Improved Emulsifying Properties of β-Barrel Domain Peptides Obtained by Membrane-Fractionation of a Limited Tryptic Hydrolysate of β-Lactoglobulin

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Fragments of the β-barrel domain of β-lactoglobulin were obtained by membrane fractionation of a limited proteolysate prepared with an immobilized trypsin bioreactor. Analysis of this fraction by size-exclusion chromatography under physiological conditions indicated that the fraction contained a predominant peptide (50%) with a size of 8400 Da and several other peptides with sizes ranging from 2000 to 30 700 Da. Analysis of reductively denatured peptides by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol indicated the presence of a major peptide with a size of 6400 Da, suggesting that a small peptide was linked to the 8400-Da peptide by a disulfide bond. Comparison of the surface and emulsifying properties of the peptide fraction with those of intact β-lactoglobulin indicated that the domain peptides have a lower surface hydrophobicity and a slightly higher surface and interfacial tension. Furthermore, the emulsifying activity index for the domain peptides was twofold larger than that of the intact protein. Examination of the emulsion by scanning electron microscopy revealed that the oil droplets formed with the domain peptides were smaller than those formed with intact protein.

Keywords: *β-Lactoglobulin; tryptic hydrolysate; limited proteolysis; immobilized trypsin; emulsifying properties; β-barrel domain*

INTRODUCTION

Because of their amphipathic nature, proteins adsorb at oil/water interfaces, thereby lowering the surface tension and retarding coalescence of the droplets of the dispersed phase. The emulsifying activity of a protein depends on molecular properties, such as size and amphiphilicity, solubility, structural flexibility, charge distribution, and surface hydrophobicity, and on environmental factors, such as ionic strength, pH, temperature, and the nature of the solvent. Kinetic studies indicate that formation and stabilization of an oil-in-water emulsion are governed by the overall ability of the protein to diffuse to the oil/water interface, adsorb, unfold, rearrange, and spread to form a continuous cohesive film at the interface (Phillips, 1981; Kinsella and Whitehead, 1989). In general, those proteins with a flexible structure display a better emulsifying activity than those with a rigid structure (Phillips, 1981; Shimizu *et al*., 1981; Kato *et al*., 1985).

Because limited proteloysis modifies the emulsifying properties of proteins, the effect of such treatment of whey proteins, particularly β-lactoglobulin (β-Lg), has been examined as a potential means for improvement of this functional property. Jost and Monti (1982) found that an oligopeptide preparation obtained by ultrafiltration of a tryptic hydrolysate of whey had improved emulsifying properties, and the emulsions exhibited more creaming stability. Treatment of whey proteins with trypsin, pepsin, or chymotrypsin yielded hydrolysates with improved foaming and emulsifying properties and higher emulsion stabilities compared with untreated proteins (Lakkis and Villota, 1990). However, detrimental effects of proteolysis of whey proteins on their emulsifying properties have also been reported. For example, Kuehler and Stine (1974) observed that limited proteolysis of whey with prolase, pepsin, or pronase resulted in improved foaming ability but caused a decrease in emulsifying activity and emulsion stability. Moreover, a recent study by Turgeon *et al*. (1991) revealed that treatment of whey protein concentrate with trypsin or chymotrypsin caused a decrease in emulsifying capacity when the total hydrolysate was used; however, after removal of free amino acids and small peptides by ultrafiltration, an oligopeptide fraction was obtained that exhibited improved emulsifying capacity. In a subsequent study (Turgeon *et al*., 1992), a similar result was obtained with a β-Lg hydrolysate; however, fractionation of the hydrolysate yielded an oligopeptide, β-Lg(41−60), with a size of 2300 Da that possessed improved surface activity properties and emulsifying capacity. The reasons for these contradictory results are not clear; however, the characteristics of the substrates and enzymes used and, especially, the extent of hydrolysis may play an important role.

As the major protein component of whey, β-Lg is responsible for much of the functional behavior (Mulvihill and Kinsella, 1987). The three-dimensional structure of the crystal form of β-Lg reveals that the core of the molecule consists of eight strands of anti-parallel β-structure that form a calyx with a short length of α-helix on the surface (Monaco *et al*., 1987). It has been suggested that the core β-barrel domain is the hydrophobic region that binds small hydrophobic molecules, such as fatty acids and retinol (Wishnia and Pinder, 1966; Monaco *et al*., 1987; Chen *et al*., 1993; Cho *et al*., 1994). In previous studies, we have shown that major fragments of the β-barrel domain can be produced by limited tryptic hydrolysis (Chen *et al*., 1993; Huang, *et al*., 1994). Characterization of the 7.9 and 8.4 kDa oligopeptides revealed that much of the original β-barrel structure was retained, although the structural stability

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was lower than that of the intact protein. Thus, these domain fragments may have unique structures with increased flexibility.

