 1984; Leman and Kinsella, 1989). The degree of unfolding at the interface is determined by the surface area present relative to the amount of protein available and the flexibility of the protein. At high oil volume fraction (ϕ), the protein unfolds as much as possible to cover maximum surface area. If the amount of protein present is relatively large, unfolding will not be as complete (Phillips, 1981). The capacity of a protein to cover maximum interfacial area greatly reflects (among other factors) its conformational flexibility; e.g., the flex-

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ible β-casein readily spreads, whereas the extensively disulfide-linked lysozyme fails to unfold (Graham and Phillips, 1979). Thus, disulfide bonds contribute to the rigidity of proteins and prevent complete protein unfolding at the interface (Phillips, 1981). Therefore, cleavage of disulfide bonds should improve the emulsifying properties of proteins.

The 17 disulfide bonds present in the BSA molecule are important to the stability of BSA (Peters, 1985). We earlier reported that oxidative sulfitolysis of BSA altered its conformation (Kella et al., 1989). The objectives of this study were to compare the emulsifying properties of native BSA and BSA with 10 and 17 of its disulfide bonds cleaved and determine the effects of different oil/water ratios on the emulsifying properties of the modified protein.

EXPERIMENTAL SECTION

Materials. The emulsions used in this study were stabilized by bovine serum albumin (BSA). BSA (Cohn fraction V) and imidazole were obtained from Sigma Chemical Co. (St. Louis, MO). The disulfide bonds of BSA were cleaved with sulfite reagent as described (Kella and Kinsella, 1985). The degree of disulfide bond cleavage, measured by the method of Thannhauser et al. (1984), was 60% (10 disulfide bonds) and 100% (17 disulfide bonds). Native BSA was used for comparison. Emulsions were made with peanut oil that did not contain added emulsifiers (Haque and Kinsella, 1988). The specific gravity of this oil was 0.91.

To accentuate contrast in the microscope, the emulsions were stained, using a staining solution of 0.1% Congo red (a hydrophilic dye) containing 5% gelatin (bovine skin, type III) that was buffered to pH 6.8 with 0.05 M imidazole buffer. The staining solution was maintained as a liquid at 90 °C. The gelatin was obtained from Sigma Chemical Co., and Congo red was obtained from Electron Microscopy Science (Fort Washington, PA). All other chemicals used were reagent grade, and distilled deionized water was used throughout this study.

The imaging system consisted of a light-sensitive diode array, from Micron Technology, Inc. (Boise, ID), mounted atop the right eyepiece of an American Optical AO-150 light microscope. The light microscope was equipped with a 15× eyepiece for examining fine emulsions and a 10× eyepiece for examining coarse emulsions. The diode array is composed of a series of 32 768 pixels, 256 per horizontal line and 128 per vertical line. The combined magnification of the diode array and the light microscope was 5500-fold when the 15× eyepiece was used. The diode array was interfaced to a Turbo-XT computer via an input/output board. Data from the diode array consisted of binary data reflecting the threshold light levels on each of the pixels. The computer converted these data to an image that was a magnification of the emulsion as seen through the eyepiece. This image micrograph was printed out as hard copy for analyses (Figure 1).

The imaging system was calibrated with an American Optical stage micrometer marked in 0.1-μm divisions. The calibration was confirmed by using latex microspheres of a known diameter, 2.04 μm (Epics division of Coulter Corp., Hialeah, FL).

Methods. *Emulsion Preparation.* Solutions of 0.3% bovine serum albumin, both native and modified, in 50 mM imidazole (pH 6.8) were added to peanut oil to give volume fractions of oil (ϕ) of 0.22, 0.42, and 0.62. The emulsions were made by initially dispersing the protein solution and oil with a Janke-Kunkel TP 18-10 turbo blender for 5 s prior to passage through the valve homogenizer as described by Haque and Kinsella (1988). The homogenizing system was kept constant at 25 °C with a circulating water bath. Emulsions were obtained by passing the protein solution and oil mixture through a valve homogenizer for 480 strokes, corresponding to an energy input of 171 × 10^7 J·m^{-3} (Klemaszewski et al., 1989).

The initial droplet size distribution of the emulsions was determined with the imaging system. Aliquots of the emulsion at 25 °C were removed and suitably diluted with the staining solution. The stained emulsion was immediately placed on a microscope slide and spread thinly to obtain a layer of emulsion. A cover slip was placed on the slide, and the stained emulsion sample was then cooled on ice to solidify the warm gelatin and prevent Brownian motion of droplets. Emulsions were then viewed with the imaging system, and the image was printed out for measurement of the droplet diameters.

