Effect of Transglutaminase-Catalyzed Polymerization of β-Casein on Its Emulsifying Properties

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β-Casein was polymerized to various extents by the transglutaminase-catalyzed cross-linking reaction. Under the reaction conditions used, dimer, trimer, and tetramer of β-casein were produced as predominant species at short reaction times (20−90 min) and large polymers at longer reaction times (3−24 h). Examination of the emulsifying properties of these cross-linked β-casein polymers showed that although the emulsifying activity index decreased, the storage stability of the emulsions increased with increasing degree of polymerization. Experiments with mixtures of native and polymerized β-casein also showed that increasing the fraction of polymerized β-casein in the mixture increased the emulsion stability. This enhancement of emulsion stability might be due either to an enhancement in steric stabilization, attributable to the branched nature of the trasgutaminase-catalyzed polymers, or to stronger cohesive interactions between polymerized β-casein molecules than between monomeric β-casein molecules in the film.

Keywords: *β-Casein; polymerization; transglutaminase; protein cross-linking; emulsion stability*

INTRODUCTION

The stability of a protein-stabilized oil-in-water emulsion is fundamentally related to the mechanical/viscoelastic and steric properties of the adsorbed protein film. Generally, phase separation in emulsions is initiated by flocculation/aggregation of the emulsion droplets via interfilm interactions. The rate and extent of flocculation/aggregation are dependent on the relative magnitude of attractive and repulsive forces between the particles. Once a floc or an aggregate is formed, the rate of coalescence and eventual phase separation is dependent on the mechanical/viscoelastic properties of the adsorbed protein film. These properties are dependent on cohesive interactions between protein molecules in the adsorbed film (Damodaran, 1997). Thus, the stability of a protein-stabilized emulsion can be improved either by increasing the repulsive interactions between the particles or by increasing the cohesive interactions between protein molecules within the film.

In the case of flocculation/aggregation of emulsion particles, the attractive interactions emanate from van der Waals and hydrophobic interactions and the repulsive interactions arise from electrostatic and steric repulsion between the oil droplets and the adsorbed protein layers (Damodaran, 1997). Above 0.1 M ionic strength, where electrostatic repulsion between emulsion particles is negligible, owing to electrostatic screening by counterions, steric repulsion between the adsorbed protein layers becomes the most important force opposing flocculation of emulsion particles. The steric repulsion arises because of overlapping between the protruding protein chains of the adsorbed protein films as they approach each other. The magnitude of this steric repulsion is proportional to the thickness of the layer of protruding chains and the number of polymer chains adsorbed per unit area of the emulsion particle surface (Damodaran, 1997). It follows then that, all other things being constant, an increase in the effective thickness of the layer of protruding chains of the adsorbed protein would greatly improve the stability of an emulsion.

The thickness of the layer of protruding chains of an adsorbed protein is dependent on the conformational characteristics of the protein. Generally, for a given protein under a given set of solution conditions (e.g., pH and ionic strength), the thickness of the layer of the protruding chains cannot be easily manipulated unless its conformation is altered prior to adsorption. In this respect, it would be interesting to evaluate the emulsifying properties, especially emulsion stability, of transglutaminase-catalyzed polymers of proteins. Transglutaminase (EC 2.3.2.13) catalyzes an acyl transfer reaction between the γ-carboxamide group of glutamine residues and the ε-amino group of lysine residues of peptide chains (Folk, 1983). Because the cross-linking occurs at the side chains of these residues, the transglutaminase reaction produces branched polymers, rather than linear polymers. It is easy to visualize that, because of steric constraints created by branching, when such a polymer adsorbs to an interface, a majority of the peptide segments of the branched polymer may not physically make direct contact with the interface. As a result, much of the branched polypeptide chain may form a thick layer protruding from the surface of the oil droplet.

To elucidate if emulsifying properties of a protein can be improved by polymerization of the protein using transglutaminase, we studied the emulsifying properties of native and polymerized β-casein. In this paper we show that transglutaminase-catalyzed polymerization of β-casein significantly improves the stability of a β-casein-stabilized emulsion.

