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Functional Properties of Cross-Linked Lysozyme and Serum Albumin

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The importance of the flexibility of protein molecules in determining their foaming and emulsifying properties was investigated using egg white lysozyme and bovine serum albumin. Samples of the two proteins, which differ in their flexibility and functional properties, were cross-linked intramolecularly by treatment with iodine or a diphenyl sulfone reagent. The properties of native and treated proteins were compared. The monomeric cross-linked proteins were resistant to heat-induced conformational changes, as indicated by circular dichroism, and were also resistant to hydrolysis by chymotrypsin. These results indicate reduced flexibility. The cross-linking greatly reduced the foaming power and foam stability of both proteins. There was a similar but less marked change in the emulsifying activity and emulsion stability. The findings confirm the importance of protein flexibility in determining foaming and emulsifying properties of proteins.

INTRODUCTION

Since the determination method of protein surface hydrophobicity was established (Kato and Nakai, 1980), a number of studies (Kato et al., 1983a; Nakai, 1983; Shimizu et al., 1983) have been done on the relationship between protein structural and functional properties such as emulsifying and foaming properties. It is reasonable to assume that protein surface hydrophobicity plays a governing role in emulsification and foaming, because amphiphilic proteins possessing high surface hydrophobicity are strongly adsorbed at the interface between oil or air and water to cause a pronounced reduction of interfacial or surface tension that readily facilitates emulsification and foaming. There are apparent relationship between the hydrophobicity and functional properties, but other factors are also involved in addition to this characteristic. We have reported that the flexibility of protein structure detected by protease digestion method is also an important structural factor governing the emulsion and foaming (Kato et al., 1985). If proteins, though the surface hydrophobicity is originally low, are susceptible to denaturation at the oil–water and air–water interface, the surface hydrophobicity may increase at the interface to result in good foaming and emulsifying properties.

The flexibility of proteins can be defined as the denaturation equilibrium under physiological conditions. A considerable N $\rightleftharpoons$ D transition in proteins is observed even under physiological conditions. The equilibrium constant (K_D) for the N $\rightleftharpoons$ D transition in native proteins is in the range of about 10^{-5}–10^{-6}. Even if the K_D value increases in the range of 10^{-2}–10^{-3} under mild denaturation conditions, e.g. at oil or air and water interface, the amounts of proteins in the D state are less than 1%. Therefore, the flexibility is hardly detectable by routine optical methods. This is possible to detect by the H–D exchange technique and the protease probe method (Imoto et al., 1976; Ueno and Harrington, 1984). It was suggested in the previous paper (Kato et al., 1985) that there was an apparent relationship between the flexibility and functional properties of proteins. However, there is little information on protein flexibility at oil or air and water interface, and it is desirable to elucidate directly an importance of protein flexibility in foam and emulsion formation. This may be proven by using intramolecularly cross-linked protein inhibiting the flexibility.

Imoto et al. (1973) reported that lysozyme was cross-linked intramolecularly at the specific sites by iodine oxidation. Wold (1961) reported that bovine serum albumin was also intramolecularly cross-linked by the bifunctional reagent. These cross-linked proteins seem to decrease the flexibility.

This paper describes the foaming and emulsifying properties of cross-linked lysozyme and albumin and discusses the importance of protein flexibility in foaming and emulsification.

MATERIALS AND METHODS

The preparation of cross-linked lysozyme was carried out by the method of Imoto et al. (1973). Lysozyme was prepared from fresh egg white by a direct crystallization method and recrystallized five times. Lysozyme, 200 mg/10 mL of water, was reacted for 4 h at room temperature with 0.6 mol of I_2/mol of protein added in five portions of 0.04 M I_2 /.48 M KI solution. The pH was kept at 5.5 during reaction by the addition of NaOH solution. Reaction of each portion of triiodine solution was complete before addition of the next, as judged by base uptake and disappearance of iodine color. During this reaction, the oxindole C-2 of tryptophan 108 forms an ester cross-link between the carboxyl group of glutamic acid 35. The products were dialyzed against distilled water and lyophilized. The lyophilized products were purified by ion-exchange chromatography on a column (1.7 × 100 cm) of Bio-Rex 70, equilibrated with 0.013 M sodium borate/0.037 M sodium carbonate buffer, pH 10.0, and eluted with a linear gradient over 2 L to 0.08 M NaCl in the same buffer. The slowest peak of three major peaks was the lysozyme cross-linked between Glu-35 and Trp-108. This peak was

