Formation of Stable Protein Foam by Intermolecular Disulfide Cross-Linkages in Thiolated α_{s1}-Casein as a Model

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Relationships between the disulfide bond formation of protein and the foam stability of a solution of the protein were examined with α_{s1}-casein into which thiol groups had been introduced as a model. When a 0.1% solution of the thiolated α_{s1}-casein was oxidized with potassium ferricyanide and foamed after 1 min, there was a 1.8-fold increase in the foam stability compared to that of the nonoxidized system. HPLC analyses and results of dithiothreitol treatment showed that this increase is due to the formation of a protein polymer through intermolecular disulfide bonds. The foam of the oxidized protein solution was stabilized more as the amount of protein polymer with higher molecular weight increased. A very stable foam was obtained when the thiolated α_{s1}-casein solution was treated with potassium ferricyanide just after foaming; there was a 4-fold increase in the half-life compared to that of oxidization before foaming. Small numbers of thiol groups introduced into the protein improved the stability of the foam. To judge from these results, the stabilization of the protein foam seemed to involve disulfide bond formation before or just after foaming.

Thiols and disulfides have essential roles in both the structure and function of proteins, including the formation and stabilization of the higher order structure, expression of enzyme activity, and production of rheomechanical properties in food proteins. In food proteins in particular, these groups are related to various functional properties, such as dough formation by wheat-flour proteins (Huebner et al., 1977). Foaming is an important functional property of food proteins also much influenced by thiols and disulfides. Foam stability is affected by the tertiary structure of proteins (Graham and Phillips, 1979), which is stabilized by disulfide bonds. There have been some reports on the effects of disulfide bonds on the foam stability of proteins. For example, controlled reduction of the number of disulfide bonds in glycinin improved its foam stability by facilitating greater protein–protein interaction (Kim and Kinsella, 1987); on the other hand, glutenin, which has broken disulfide bonds, had decreased foam stability because of the lowering of the surface viscosity (Mita et al., 1978).

Many studies of the rheomechanical characterization of food proteins have analyzed component proteins in their native state. However, when proteins are considered a functional material used for purposes other than as a food ingredient, one fruitful approach may be to modify the protein function by chemical or enzymatic methods. The results obtained from chemically modified proteins could also provide information for the design of new functional proteins in protein engineering. One such approach was called "protein tailoring" in a review by Feeney (1986).

Milk casein, especially α_{s1}-casein, a familiar animal protein, exists in a partially unfolded state and is a so-called flexible protein. α_{s1}-Casein is well-characterized and easy to handle and has been cloned in *Escherichia coli* by Nagao et al. (1984), so it can be used as a basal protein or a template for tailoring by protein engineering. As α_{s1}-casein has no cysteine residue, α_{s1}-casein into which thiol groups have been introduced would also be a convenient material for examination of the role of thiol groups in the expression of protein function. Using thiolated α_{s1}-casein, we have found that disulfide bond formation just after foaming enhances the stability of protein foams (Okumura et al., 1989). In this study, we further investigated the relationship between the stability of protein foams and the protein polymer formed by the oxidation of thiol groups. Our system with α_{s1}-casein with thiol groups chemically introduced into its lysine residues and oxidized by potassium ferricyanide before or after foaming is one model for the study of protein functionality expressed by disulfide bonds.

MATERIALS AND METHODS

Materials. α_{s1}-Casein was prepared from fresh cow's milk by the method of Zittle and Custer (1963). *N*-Acetyl-D,L-homocysteine thiolactone (AHTL) was purchased from Nacalai tesque, Inc. (Kyoto, Japan). Other chemicals were of guaranteed reagent grade. Two buffers were used: buffer A was 100 mM sodium phosphate buffer (pH 8.0), and buffer B was 50 mM sodium phosphate buffer (pH 7.0).