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MATERIALS AND METHODS

β-Casein, CBZ-L-glutaminylglycine, and hydroxylamine were purchased from Sigma Chemical Co. (St. Louis, MO). Transglutaminase from a new *Streptomyces* species was produced in our laboratory. The enzyme preparation was at least 70% pure as judged by SDS−PAGE. The enzyme preparation did not contain proteolytic activity as judged from no loss of transglutaminase activity over an extended period of storage at 4 °C. The specific activity of the enzyme preparation was 1.37 units/mg. Canola oil, obtained from a local store, was used in oil-in-water emulsions. All other chemicals used in this study were of analytical grade.

Assay of Transglutaminase Activity. Transglutaminase activity was measured using the colorimetric hydroxamate assay method described by Folk and Cole (1965). The final reaction mixture contained 0.1 M Tris−acetate buffer, pH 6.0, 30 mM CBZ-L-glutaminylglycine, 0.1 M hydroxylamine, 10 mM glutathione, and 5 mM $CaCl_2$. After 10 min of incubation with enzyme solution at 37 °C, ferric chloride/trichloroacetic acid reagent (0.7% w/v final concentration) was added to stop the reaction. The precipitate was removed by centrifugation, and the resulting red color was measured at 525 nm. L-Glutamic acid/γ-monohydroxamic acid was used as a standard for calibration. One unit of transglutaminase activity was defined as the amount of enzyme needed to produce one micromole of hydroxamic acid per minute.

***β*-Casein Polymerization by Transglutaminase.** Transglutaminase-catalyzed polymerization of β-casein was carried out as described previously (Han and Damodaran, 1996). A typical reaction system contained 1% β-casein and an enzyme level of 4.25 units/g of protein substrate in 0.1 M Tris-HCl buffer, pH 7.5. Because the microbial transglutaminase used in this study did not require a reductant, such as glutathione or dithiothreitol (DTT), for its activity, no reductant was used in the reaction buffer. The reaction mixture was incubated for various time intervals at 37 °C, and the reaction was terminated by heating the solution at 85 °C for 5 min.

SDS−PAGE. SDS−PAGE was performed using a discontinuous buffer system as described by Laemmli (1970) on a 5−20% linear gradient slab gel. Protein samples were mixed directly with the SDS−PAGE sample buffer solution containing 5% β-mercaptoethanol and 2% SDS. About 50 μg of protein was loaded per well. The gel electrophoresis was carried out at 120 V constant voltage. The gel was stained with 0.1% Coomassie Brilliant Blue R250. Molecular weight marker proteins were run along with test samples.

Surface Hydrophobicity Measurements. The surface hydrophobicity of native β-casein and cross-linked β-casein polymers was determined according to the procedure of Kato and Nakai (1980) using *cis*-parinaric acid as the fluorescent probe. The fluorescent measurements were made using a Perkin-Elmer model LS-5 luminescence spectrophotometer. Excitation and emission wavelengths were 325 and 410 nm, respectively. The slope of the fluorescence intensity versus protein concentration plot was taken as a measure of relative surface hydrophobicity.

Emulsion Preparation. In a typical experiment, to 4 mL of a 0.05% β-casein in 10 mM phosphate buffer, pH 7.0, containing 0.02% NaN_3 was added 1.33 mL of canola oil (25% oil volume fraction), and the mixture was stirred vigorously using a magnetic stirrer. The mixture was then sonicated in a Branson 450 sonifier using a microtip horn. In all emulsion preparations, an energy input setting of 2 on the sonifier and a emulsification time of 3 min were used as standard conditions. During emulsification, the temperature of emulsions was kept constant at 25 °C by circulating water from a thermostated water bath.

