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Stabilization of Oil-in-Water Emulsions by β-Lactoglobulin−Polyethylene Glycol Conjugates

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The disulfide bonds of β-lactoglobulin (β-lg) were modified by oxidative sulfitolysis to generate β-lgSO_3. The native protein (β-lg) and the modified protein (β-lgSO_3) were conjugated to activated polyethylene glycol (PEG) to generate β-lgPEG and β-lgSO_3PEG, respectively. Oil-in-water (o/w) emulsions containing 1% β-lg or β-lg conjugates were prepared at pH 2.8, 5.0, and 7.0. Emulsion droplet diameters and zeta potentials were measured. For the same emulsifier, emulsion droplet diameters decreased when emulsion pH increased. Zeta potentials of emulsion droplets increased with pH for β-lg and β-lgSO_3. Zeta potentials of β-lgPEG and β-lgSO_3PEG approached zero, suggesting that the protein molecule was covered by PEG chains. Accelerated and 7-day storage stabilities at 21 °C of the emulsions were monitored. The emulsifying activity index (EAI) of β-lgPEG was not significantly different from the EAI of β-lg. The EAI of β-lg was enhanced following sulfitolysis of β-lactoglobulin. The emulsifying activity increased more when the oxidatively modified protein was conjugated to polyethylene glycol. Emulsions made with β-lgSO_3PEG were more stable than emulsions made with β-lg, β-lgPEG, or β-lgSO_3 under accelerated stability study and for 7 days at 21 °C. The stability of o/w emulsions stabilized with β-lgSO_3PEG increased because individual droplets were better protected, against protein bridging or coalescence, by the thick adsorbed protein−PEG layer.

KEYWORDS: β-Lactoglobulin; polyethylene glycol; emulsions

INTRODUCTION

β-Lactoglobulin (β-lg), a major constituent of whey protein, is a globular protein of 162 amino acids residues with two disulfide bridges between residues 106−119 and 66−160 and a free thiol group at residue 121, which all provide stability and a potential for inter- and intramolecular disulfide linkages during conformational changes associated with pH alteration or heat treatment (*1*).

Resistance to surface denaturation due to intramolecular disulfide bonds and the complex aggregation properties of β-lg as a function of pH have been suggested as the most important factors associated with the poor functionality of β-lactoglobulin at acidic pH (*2*, *3*). The folded structure of β-lg is only slightly affected at pH values as low as 2 (*4*, *5*). Emulsifying properties of β-lg include its pH dependency, its fairly coarse droplet formation, and its ability to undergo rapid creaming at pH 4.4−5.0 (*6*). Disulfide bonds contribute to the protein rigidity and prevent protein unfolding at the interface. Controlled cleavage of disulfide bonds with simultaneous blockage of the newly formed sulfhydryl groups was reported as a desirable approach to obtain soluble end-products with minimum side reactions in the absence of denaturant (*7*). Reddy et al. (*4*) demonstrated that cleavage of S−S bonds caused extensive changes in β-lg conformation and was more effective than heating at 90 °C in decreasing the resistance to gastric proteolysis. The effects of sulfitolysis on the emulsifying properties of bovine serum albumin (BSA) were studied by Kella et al. (*8*) and Klemaszewski et al. (*9*). These authors speculated that enhanced protein flexibility associated with sulfitolysis facilitated the formation of a stronger interfacial film and increased the net negative charge around emulsion droplets, which retarded the rate of coalescence. The emulsion made using BSA in which all of the disulfide bonds were cleaved had a maximum stability of 2−3 days (*10*).

Polyethylene glycol (PEG) is a nontoxic, water-soluble polymer widely used for stabilizing colloids in foods and paints and in formulating pharmaceuticals and cosmetics because PEG acts as a dispersant and yet is inert (*11*). Covalent chemical modification of proteins with PEG has become a powerful complementary approach to site-directed mutagenesis with numerous medical and biotechnological applications as a result of the unique properties of PEG and its general biocompatibility with biological systems (*12*, *13*).