Preparation of Thiolated α_{s1}-Casein. α_{s1}-Casein was thiolated basically by the method of Benesch and Benesch (1958). Typical experimental conditions were as follows: a solution of α_{s1}-casein (450 mg) in buffer A (150 mL) containing 8 M urea bubbled with N_2 was mixed with AHTL (2.3 g) and incubated at 37 °C for 3 days. The solution was concentrated to 10 mL by ultrafiltration (UHP-43K, Advantec Toyo, Tokyo, Japan) and dialyzed against buffer A. Some of the thiol groups introduced into the casein were oxidized by air during this treatment, so they were reduced with 1 M mercaptoethanol in the presence of 8 M urea and then desalted immediately at 4 °C just before use. The number of thiols was estimated by the method of Sedlak and Lindsay (1968) with Ellman's reagent. Under the conditions used, 7.5 mol of thiol groups was introduced per mole of casein. The number of thiol groups introduced was changed by adjustment of either the ratio of α_{s1}-casein to AHTL or the reaction time. The protein concentration was measured with a Bio-Rad assay kit.

Foaming and Estimation of Foam Stability. Foam was produced by stirring on a small scale. Two milliliters of a 0.1% protein solution in buffer A was placed in a round-bottom tube

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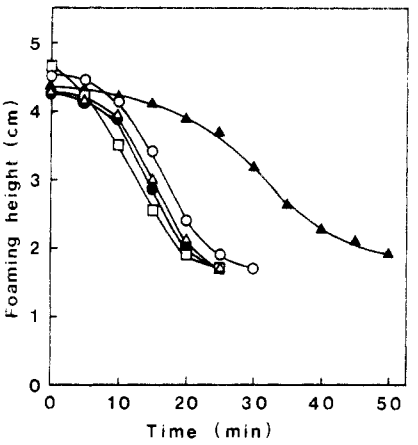

Figure 1. Foam stability of thiolated α_{s1}-casein oxidized with potassium ferricyanide. Thiolated α_{s1}-casein solution was foamed after the addition of various reagents: 5 mM $K_3Fe(CN)_6$ (▲), 5 mM DTT (●), both 5 mM $K_3Fe(CN)_6$ and DTT (△), and none of these (○). To thiolated α_{s1}-casein modified with iodoacetamide were added both 5 mM $K_3Fe(CN)_6$ and DTT (□).

(13 × 60 mm). A homogenizer (NK Micronizer; Nihon Seiki Kaisha Ltd., Tokyo, Japan) with rotating two-bladed knives was used at full speed (15 000 rpm) for 1 min. After the stirring, the knives were withdrawn and the height of the foam was measured at room temperature. The foam stability was defined as the half-life ($T_{1/2}$; in minutes) of the sum of the height of the initial solution plus the height of the foam just after the stirring.

Oxidation of Thiolated α_{s1}-Casein. Protein–thiol groups were oxidized by the addition of 5 mM potassium ferricyanide as an oxidant. The timing of the formation of disulfide bonds was varied by the addition of an oxidant before or just after foaming. When oxidation was to occur before foaming, potassium ferricyanide was added to the protein solution and the solution was foamed after 1 min. Almost all of the thiol groups of protein were oxidized under these conditions. When oxidation was to occur just after foaming, the protein solution was stirred for 1 min, potassium ferricyanide was added immediately, and stirring was done for 10 s for uniform dispersal of the oxidant.

HPLC Analysis. A Tosoh liquid chromatograph system with a Model CCPM pump, a Model PX-8000 controller, and a Model UV-8000 variable-wavelength detector was used. The signal was electronically integrated by a Sic Chromatocorder 12 integrator. Five to 20 μL of the protein solution was injected, and separation was done on a 300 × 7.8 mm (i.d.) column packed with TSK gel G3000SW_{XL}. The column was developed with buffer B. The flow rate was 1 mL/min, and detection was at 280 nm.

RESULTS AND DISCUSSION

We used AHTL, a convenient chemical modifier that attacks the amino groups of proteins, as the thiolation agent. In almost all of the experiments, thiolated α_{s1}-casein into which 7.5 mol of thiol groups was introduced per mole of protein was used, except for the experiment in which different numbers of thiol groups were introduced.