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collected and lyophilized after dialysis against distilled water.

The cross-linked BSA (bovine serum albumin) was prepared by the method of Wold (1961). BSA was purchased from Sigma Chemical Co. (St. Louis, MO). To a 1% protein solution (15 mL) in 1% carbonate buffer, pH 10.7, was added 0.5 mL of acetone solution containing 7.8 mg of FNPS [bis(4-fluoro-3-nitrophenyl) sulfone]. The reaction mixture was left at room temperature by stirring for 2 h. The product was dialyzed at 4 °C against deionized water for 24 h and lyophilized. The lyophilized product was further purified by a gel filtration on a Sephadex G-100 column (2 × 100 cm). The monomer cross-linked BSA was collected and lyophilized after dialysis against distilled water.

Protease digestion was performed as follows: To 4 mL of 0.1% protein solution in 0.05 M Tris-HCl buffer, pH 8.0, was added 250 μL of 0.1% α-chymotrypsin.

Enzymatic reaction was carried out at 38 °C for a given time in the protein to enzyme ratio of 16:1. After protease digestion, 4 mL of 10% aqueous trichloroacetic acid was added to remove the native protein, and then the precipitates were removed by filtration with filter paper (Toyo Roshi Ltd.; No 5b). The amount of amino acids and peptides in the filtrate was estimated by measuring the absorbance at 280 nm.

Cross-linked preparations of lysozyme and bovine serum albumin were analyzed for polymers by gel filtration using high-performance liquid chromatography (HLC-805; Toyo Soda Co.) on a TSK Gel G3000SW column (Toyo Soda Co.; 0.75 × 30 cm). Protein solutions (0.1%) in 0.2 M phosphate buffer, pH 6.9, were applied on a column at a flow rate of 0.8 mL/min. A variable-length UV detector (SF 770; Toyo Soda Co.) was used to monitor the effluent at 280 nm.

Circular dichroism of proteins was measured with a Jasco Model J-20C spectropolarimeter. Samples were heated in an incubator at the rate of 2 °C/min from 30 °C to a given temperature and then immediately cooled to room temperature in cold water. The helix content of proteins was estimated from the values of the ellipticity at 222 nm at 20 °C.

The foaming power and foam stability of proteins were determined by measuring the electric conductivity of foams when air was introduced into 5 mL of 0.1% protein solution in 0.1 M phosphate buffer, pH 7.4, in a glass filter (G-4) at a constant flow rate, 90 cm^3/min, for 15 s (Kato et al., 1983b). After air was introduced into proetin solution for 15 s, the conductivity of foams was measured with time. Foaming power was indicated as the conductivity of foams produced immediately after the introduction of air into protein solution for 15 s.

The emulsifying activity and emulsion stability of proteins were determined by the method of Pearce and Kinsella (1978). To prepare emulsions, 1.3 mL of corn oil and 4 mL of 0.1% protein solution in 0.1 M phosphate buffer, pH 7.4, were homogenized in Ultra Turrax (Hansen & Co.) at 12000 rpm for 1 min at 20 °C. A $^1/_{10}$-mL portion of emulsion was taken from the bottom of the container at 1-min intervals immediately after homogenization and diluted with 5 mL of 0.1% SDS solution. The turbidity of diluted emulsion was then measured at 500 nm with time.