Covalent attachment of activated PEG to proteins alters protein properties, such as increased solubility and stability in organic solvents, increased thermal stability, and reduced immunogenicity and antigenicity, in ways that extend their potential uses (*14*−*17*). The chemistry of synthesizing protein−PEG conjugates involves the preparation of an activated PEG

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containing a reactive terminal group that can be readily coupled to a nucleophilic group on the protein.

The literature is well established that generally when a protein and a small uncharged surfactant are added separately to an emulsion the protein may be displaced from the interface of o/w emulsions by the small uncharged surfactant. As a result, the steric and charge stabilization effects provided by the protein layer may be reduced (*18*). There is no information on the effect of a conjugate containing a protein and a small surfactant on the stabilization/destabilization of o/w emulsions.

The disulfide bridges of β-lg were broken by sulfitolysis. The sulfitolyzed protein was pegylated at the ϵ-amino groups of lysine and other nucleophilic groups in order to provide a β-lgPEG conjugate with improved emulsifying properties at acidic pH according to the equation:

$$(\mathrm{Pr{-}S{-}S{-}Pr})_n + 2n\mathrm{SO_3}^{2-} \rightarrow 2n\mathrm{Pr{-}S{-}SO_3}^{-} \quad (1)$$

$$p\text{-NPC} + \text{PEG-OH} \rightarrow p\text{NPC-PEG} \quad (2)$$

$$p\text{-NPC{-}PEG} + \mathrm{H_2NPr{-}S{-}SO_3} \rightarrow \mathrm{PEG{-}O{-}CONHPr} \quad (3)$$

where Pr represents protein and *p*-NPC represents *p*-nitrophenylchloroformate.

MATERIALS AND METHODS

Materials. β-Lactoglobulin A (molecular weight of 18500), monomethoxypolyethylene glycol or PEG (MW of 5000), *p*-nitrophenylchloroformate, pepsin (from hog mucosa, 1:60000, 2× crystallized), lysozyme, bovine serum albumin, lactoferrin, bovine IgG, and Nonidet P-40 were purchased from Sigma Chemical Co. (St. Louis, MO). Chicken immunoglobulin IgY was purified according to the method of Polson et al. (*19*). Sephacryl 300HR was from Amersham Pharmacia Biotechnology (Quebec, Canada). Ellman reagent was purchased from Pierce Chemical Co. (Rockford, IL). Sodium sulfite was purchased from Fisher Scientific (Ottawa, Canada). SpectraPor dialysis membrane was from Spectrum Medical Industries, Inc. (Houston, TX). Corn oil was purchased from a local store. All other chemicals were of reagent grade.

Methods. *Preparation of Activated Polyethylene Glycol.* Activated PEG was prepared according to the method of Veronese et al. (*20*). To a solution of 0.6 g of *p*-nitrophenylchloroformate in 50 mL of acetonitrile were added 5 g of PEG and 0.29 g of triethylamine. After 24 h of stirring at 21 °C, the precipitated triethylammonium chloride was filtered, 500 mL of ethyl ether was added, and the solution was left to crystallize overnight at 4 °C. The product was filtered, washed with ether, and recrystallized from acetonitrile/ether. The activated PEG was extensively dialyzed against water at pH 6 and lyophilized.

