ENHANCEMENT OF HEAT-INDUCED GELATION OF FOOD PROTEINS BY MILK α-LACTALBUMIN

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ABSTRACT

α-Lactalbumin is one of the major component of milk whey proteins. This study was conducted to elucidate the contribution and mechanism of α-lactalbumin in heat-induced gelation of the mixed-proteins. At concentration of 8%, α-lactalbumin alone does not form gel, but it mixtures with whey protein isolate, whey protein concentrate, β-lactoglobulin, bovine serum albumin, egg white protein, lysozyme and ovalbumin resulted in the gel strength of 378.5, 270.6, 380.8, 310.2, 215.6, 0.0 and 400.9 in 10^4 dyne/cm^2, respectively. The mixture of α-lactalbumin and lysozyme did not form a gel, because α-lactalbumin and lysozyme appear to be two very closely related protein both in primary and three-dimensional structures. Determination by electrophoresis, chromatography, immunoassay, surface hydrophobicity and amino acid sequencing indicated that a specific disulfide bond (Cys6-Cys120) in α-lactalbumin contribute to heat-induced gelation of the mixed proteins. Reducing one, two and three of four disulfide bonds in α-lactalbumin confirmed the enhancement of heat-induced gelation of the mixed proteins. It was postulated that the enhancement mechanisms of α-lactalbumin in heat-induced gelation of the mixed proteins was mainly through disulfide bond and hydrophobicity interactions.

INTRODUCTION

Proteins are important macro nutrients of foods. The functional properties of proteins are defined as those physicochemical properties that enable proteins to affect the characteristics of food during processing, storage, preparation and consumption (Rupnow, 1992). Gelation is an important functional property of proteins in food systems. Different proteins produce gels which vary in textural characteristics (Aguilera, 1995; Legowo *et al.*, 1996).

Milk whey protein consist several proteins such as α-lactalbumin, β-lactoglobulin, bovine serum albumin (BSA), and some others (Farrell et al., 2004), which contributes to the gelation properties (Aguilera, 1995). The monomeric α-

lactalbumin is characterized conformationally stable protein because of four disulfide bonds. Matsudomi *et al.* (1992) reported that formation of soluble aggregates with α-lactalbumin mainly through a sulfhydryl (SH)-disulfide interchange reaction during heating. The aggregation rate of α-lactalbumin increased when heated in combination with –lactoglobulin (Hines and Foegeding, 1993; Legowo *et al.*, 1993). However, the contributions of disulfide bonds in α-lactalbumin to the mechanism of gelation are not clearly yet.

Thus, a study on the interactions of α-lactalbumin with other proteins containing sulhydryl groups in heat-induced gelation is interesting to be focused. β-Lactoglublin, an abundant protein in whey, is a protein containing one free SH group (Kontopidis et al., 2004). Egg white contains about 40 different proteins (Osuga and Feeney, 1977). Ovalbumin, the most abundant protein in egg white, having 385 amino acids with one disulfide bond and four SH groups (Nisbet *et al.*, 1981). Lysozyme is one of egg white proteins, which is having similarity structure to α-lactalbumin. Choosing of all of these proteins is presumably a proper way for investigating the contribution of disulfide bonds in α-lactalbumin to the protein interactions during gelation of α-lactalbumin mixed gels.

MATERIALS AND METHODS

Preparation of proteins

α-lactalbumin and β-lactoglobulin were prepared from milk whey by the ammonium sulfate precipitation (Legowo *et al.*, 1993). Egg white protein was obtained by separation of yolk from whole hen egg, subsequently homogenized at 1,000 rpm for 5 min under cooling. Homogenized egg white was diluted with an equal volume of distilled water and dialyzed against deionized water at 4^0C for two days (3 changes). The egg white solution was freeze-dried after removing some precipitates by centrifugation. Ovalbumin was prepared from hen egg white by ammonium sulfate preparation (Legowo *et al.*, 1996). Bovine serum albumin and lysozyme were purchased from Sigma Chemical Co. (St Louis, MO). Whey protein isolate (WPI) and whey protein concentrate (WPC) were the products of Meiji Milk Products Co., Japan. The 3SSα-lactalbumin reduced at a specific disulfide bond, Cys6-Cys120 (it

was confirmed by amino acid sequencing), was prepared by the treatment of dithiothreitol in the presence of $CaCl_2$ followed by blocking free SH with iodoacetamide (Kuwajima *et al.*, 1990).