In this study, β-Lg was subjected to limited proteolysis with an immobilized trypsin bioreactor under conditions that optimize production of these domain fragments, and these fragments were subsequently isolated by membrane-fractionation technology. The emulsifying properties of these oligopeptides were compared with those of intact β-Lg and egg white protein.

MATERIALS AND METHODS

Chemicals. All chemicals were of reagent grade, and distilled-deionized water was used throughout.

Preparation of Celite Derivatives. Celite beads (R648, 30/50 mesh, Celite Corp., Lompoc, CA) were silanized by the method of Janolino and Swaisgood (1982). Surface amino groups were assayed quantitatively by the *o*-phthalaldehyde (OPA) method of Janolino and Swaisgood (1992). Aminopropyl Celite beads were subsequently succinylated by the nonaqueous method of DuVal *et al*. (1984). The completion of the reaction was determined by quantifying the disappearance of amino groups by the OPA method with aminopropyl beads as a reference.

Immobilization of Trypsin. Trypsin (type XIII, TPCK treated, from bovine pancrease, Sigma) was immobilized on succinamidopropyl Celite (SAPC) beads at 4 °C by the method of Taylor (1979). Deaerated SAPC beads (100 mL) were activated for 30 min with 500 mL of 0.02 mM 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) at pH 7.0. Then, 500 mL of trypsin solution (4 mg/mL in distilled-deionized water containing 0.04 M $CaCl_2$, pH 7.0) was added to the mixture and allowed to react for 24 h. After reaction, the immobilized enzyme was washed with 2 M urea in phosphate buffer, pH 7.5, containing 0.02% NaN_3 to remove noncovalently bound enzyme. Finally, the immobilized enzyme was washed with the phosphate buffer and stored at 4 °C.

The activity of the immobilized trypsin was determined according to Taylor and Swaisgood (1980) with *p*-tosyl-L-arginine methyl ester (TAME) as the substrate. The estimated activity was 24.6 μmol min^{-1} mL^{-1} of beads.

Preparation of β-Lactoglobulin. Bovine β-Lg was purified from milk according to the method of Fox *et al*. (1967). Fifty-seven liters of skim milk, obtained from the North Carolina State University Dairy Plant, was adjusted to pH 4.6 with 4 M HCl, and 115 g of $CaCl_2$ were added. After allowing the milk to stand for 6 h at 15 °C, the casein precipitate was removed by filtration through four layers of cheesecloth, and protein in the resulting whey was concentrated fivefold with a Millipore 10-kDa retention membrane. Trichloroacetic acid (TCA; 3%, w/v) was added to the concentrated whey solution to precipitate all proteins except β-Lg. The resulting precipitate was removed by centrifugation at 10000*g* for 20 min at 4 °C. The supernatant solution was adjusted to pH 2−3 with 1 M NaOH, concentrated, and dialyzed against distilled-deionized water, with the Millipore Concentrator/Dialyzer system, until the pH reached 6−7. The concentrated solution was lyophilized, and the protein powder was stored at −22 °C. The purity of the preparation was estimated to be >96% as determined by size-exclusion chromatography.

Preparation of the Oligopeptide Fraction. The bioprocess combining the immobilized trypsin bioreactor with membrane fractionation of the products is illustrated in Figure 1. Four hundred milliliters of β-Lg solution (5 mg/mL in 10 mM ammonium acetate buffer, pH 7.6) were recirculated through a fluidized-bed bioreactor containing 100 mL of immobilized trypsin for 60 min at 24 °C. The resulting limited hydrolysate was first fractionated with a 30-kDa retention membrane (YM30, Amicon, Inc., Beverly, MA), and the resulting permeate was further fractionated with a 3-kDa retention membrane (YM3, Amicon); both processes were at 4 °C. The retentate from the YM3 membrane, representing the domain fragment oligopeptides, was lyophilized, and the powder was stored at −22 °C.

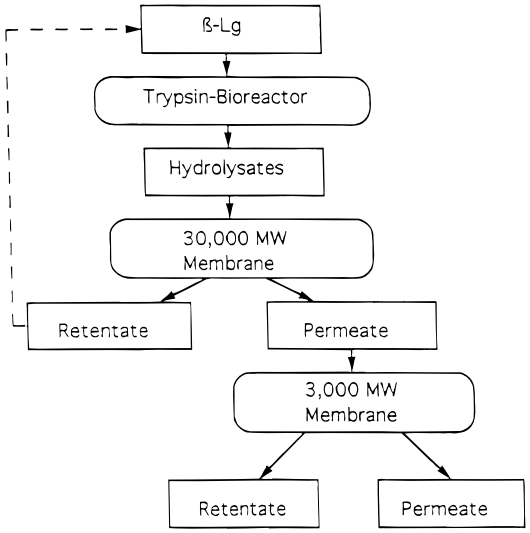

Figure 1. Schematic illustration of an immobilized enzyme-membrane fractionation bioprocess for preparation of the domain fraction of β-Lg.