Sulfitolysis of β-Lactoglobulin and Coupling to Activated Polyethylene Glycol. Disulfide bonds in β-lg were cleaved according to the method of Weil and Seibles (*21*). To 1 g of β-lg dissolved in 50 mL of water, by adjusting the pH to 9.0 with 0.5 N NH_4OH, was added 40 mL of cupric ammonium solution (containing 0.249 g of $CuSO_4 \cdot 5H_2O$ dissolved in water and adjusted to pH 9.0 with concentrated NH_4OH) and 10 mL of Na_2SO_3 solution (containing 0.83 g). After 2 h of stirring at room temperature, the reaction mixture was successively dialyzed against 0.1 M sodium citrate at pH 7.0 and against water and lyophilized. The number of intact disulfide bonds and free sulfhydryl groups was determined using Ellman reagent. A conjugate of β-lg with PEG or β-lgSO_3 with PEG was prepared as follows: To a solution of 100 mg of β-lg or β-lgSO_3 in 5 mL of 0.1 M borate buffer at pH 9.4 was added a 6-fold molar of activated PEG. The mixture was stirred overnight at room temperature, dialyzed against distilled water using a SpectraPor membrane with a molecular weight cutoff of 25000 Da, and lyophilized. The lyophilized β-lgPEG or β-lgSO_3PEG was stored at −20 °C until use.

Molecular Weight of β-Lactoglobulin−PEG Conjugates. The molecular weight of β-lg conjugated to PEG was determined by gel permeation chromatography using an FPLC system from Amersham Pharmacia Biotechnology. FPLC was carried out on 200 μL of 5 mg/mL protein or conjugate on Sephacryl 300HR (60 cm × 1 cm) column in 0.15 M phosphate buffer at pH 8.0 and containing 8 M urea at a flow rate of 1 mL/min. The absorbance of the eluate was monitored at 280 nm. Standard proteins (lysozyme, β-lg, BSA, lactoferrin, IgG, and IgY) were eluted under the same conditions.

Preparation of O/W Emulsions. All of the emulsion preparations contained 10% w/w corn oil, (90 − *X*)% w/w of 0.05 M Na_2HPO_4 previously adjusted to pH 2.8, 5.0, or 7.0, and *x*% w/w (1%) of β-lg, β-lgPEG, β-lgSO_3, or β-lgSO_3PEG dissolved in the aqueous buffer. The mixture was homogenized at 21 °C for 5 min using a Polytron Ultra Turrax homogenizer (Jankel and Kunkel) equipped with a Tekmar model TR-10 power control at full speed. Emulsions were divided into aliquots and stored at 21 °C for up to 7 days.

Surface Properties of Emulsions. The zeta potential of emulsion droplets was determined by measuring the particle electrophoretic mobility using a zetamaster (Malvern Instrument, Southboro, MA), which provides an absolute determination of zeta potential with no calibration required. The droplet size distribution was also measured using a zetamaster. The sample was dispersed in Milli-Q water with a dilution factor of 200. The droplet size distribution was measured within 1 h after homogenization and within 24 h after homogenization.

Emulsifying Properties of β-Lactoglobulin and β-Lactoglobulin Conjugates. The emulsifying activity index (EAI) was measured according to the method of Pearce and Kinsella (*22*) with slight modifications. Aliquots of emulsions were drawn from the test tube bottom immediately after emulsion preparation and diluted 1000-fold with 0.1% of Nonidet P-40 (for emulsions prepared at pH 2.8) or 0.1% SDS (for emulsions prepared at pH 5.0 and 7.0). The absorbances of the diluted emulsions were measured at 500 nm in a Shimadzu 160 UV−visible spectrophotometer. The oil volume fraction of each emulsion and the EAI were determined using the corrected formula of Cameron et al. (*23*). Accelerated emulsion stability was determined by centrifugation. Five milliliter aliquots of each emulsion were dispensed in 12 × 75 mm polystyrene tubes and spun for 25 min at 1000*g* in a Sorvall RC 5B Plus centrifuge. Following centrifugation the height of the creamed layer was measured and expressed as a percentage of initial height. Emulsions were stored at 21 °C for up to 7 days. EAI was measured each day for 7 days and values are presented as "ES", which is defined as the percentage of EAI at any day relative to the initial value of EAI on the day the emulsion was made.