Gel preparation and gel strength determination

The α-lactalbumin and other protein at pH 7.0 were mixed at ratios of 1:1. The final content of mixed solution was 8%. Aliquots of the various mixed solutions were placed in small petridish covered with silicon and glass plate, and then heated in a water bath at 80^0C for 15 min. Gel strength was determined using a rheometer of Yamaden RE-3305 Rheoner with a 0-50 g load cell (Legowo *et al.*, 1996). Gel strength was expressed as the force in dyne/cm2 applied to the probe edge when the surface yield point was reached. Determination of gel strength was repeated at least 6 times for each sample.

Electrophoresis

The mixture of α-lactalbumin and other protein at pH 7.0 in the same ratio at final concentration of 8% protein was heated at 80^0C for 15 min. Aliquot of heated protein was applied to sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) in the presence and absence of 2-mercaptoethanol. The acrylamide gel concentration was 4.5% for stacking gel and 12.5% for the running gel. The gel was stained with coomassie brilliant blue R-250 and destained by diffusion in isopropanol-acetic acid-water (7:5:8, v/v).

Gel filtration chromatography

The gels of the mixture of α-lactalbumin and ovalbumin were crumbled, followed by ultra-centrifugation at 100,000 g at 20^0C for 1 hr. The liquid of gels were eluted on a Sephacryl S-100 column with PBS-buffer at pH 7.0 as eluent. The fractions were collected and measured.

Immunoassay method

The immuno assay method to detect the protein interaction was iniated by the production of monoclonal antibodies. The antibodies were produced by the laboratory of Utilization of Animal Products, Faculty of Agriculture, Kagawa University

according to the method of Kusmanoff *et al.* (1990). Mice (BALB/c) were immunized with α-lactalbumin. Mouse spleen cell were fused with myeloma (Sp2/O) cells with PEG, and the hybridoma cells were selected in HAT medium. Three monoclonal antibodies designated Mab2, Mab3, and Mab4 were obtained and characterized by ELISA, isotyping and western blotting.

The reactivities of three monoclonal antibodies with α-lactalbumin were examined. The selected antibody of Mab2, because it had high activities to bind α-lactalbumin molecule, was used in Competitive ELISA for detection of α-lactalbumin interaction with other proteins. The proteins (α-lactalbumin, 3SSα-lactalbumin, and ovalbumin) were mixed and conditions used for gel strength determination. About 20 μL α-lactalbumin per mL of these mixed solutions was examined by Competitive ELISA (Friguet *et al.*, 1989; Kaminogawa *et al.*, 1989). The binding reactivity of protein with antibody was expressed as % inhibition, which was calculated by the following equation:

$$\% \text{ Inhibition} = \frac{(Ap-Aa) - (As-Aa)}{(Ap-Aa)} \times 100\%$$

where Ap is absorbance for controls in the presence and Aa, in the absence of coated antigen and As is absorbance of the sample.

Hydrophobicity determination

Hydrophobicity was determined using a hydrophobic fluorescence probe 8-anilino-1-naphtalene sulfonate (ANS). Determination was performed according to the method of Hayakawa and Nakai (1985). Each protein sample (3 mL) was nserially diluted with 50 mM Tris-HCl buffer (pH 7.5) to obtain protein concentration range 0.01-0.05%. After addition of 15 μL ANS (8 mM in 50 mM Tris-HCl buffer, pH 7.5), the fluorescence intensity (FI) was measured with a spectrofluoro-photometer (Shimadzu RF 510) at λ_{EX} of 390 nm and λ_{EM} of 470 nm. FI reading was standardized by adjusting the reading of fluorometer to 30% full scale for ANS in methanol. The initial slope of the FI versus protein concentration (%) plot was

calculated by linear regression analysis, and was used as an index of the protein hydrophobicity.