Determination of the Degree of Hydrolysis. The degree of hydrolysis was determined by measuring the increase in concentration of amino groups resulting from proteolysis. Lyophilized powder from 50 mL of the hydrolysate and lyophilized native β-Lg as a reference were dissolved in 40 mM imidazole buffer, pH 7.5, and the fluorescence intensities were measured after the OPA reaction. The fluorescence of a 0.1-μg/mL solution of quinine sulfate was used as a reference to calculate the relative fluorescence intensity (RFI). The calculated RFI was converted to amino group concentration with a specific RFI value of 2.71×10^8/mol of lysyl residues in β-Lg (Goodno *et al*., 1981). The degree of hydrolysis was calculated from the increase in amino group concentration by the relationship

$$DH(\%) = N_t/N_0 \times 100 \quad (1)$$

where N_t is the increase in concentration of amino groups due to proteolysis at time t and N_0 is the total amino group concentration from all residues in the protein if complete hydrolysis occurred (both values are expressed as mol NH_2/g of protein).

Determination of Protein Concentration. The concentration of soluble protein was assayed by the dye-binding method of Bradford (1976) with bovine serum albumin (BSA, Sigma) as a standard.

Fast-Protein Liquid Chromatography (FPLC) Size-Exclusion Chromatography. Molecular weights of the oligopeptides in the hydrolysate and in the isolated fractions were estimated by size-exclusion chromatography with a Superose TM HR 10/30 column. The FPLC system used consisted of a model 510 pump, a model UK6 injector, and a model 990 photodiode array detector (Waters, Milford, MA). The detector was equipped with an APC IV series computer (NEC Information System, Inc., Foxborough, MA) for data acquisition and spectral analysis. The elution buffer was 20 mM Tris-HCl, pH 7.0, containing 150 mM NaCl and 0.02% NaN_3. BSA (66 000 Da), β-Lg (dimer, 30 000 Da), α-lactalbumin (14 200 Da), and vitamin B_{12} (1355 Da) were used as the molecular weight standards.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). The domain oligopeptide fraction was analyzed by SDS-PAGE using an LKB 2050 Midget Electrophoresis unit (LKB Produkter AB, Bromma, Sweden) with a gradient gel of 16−27% acrylamide (Huang *et al*., 1994). The protein or peptide bands were visualized by staining with Coomassie brilliant blue R250.

Measurement of Surface Hydrophobicity. Surface hydrophobicity was determined according to the method of Kato and Nakai (1980) with 1-anilino-8-naphthalenesulfonate