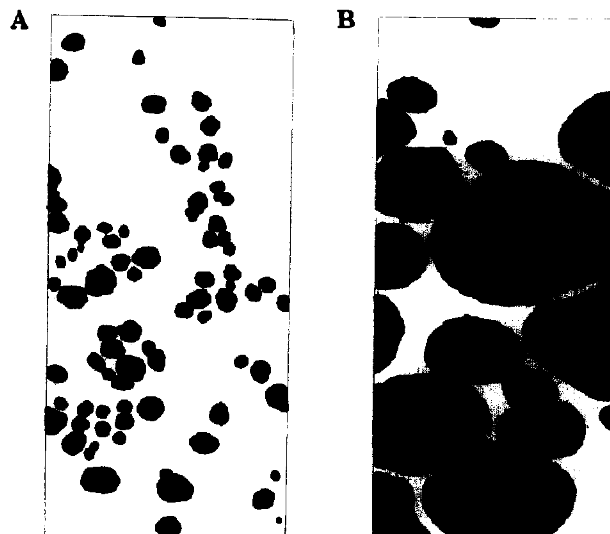

Figure 1. Typical image micrographs of oil in water emulsions stabilized by 0.3% native BSA obtained with a computerized diode array imaging system to magnify light microscope image at oil volume fractions of (A) ϕ = 0.22 and (B) ϕ = 0.62. Original magnification = 2940 in x direction and 4900 in y direction. The micrographs have been reduced to 30% of their original magnification.

Calculations. A histogram of the droplet size distribution for each emulsion was prepared by measuring the diameter of the droplets on an imaging micrograph printout. Approximately 200 randomly selected droplets had to be measured to indicate a statistically significant difference between samples. The number-average droplet diameter (d_m) of the emulsions was determined by using the equation

$$d_m = \sum n_i d_i / \sum n_i \tag{1}$$

The surface area of emulsion droplets was calculated as emulsifying activity index (EAI), defined by Pearce and Kinsella (1978) as surface area ($meter^2$) created during homogenization by unit mass protein. From the data of droplet size distribution the average volume surface diameter (d_{vs}) was calculated from the equation

$$d_{vs} = \sum n_i d_i^3 / \sum n_i d_i^2 \tag{2}$$

d_{vs} is related to area created, $meter^2$ per milliliter of oil (A), by the equation

$$A = 6/d_{vs} \tag{3}$$

when d_{vs} is expressed in microns (μm). Since experiments were conducted at different phase volume ratios of the oil, the varying amounts of oil and protein need to be taken into account when the surface area per gram of protein is calculated. If V_o is the volume of oil (mL) in total volume V_t (mL) of emulsion, then

$$\text{total area of oil} = 6V_o/d_{vs} \quad (m^2) \tag{4}$$

$$\text{total protein in the emulsion} = C(V_t - V_o)/100 \quad (g) \tag{5}$$

where C is the concentration of protein in g/100 mL of aqueous solution. Therefore according to Pearce and Kinsella (1978)

$$\text{EAI} = (6V_o/d_{vs})[100/C(V_t - V_o)] \tag{6}$$

which can be simplified to

$$\text{EAI} = 600\phi/Cd_{vs}(1-\phi) \quad (m^2/\text{g of protein}) \tag{7}$$

where oil volume fraction $\phi = V_o/V_t$. EAI calculated from this

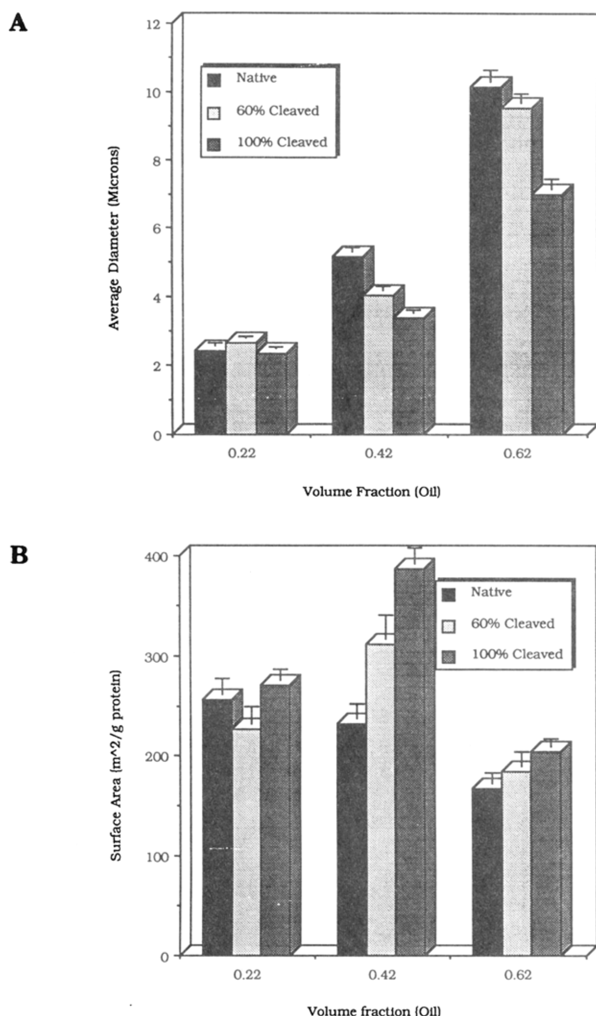

Figure 2. Histograms showing number-average droplet diameter (A) and surface area (B) of emulsions as functions of volume fraction of oil and extent of cleavage of BSA.

equation allows for the different oil/protein ratios in the emulsions of different oil volume fractions.