Foam Stability of Thiolated α_{s1}-Casein Oxidized by Potassium Ferricyanide. When 5 mM potassium ferricyanide was added to a 0.1% solution of thiolated α_{s1}-casein and the solution was foamed after 1 min, there was a 1.8-fold increase in the foam stability (Figure 1) as calculated from the half-life. This effect was not observed when 5 mM dithiothreitol was added to the protein solution before foaming or when thiolated α_{s1}-casein chemically modified with iodoacetamide was used as a protein. This indicates that the foam stabilization by the oxidant was due to disulfide bond formation.

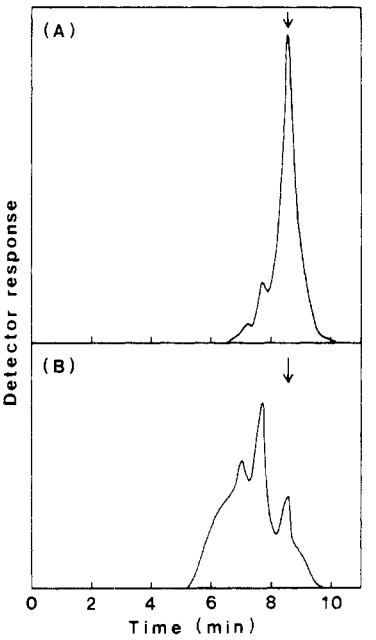

Figure 2. HPLC elution profiles on a TSK gel G3000SW_{XL} column of the foam protein from systems untreated (A) or treated (B) with potassium ferricyanide. The position of thiolated α_{s1}-casein is indicated with the arrow.

The foam was removed with a pipet after 20 min and was centrifuged at 2000*g* for 5 min at 20 °C, which caused the foam to collapse and resulted in a solution without precipitate. This solution was analyzed by HPLC with a TSK gel G3000SW_{XL} column. Figure 2 shows HPLC profiles of the foam protein of thiolated α_{s1}-casein solution untreated or treated with potassium ferricyanide. When potassium ferricyanide was added, protein polymer formed in the foam protein (Figure 2B). This protein polymer did not form when dithiothreitol was added (data not shown). These results suggested that the increased foam stability arising from the oxidation was based on the formation of protein polymer through intermolecular disulfide bonds.

Relationship between Protein Polymerization and Foam Stability. The effects on foam stability of polymer formation in the protein solution were examined by use of thiolated α_{s1}-casein of high polymerization. To different concentrations of thiolated α_{s1}-casein (7.5 mol of thiol groups/mol of casein; 1.0, 3.0, or 15 mg/mL) was added potassium ferricyanide, and the mixture was diluted to 1.0 mg/mL casein with buffer A after 1 min. These solutions were then analyzed by HPLC with a TSK gel G3000SW_{XL} column and were stirred at this protein concentration of 1.0 mg/mL for comparison of the foam stability. Figure 3 shows the HPLC profiles of the solutions. As the protein concentration increased, the protein detected was more highly polymerized. The foam was more stable as the amount of protein polymer in the solution increased (Table I). These results indicated that the foam of the protein solutions was stabilized by protein polymers of high molecular weight. The mechanism probably involves an increase in the viscosity of the bulk solution inside the lamellae forming a bubble because of protein polymer formation arising through intermolecular disulfide bonds. Most of the sulfhydryl groups of the protein were confirmed to be oxidized immediately under these conditions. The initial height of foam was lower as the amount of protein polymer in the solution increased (Table I and Figure 3).