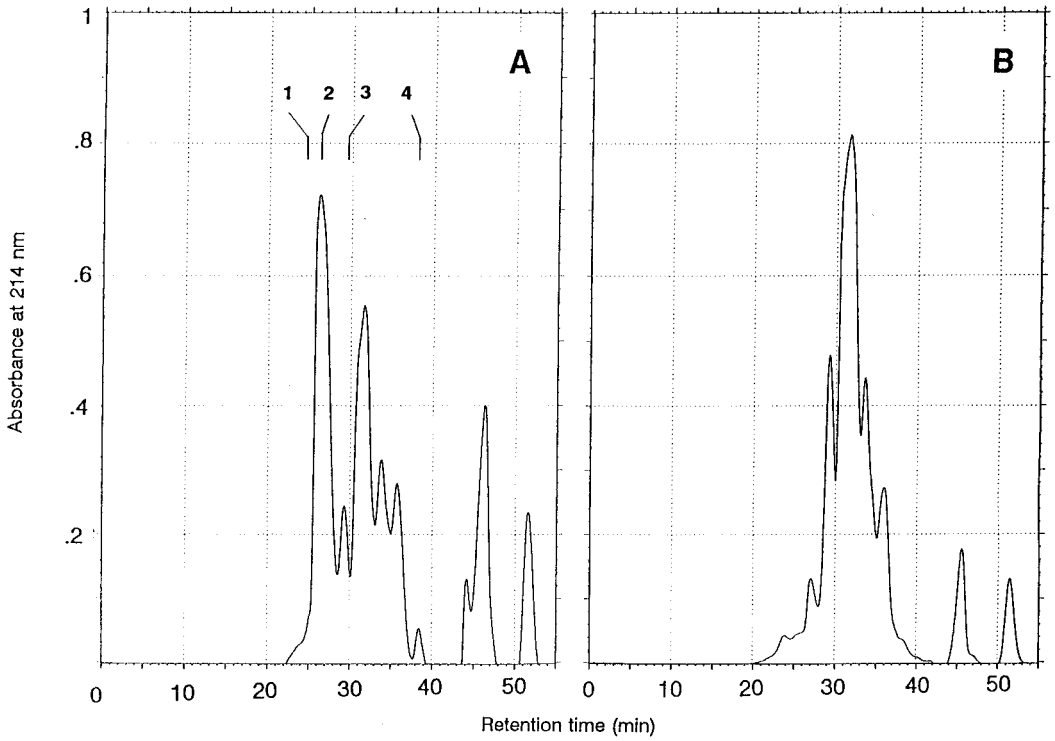

Figure 2. Typical elution profiles of limited tryptic hydrolysates of (A) β-Lg and (B) 3-kDa membrane retentate (domain fraction) obtained from size-exclusion chromatography (Superose TM HR 10/30). The elution buffer was 20 mM Tris-HCl, pH 7.0, containing 15 mM NaCl and 0.02% NaN_3. Molecular weight markers were (1) bovine serum albumin (MW = 66 000), (2) β-Lg (dimer, MW = 36 700), (3) α-lactalbumin (MW = 14 200), and (4) vitamin B_{12} (MW = 1355). The markers were chromatographed individually.

(ANS, Sigma). Each protein sample (2 mL) was serially diluted with 10 mM phosphate buffer, pH 7.0, to give protein concentrations ranging from 0.001 to 0.03% (w/v). Ten microliters of ANS solution (8.0 mM in phosphate buffer) were added to 2 mL of the diluted protein solutions, and the fluorescence intensities (FI) were measured with a spectrofluorometer (Optical Technology Devices, Inc., Elmford, NY) at excitation and emission wavelengths of 390 and 470 nm, respectively. The 30% FI value of ANS in methanol was used as a reference value for calculating the RFI of the protein-ANS solutions. The RFI for the protein-ANS interaction was obtained by subtraction of the RFI of a control protein solution without ANS. Slopes of plots of RFI versus protein concentration, designated S_0, were calculated by linear regression.

Measurement of Surface and Interfacial Tension. Surface tension of 0.5% (w/v) protein solutions in 10 mM phosphate buffer, pH 7.0, was measured at 24 °C with an interfacial tensiometer (CSC NOS70545, CSC Scientific Company, Inc., Fairfax, VA) equipped with a 55 × 28-mm glass plate. The interfacial tension between the protein solution and peanut oil was also measured under similar conditions. The tensiometer was calibrated, and the plate was cleaned according to instructions provided by the manufacturer.

Determination of the Emulsifying Activity Index. The emulsifying activity index (EAI) was determined according to the turbidimetric method of Pearce and Kinsella (1978). Pure peanut oil (0.5 mL, Sigma) was dispersed in 1.5 mL of protein solution (0.5% w/v in 10 mM phosphate buffer ranging in pH from 3.0 to 9.0) in a 13 × 100-mm culture tube with a Tissumizer (Tekmar Company, Cincinnati, OH) operated at 12 000 rpm for 1 min. Aliquots (5 mL) of the emulsions were immediately diluted 1000-fold with 0.1% SDS solution in 10 mM phosphate buffer, pH 7.5, and the turbidity was measured at 500 nm. The EAI was calculated with the relationship

$$\mathrm{EAI} = 2\tau/\phi C \qquad (2)$$

where turbidity $\tau = 2.303\ A_{500}/l$, l is the cuvette pathlength, ϕ is the volume fraction of the oil phase, and C is the protein concentration.

Determination of Emulsion Stability. Stability of the protein emulsions was evaluated according to the method of Britten and Giroux (1991) with some modifications. Emulsions prepared as just described were incubated and agitated in a temperature-controlled shaker (37 °C, 150 rpm) to facilitate and increase collisions between emulsion droplets, thus accelerating coalescence. Aliquotes (5 μL) of the emulsion were withdrawn from the bottom layer at various times and diluted 500 to 1000-fold with 0.1% SDS solution, and the turbidity was determined at 500 nm as described for EAI measurements. Because coalescence results in a decrease in turbidity with time, τ_0/τ_t was plotted as a function of time, where τ_0 is initial the turbidity and τ_t is the turbidity at time t. The slope of this linear plot obtained by least squares linear regression was used as an index of the stability.

Scanning Electron Microscopy (SEM). Samples for SEM were prepared as described by Liboff *et al.* (1988). Freshly prepared emulsions made with β-Lg or the domain oligopeptide fraction (0.5% w/v) were gently mixed (1:1) with a warm 1% agarose solution. After gentle stirring with a wooden stick, the dispersion was allowed to gel at 24 °C. The resulting gels were cut into cubes (≅1 mm^3) and fixed with 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, overnight. The fixed gels were dehydrated with a graded series of methanol solutions, critical point dried, mounted on aluminum stubs, coated with gold/palladium, and photographed at various magnifications in a ETEC autoscan microscope (SEM 505 T, Phillips, Holland). Duplicate samples were analyzed with the selection of at least five different fields for photography.