Determination of Protein Load at the Emulsion Interface. An aliquot (1 mL) of freshly prepared emulsion was centrifuged in a Marathon 21K/R centrifuge (Fisher Scientific Co., Pittsburgh, PA) at 11800 rpm for 15 min at 25 °C. No protein pellet was found after centrifugation, which indicated no precipitation of the protein during emulsification. The bottom aqueous phase was removed using a syringe, and the protein content was determined according to the Lowry et al. (1951) method. The adsorption load, Γ (amount adsorbed in mg/m^2 surface area), was calculated as

$$\Gamma = (C_i - C_f)V_p/\mathbf{a} \quad (1)$$

where C_i and C_f are the initial and final protein concentrations (mg/mL) in the aqueous phase, V_p is the volume of the aqueous phase in emulsion, and **a** is the total interfacial area of the emulsion. The interfacial area **a** was calculated on the basis of the Mie theory of light scattering, according to which the interfacial area of an emulsion is 2 times its turbidity. The turbidity T of an emulsion is given by $T = 2.303A/l$, where A is the absorbance and l is path length. The interfacial area **a** of emulsions was determined by measuring the turbidity at 500 nm. The emulsifying activity index (EAI) of proteins was determined from the relation (Pearce and Kinsella, 1978)

$$\mathrm{EAI} = 2T/(1 - \phi)c \quad (2)$$

where ϕ is the volume fraction of the oil and c is the protein concentration in mg/mL.

Emulsion Stability. An aliquot (5 mL) of each emulsion taken in a capped glass vial was kept in constant gentle agitation in a TubeRocker (American Dade, Miami, FL) placed inside an incubator maintained at 25 °C. The tube rocker speed was set at ∼10 cycles per minute. Aliquots (0.5 mL) were withdrawn at different time intervals over a period of 2−5 days. These aliquots were accurately diluted with 0.1% SDS to give an absorbance value of <0.7 at 350 nm. The turbidity spectra were recorded in the visible range from 400 to 700 nm in a Beckman DU-60 spectrophotometer. The emulsion stability was analyzed according to the method of Reddy and Fogler (1981)

$$N_t/N_0 = \frac{1}{r_0^3}\left(\frac{\lambda^{m_t - m_0} r_0^{\,m_0 - 1}}{T_0 T_t}\right)^{3/m_t - 1} \quad (3)$$

where N_0 and N_t are the initial number concentrations of emulsion droplets at zero time and at time t, respectively; T_0 and T_t are turbidities at $t = 0$ and $t = t$, respectively; m_0 and m_t are the slopes of a plot of ln T versus ln λ of an emulsion (where λ is the wavelength) at zero time and at time t, respectively; and r_0 is the initial average droplet radius. A very approximate value of r_0 was obtained from (Pearce and Kinsella, 1978)

$$r_0 = 3\phi_0/2T_0 \quad (4)$$

where ϕ_o is the initial volume fraction of the oil phase.

Light Scattering Measurements. Light scattering experiments were performed on polymerized protein solutions using a Malvern 4700c particle analyzer (Spring Lane, U.K.) with a 128-channel autocorrelator and a Lexel model 95-2 argon-ion laser (Fremont, CA) equipped with an Etalon and operated at a power of 75−200 mW and at a wavelength of 488 nm. To reduce stray light, the equipment was enclosed in a black box. The various transglutaminase-treated β-casein solutions were diluted to a final concentration of 0.5 mg/mL with 0.1 M Tris-HCl buffer, pH 7.5, which had been previously filtered through a 0.2 μm filter. The diluted solution was transferred to a precleaned, dust-free cuvette and placed in a thermostated sample holder that contained decahydronaphthalene as a refractive index matching fluid. The sample was maintained at 25 °C by using a temperature-controlled circulating water bath.