RESULTS AND DISCUSSION

Bovine serum albumin has been shown in the previous papers (Kato and Nakai, 1980; Kato et al., 1985) to have excellent foaming and emulsifying properties. These good functional properties may be due to the high surface hydrophobicity or the flexible conformation. On the other hand, lysozyme has poor foaming and emulsifying properties. These proteins, which differ in their properties, can be cross-linked intramolecularly. Lysozyme is cross-linked between Glu-35 and Trip-108 by iodine oxidation (Imoto et al., 1973). Bovine serum albumin is also intramolecularly cross-linked between lysine and tyrosine or histidine which may be located in the suitable position to form covalent bridges between distant parts of the polypeptide chain by the bifunctional reagent FNPS (Wold, 1961). These cross-linked proteins prepared in this experiment were confirmed to be in monomeric form from the patterns of high-performance liquid chromatography. Figure 1 shows changes in the ellipticity at 222 nm of circulate dichroism in native and cross-linked lysozyme and bovine serum albumin during heating. This result suggests that lysozyme and bovine serum albumin were converted to stable forms to heating by iodine and FNPS treatments, respectively. Thus, it was suggested that lysozyme and bovine serum albumin are intramolecularly cross-linked. This is further confirmed from the susceptibility to protease (Figure 2). The cross-linked lysozyme and serum albumin were resistant to protease, as shown in Figure 2. Flexible serum albumin may be converted to rigid form by cross-linking, and rigid lysozyme may be converted to more rigid form by corss-linking.

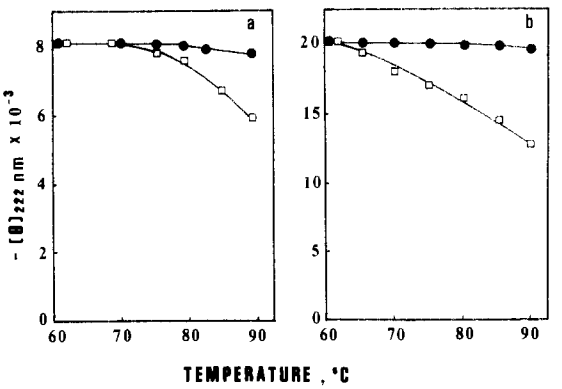

Figure 1. Thermal denaturation curves of cross-linked lysozyme and serum albumin. The ellipticity at 222 nm of circular dichroism was used as an indication of denaturation and measured after samples heated at a given temperature were cooled to 20 °C: a, lysozyme; b, serum albumin. Key: □, native protein; ●, cross-linked protein.

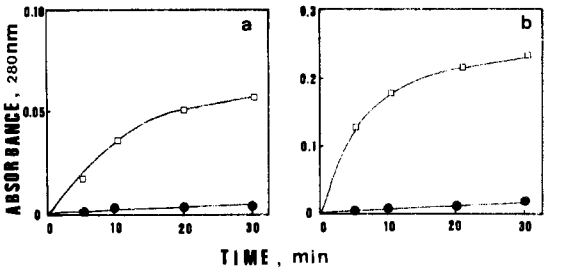

Figure 2. Time course of Proteolytic digestion in cross-linked lysozyme and serum albumin. The absorbance indicates the reading of TCA filtrates: a, lysozyme; b, serum albumin. Key: □, native protein; ●, cross-linked protein.

The functional properties of cross-linked proteins were investigated to elucidate the importance of flexibility. As shown in Figure 3, the foaming properties of cross-linked lysozyme and bovine serum albumin greatly decreased. The foaming power of lysozyme is originally low (480 μv/cm) and was greatly lowered (50 μv/cm) by cross-linking. The foam stability of cross-linked lysozyme is extraordinarily low, and the foam was diminished with in

