Polymerization of Whey Proteins in Whey Protein-Stabilized Emulsions

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In emulsions stabilized by whey protein isolate, selective adsorption of β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) over bovine serum albumin and immunoglobulins occurred at the oil–water interface. High molecular weight protein polymers were progressively formed at the oil–water interface with increasing time following emulsion preparation. The formation of the high molecular weight protein polymers correlated with the disappearance of β-Lg ($r = -0.98$) and α-La ($r = -0.97$) over the 7-day storage period. The β-Lg concentration decreased from 50.7% of total whey proteins adsorbed at the interface immediately after emulsion preparation to 24.6% after 24 h, and α-La decreased from 14.7% initially to 10.3% after 24 h. Polymerization involved the formation of intermolecular disulfide cross-links between the monomeric proteins.

INTRODUCTION

Whey proteins are utilized in food products for both functional and nutritional purposes (Kinsella et al., 1989). Factors that affect the functional properties of whey proteins, such as gelling, emulsifying, and foaming ability, have been extensively reviewed (Kinsella and Soucie, 1989; De Wit, 1989). The relationship between the physicochemical properties of whey proteins and their ability to form and stabilize emulsions has been studied (Pearce and Kinsella, 1978; Halling, 1981; Leman and Kinsella, 1989; Dickinson, 1992). In emulsions, the formation of a stable protein film at the oil–water interface is dependent on protein diffusion, adsorption, spreading, and partial unfolding to allow rearrangement, reorientation, and interactions in the interfacial surface (Kinsella and Whitehead, 1987). Both surface hydrophobicity, which affects the affinity of the protein for the oil–water interface, and molecular flexibility, which influences the ability to unfold and interact with other proteins, are important factors determining the emulsifying activity of whey proteins (Shimizu et al., 1985; Damodaran, 1989).

Native β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), and immunoglobins (Ig) exist as globular proteins which have a tertiary structure stabilized by intramolecular disulfide bonds between cysteine residues (Kinsella and Whitehead, 1989). β-Lg, α-La, BSA, and immunoglobulin G (IgG) have 5, 8, 35, and 64 cysteine residues, respectively (Kinsella et al., 1989); β-Lg and BSA each have a free sulfhydryl group. In native dimeric β-Lg the sulfhydryl group is located in the interior of the globular protein and is therefore unavailable for interaction with free sulfhydryl groups or disulfide groups on other protein molecules. At pH 6.7 and above, the reactivity of the single thiol group of β-Lg is increased and intra- or intermolecular disulfide exchange reactions are induced (Dunnill and Greene, 1966). If the protein is denatured by heating, it unfolds and the free sulfhydryl group is then available for interaction (Kella and Kinsella, 1989; Kinsella and Whitehead, 1989). Previous studies have shown that disulfide bond formation is involved in gelation of heated whey protein solutions (Rector et al., 1989; Mulvihill et al., 1991), in urea-induced gelation of whey proteins (Xiong and Kinsella, 1990), and in the time-dependent polymerization of β-Lg and α-La in emulsions (Dickinson and Matsumura, 1991).

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The objective of the present study was to examine the effect of emulsion storage time on polymerization and the relative contributions of individual whey proteins to the polymerization reaction at the oil–water interface of a whey protein isolate (WPI)-stabilized emulsion.

MATERIALS AND METHODS

Reagents. WPI (>97% protein) was obtained from Le Sueur Isolates, Le Sueur, MN. *n*-Hexadecane, *N*-ethylmaleimide (NEM), and sodium azide were purchased from Sigma Chemical Co. Ltd., St. Louis, MO. Mono- and dibasic sodium phosphate were purchased from Fisher Scientific, Pittsburgh, PA.