Static light scattering (SLS) experiments were performed essentially as described by Tomski and Murphy (1992). The scattered light intensity of the sample was collected for 20 s at 21 different angles between 20° and 140°. Each measurement was an average of 10 measurements. The scattered light intensity of buffer blank was then measured under the same conditions. The results were normalized with respect to intensity data from spectrophotometric grade toluene (Aldrich,

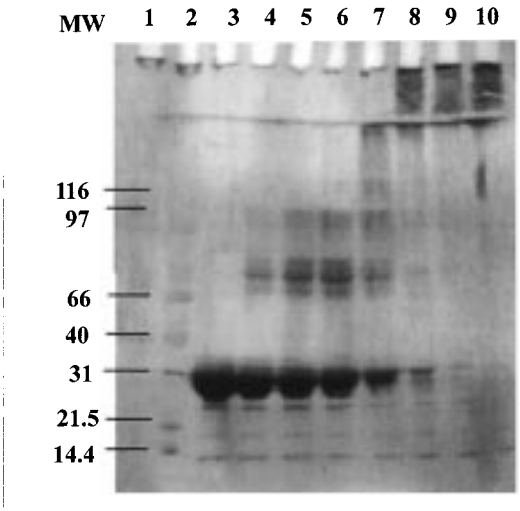

Figure 1. SDS−PAGE profiles of transglutaminase-catalyzed polymers of β-casein. Lanes 1 and 2 are protein markers. Lanes 3−10 represent β-casein treated with transglutaminase for 0, 20, 30, 40, and 90 min, and 3, 5, and 24 h, respectively.

Milwaukee, WI) to obtain the Raleigh ratio of the sample $R_{s(q)}$

$$\frac{Kc}{R_{s(q)}} = \frac{1}{\langle M \rangle_{w,app}} 1 + \frac{1}{3} q^2 \langle R_g^2 \rangle_z \quad (5)$$

where $K = 4\pi^2 n^2 (dn/dc)^2 / N_A \lambda_0^4$, n is the refractive index of the solvent, dn/dc is the refractive index increment, N_A is Avogadro's number, λ_0 is the wavelength in a vacuum, c is protein concentration in g/mL, $q = 4\pi n/\lambda_0 \sin(\theta/2)$ is the scattering vector, θ is the angle, $\langle M \rangle_{w,app}$ is the apparent weight-averaged molecular weight, and $\langle R_g^2 \rangle_z$ is the light-scattering-averaged squared radius of gyration. A plot of $Kc/R_{s(q)}$ versus q^2 yields an intercept corresponding to the inverse of $\langle M \rangle_{w,app}$ and a slope from which $\langle R_g \rangle_z$ can be calculated.

RESULTS AND DISCUSSION

Figure 1 shows the effect of reaction time on the extent of polymerization of β-casein by transglutaminase. In the SDS−PAGE, the native β-casein monomer migrated as a ∼31 kDa protein. The transglutaminase-treated samples contained, in addition to β-casein monomer, new protein bands corresponding to molecular masses of ∼72, ∼110, and ∼150 kDa. These protein species appear to be dimer, trimer, and tetramer of β-casein, produced as a result of transglutaminase-catalyzed cross-linking. The intensity of the dimer band increased with reaction time up to 40 min, decreased thereafter, and disappeared after 3 h. The trimer appeared only after 20 min of reaction, transiently accumulated up to 90 min of reaction time, and then disappeared. The transient accumulation of the tetramer species occurred only after 40 min of reaction and disappeared after 90 min. The 3, 5, and 24 h polymerized samples contained only polymers that could not enter the separating and stacking gels. The monomeric β-casein disappeared completely after a reaction time of ∼3 h. The data in Figure 1 suggest that, under the reaction conditions used, cross-linking of β-casein by transglutaminase followed a general reaction model