RESULTS AND DISCUSSION

Gel strength of the mixed proteins

Table 1 shows the gel strength of the mixture of α-lactalbumin and other proteins. At concentration of 8%, α-lactalbumin alone did not form gel. Lysozyme also did not form gel. Although α-lactalbumin alone did not form gel, the mixture of α-lactalbumin and other proteins, in ratio of 1:1, resulted gels with gel strength almost same to other protein alone.

Table 1. The gel strength of the mixture of α-lactalbumin and other proteins

No	The Proteins	Average Gel Strength (10^4 dyne/cm2)
1	α-Lactalbumin	Not detected
2	Whey protein isolate (WPI)	390.8
3	Whey protein concentrate (WPC)	287.2
4	β-lactoglobulin	392.5
5	Bovine serum albumin (BSA)	330.1
6	Egg white protein	225.3
7	Lysozyme	Not detected
8	Ovalbumin	220.5
9	α-Lactalbumin + WPI	378.5
10	α-Lactalbumin + WPC	270.6
11	α-Lactalbumin + β-lactoglobulin	380.8
12	α-Lactalbumin + BSA	310.2
13	α-Lactalbumin + egg white protein	215.6
14	α-Lactalbumin + lysozyme	Not detected
15	α-Lactalbumin + ovalbumin	400.9

The whey protein isolate, whey protein concentrate, β-lactoglobulin, bovine serum albumin, egg white protein, and ovalbumin resulted in the gel strength of 390.8, 287.2, 392.5, 330.1, 225.3 and 220.5 respectively. The mixtures of α-lactalbumin and whey protein isolate, whey protein concentrate, β-lactoglobulin,

bovine serum albumin, egg white protein, lysozyme and ovalbumin resulted in the gel strength of 378.5, 270.6, 380.8, 310.2, 215.6, 0.0 and 400.9 in 10^4 dyne/cm^2, respectively. The mixture of α-lactalbumin and lysozyme did not form a gel, because α-lactalbumin and lysozyme appear to be two very closely related protein both in primary and three-dimensional structures.

In case of the mixture of α-lactalbumin and ovalbumin (8% protein concentration), a high gel strength of 400.9 x 10^4 dyne/cm^2 was obtained. This gel strength was almost double that of 8% ovalbumin alone. This result indicated that α-lactalbumin interacted with ovalbumin, and subsequently enhanced the gelation of ovalbumin-α-lactalbumin mixed gel.

Interaction of α-lactalbumin and other proteins

SDS-PAGE patterns showed that the mixture of α-lactalbumin and all other proteins, except lysozyme, resulted in high molecular weight of polymerized protein bands. Molecular weight of proteins which remained on top of the stacking gel were higher than that of α-lactalbumin. High molecular weight of fraction was presumably resulted from polymerization of α-lactalbumin and other proteins during heating. In the presence of 2-mercaptoethanol, the polymerized protein dissociated almost completely to the monomers of α-lactalbumin and other protein. The 2-mercaptoethanol is one reducing agent which has capability to reduced disulfide bonds in a protein. Heated α-lactalbumin alone did not show the polymerized protein band as well as the mixture of α-lactalbumin and lysozyme. Therefore, it can be supposed that the polymerized protein band of for the mixture of α-lactalbumin and other protein represents aggregates of α-lactalbumin and other protein. For further examination, ovalbumin was choosed as a model, because the mixture of α-lactalbumin and ovalbumin relted in the highest gel strength (Table 1).

The chromatogram of the liquid gel for the mixture of α-lactalbumin and ovalbumin indicated the highly polymerized protein and a small peak of α-lactalbumin. Aguilera (1995) suggested that the heat-induced gelation of protein is characterized by the extent of formation aggregates and subsequent polymerization.

This suggestion relate to the result of the present study. A molecule of α-lactalbumin contains four disulfide bonds, whereas ovalbumin has four SH groups and only one disulfide bond (Nisbet et al., 1981). Therefore, it was possible that α-lactalbumin and ovalbumin were interacting in heat-induced gelation through intermolecular SH-disulfide bond exchange interactions and resulted the highly polymerized proteins.