RESULTS AND DISCUSSION

Molecular Weight of β-Lactoglobulin—PEG Conjugate. An active ester of PEG was prepared by reacting PEG and *p*-nitrophenylchloroformate. The active ester is nontoxic and stable and selectively reacts with amino groups and other nucleophilic groups. The molecular weight profile of β-lgPEG and β-lgSO_3PEG conjugates is shown in **Figure 1**. Conjugation of β-lg or β-lgSO_3 with PEG resulted in polydispersed molecular weight conjugates that eluted as discrete peaks ranging from about 34000 to 94000 Da as determined by gel permeation. Conjugation of PEG to proteins can occur through the ϵ-amine groups of lysine and other potential nucleophilic groups such as Glu, Asp, Thr, Ser, or Tyr on the surface of the protein. Due to the nature of most proteins and the presence of the nucleophilic groups on the surface of the proteins the attachment of PEG to protein can be considered nonspecific. This type of attachment of PEG to protein results in several degrees of heterogeneity that include (1) the number of PEG molecules attached per β-lg molecule, (2) the site of PEG attachment, and (3) the polydispersity nature of PEG. The polydispersity of protein−PEG conjugates has been demonstrated using MALDI-MS (*24*). Gel filtration of β-lgSO_3 and β-lgSO_3PEG showed that these modified proteins eluted earlier than their native counterpart β-lg and appeared to be significantly larger than their true molecular weight (**Figure 1**). The cleavage of disulfide

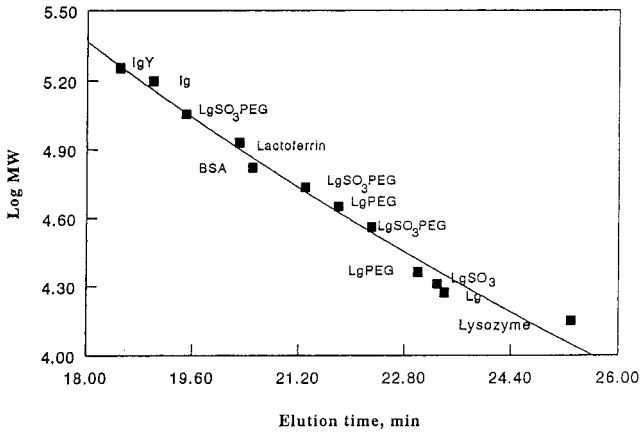

Figure 1. Molecular weight profile of β-lg conjugated to PEG as determined by gel filtration using FPLC system. Gel filtration chromatography was carried out on 200 μL of 5 mg/mL protein standards, β-lg, β-lgPEG, β-lgSO$_3$, or β-lgSO$_3$ PEG conjugates, on a Sephacryl 300 HR (60 cm × 1 cm) column in 0.15 M phosphate buffer at pH 8.0 containing 8 M urea at a flow rate of 1 mL/min.

Table 1. Effect of Sulfitolysis and Conjugation of β-Lactoglobulin to PEG on Zeta Potential of Emulsion Droplets Formed at Different pH Values[a]

pH	β-lg	β-lgPEG	β-lgSO$_3$	β-lgSO$_3$PEG
2.8	−19.23 ± 0.15	−15.10 ± 0.41	−35.04 ± 0.17	−17.06 ± 0.32
5.0	−35.42 ± 0.43	−10.20 ± 0.26	−45.44 ± 0.26	−12.78 ± 0.46
7.0	−50.31 ± 0.38	−10.05 ± 0.15	−59.04 ± 0.12	−11.54 ± 0.22

[a] Results are mean ± standard deviation of triplicate determinations.

bonds followed by the introduction of negatively charged sulfonated SO_3^- groups caused an expansion in the hydrodynamic volume of the pegylated β-lg and therefore the protein size. As a result, the difference in molecular size of β-lg and β-lgSO$_3$, in **Figure 1**, should appear larger than the true molecular weight difference, which is 80, between the native and the sulfitolyzed β-lg. However, because the K_{av} of Sephacryl 300HR is shallow, those differences become very subtle on the graph (**Figure 1**). Same explanation applies for comparison of β-lgPEG versus β-lgSO$_3$PEG. Malhotra and Sahal (*25*) observed similar chromatographic elutions following sulfitolysis of BSA, ovalbumin, aldolase, ribonuclease, and a recombinant fusion protein XA. Malhorta and Sahal also reported that the expansion in the hydrodynamic volume of sulfitolyzed protein, which is accentuated by the introduction of negative charges on the BSA protein molecule, was associated with retarded mobility of sulfitolyzed BSA on SDS-PAGE.