$$M_n + M_m \rightarrow M_{n+m} \quad (6)$$

Table 1 shows the apparent weight-averaged molecular weight, the radius of gyration (as determined by light scattering), and the surface hydrophobicity (as determined by the fluorescent probe method) of the cross-linked β-casein polymers. The weight-averaged molecular weight of β-casein polymers increased with increasing reaction time. However, the apparent molecular weight of the 20 min (P20) and 40 min (P40) samples did not agree with the SDS−PAGE results, which showed only the presence of dimers and trimers but not polymers with molecular mass in the millions. It should be noted that no trace of high molecular mass polymers that could not penetrate the stacking or separating gels was found in the SDS−PAGE (Figure 1) of these samples. It is probable that this discrepancy might be due to aggregation of the dimers and trimers via noncovalent interactions. It is also possible, although unlikely, that the presence of a small fraction of highly polymerized species may skew the light scattering results. Thus, the molecular mass and the radius of gyration values shown in Table 1 may reflect the size of the aggregates, not the actual size of cross-linked polymers. However, because the protein concentration in the samples for light scattering experiments was ∼0.5 mg/mL, it is questionable whether extensive aggregation of cross-linked β-casein can occur at this low concentration. It should be noted that although light scattering predicts a reasonable molecular mass of ∼17200, as opposed to 24000 for the β-casein monomer, it gives a very high radius of gyration value of 164 nm for native β-casein. This highlights the limitations of the use of the light scattering technique to determine molecular masses of transglutaminase-treated β-casein polymers. The data in Table 1 shows that the surface hydrophobicity of the cross-linked polymers was not affected significantly by the extent of polymerization.

Table 1. Some Properties of the Cross-Linked β-Casein Polymers Produced by the Transglutaminase Reaction[a]

reaction time	mol mass (kDa)	$\langle R_g \rangle$ (nm)	surface hydrophobicity
0 min	17.2	164	1.22
20 min	2810	392	1.41
40 min	3220	368	1.24
90 min	3420	357	1.46
5 h	11900	583	1.70
24 h	18800	576	1.13

[a] The data are means of triplicate measurements.

Table 2. Emulsifying Properties of Cross-Linked β-Casein Polymers[a]

reaction time	surface load (mg/m^2)	% total protein unadsorbed	EAI (cm^2/mg)
0 min	1.50	1.0	2946
20 min	1.13	1.7	3462
40 min	1.53	3.62	2977
90 min	1.69	5.43	2565
5 h	3.34	4.86	1624
24 h	2.83	5.24	1535
LSD[b]			1083

[a] The data are averages of triplicate measurements. [b] Least significant difference at 5% level. Differences of two means exceeding this value are significant.

Emulsifying Properties. Table 2 shows the emulsifying properties of cross-linked β-casein polymers. In all cases, the amount of total protein unadsorbed in the aqueous phase of the emulsion was in the range of 1−5%, indicating that roughly almost all protein was adsorbed to the oil droplet surface. This is expected because the initial protein concentration in the aqueous phase was only 0.05%. This low concentration was deliberately selected to create emulsions with only a

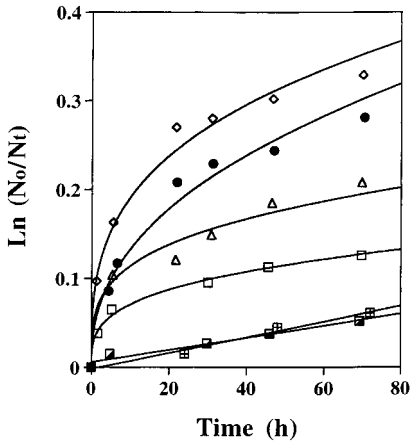

Figure 2. Variation of $\ln(N_0/N_t)$ as a function of storage time of β-casein-stabilized emulsions: (●) native β-casein; (◇) P20 polymer; (△) P40 polymer; (□) P90 polymer; (⊞) P5h polymer; (◪) P24h polymer. The initial aqueous phase concentration of β-casein was 0.05% (w/v), and the volume fraction of the oil phase was 25% (v/v).