Immunoassay to detect protein interaction

To determine the binding reactivity of α-lactalbumin with antibody in heat-induced gelation, Competitive ELISA was performed. ELISA values of the mixture of α-lactalbumin and other proteins was shown in Table 2.

Unheated α-lactalbumin showed a high inhibition, reflecting its high binding reactivity with the antibody. A similar result was also shown by the heated 8% α-lactalbumin. However, the mixture of α-lactalbumin and ovalbumin exhibited decreasing reactivity upon heating, hence this indicated that reactivity markedly decreased.

Table 2. Competitive ELISA profiles of the mixture of α-lactalbumin and ovalbumin

Proteins	α-lactalbumin concentration (μg/mL)	Inhibition (%)
Unheated α-lactalbumin	100	99.85
	50	99.05
	1	98.70
	0.1	96.94
	0.01	95.01
Heated α-lactalbumin	100	94.53
	50	99.16
	1	98.65
	0.1	96.30
	0.01	94.75
The mixture of α-lactalbumin and ovalbumin	100	93.58
	50	76.80
	1	34.95
	0.1	10.22
	0.01	0.0

Hydrophobicity values of the proteins

The ANS is a useful fluorophore that is frequently employed to probe hydrophobic sites on proteins (Musci and Berliner, 1985; Hayakawa and Nakai, 1985). As shown in Table 3, ovalbumin exhibited alarge increase in hydrophobicity by heating. The high hydrophobicity value indicated the exposure of hydrophobic amino acid residues to the surface after the protein is treated (Tani *et al.*, 1995). However, the hydrophobicity of both α-lactalbumin and 3SSα-lactalbumin did not changes even the proteins were heated.

The hydrophobicity of the mixture of the separately heated α-lactalbumin and ovalbumin was almost the same as that of the mixture of separately heated 3SSα-lactalbumin and ovalbumin (Table 3). The hydrophobicity of the heated mixture of 3SSα-lactalbumin and ovalbumin was relatively close to that of the mixture of separately heated 3SSα-lactalbumin and ovalbumin. In contrast, the heated mixture of α-lactalbumin and ovalbumin resulted in larger hydrophobicity value than the mixture of separately heated proteins. This indicated that the heated mixture of α-lactalbumin and ovalbumin resulted in a large changes of conformational properties. This was also related to the interactions of α-lactalbumin and ovalbumin which could form a polymerized proteins and high gel strength.

Table 3. Hydrophobicity of α-lactalbumin, 3SSα-lactalbumin and protein mixture

No	Proteins	Hydrophobicity Value
1	Unheated α-lactalbumin	35
2	Heated α-lactalbumin	37
3	Unheated 3SSα-lactalbumin	28
4	Heated 3SSα-lactalbumin	29
5	Unheated ovalbumin	3
6	Heated ovalbumin	335
7	Mixture of unheated α-lactalbumin and ovalbumin	10
8	Mixture of unheated 3SSα-lactalbumin and ovalbumin	11
9	Mixture of separately heated α-lactalbumin and ovalbumin	172
10	Mixture of separately heated 3SS α-lactalbumin & ovalbumin	170
11	Heated mixture of α-lactalbumin and ovalbumin	202
12	Heated mixture of 3SS α-lactalbumin and ovalbumin	167

CONCLUSIONS

α-Lactalbumin improved the heat-induced gelation of some proteins (whey protein isolate, whey protein concentrate, egg white protein, β-lactoglobulin, bovine serum albumin) and enhanced markedly the gelation of ovalbumin. It was postulated that α-lactalbumin and other proteins, especially ovalbumin, were possibly interacting through SH-disulfide bond reactions which were presumed to occur in heat-induced gelation. α-Lactalbumin interacted with ovalbumin at certain concentration, which characterized by formation of highly polymerized proteins. The contribution of a spesific disulfide bond Cys6-Cys120 in α-lactalbumin was implied by its participation in the enhancement of gel strength of the mixed proteins.

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