Foam Stability of Thiolated α_{s1}-Casein Oxidized after Foaming. The foam oxidized just after foaming

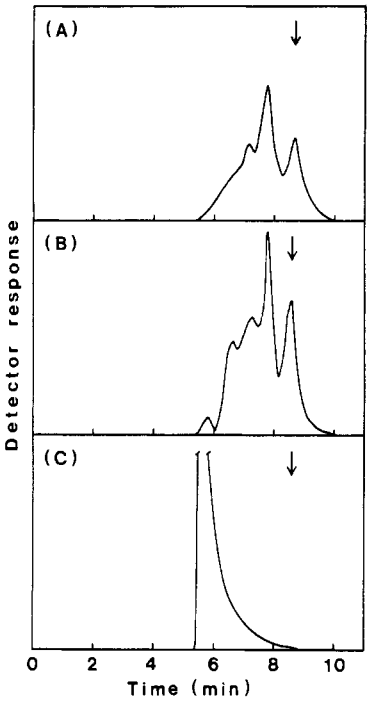

Figure 3. HPLC elution profiles on a TSK gel G3000SW$_{XL}$ column of thiolated α_{s1}-casein oxidized at different concentrations: (A) 1.0, (B) 3.0, and (C) 15 mg/mL. The position of thiolated α_{s1}-casein is indicated with the arrow.

Table I. Relationship between Formation of Protein Polymer and Foam Stability[a]

protein concn, mg/mL			
oxidized	foamed	$T_{1/2}$, min	initial foaming height, cm
	1.0	16.6	4.5
1.0	1.0	32.8	4.3
3.0	1.0	45.9	4.1
15	1.0	106	3.9

[a] Different concentrations of thiolated α_{s1}-casein solution were oxidized, and then each solution was diluted to 1 mg/mL and foamed.

Table II. Foam Stability of Thiolated α_{s1}-Casein Oxidized with Different Timing

timing of oxidation by 5 mM $K_3Fe(CN)_6$	addition of 5 mM DTT	$T_{1/2}$, min
thiolated α_{s1}-casein	−	16.6
before foaming	−	32.8
before foaming	+	15.3
just after foaming	−	120
just after foaming	+	17.0

was more stable than that oxidized before foaming, with about a 4-fold increase in the half-life (Table II). Foam stability was strongly affected by the timing of the formation of disulfide bonds. To find whether a highly polymerized protein is formed in foam oxidized just after foaming, the foam protein was analyzed by HPLC with a TSK gel G3000SW$_{XL}$ column as described above and compared with the foam protein oxidized before foaming (Figure 4). The HPLC profiles of the two systems were similar. These results indicated that the molecular sizes of the protein formed by addition of potassium ferricyanide are similar whether oxidation occurs before or after foaming.

Mechanism of Stabilization of the Foam Containing Disulfide Cross-Linked Protein. A diagram of the possible mechanism of the enhanced stability of protein foam arising from disulfide bond formation before or just after foaming is shown in Figure 5. α_{s1}-Casein has an unfolded structure and self-associates (Chiba et al., 1970),

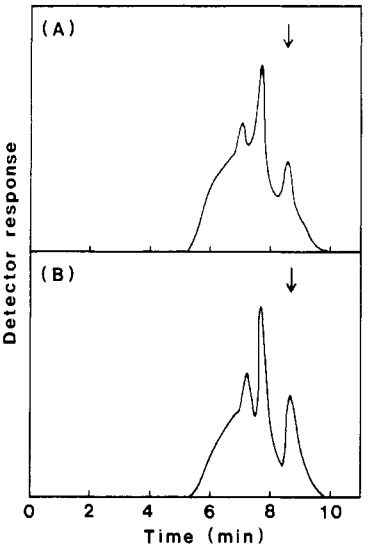

Figure 4. HPLC elution profiles on a TSK gel G3000SW$_{XL}$ of the foam protein from the systems oxidized before (A) or just after (B) foaming. The position of thiolated α_{s1}-casein is indicated with the arrow.