Surface Properties of Emulsions. The effect of oxidative sulfitolysis and β-lg conjugation to PEG on zeta potential of emulsion droplets at different pH values is given in **Table 1**. Zeta potential was measured immediately after emulsion preparation. Zeta potential of β-lg and β-lgSO$_3$ increased with pH. Sulfitolysis led to an increase in net negative charge. Zeta potential of β-lgPEG decreased from −15 to −10 mV when the pH increased from 2.8 to 7.0. Zeta potential of β-lgSO$_3$-PEG also decreased as pH increased. At the same pH the zeta potential value was β-lgPEG < β-lgSO$_3$PEG < β-lg < β-lgSO$_3$ (**Table 1**). The negative charge added on the protein molecule by sulfitolysis may have been covered by the PEG molecules, and as a result the zeta potential value decreased. Sulfitolysis led to increased protein flexibility, suggesting a change in protein secondary structure. Although no circular dichroism study was carried on sulfitolyzed β-lg, it is known that sulfitolysis, which

Table 2. Change in Emulsion Mean Droplet Diameters (Micrometers), within 24 h, of Emulsions Stabilized with β-Lactoglobulin or Its Conjugates[a]

pH	β-lg	β-lgPEG	β-lgSO$_3$	β-lgSO$_3$PEG
2.8	3.63–6.81	3.98–5.32	3.02–3.46	3.55–3.67
5.0	4.27–5.85	4.76–5.10	2.13–2.74	2.07–2.16
7.0	2.11–3.43	2.26–3.57	2.01–2.28	2.10–2.20

[a] Mean droplet diameters taken at 0 h (left value) and 24 h (right value) after emulsion preparation. Values of droplet size are means of triplicate determinations under the same experimental conditions.

Table 3. Emulsifying Activity Index (Square Meters per Gram) of β-Lactoglobulin and Its Conjugates at Different pH Values[a]

pH	β-lg	β-lgPEG	β-lgSO$_3$	β-lgSO$_3$PEG
2.8	55.2 ± 3.0[a]	57.6 ± 1.4[a]	37.7 ± 1.3	52.2 ± 1.8
5.0	45.6 ± 1.2[b]	43.1 ± 1.1[b]	76.3 ± 2.1	114.3 ± 5.5
7.0	72.4 ± 2.2[c]	73.3 ± 1.5[c]	115.3 ± 5.3	153.7 ± 5.7

[a] Results are mean values of triplicate determinations ± standard deviation. Values followed by the same letters are not significantly different ($P < 0.05$).

Table 4. Accelerated Emulsion Stability of β-Lactoglobulin or Its Conjugates, Expressed as Percent of Creaming

pH	β-lg	β-lgPEG	β-lgSO$_3$	β-lgSO$_3$PEG
2.8	65.60 ± 3.20	61.33 ± 3.33	57.71 ± 4.33	3.00 ± 0.60
5.0	54.40 ± 2.20	46.24 ± 2.42	26.20 ± 3.80	0.50 ± 0.02
7.0	37.60 ± 1.20	35.33 ± 3.33	23.30 ± 4.10	0.51 ± 0.01

is associated with the breakage of disulfide bonds, is also associated with reduced rigidity of the protein molecule. Circular dichroism analysis of sulfitolyzed BSA indicated significant loss in α-helix content (*25*). Covalent attachment of PEG to β-lg at the positively charged ε-amino groups of lysine led to an increase in protein net negative charge as the pH increased away from the p*I* of β-lg.