monolayer protein coat around oil droplets and hasten the rate of coalescence. The EAI values of the β-casein polymers obtained at 20 min (P20), 40 min (P40), and 90 min (P90) reaction times were not statistically different from that of native β-casein. However, the EAI values of the 5 h (P5h) and 24 h (P24h) polymers were significantly lower than those of the other polymers. The adsorption load, however, exhibited an inverse relationship with EAI. That is, the adsorption loads of the P20, P40, and P90 polymers were very similar to that of native β-casein, whereas those of the P5h and P24h polymers were ~2-fold higher than those of the other polymers. It should be pointed out that during the 20−90 min of reaction, only dimer, trimer, and tetramer appear as the major cross-linked species, whereas at 5 and 24 h reaction times the reaction product contained only high molecular mass polymers that could not enter the stacking and separating gels (Figure 1). Taken together, the data indicate that the low molecular mass cross-linked polymers form a thin protein film at the air−water interface and therefore are able to create a large interfacial area per milligram of protein. This also supports the earlier argument that the P20 and P40 polymers are not really huge cross-linked species, as suggested by the light scattering data (Table 1), but are mainly dimers and trimers of β-casein. On the other hand, because of their inability to dissociate at the interface, the high molecular mass cross-linked polymers, P5h and P24h, are unable to create a large interfacial area per milligram of protein and therefore form a thick protein film around the emulsion droplets. The inability to create a large interfacial area might be related to structural constraints imposed on the peptide segments to effectively interact with the interface. In other words, much of the protein mass of the highly cross-linked β-casein film at the oil−water interface might not be *at* the interface but might be in the form of "loops" suspended into the aqueous phase, forming a thick protein layer.

Emulsion Stability. Figure 2 shows the stability of emulsions formed with P20, P40, P90, P5h, and P24h polymers. Generally, instability of an emulsion follows first-order kinetics (Das and Chattoraj, 1982). In fact, emulsions stabilized by globular proteins exhibit first-order decay (Das and Kinsella, 1989; Damodaran and Anand, 1997). However, in the case of β-casein polymers, except those of P5h and P24h, the rate of decrease in the number of emulsion particles did not follow first-order kinetics. Neither did the data fit second-order kinetics. The emulsions made with P5h and P24h β-casein polymers were more stable than those made with the other polymers. Except for the emulsion made with the P20 polymer, the rate of decrease of particle number decreased with increasing degree of polymerization. Visual observation of the emulsions also confirmed that oil separation in the P5h and P24h emulsions were very low compared to that in other emulsions during the storage period. Oil separation in emulsions is generally preceded by flocculation and creaming (Damodaran, 1997). However, because the emulsions were gently agitated in a tube rocker, creaming of the emulsion was not possible. Thus, the rate of decrease of particle number must be related to flocculation/clustering of the particles. Because oiling-off is seen in these emulsions, the aggregated/clustered particles must be coalescing (i.e., small droplets fusing to become large particles) and breaking with time. This process also might be responsible for the rate of decrease of particle number.

As noted earlier, the stability of protein-stabilized emulsions is fundamentally related to the mechanical/viscoelastic/steric properties of the protein film (Damodaran, 1997). Thus, the increase in the stability of a β-casein emulsion with increasing degree of polymerization might be related either to formation of a more cohesive and viscoelastic protein film around the oil droplet or to an increase in steric repulsion between droplets as a result of formation of a thicker layer of protruding branched polymer chains. It is difficult to predict which of these factors actually imparts stability to these emulsions. In general, in the case of native proteins, steric repulsion between adsorbed protein layers is regarded as the most important factor stabilizing emulsion (Dickinson, 1992; Damodaran, 1997). The steric repulsion arises because of the overlapping of the protruding peptide chains of the adsorbed protein layers. The steric repulsive energy per unit area of the interacting emulsion surfaces is (de Gennes, 1987)