Emulsion Preparation. Oil-in-water emulsions (21.7 wt % *n*-hexadecane in 0.05 M phosphate buffer, pH 7.01, containing 1 wt % WPI) were prepared using a Waring Blendor and a Rannie high-pressure laboratory homogenizer (five passes at 160 bar) (Klemaszewski et al., 1990). Sodium azide (0.02 wt %) was included in the buffer to inhibit microbial growth. After preparation, emulsions were diluted with buffer alone or with buffer containing appropriate concentrations of the sulfhydryl group blocking agent, NEM, to give 20.0 wt % oil-in-water emulsions with 0, 0.10, 0.25, and 0.50 mM NEM in the aqueous phase. Aliquots (4 mL) of each emulsion were stored at 20 °C in screw-cap vials (Fisher Scientific) for up to 7 days.

SDS–PAGE. Following storage for specific time periods, NEM (20 mM) was incorporated into emulsion samples to stop sulfhydryl group-mediated polymerization reactions. Proteins adsorbed at the oil–water interface were separated as described by Dickinson and Matsumura (1991). The quantity of protein in the unadsorbed and adsorbed protein fractions was determined according to the Lowry method (Lowry *et al.*, 1951). The protein fractions were analyzed by SDS–PAGE, without 2-mercaptoethanol, on 12.5% acrylamide gels following the procedure of Laemmli (1970). Gels were run on a Hoefer Mighty Small vertical slab electrophoresis unit (Hoefer Scientific Instruments, San Francisco). Relative concentrations of proteins and polymers were determined on an LKB Ultrascan XL scanning densitometer. The experiment was performed in triplicate.

RESULTS

Increasing amounts of large protein polymers accumulated with time after emulsion preparation for up to 7 days (Figure 1, lanes 1–9). These proteins formed a continuous band between BSA (MW 66 200) and Ig (MW 160 000), while the higher molecular weight polymeric forms (MW >160 000) remained at the top of the gel. The formation of the high molecular weight proteins occurred at the expense of the monomeric whey proteins, particularly α-La and β-Lg (Figure 1). The changes in the profiles of the adsorbed protein fractions were most dramatic

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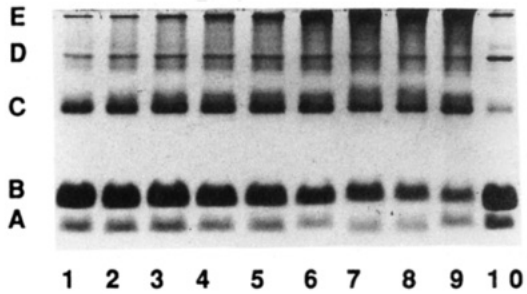

Figure 1. SDS–PAGE of adsorbed proteins from a WPI-stabilized emulsion at various time intervals after emulsion formation: (lane 1) adsorbed protein immediately after emulsion formation; (lanes 2, 3, 4, 5, 6, 7, 8, 9) adsorbed protein obtained after the emulsions were stored at 20 °C for 1, 2, 4, 6, 24, 48, 72, and 168 h, respectively; (lane 10) WPI solution used in emulsion preparation. (A) α-La (MW 14 200); (B) β-Lg (MW 18 400); (C) = dimers of α-La and β-Lg; (D) BSA (MW 66 200); (E) = Ig (MW 160 000).

