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Full Length Research Paper

Hydrogen peroxide and ferulic acid-mediated oxidative cross-linking of casein catalyzed by horseradish peroxidase and the impacts on emulsifying property and microstructure of acidified gel

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Horseradish peroxidase (HRP, EC 1.11.1.7) was used in this study to catalyze oxidative cross-linking of casein in the presence of hydrogen peroxide and cross-linker ferulic acid. Cross-linking of casein was demonstrated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and ultraviolet absorbance analysis. Oxidative cross-linked casein mediated by hydrogen peroxide and ferulic acid was prepared at casein concentration of 5% (w/ w), HRP addition of 3 μkat·g^{-1} proteins, ferulic acid addition of 6 mmol·l^{-1}, 3% (w/w) H_2O_2 addition of 1 ml, reaction temperature 37°C and reaction time 3 h. Analysis results showed that the emulsifying activity index and emulsifying stability index of cross-linked casein prepared were enhanced compared to that of untreated casein totally. Microstructure of chemical acid-induced gel of cross-linked casein was observed under scanning electron microscopy and appeared to be more compact and uniform than that of casein. Hydrogen peroxide and ferulic acid-mediated oxidative cross-linking of casein catalyzed by horseradish peroxidase might have a beneficial to the emulsifying property or gelation of casein.

Key words: Casein, horseradish peroxidase, cross-linking, emulsifying property, ferulic acid, acidified gel, microstructure.

INTRODUCTION

Treatment of food proteins with specific enzymes is employed in recent years to modify their functional properties because enzymatic treatments can be carried out under relatively mild conditions and specificity of the reactions will not likely lead to the production of toxic products. Among the reactions catalyzed by enzymes, it was found that some could lead to intra and inter-molecular cross-linking of food proteins and detailed mechanism had been proposed in some literatures (Matheis and Whitaker, 1987; Gerrard, 2002; Strauss and Gibson, 2004). Transglutaminase has good ability to cross-link food proteins. Transglutaminase allows modification in the structure of food proteins at molecular level and will influence some functional properties of food proteins treated (Motoki and Seguro, 1998; Nielsen, 1995). The impacts of transglutaminase treatment on milk proteins, especially casein, had been extensively studied in the past. It was confirmed that cross-linking of casein by transglutaminase led to the modifications in rheological properties and microstructure of chemically acidified gels (Myllalrinen et al., 2007), syneresis of neutral and acidic gels (Partanen et al., 2008) and ethanol stability of casein micelles (Huppertz and de Kruif, 2007). Also, treatment of milk proteins with transglutaminase would improve the rheological properties of stirred yoghurt with no syneresis (Bonisch et al., 2007), or could incorporate whey proteins into cheese curd to obtain whey- protein- enriched dairy products (Cozzolino et al., 2003). There are other enzyme systems that are capable of cross-linking side chains of food proteins, such

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Abbreviations: HRP, Horseradish peroxidase; **SDS-PAGE,** sodium dodecyl sulfate - polyacrylamide gel electrophoresis; **SPI,** soybean protein isolate; **EAI,** emulsifying activity index; **ESI,** emulsion stability index; **SEM,** scanning electron microscopy.

Table 1. Preparing conditions of cross-linked casein used in this study.

Parameters selected	Optimal conditions
Casein concentration	5% (w/w)
Addition of HRP	3 μkat·g^{-1} proteins
Addition of ferulic acid	6 mmol·L^{-1}
Reaction temperature	37°C
Reaction time	3 h
Addition of 3% (w/w) H_2O_2	1 ml

Each evaluation was carried out triplicate and the results are reported as mean(standard error. The ESI of casein or cross-linked casein at 0.01% (w/w) are saved as control. One and two star marks above columns indicate that one-way ANOVA of means are significantly different ($P < 0.05$ and $P < 0.01$).

as peroxidase. There exists a need to study the impacts of these enzyme systems on the functional properties of food proteins and potential applications.