$$E_{SR} = (kT\Gamma L/s)[(2L/D^{2.25} - (D/2L)^{0.75}] \qquad (7)$$

where k is the Boltzman constant, T is the temperature, Γ is the protein load, s [$= \sqrt{(1/\Gamma)}$] is the mean distance between the polymers in the protein film, L is the thickness of the layer of protruding chains, and D is the distance between the interacting surfaces. According to eq 7, E_{SR} is a function of both Γ and L, and it increases steeply when $D < 2L$. The thickness of the protruding peptide chains ("hairs") on the surface of an emulsion droplet depends on conformational characteristics of the adsorbed protein. Dynamic light scattering measurements have shown that the thickness of the adsorbed layer of β-casein on polystyrene latex particles is ~10−15 nm from the hydrophobic particle surface (Dalgleish, 1990). Similarly, the thickness of the adsorbed layer of sodium caseinate at the oil−water interface of emulsions also was found to be ~5 nm at $\Gamma = 1$ mg/m^2 and reaches a maximum value of ~10 nm at $\Gamma \geq 1.8$ mg/m^2 (Fang and Dalgleish, 1996). Theoretical calculations using the self-consistent-field theory (Fleer et al., 1993) also have shown that the β-casein monolayer at an interface has a dense inner layer up to 2 nm from the interface and

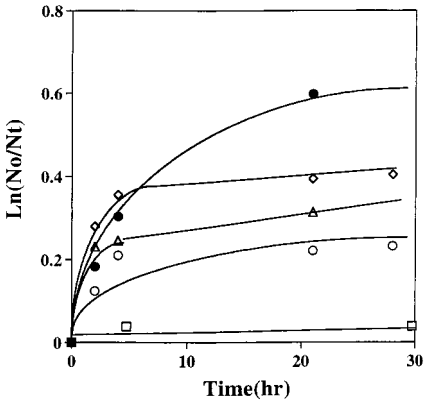

Figure 3. Variation of $\ln(N_0/N_t)$ as a function of storage time of emulsions formed by mixtures of native β-casein and P24h β-casein polymer: (●) 100% native β-casein; (◇) 75% native β-casein + 25% P24h polymer; (△) 50% native β-casein + 50% P24h polymer; (○) 25% native β-casein + 75% P24h polymer; (□) 100% P24h polymer. The total initial protein concentration in the aqueous phase was 0.05% (w/v), and the volume fraction of the oil phase was 25% (v/v).

a dilute layer up to 10 nm (Atkinson et al., 1997). It should be emphasized that sodium caseinate contains only linear polypeptides, whereas transglutaminase-catalyzed polymers of β-casein are branched polymers. The branching occurs at the lysine and glutamine sites. Because of configurational constraints, the orientation of these branched polymers at the oil−water interface may be very different from that of linear casein polypeptides. Thus, for a given Γ value, the branched polymers of β-casein may form a thicker protein layer than linear polypeptides. In other words, although the protein loads of emulsions produced by native, P20, P40, and P90 β-casein polymers are in the range of 1.13−1.7 mg/m^2 (Table 2), it is likely that the thickness of the protein layer in the P20-, P40-, and P90-stabilized emulsions may be only slightly greater than 5−10 nm expected for the native β-casein-stabilized emulsion. In contrast, because the protein loads of the P5h- and P24h-stabilized emulsions are ∼2-fold greater than those of other emulsions, and because the extent of polymerization in these samples is much higher than in others, it is likely that the protein layer in the P5h and P24h emulsions may be thicker than in others. However, it is difficult to conclude whether the enhancement of emulsion stability of the P5h- and P24h-stabilized emulsions is related to their rheological properties or to their steric repulsive qualities. This ambiguity arises because of the fact that for a protein layer to impose steric repulsion, its protruding chains must be in a state of high entropy. It is debatable whether the entropy of the protruding chains of the branched polymers would be as high as that of native β-casein monomer. Moreover, it is easy to visualize that, because of steric constraints created by branching, all hydrophobic segments of the polymer may not be physically in direct contact with the interface, and hence some of the hydrophobic segments may be forced into the aqueous phase around the oil droplet. Attractive interactions between these segments would not only further reduce the entropy of the protruding chains but also create a mechanically stronger protein film. Because of these considerations, it is reasonable to assume that the increase in emulsion stability might be due to an improvement in both the viscoelastic and steric properties of the protein film.