The mean diameter of emulsion droplets is given in **Table 2**. Mean diameters of emulsion droplets were taken at two different times: immediately after emulsion formation and 24 h after emulsion formation. Mean diameters of emulsion droplets stabilized by β-lg or β-lgPEG increased from pH 2.8 to pH 5.0 and decreased from pH 5.0 to pH 7.0. Below pH 3.5 β-lg exists as a monomer of ∼18.5 kDa. Between pH 3.5 and pH 5.2, especially at pH 4.6, all β-lg variants exist as octamers of molecular mass of 148 kDa, and between pH 5.5 and pH 7.5 all β-lg variants form dimers of molecular mass of 37 kDa. Above pH 7.5 β-lg exists as a monomer. The dimers are formed between monomers of the same variant and not between monomers of A and B variants. Mean droplet diameters of β-lg-stabilized emulsions was higher at pH 5.0 than at pH 2.8 and was lower at pH 7.0 than at pH 2.8 or pH 5.0 (**Table 2**). The rigid structure of β-lg is known to exist at pH values as low as 2.0. When native β-lg was pegylated (β-lgPEG), mean droplet diameters of emulsions stabilized by β-lgPEG followed the same trend as the mean droplet diameters of emulsions stabilized by β-lg. Pegylation of β-lg did not significantly improve the flexibility of the protein molecule compared to the control protein. When β-lg was sulfitolyzed to β-lgSO$_3$, the mean droplet diameters of emulsions decreased as pH increased from pH 2.8 to pH 5.0 and 7.0. The same trend was observed when emulsions were stabilized by β-lgSO$_3$PEG. Sulfitolysis is known to be associated with change in the secondary structure of the protein and a shift of protein p*I* value to a lower p*I* value (*7*,