Gerrard (2002) reviewed various enzymatic reactions that might induce cross-linking of proteins. Matheis and Whitaker (1987) proposed that some oxidoreductase should be considered for oxidative cross-linking of food proteins. An important product of protein oxidation is the formation of protein dimer, which can be formed by the catalysis of peroxidase (Li and Nicell, 2008) or tyrosinase (Thalmann and Lotzbeyer, 2002). Horseradish peroxidase (HRP, EC 1.11.1.7) represents an interesting biocatalyst that has potential application in cross-linking of food proteins. HRP could induce cross-linking of some proteins in the presence of H_2O_2 and a low molecular weight hydrogen donor (Stahmann et al, 1977). Enzymatic oxidation of proteins with peroxidase together with H_2O_2 at basic pH led to an oxidative phenolic coupling of adjacent tyrosine residues to form cross-linked proteins (Aeschbach et al, 1976). Stuchell and Krochta (1994) had treated soy protein isolate with horseradish peroxidase and hydrogen peroxide after soy protein isolate solution was heated at 80°C, but found that the oxidation of protein residues to promote cross-linking was not specific enough for the preparation of edible films. Boeriu et al. (2005) also used horseradish peroxidase to crosslink wheat arabinoxylan with β-casein. Recently, a microbial enzyme *Coprinus cinereus* peroxidase could be employed as an environmentally friendly protein polymerization catalyst to induce cross-linking of casein (Steffensen et al., 2008).

To cross-link food proteins, phenols can be used as cross-linker. Strauss and Gibson (2004) had used plant-derived phenolic acids and flavonoids to prepare cross-linked gelatin and found that cross-linked gelatin had greater mechanical strength, reduced swelling and fewer free amino groups. Ou et al. (2005) employed ferulic acid as cross-linker to treat soybean protein isolate (SPI) in the presence of H_2O_2 to prepare SPI-based edible films and found that the SPI films cross-linked with ferulic acid demonstrated increased tensile strength, increased percent elongation at break and decreased oxygen permeability. Cao et al. (2007) also found that ferulic acid and tannin acid could act as cross-linkers to cross-link gelatin film.

In the presented study, HRP was used as catalyst, hydrogen peroxide as oxidant and ferulic acid as a hydrogen donor (i.e. a cross-linker) to cross-link casein. The impacts of oxidative cross-linking on the emulsifying property and microstructure of acidified gel of casein was analyzed and compared. The oxidative cross-linking of casein by HRP in the presence of H_2O_2 and ferulic acid was confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or ultraviolet absorbance (UV) analysis of casein samples treated. Emulsifying activity index (EAI) and emulsion stability index (ESI) of cross-linked casein were evaluated. The microstructures of chemical acidified gels of cross-linked casein and casein were observed with scanning electron microscopy (SEM) and compared to show the impacts of oxidative cross-linking treatment.

MATERIALS AND METHODS

Materials and chemicals

Casein (93.8% protein content on dry basis) was from Sigma-Aldrich Co. (St. Louis, MO, USA). Horseradish peroxidase used was purchased from Shanghai Guoyuan Biotech Inc (Shanghai, China) with enzyme activity 3670 μkat·g^{-1}. All chemicals used were analytical reagents. Highly purified water prepared with Milli-Q PLUS (Millipore Corporation, New York, NY, USA) was used for the preparation of all buffers and solutions.

Cross-linking of casein catalyzed by HRP in the presence of ferulic acid

Casein solution was prepared by dispersing casein powder 5.4 g in 100 ml Na_2CO_3- $NaHCO_3$ buffer (0.2 mol·l^{-1}, pH 9.5) and keeping at 4°C overnight for rehydration. Some potassium sorbate was added to inhibit microbial growth. Casein solution was heated in a water bath at 70°C for 5 min and cooled naturally to ambient temperature (about 20°C) prior to cross-linking. 1 ml of 0.8 mol L^{-1} ferulic acid solution was added to casein solution. After withdrawing a 10 ml portion (zero time sample) from casein solution, the cross-linking reaction was started by addition of 1.0 ml of 3% (w/w) H_2O_2 and 1.0 ml HRP solution to the casein solution, which gave an enzyme concentration approximately 7.3 μkat·g^{-1} proteins, casein content about 5% (w/w) and ferulic acid content about 8 mmol·l^{-1}. The reaction was carried out at ambient temperature with continuous agitation. 10 ml of samples were withdrawn from reaction system after 0.5, 1, 1.5, 2, 2.5 and 3 h, respectively. The HRP in the separated casein samples was inactivated immediately by heating casein solution samples at 85°C for 10 min. The solution of HRP-treated casein was then freeze-dried and stored at -18°C for further SDS-PAGE and UV analysis.