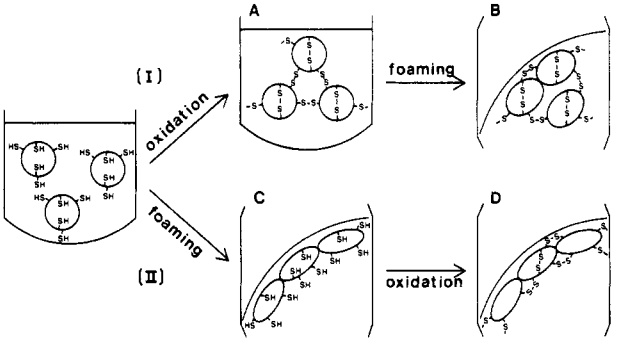

Figure 5. Possible changes in disulfide cross-linked protein at the air–water interface depending on timing of oxidation and foaming.

but in the figure, thiolated α_{s1}-casein is shown as a globular shape, for convenience. A protein foam is formed via several processes (Phillips, 1981). First, protein molecules reach the air–water interface through diffusion and adsorption. Next, they penetrate into the surface and are rearranged by conformational changes and protein–protein interactions (Kitabatake and Doi, 1987). By treatment with potassium ferricyanide before foaming, thiolated α_{s1}-casein was polymerized in the solution by the formation of intermolecular disulfide bonds (Figures 5IA). During foaming, the polymerized proteins diffuse and adsorb at the air–water interface, where they are rearranged with conformational changes (Figure 5IB). However, with treatment just after foaming, thiolated α_{s1}-casein is first rearranged at the air–water interface with conformational changes and the protein–protein interaction (Figure 5IIC). By oxidation in the next step, disulfide networks are formed, giving a stable protein film (Figure 5IID).

α_{s1}-Casein has a flexible amphipathic structure, so it needs little specific unfolding at the surface for high foamability (Kitabatake and Doi, 1982). In our system containing modified casein, however, intramolecular disulfide bonds may decrease the protein flexibility and the conformational change, particularly with oxidation before foaming [Figure 5IB; see also Kato et al. (1986)]. Perhaps from stability was increased with oxidation before foaming in spite of decreased protein flexibility because foam collapse would be inhibited by the increased vis-

Table III. Relationship between the Number of Thiol Groups Introduced and Foam Stability[a]

thiol groups introduced, mol/mol of α_{s1}-casein	ratio[b] of $T_{1/2}$
1.0	0.75
1.4	2.3
2.7	2.3
7.5	6.7
8.9	12
11	5.0

[a] Thiolated α_{s1}-casein with different numbers of thiol groups was prepared and the foam stability was compared, with oxidation with potassium ferricyanide done just after foaming. [b] Ratio of $T_{1/2}$ = ($T_{1/2}$ of the system treated with $K_3Fe(CN)_6$)/($T_{1/2}$ of the untreated system).

cosity of the bulk solution inside the lamellae, which contain the protein polymer.

Number of Thiol Groups Needed for the Enhancement of Foam Stability. Thiolated α_{s1}-caseins with various numbers of thiol groups introduced were prepared, and foam stability was examined in samples oxidized just after foaming. The introduction of 1.4 mol of thiol groups/mol of casein stabilized the foam (Table III). The foam obtained was most stable when 8.9 mol of thiol groups was introduced into the casein. The lowering of the foam stability of the most thiolated casein prepared (11 mol of thiol groups introduced per mole of casein) might arise from protein flexibility being decreased through the formation of an excess of intramolecular disulfide bonds. A small degree of modification to improvement of a functional property of the food protein is of practical importance for protein engineering, although α_{s1}-casein self-associates, and an aggregate of the associated casein into which a few thiol groups have been introduced may have several such groups.

The enzymatic introduction of cysteine residues into protein has been reported by Ikura et al. (1981); in our experiment, chemical modifier was used for the protein thiolation. For the disulfide formation, we used potassium ferricyanide as the oxidant. The same results described here were also obtained with *o*-iodosobenzoic acid as the oxidant (data not shown). As an enzymatic modifier, sulfhydryl oxidase may be used for this purpose (Janolino and Swaisgood, 1975). There have been some reports of the improvement of protein functionality by chemical and enzymatic modification (Whitaker, 1986; Arai et al., 1986; Chiba et al., 1983). By these various techniques, novel proteins with useful properties may be constructed.

ABBREVIATIONS USED

AHTL, *N*-acetyl-D,L-homocysteine thiolactone; buffer A, 100 mM sodium phosphate, pH 8.0; buffer B, 50 mM sodium phosphate, pH 7.0.

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