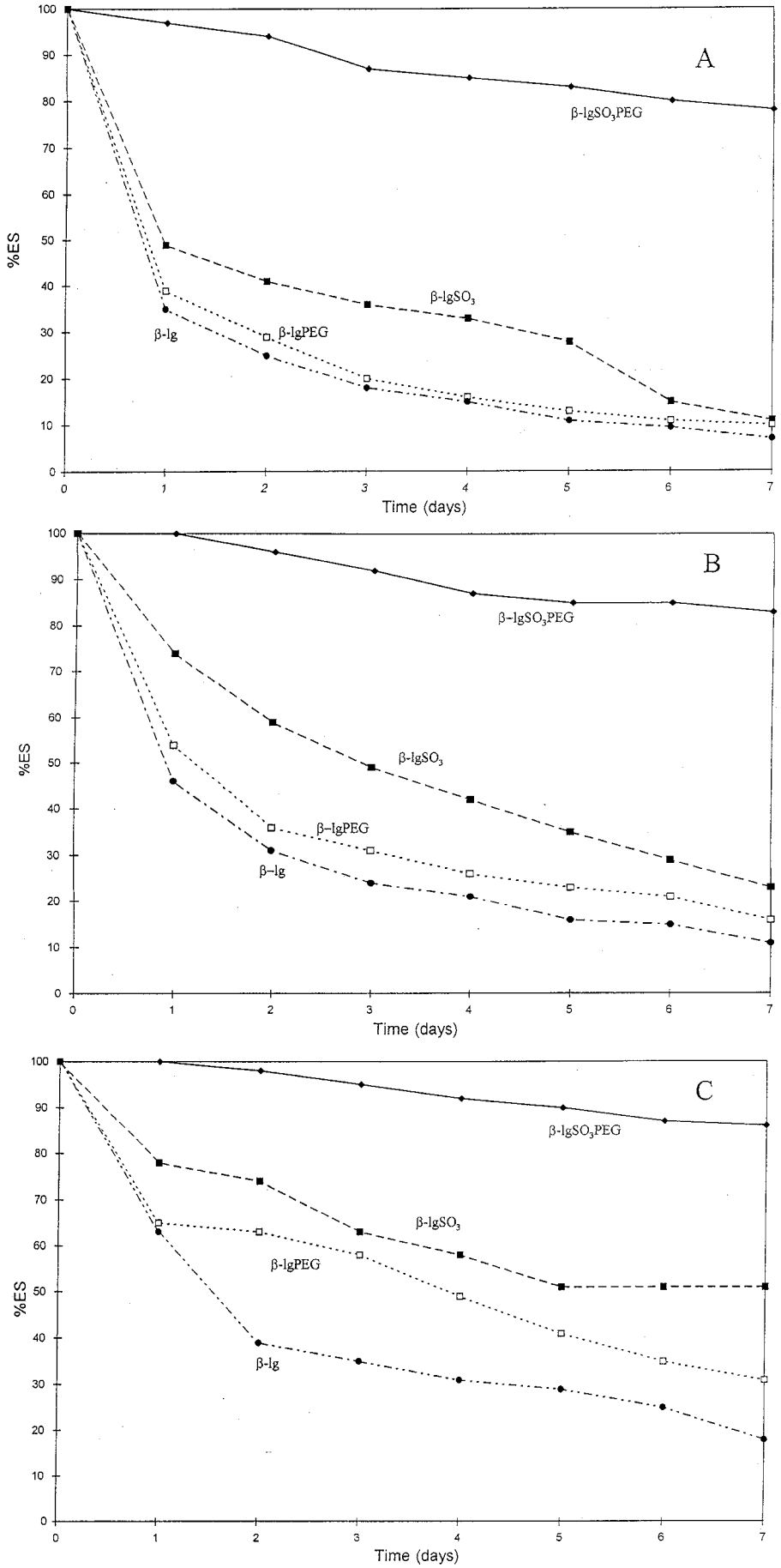

Figure 2. Stability of emulsions prepared with β-lg, β-lgPEG, β-lgSO_3, or β-lgSO_3 PEG conjugates stored at 21 °C for up to 7 days at (A) pH 2.8, (B) pH 5.0, and (C) pH 7.0.

25). Sulfitolysis of β-lg may have shifted the p*I* value of the conjugate(s) to a lower value. At the same pH value, emulsion mean droplet diameters increased as β-lgSO_3PEG < β-lgSO_3 < β-lgPEG < β-lg. Addition of negatively charged sulfonate groups increased the negative charge around emulsion droplets. The effect was observed as emulsions stabilized by β-lgSO_3 had a slight increase in mean droplet diameters 24 h after emulsion preparation. Pegylation of β-lgSO_3 to form β-lgSO_3-PEG provided PEG molecules that added an emulsion-stabilizing effect to the sulfitolyzed protein.

Emulsifying Activities. The emulsifying activity indices (EAI, $m^2\ g^{-1}$) of β-lg, β-lgPEG, β-lgSO_3, and β-lgSO_3PEG are given in **Table 3**. The EAI of β-lg at different pH values can be explained by the quaternary structure of β-lg, assuming that all of the β-lg is adsorbed to the interface. Modification of the protein by pegylation may have altered the fraction of β-lg that adsorbed to the interface. Pegylation of β-lg did not improve the EAI of the protein. EAI values of β-lg and β-lgPEG were not significantly different at each pH considered. Conjugation of PEG to intact β-lg without cleavage of disulfide bonds was not effective in improving the EAI of the conjugate compared to intact β-lg. The decrease in EAI may also be explained by the effect of pH on the quaternary structure of β-lg. The rigidity associated with the disulfide bridges and the size of β-lg as a function of pH is an important factor when emulsion stabilization by β-lg is considered. The EAI of β-lgSO_3 and β-lgSO_3PEG increased with pH. Sulfitolysis changes the protein secondary structure and the protein p*I*. Shimizu et al. (*3*) reported that disulfide bonds in β-lg were the major factor associated with its low emulsifying ability at acidic pH because the protein has a rigid conformation. The result from this work and the information on the quaternary structure of β-lg provide evidence that within the acidic pH region, β-lg performs worse closer to its natural p*I* than far away from pH 5.3. At the same pH, reducing the rigidity of β-lg by sulfitolysis, and especially by sulfitolysis followed by pegylation, significantly improved the EAI of the protein (**Table 3**).