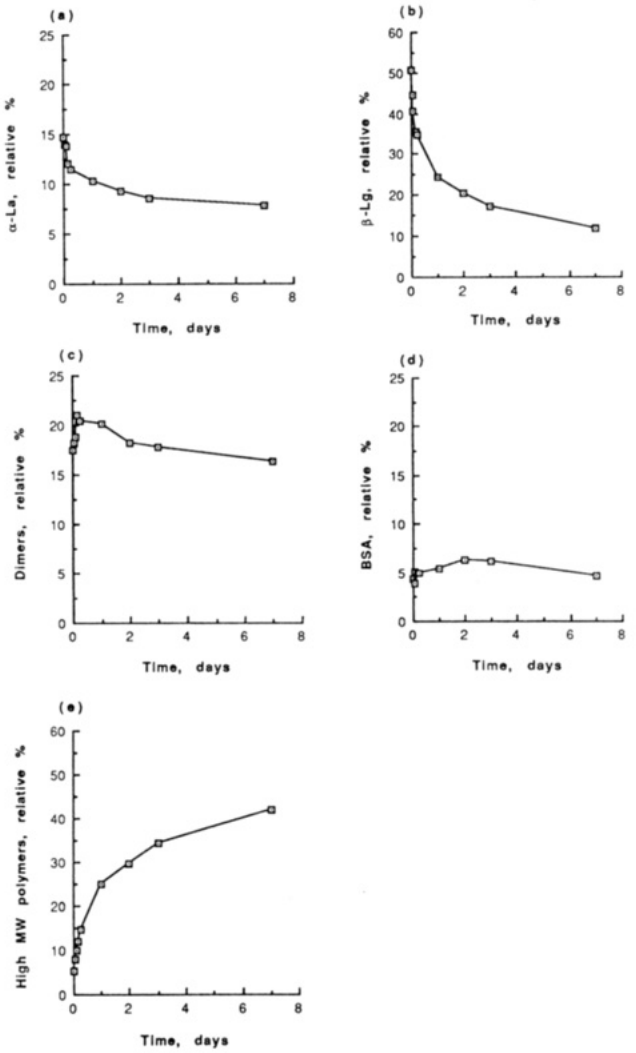

Figure 2. Effect of storage time on the relative concentrations of (a) α-La, (b) β-Lg, (c) dimeric proteins, (d) BSA, and (e) high molecular weight polymers (MW > 160 000) in the adsorbed protein fraction of a WPI-stabilized emulsion.

during the first 24 h after emulsion preparation and particularly in the first 2 h (Figure 2). The relative β-Lg concentration in the adsorbed protein fraction decreased from 50.7% to 24.6% in the first 24 h after emulsion formation and α-La concentration from 14.7% to 10.3% over the same period (Figure 2a,b). After 7 days of storage, β-Lg and α-La concentrations had fallen to 11.9% and 7.8%, respectively. Dimeric proteins increased in the first

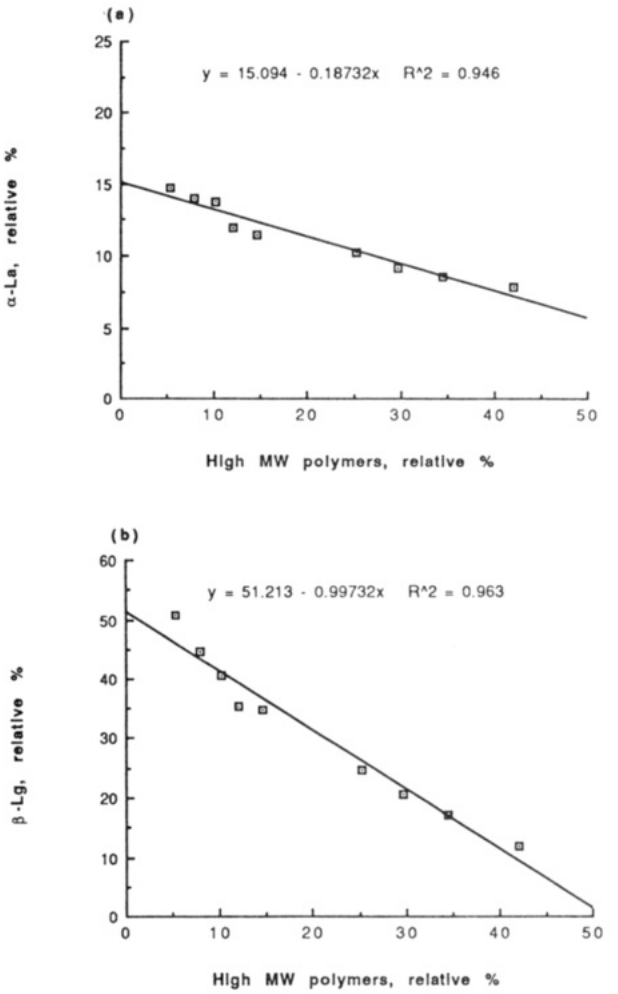

Figure 3. Relationship between relative concentrations of (a) α-La and (b) β-Lg and high molecular weight polymers in the adsorbed protein fraction of a WPI-stabilized emulsion stored for up to 7 days.