RESULTS

Preparation of β-Barrel Domain Fragments. β-Lg was subjected to limited proteolysis with the immobilized trypsin bioreactor at 24 °C for 60 min. A typical elution profile from FPLC size-exclusion chromatography of the limited hydrolysate is shown in Figure 2A. Analyses of the data from chromatography

Table 1. Molecular Size Distribution of the 3-kDa Membrane-Fractionated Hydrolysate of β-Lactoglobulin As Determined by Size-Exclusion HPLC[a]

	peak area (%)	
molecular weight (Da)	retentate	permeate
30 700	4	0
16 400	12	0
8 400	50	0
4 700	14	16 (MW > 3000)
<3 000	20	84

[a] Mean of duplicate measurements; the elution profile was monitored at 214 nm, and the percentage of each peak represents the ratio based on the total area of all peaks.

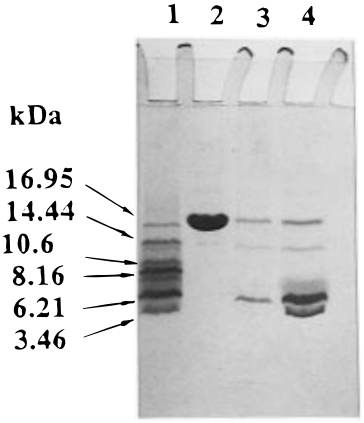

Figure 3. SDS-PAGE of β-Lg and the domain fraction in the presence of 2-mercaptoethanol. Lane 1, protein markers; lane 2, native β-Lg (5 μg); and lanes 3 and 4, the domain fraction (4 and 12 μg, respectively).

and OPA assays indicated that ~55% of the original β-Lg was hydrolyzed with a degree of hydrolysis (DH) of 2.4%. The remaining 45% of intact protein was retained by the 30-kDa membrane and could be recycled through the bioreactor. Membrane fractionation of the hydrolysate, as outlined in Figure 1, yielded the domain fragments in the 3-kDa membrane retentate. Estimation of the molecular size by size-exclusion chromatography under physiological conditions indicated peptides ranging from <3 to 30.7 kDa (Figure 2B and Table 1). The predominant domain fragment was the 8.4 kDa oligopeptide (50%), whereas the permeate contained small peptides and amino acids (Table 1). Under physiological conditions, β-Lg exists as a dimer; therefore, the 30.7 kDa fragment may represent the dimer with a small peptide released or it could result from association of oligopeptides. The small peptides in the retentate, <3 kDa, could be reduced in concentration by diafiltration with buffer.

Further analysis of the retentate fraction by SDS-PAGE in the presence of 2-mercaptoethanol revealed a major band with a size of 6.4 kDa (Figure 3). The difference in size as determined by size-exclusion under physiological conditions and by SDS-PAGE after disulfide reduction suggests that a small peptide is attached to the 6.4 kDa fragment via a disulfide bond. Analysis of the *N*-terminal amino acids of the purified 8.4 kDa fragment indicated two cleavage sites; *viz*., Arg_{40} and Arg_{148}, suggesting that peptide β-Lg(f149−162) is attached to the 6.4 kDa fragment via the disulfide bond Cys_{66}−Cys_{160}. A similar peptide produced by limited proteolysis was previously characterized (Chen *et al*., 1993; Huang *et al*., 1994). This fragment yielded an estimated 6.8 kDa peptide upon reduction and *C*-terminal amino acid, and mass spectrometric analyses

Table 2. Surface Properties of Native β-Lactoglobulin and the Domain Fraction

solution	hydrophobicity (S_0)[a]	surface tension[b]	interfacial tension[c]
β-lactoglobulin	5.24 ± 1.48	51.7 ± 0.4	13.7 ± 0.3
domain fraction	0.16 ± 0.02	56.7 ± 0.2	15.8 ± 0.4
phosphate buffer (10 mM; pH 7.0)	—	76.6 ± 1.2	20.9 ± 0.3

[a] Surface hydrophobicity was determined by the ANS probe method at protein concentrations ranging from 0.001 to 0.03% (w/v) in 10 mM phosphate buffer, pH 7.0; mean ± standard deviation was calculated from triplicate measurements. [b] Surface tension (dynes/cm) was determined with a surface tensometer at a protein concentration of 0.5% (w/v) in 10 mM phosphate buffer, pH 70; mean ± standard deviation was calculated from triplicate measurements. [c] Interfacial tension (dynes/cm) was determined at the interface between peanut oil and protein solution (0.5%, w/v, in 10 mM phosphate buffer, pH 7.0); mean ± standard deviation was calculated from triplicate measurements.

indicated that the oligopeptide was β-Lg(f41−100 + 149−162) with a molecular weight of 8.6 kDa (Chen *et al*., 1993). Characterization of the oligopeptide suggested that native secondary and tertiary structure, which would correspond to a five-stranded anti-parallel β-barrel fragment (Chen *et al*., 1993), was retained. Hence, the 3-kDa membrane retentate fraction will be referred to as the domain fraction in the remaining discussion.