RESULTS AND DISCUSSION

Representative micrographs of the emulsions from these experiments revealed that these were composed of heterogeneous populations of oil droplets dispersed in water at all oil volume fractions (ϕ) whether stabilized by native or modified BSA solutions (Figure 1). As ϕ was increased, the emulsions became more viscous. At $\phi = 0.62$, the emulsion was extremely viscous and passed through the homogenizer with difficulty.

The micrographs show droplets with smaller diameters (approximately 2.5 μm) in emulsions made at the lowest ϕ, i.e., 0.22 (Figure 1A), and revealed the spherical nature of most droplets. When ϕ was increased to 0.62 (Figure 1B), larger nonspherical droplets were observed. The deformed oil droplets at this high oil volume fraction could be the result of tight packing, the high oil content, or coalescence.

The data for the number-average droplet diameter of emulsions stabilized by native and sulfitolyzed BSA are presented in Figure 2A. Above an oil phase volume ϕ = 0.22, the average droplet diameter of the emulsions stabilized by BSA decreased as the number of cleaved disulfide bonds increased. Disulfide bond cleavage did not significantly affect the average droplet diameter at the low oil volume fraction of 0.22. However, at higher oil volume fractions, the average droplet diameter was significantly smaller for emulsions made from the modified BSA than for native BSA. At the highest oil volume fraction ($\phi = 0.62$), the average diameter of the droplets in the native BSA stabilized emulsion was 10.15 μm, which compares to 9.52 and 7.00 μm for droplets in the emulsions made with BSA with 60% and 100% disulfide bonds cleaved (Figure 2A).

Kella et al. (1989) reported that the conformation of BSA changed considerably on oxidative sulfitolysis. As SO_3H groups were introduced into the protein molecule, they imparted a net negative charge and rendered the modified BSA molecule more flexible and more capable of unfolding. This is consistent with earlier observations that the Stokes radius of BSA increased with disulfide bond cleavage (Habeeb and Borella, 1966). Cleavage of disulfide bonds also increased surface hydrophobicity (Kella et al., 1989).

The surface areas of the respective emulsions, in terms of EAI calculated from droplet size data, are presented in Figure 2B. The EAI values of emulsions made from native and cleaved BSA were similar to each other at $\phi = 0.22$. However, at $\phi = 0.42$ and 0.62, the EAI values of emulsions made with disulfide-cleaved BSA were higher than those prepared with native BSA. The EAI increased as more disulfide bonds were cleaved. At $\phi = 0.22$, because of the relatively low fat/protein ratio, an appreciable amount of available protein remained unadsorbed. Since EAI is usually expressed as area created by the total amount of protein, rather than the amount of adsorbed protein, sulfitolysis of the BSA had no apparent effect on the surface area at $\phi = 0.22$. Also since there was an excess of protein, unfolding of the adsorbed protein may not have occurred at the oil/water interface. As the fat/protein ratio was increased, e.g., $\phi = 0.42$, the amount of unadsorbed protein decreased, and therefore EAI reflected the protein load on the interface. The data indicate that the cleavage of disulfide bonds increased the surface area of the emulsion. This is a reflection of the greater unfolding at the interface because of the loss of structural integrity and enhanced conformational flexibility as disulfide bonds of BSA were progressively cleaved. At $\phi = 0.62$, the trend was similar, though the total interfacial areas were smaller than that at $\phi = 0.42$. This was because of rapid recoalescence of the emulsion droplets at these high fat contents where the droplets were tightly packed.

Therefore, the surface area data suggest considerable unfolding of BSA as the disulfide bonds were cleaved, which is consistent with the analytical data of Kella et al. (1989). Unfolding of BSA could expose interior hydrophobic segments that could align toward the lipid phase, lower the interfacial tension, and form more spread interfacial films.

In conclusion, the capacity of bovine serum albumin to stabilize emulsions was enhanced following sulfitolysis of disulfide bonds. Because there was ample protein present at $\phi = 0.22$, unfolding was unnecessary and sulfitolysis had little apparent effect on the EAI of BSA. At higher volume fractions, the modified BSA formed emulsions with smaller droplets and larger surface areas than did native BSA. The emulsifying ability of BSA was enhanced by the increased flexibility and possibly by exposure of normally hidden hydrophobic side groups of the protein resulting from the cleavage of disulfide bonds.

ACKNOWLEDGMENT

This work was supported in part by the Wisconsin Milk Marketing Board.

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Received for review August 9, 1988. Revised manuscript received November 27, 1989. Accepted December 4, 1989.