To confirm whether polymerized β-casein indeed imparts stability to emulsions, experiments were conducted on the stability of emulsions produced by mixtures of native β-casein and P24h β-casein polymer. The data in Figure 3 show that when the P24h β-casein polymer was mixed with native β-casein at various ratios (w/w), the kinetic stability of emulsions prepared using these mixtures progressively increased with increasing percentage of P24h polymer in the mixture. This confirms that branched β-casein polymers produced by the transglutaminase-mediated cross-linking reaction have the ability to stabilize emulsions.

The results presented here suggest that the emulsifying properties of a protein can be improved by controlled polymerization of the protein using transglutaminase. The improvement in the stability of oil-in-water emulsions formed by these polymers seems to be due to the branched nature of the polymers. The mechanism may involve both an increase in the thickness of the layer of protruding protein chains and an improvement in the mechanical/viscoelastic properties of the adsorbed protein layers.

LITERATURE CITED

Atkinson, P. J.; Dickinson, E.; Horne, D. S.; Leaver, J.; Leermakers, F. A. M.; Richardson, R. M. In *Food Colloids: Proteins, Lipids and Polysaccharides*; Dickinson, E., Bergernstahl, B., Eds.; Royal Society of Chemistry: Cambridge, U.K., 1997; p 217.

Dalgleish, D. G. The conformations of proteins on solid/water interfaces—Caseins and phosvitin on polystyrene latices. *Colloids Surf.* **1990**, *46*, 141−155.

Damodaran, S. Protein-stabilized foams and emulsions. In *Food Proteins and Their Applications*; Damodaran, S., Paraf, A., Eds.; Dekker: New York, 1997; pp 57−110.

Damodaran, S.; Anand, K. Sulfhydryl-disulfide interchange-induced interparticle polymerization in whey protein-stabilized emulsions and its relation to emulsion stability. *J. Agric. Food Chem.* **1997**, *45*, 3813−3820.

Das, K. P.; Chattoraj, D. K. Kinetics of coalescence of polar oil/water emulsions stabilized by ionic detergents and proteins. *Colloids Surf.* **1982**, *5*, 75−78.

Das, K. P.; Kinsella, J. E. PH dependent emulsifying properties of β-lactoglobulin. *J. Dispersion Sci. Technol.* **1989**, *10*, 77−102.

de Gennes, P. G. Polymers at an interface: a simplified view. *Adv. Colloid Interface Sci.* **1987**, *27*, 189.

Dickinson, E. *An Introduction to Food Colloids*; Oxford Science: Oxford, U.K., 1992.

Fang, Y.; Dalgleish, D. G. Competitive adsorption between dioleoylphosphatidylcholine and sodium caseinate on oil−water interfaces. *J. Agric. Food Chem.* **1996**, *44*, 59−64.

Fleer, G. J.; Stuart, M. A. C.; Scheutjens, J. M. H. M.; Cosgrove, T.; Vincent, B. *Polymers at Interfaces*; Chapman and Hall: London, U.K., 1993.

Folk, J. E. Mechanism and basis for specificity of transglutaminase-catalyzed ϵ-(γ-glutamyl)lysine bond formation. *Adv. Enzymol* **1983**, *54*, 1−57.

Folk, J. E.; Cole, P. W. Structural requirements of specific substrates for guinea pig liver transglutaminase. *J. Biol. Chem.* **1965**, *240*, 2951−2960.

Han, X.-Q.; Damodaran, S. Thermodynamic compatibility of substrate proteins affects their cross-linking by transglutaminase. *J. Agric. Food Chem.* **1996**, *44*, 1211−1217.

Kato, A.; Nakai, S. Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochim. Biophys. Acta* **1980**, *624*, 13−20.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem*. **1951**, *193*, 265−275.

Pearce, K. N.; Kinsella, J. E. Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *J. Agric. Food Chem*. **1978**, *26*, 716−723.

Reddy, S. R.; Fogler, H. S. Emulsion stability: Determination from turbidity. *J. Colloid Interface Sci*. **1981**, *79*, 101−104.

Tomski, S. J.; Murphy, R. M. Kinetics of aggregation of synthetic β-amyloid peptide. *Arch. Biochem. Biophys*. **1992**, *294*, 630−638.

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