24 h and then declined (Figure 2c). This is consistent with the view that dimers form initially from the interaction of monomeric whey proteins and subsequently interact with each other and other whey proteins to form larger polymers (Rector et al., 1989).

The reduction in concentrations of monomeric β-Lg and α-La with time was shown to be directly related to formation of the high molecular weight polymers (Figure 3). The data indicated a strong negative correlation between β-Lg ($r = -0.98$) and α-La ($r = -0.97$) concentration in the adsorbed whey protein fraction and high molecular weight polymer formation.

Using the sulfhydryl blocking agent NEM, the role of free sulfhydryl groups on polymerization of adsorbed proteins was investigated (Figure 4). Addition of 0.1 mM NEM the emulsion immediately after preparation led to a reduction in the rates of disappearance of β-Lg (Figure 4a) and α-La (Figure 4b) and a reduction in the rate of formation of the high molecular weight proteins (Figure 4c). At 0.25 mM NEM, changes in the concentrations of β-Lg, α-La, and the high molecular weight protein components with time were virtually eliminated. At 0.5 mM NEM polymerization was completely inhibited.

A comparison of the electrophoretic pattern of the WPI in solution prior to emulsion formation (Figure 1, lane 10) with that of the absorbed protein immediately after emulsion preparation (Figure 1, lane 1) showed more intense dimeric protein banding and less intense BSA and

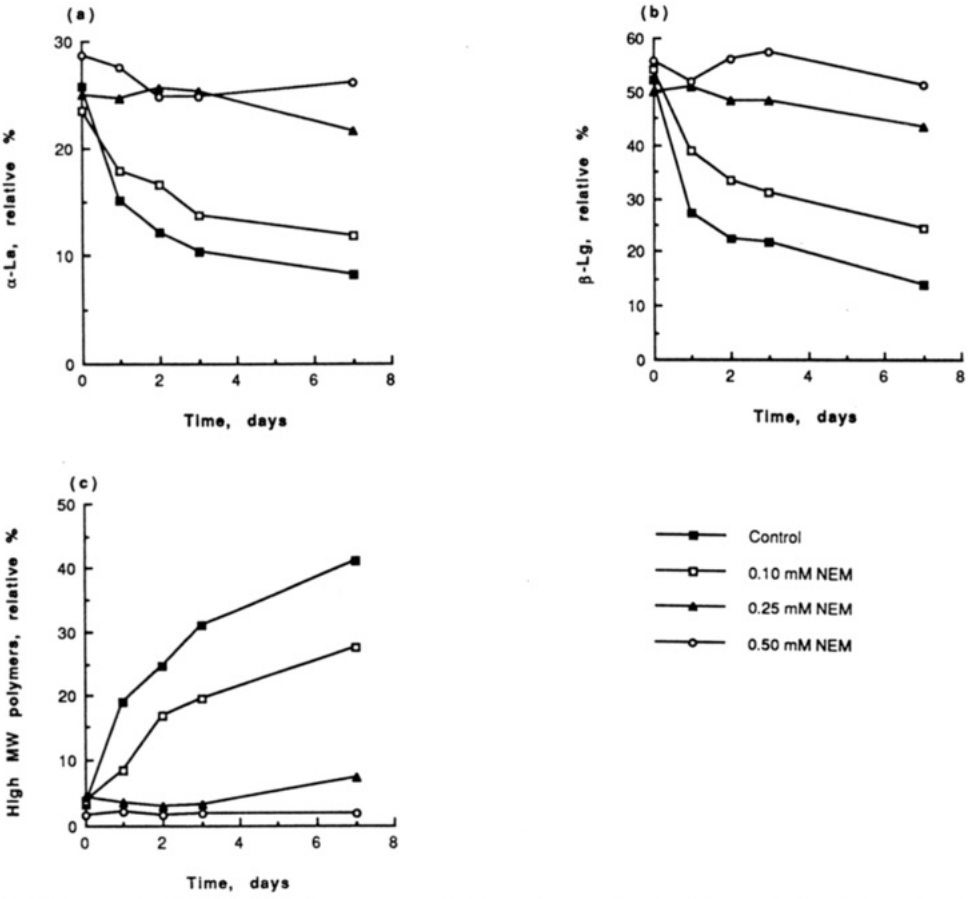

Figure 4. Effect NEM concentration on the relative concentrations of (a) α-La, (b) β-Lg, and (c) high molecular weight polymers in the absorbed protein fraction of a WPI-stabilized emulsion for up to 7 days.