Comparison of the Surface Properties of the Domain Fraction with Those of Proteins. Some of the surface properties of the domain fraction are compared with those of intact β-Lg in Table 2. The surface hydrophobicity, as measured by binding of ANS, was 33-fold lower for the domain fraction compared with that of the intact protein. The value obtained for the intact protein is in agreement with the value (7.0) reported by Shimizu *et al*. (1985), which was obtained under similar conditions, but lower than the values of 18 and 30 found by Hayakawa and Nakai (1985) and Tsutsui *et al*. (1986), respectively.

Values for the surface and interfacial tensions of solutions of the domain fraction are also compared with those of β-Lg in Table 2. Both solutes lowered the surface activity by nearly the same amount on a weight concentration basis; however, there appears to be an inverse relationship between surface hydrophobicity and the lowering of surface and interfacial tensions. A similar conclusion was reached by Keshavarz and Nakai (1979) from measurements of interfacial tensions and surface hydrophobicities of various proteins.

Emulsifying Activities of Proteins Compared with That of the Domain Fraction. The EAI of the domain fraction is compared with the activities of intact β-Lg and egg white protein as a function of pH in Figure 4. Both the domain fraction and intact β-Lg exhibited increasing EAI values with increasing pH; however, values for egg white protein decreased with increasing pH. The emulsifying activity of the domain fraction was greater than those of both intact proteins throughout the entire pH range. The values obtained for the intact protein as a function of pH are in agreement with those reported by Shimizu *et al*. (1985) and by Phillips *et al*. (1994). However, the value at pH 7.5 obtained here (30 m^2/g) is substantially lower than that obtained by Pearce and Kinsella (1978) under similar conditions (153 m^2/g) except for the type of blender used.

A turbidometric method was used to measure the stability of the emulsions. The change in the relative turbidity (τ_0/τ_t) is given as a function of time in Figure

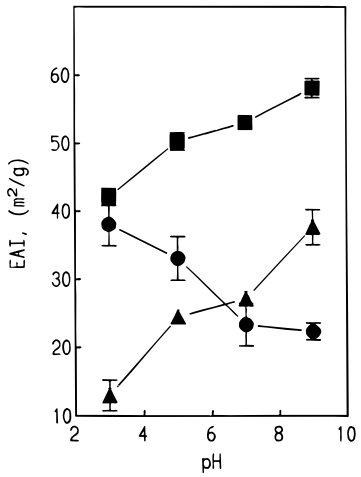

Figure 4. Effect of pH on the emulsifying activity of egg white protein (●), β-Lg (▲), and the domain fraction (■). Peanut oil (0.5 mL) was dispersed in 1.5 mL of protein solution (0.5%, w/v) with a tissumizer. The emulsion was diluted 1000-fold with 0.1% SDS solution, and the turbidity was measured at 500 nm. Each data point represents the mean of three independent measurements.

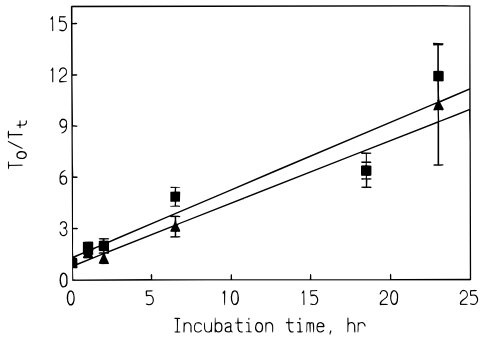

Figure 5. Changes in the turbidity ratio (τ_0/τ_t) of β-Lg emulsions (▲) and domain fraction emulsions (■) as a function of time at 37 °C with agitation at 150 rpm. The least-squares linear regression lines for β-Lg and domain fraction emulsions are $\tau_0/\tau_t = 0.37t + 0.81$ ($r = 0.98$, $n = 6$) and $\tau_0/\tau_t = 0.39t + 1.31$ ($r = 0.95$, $n = 6$), respectively.