Creaming Sability. Using the accelerated stability assay, the emulsion stabilities of β-lg and its conjugates were compared (**Table 4**). Emulsion stability followed the order β-lg < β-lgPEG < β-lgSO_3 < β-lgSO_3PEG. Conjugation of PEG to β-lg, prior to sulfitolysis, did not significantly improve the stability of the emulsion compared to emulsions prepared with β-lg. β-lgSO_3 assumes a more open flexible structure than β-lg or β-lgPEG. Klemaszewski et al. (*9*) improved the flexibility of BSA by controlled oxidative sulfitolysis and reported that the enhanced flexibility may have facilitated the formation of a stronger interfacial film with more favorable alignment of the hydrophobic/hydrophilic region of the protein and/or that the increased net negative charge retarded the rate of coalescence. The orientation of PEG molecules grafted on the β-lg surface, toward the interface between adjacent droplets, may have been responsible for enhanced emulsion stability by reducing the probability of collision between droplets. When PEG was conjugated to β-lgSO_3, emulsion stability increased relative to emulsions containing only β-lgSO_3 or β-lg (**Figure 2**). The zeta potential values (**Table 1**) suggest more stable emulsions staibilized by β-lgSO_3 followed by β-lg. Although the viscosity of the emulsion was not measured, it can be said from Rha and Pradipasena (*26*) that sulfitolyzed β-lg, because of its extended linear structure, increased the viscosity of the aqueous phase more effectively than the compact β-lg. Emulsions stabilized with β-lgSO_3PEG remained stable for 7 days (**Figure 2**).The addition of 0.02% NaN_3 to the emulsions provided stability against microbial growth. At the interface PEG molecules are known to protrude into the aqueous phase between the droplet globules and cause droplet repulsion. Surfaces coated with PEG having a molecular mass of 2000 Da or higher exhibit the ability to repel proteins (*27*). As far as molecular interactions are concerned, the PEG molecules anchored to a β-lg molecule create an extended, hydrated structure around each droplet, and this prevents droplets from coming together. The order of emulsion stability at all pH values considered was β-lg < β-lgPEG < β-lgSO_3 < β-lgSO_3PEG.

PEG is highly compatible with water, whereas at the same time it creates an unfavorable free energy change when a second species interacts with a solvated PEG molecule, resulting in a statistical tendency for the second species to be excluded from the region within or near the PEG chain. As a consequence, electrostatic and van der Waals interactions are also reduced. Thus, it appears that PEG on the surface of β-lg acted to prevent adjacent droplets from coming together, therefore extending emulsion stability. At acidic pH the combined effect of protein flexibility and PEG ability to repel adjacent droplets contributed to increased emulsion stability compared to intact β-lg. Sulfitolysis, which consists of adding an SO_3^- group to an −SH group, reduces the potential of disulfide interchange. Sulfitolysis and sulfitolysis combined with covalent attachemnt of PEG to β-lg were associated with smaller emulsion droplets and better emulsifying properties. Anchoring of PEG to β-lg may have been the prerequisite to emulsion droplet stability. Conjugation of PEG to liposome surfaces via alkyl chains was associated with reduced zeta potential and increased repulsive forces between liposome droplets because of an increase of friction at the surface of liposomes and the presence of PEG on the liposome surface (*28*). In conclusion, oxidative sulfitolysis combined with covalent attachment of PEG to β-lg improved the stability of β-lg-stabilized emulsions at acidic as well as neutral pH.

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