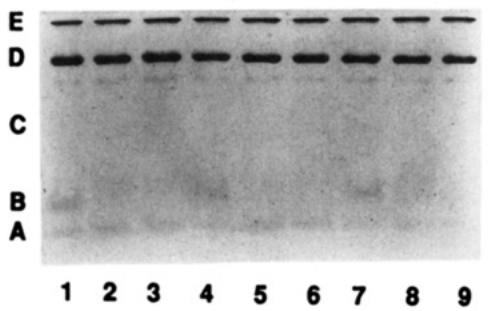

Figure 5. SDS–PAGE of unadsorbed proteins from a WPI-stabilized emulsion at various time intervals after emulsion formation: (lane 1) adsorbed protein immediately after emulsion formation; (lanes 2, 3, 4, 5, 6, 7, 8, 9) adsorbed protein obtained after the emulsions were stored at 20 °C for 1, 2, 4, 6, 24, 48, 72, and 168 h, respectively. (A) α-La (MW 14 200); (B) β-Lg (MW 18 400); (C) dimers of α-La and β-Lg; (D) BSA (MW 66 200); (E) Ig (MW 160 000).

Ig banding in the adsorbed protein compared to the original WPI solution. Densitometric analyses showed that the amount of the dimeric proteins was 2.9-fold higher in the absorbed protein fraction (lane 1) compared to the WPI solution (lane 10). The BSA band was 2.2-fold higher and the Ig band 1.9-fold higher in the WPI solution compared to the adsorbed protein fraction. A possible explanation is that selective adsorption of some whey proteins at the oil–water interface occurred during emulsion formation and accounts for the difference in electrophoretic patterns. In support of this theory SDS–PAGE analysis of the unadsorbed fractions obtained after emulsion formation (Figure 5) indicated that the unadsorbed fraction was almost exclusively composed of BSA and Ig, while the β-Lg and α-La bands were barely visible. This suggests that β-Lg and α-La, and their covalently bonded dimers, are adsorbed preferentially over the other whey protein components at the oil–water interface during emulsion preparation.

DISCUSSION

The results show that in a WPI-stabilized model emulsion system both β-Lg and α-La undergo polymerization reactions with time after emulsion formation. Dickinson and Matsumura (1991) showed that disulfide bonding between monomeric β-Lg was responsible for the formation of dimers, trimers, and other polymeric forms of β-Lg in a β-Lg-stabilized emulsion. In contrast, in an α-La-stabilized emulsion, polymerization of monomeric α-La was not detected, reflecting the absence of a free –SH group in α-La. In a model system containing equal concentrations of both α-La and β-Lg, however, α-La was shown to participate in the polymerization reaction and in doing so reduced the frequency of β-Lg–β-Lg interactions (Dickinson and Matsumura, 1991). Thiol–disulfide interchange between free –SH of β-Lg and –SS– bonds of α-La is likely to explain the involvement of α-La in the polymerization reaction in model systems containing both β-Lg and α-La. At the β-Lg/α-La ratio present in whey, our results confirm those of Dickinson and Matsumura (1991) in showing an involvement for both β-Lg and α-La in time-dependent polymerization at the oil–water interface. In addition, it appears that the relative contribution, if any, of BSA to polymerization at the oil–water interface is minimal. With regard to the Ig fraction, the banding on SDS–PAGE gels due to the high molecular weight polymers masked any changes in the intensity of Ig bands (Figure 1). Proportionately more Ig remained in the unabsorbed protein fraction compared to α-La and β-Lg.

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