5. A linear relationship was observed, and the slope was used as a measure of the stability. The slopes for the intact β-Lg and the domain fraction are 0.37 and 0.39, respectively, suggesting that these emulsions had similar stabilities. Visually, however, the two emulsions were distinct, with that formed from intact protein showing clear phase separation and that formed from the domain fraction showing no separation, perhaps because many more oil droplets were emulsified in the presence of the domain fraction.

The microstructure of the emulsions formed was examined by SEM, and photographs of the emulsions fixed in agarose gels are shown in Figure 6. Comparison at two levels of magnification indicates that more numerous and smaller droplets were formed with the domain fraction (compare Figure 6A,B to Figure 6C,D). To confirm these observations, the dried agarose gel was re-opened and coated with gold/palladium, and the freshly opened surface was examined by SEM. The β-Lg emulsions again displayed larger and fewer droplets. We also consistently observed more droplets appearing to adhere to the surface of the agarose gel with the domain emulsions. Perhaps a more hydrophilic surface with the domain emulsified droplet interacted more strongly with the hydrophilic agarose and thus was not as easily removed by washing.

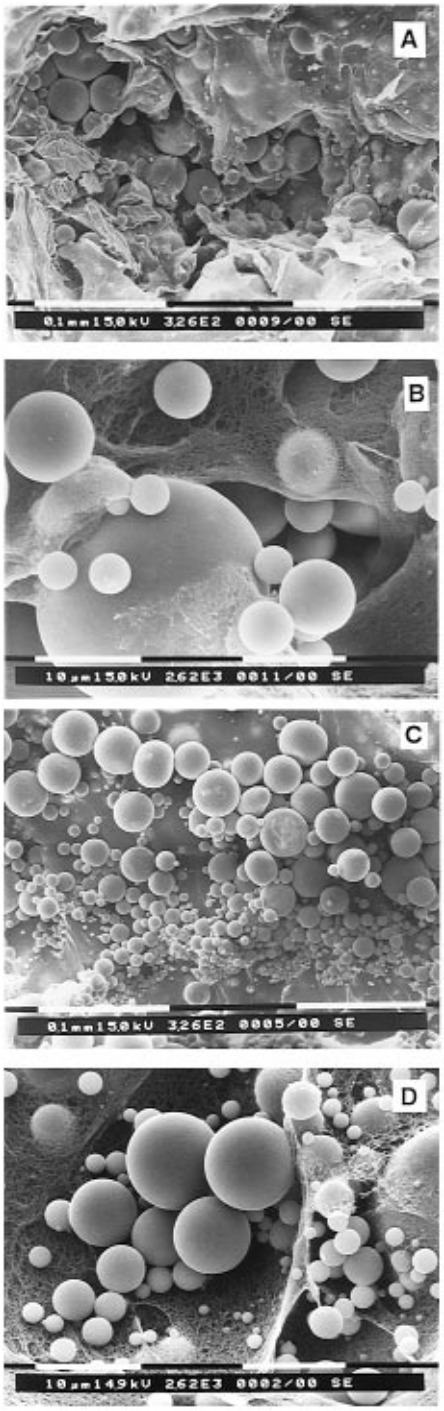

Figure 6. Scanning electron micrographs of oil/protein emulsions fixed in agarose. Peanut oil/β-Lg emulsion at magnifications of (A) ×326 and (B) ×2620 and oil/domain fraction emulsion at magnifications of (C) ×326 and (D) ×2620 are shown.

Results of the SEM analysis are consistent with the emulsifying activity and emulsion stability data. More numerous droplets are indicative of greater emulsifying activity and smaller droplets suggest greater stability (St. Angelo, 1990). A more hydrophilic surface and electrostatic repulsion between droplets would prevent coalescence (Petrowski, 1976; Kinsella and Whitehead, 1989).

DISCUSSION

Factors affecting the interfacial and emulsifying activities of proteins and peptides include the distribu-

tion of polar and nonpolar residues, flexibility of the structure, the net charge, and the molecular size (Jost and Monti, 1982; Lee *et al*., 1987; Chaplin and Andrews, 1989; Turgeon *et al*., 1991, 1992). For example, it was suggested that a minimum size of 2000 Da is essential for good emulsifying activity. The 8.6 kDa fragment of the core domain of β-Lg [β-Lg(f41−100 + 149−162)], which represents 50% of domain fraction characterized in this study, appears to possess many of these desirable properties. Examination of the primary structure (Swaisgood, 1982) indicates a charge density at pH 6.6 of 0.39 for the oligopeptide compared with 0.30 for the intact protein; thus, an increase in hydrophilicity and excellent solubility for the domain fraction would be expected. Both net charge and charge density may contribute to formation and stabilization of an emulsion. In agreement with previous observations for β-Lg (Jost and Monti, 1982; Shimizu *et al*., 1985; Das and Kinsella, 1989), we observed increasing emulsifying activity with increasing pH. Furthermore, calculation of the mean residue free energy of transfer from a hydrophobic to a hydrophilic phase using the consensus values of Eisenberg *et al*. (1982) for each amino acid indicates an increase in hydrophilicity for the domain peptide (−162 cal/mol residue for the domain fragment versus −120 cal/mol residue for the intact protein).