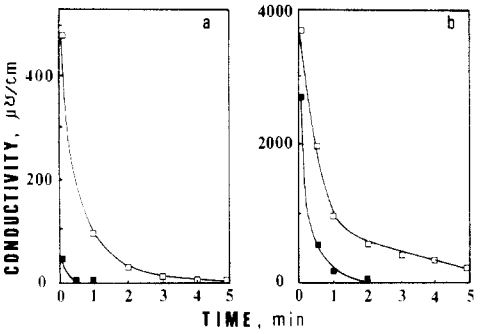

Figure 3. Foaming properties of cross-linked lysozyme and serum albumin. The foaming power and foam stability were determined by measuring the electric conductivity (μv/cm, the reciprocal of resistance) of foam: a, lysozyme; b, serum albumin. Key: □, native protein; ■, cross-linked protein.

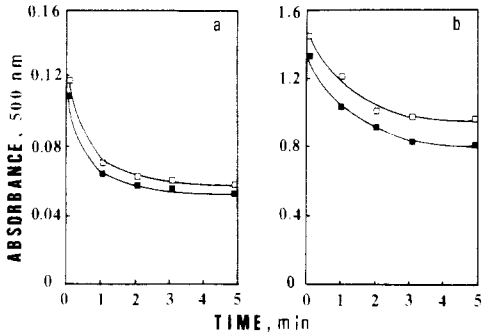

Figure 4. Emulsifying properties of cross-linked lysozyme and serum albumin. The emulsifying activity and emulsion stability were determined by measuring the turbidity at 500 nm of diluted emulsion: a, lysozyme; b, serum albumin. Key: □, native protein; ■, cross-linked protein.

30 s. The foaming power of bovine serum albumin is high (3700 μv/cm) and is significantly lowered (2750 μv/cm) by cross-linking. The foam stability of cross-linked serum albumin was greatly decreased, and the foam was diminished within 2 min.

On the other hand, the emulsifying properties of lysozyme and serum albumin were slightly decreased by cross-linking (Figure 4). The emulsifying properties of lysozyme are originally low. Therefore, the difference in the emulsifying properties between native and cross-linked lysozyme was slightly observed. However, in the case of bovine serum albumin possessing good emulsifying properties, a significant difference between native and cross-linked one was observed, although the difference was smaller than that in foaming properties.

We have proposed in a previous paper (Kato et al., 1985) that the flexibility of proteins detected by protease probe method may play an important role on the foaming and emulsifying properties. This was confirmed by using cross-linked proteins, as shown in this paper. The foaming properties may be more closely affected by the flexibility of proteins than the emulsifying properties. Bovine serum albumin is originally a hydrophobic protein. Therefore, the emulsifying properties of serum albumin may be still good, although it was cross-linked by FNPS.

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Segregation of Leptine Glycoalkaloids in *Solanum chacoense* Bitter

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Foliar leptine levels in 60 *Solanum chacoense* accessions were determined in a search for high-leptine plants that might be useful in breeding for resistance to the Colorado potato beetle, *Leptinotarsa decemlineata* Say. New methods were developed for detecting and quantifying leptine glycoalkaloids. A wide range of levels (<2–98 mg % fresh weight) was found among the accessions; most (42 of 60) apparently did not synthesize even traces of leptines. Among six sibs individually sampled from one accession, levels ranged from <2 to 306 mg % in replicated analyses. The results indicate that leptine glycoalkaloids are segregating widely among and within *S. chacoense* accessions. Tubers from high-leptine clones did not synthesize leptines even when greened or wound healed.

INTRODUCTION

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say, is becoming increasingly difficult to control with available pesticides. New control measures are needed, including the use of resistant varieties in integrated pest management programs. Present potato cvs have little or no resistance and may not be adequate as a germplasm base to improve resistance (Sanford et al., 1984). Resistant interspecific hybrids were developed in Europe in the 1940s (Schwarze, 1963; Torka, 1950), but when pesticides such as DDT became widely available, most of the European breeding research was discontinued.

Resistant wild species and their hybrids with *Solanum tuberosum* in these early European breeding studies generally had high total glycoalkaloid (TGA) levels. The re-

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