The primary structure of β-Lg(f41−100 + 149−162) is characterized by a periodic distribution of polar and hydrophobic residues (Swaisgood, 1982) that apparently is an important factor for emulsifying activity (Turgeon *et al*., 1992). The latter investigators observed good emulsifying properties with β-Lg(f41−60), which possesses this periodic distribution with clusters of hydrophobic residues. However, β-Lg(f61−70 + 149−162), which does not contain hydrophobic clusters, did not have good interfacial properties. The importance of surface hydrophobicity for good emulsifying properties of oligopeptides and proteins has been established (Keshavarz and Nakai, 1979; Kato and Nakai, 1980; Voutsinas *et al*., 1983; Saito, 1994). However, a balance of polar and hydrophobic residues is very important because a protein with good emulsifying activity must also have good solubility (Hayakawa and Nakai, 1985).

A positive relationship between surface hydrophobicity and emulsifying activity is not always observed (Shimizu *et al*., 1981, 1985). For the case of β-Lg, these authors measured greater surface hydrophobicity at pH 3; however, the emulsifying activity was much greater at pH 7. It was suggested that this observation was caused by greater structural flexibility at the higher pH as is known to be the case for this protein. Hence, the higher emulsifying activity of the domain fraction may be caused by increased flexibility, whereas its apparent surface hydrophobicity is much lower than that of the intact protein. A lower surface hydrophobicity for β-Lg(f41−100 + 149−162) may result from loss of a hydrophobic binding site between the α-helix, which is lost upon limited proteolysis (Chen *et al*., 1993), and the β-barrel domain.

The existence of structure and, perhaps more importantly, the flexibility of that structure is a key characteristic of a protein or oligopeptide with good emulsifying properties. It has long been recognized that continued hydrolysis of oligopeptides causes loss of emulsifying activity and especially emulsion stability (Kuehler and Stine, 1974; Puski, 1975; Lakkis and Villota, 1990; Turgeon *et al*., 1992). Comparison of the emulsifying properties of native and unfolded forms of BSA, formed by disulfide reduction or treatment with 8 M urea, indicated that disruption of secondary and tertiary structure eliminated emulsifying activity (Waniska *et al*., 1981). However, flexibility of the structure appears to be extremely important (Morr, 1979; Phillips, 1981; Shimizu *et al*., 1981; Kato *et al*., 1985; Shimizu *et al*., 1985; Das and Kinsella, 1989).

We have previously shown that β-Lg(f41−100 + 149−162) exhibits native-like secondary and tertiary structure corresponding to the β-barrel domain of the intact protein and that this structure, although less stable than the native protein, can be reversibly unfolded (Chen *et al*., 1993). Hence, this domain fragment, because of its solubility, amphipolarity, secondary and tertiary structure, and structural flexibility, should have excellent surface activity and interfacial stabilization characteristics.

Thus, ideal emulsifying properties of a protein or oligopeptide result from a delicate balance of charge density and net charge, distribution of polar and hydrophobic residues, structure, and structural flexibility. Charge density, net charge, and polar residues confer excellent solubility allowing rapid diffusion to the interface without excessive electrostatic repulsion, but with sufficient net charge to generate a surface repulsion that minimizes coalescence. Periodic clusters of polar and hydrophobic residues confer attraction to the interface and the capability of conformational orientation with hydrophobic clusters in the oil phase and polar clusters in the aqueous phase. A stable molecular structure buries the hydrophobic residues and prevents molecular aggregation prior to generation of an interface, whereas structural flexibility (*i.e*., a limited magnitude of structural stability) allows the molecule to rapidly undergo conformational changes to orient on the surface. Remaining structure in the aqueous phase allows interaction between molecules on the surface, thus confering stability to the emulsion.

Limited proteolysis of β-Lg with trypsin yields oligopeptides with improved emulsifying properties; however, the extent of hydrolysis is extremely important. Extensive proteolysis leads to complete loss of functionality (Saito, 1994; Turgeon *et al*., 1992). Our results suggest that the emulsifying properties of β-Lg(f41−100 + 149−162) are superior to those of β-Lg(f41−60) described by Turgeon *et al*. (1992). Use of immobilized trypsin allows precise control of the extent of hydrolysis. Furthermore, a downstream inactivation step (*e.g*., heat treatment), which would cause destruction of the desirable structure of the oligopeptide, is not required.

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