Cross-linked casein used for evaluation of emulsifying properties and microstructure of acidified gel was prepared with optimal reaction conditions. These reaction conditions were studied by response surface methodology and are not presented here, but the detailed parameters are summarized in Table 1.

SDS-PAGE and UV analysis of cross-linked casein

Cross-linking of casein was confirmed by SDS-PAGE analysis of some cross-linked casein samples under reducing conditions using separating and stacking gels containing 12 and 4% (w/w) acrylamide, respectively, as described by Laemmli (1970). The protein concentration of all samples were fixed at 5 $g \cdot l^{-1}$ and 10 µl of sample was applied in each lane for analysis. Standard protein markers used and their molecular weights were as follow: egg albumin lysozyme (14.4 kDa), trypsin inhibitor (20.1 kDa), bovine carbonic anhydrase (31.0 kDa), ovalbumin (43.0 kDa) bovine serum albumin (66.2 kDa) and phosphorylase b (97.4 kDa). The protein concentration of each marker was fixed at 0.1 $g \cdot l^{-1}$. The stacking gels were run at 120 V and the separating gels were run at 80 V. The gels were stained using 2.5% (w/v) Coomassie Brilliant Blue G250 in a 4.5:4.5:1 (v/v) mixture of deionized water, methanol and glacial acetic acid for 0.5 h, followed by repeated destaining in a mixture of 1:1:8 (v/v) methanol, glacial acetic acid and deionized water until the background of the gels became clear. The gel images were visualized and photographed by PhotoDoc-It Imaging System (UVP Inc., San Gabriel, CA, USA).

According to the method of Tenovuo and Paunio (1979), all samples from cross-linking of casein with different reaction times were diluted to a protein concentration of 1 $g \cdot L^{-1}$. The solutions were then measured at 280 and 315 nm with an UV spectrophotometer (UV-2401P, Shimadzu, Japan). The ratio of the absorbance at 315 nm to the absorbance at 280 nm was calculated thereby to show indirectly the decrease of ferulic acid in reaction mixture as cross-linking reaction progressed.

Emulsifying activity index and emulsifying stability index of cross-linked casein

The emulsifying properties of casein and cross-linked casein prepared were determined by a classic turbidimetric method (Pearce and Kinsella, 1978). To prepare emulsions, 25.0 ml of refined soybean oil and 75.0 ml of casein solution (0.01, 0.02, 0.03 and 0.04% w/w on protein basis, respectively) in 0.2 $mol \cdot l^{-1}$ Na_2CO_3-$NaHCO_3$ buffer (pH 9.5) were mixed together in a plastic tube and homogenized with a high speed homogenizer (Type DC-1, Shanghai Jingke Ltd., Shanghai, China) at 12000 rpm for 1 min. The emulsion was immediately transferred into a 250 ml capacity glass beaker. 50 µl aliquots of freshly prepared emulsion were taken at 0.5 cm from the bottom of the beaker and dispersed into 5 ml of 0.1% (w/w) SDS solution. The absorbance of the sample was measured at 500 nm against 0.1% SDS blank solution in a UV-2401 PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Repeated this analysis procedure after 10 min incubation. The absorbance of the sample was also measured at 500 nm against 0.1% SDS blank solution. Emulsifying activity index (EAI, $m^2 \cdot g^{-1}$) and emulsifying stability index (ESI, %) of casein or cross-linked casein were calculated by using equations (1) and (2), respectively. Each EAI and ESI evaluation was carried out triplicate.

$$\mathrm{EAI}(m^2 \cdot g^{-1}) = \frac{2 \times 2.303 \times A_{500} \times \mathrm{dilution}}{C \times (1-\Phi) \times 10^4} \quad (1)$$

$$\mathrm{ESI}(\%) = \frac{A_{10}}{A_0} \times 100 \quad (2)$$

Where, A_{500} represents the absorbance of analysis sample at 500 nm, C is protein concentration ($g \cdot ml^{-1}$) before emulsification, Φ is the oil volume fraction (v/v) of the emulsion (Φ = 0.25), dilution = 100, while, A_{10} and A_0 represent the absorbance at 500 nm after 10 min and at time zero respectively at 500 nm.

Microstructure of acidified gel of cross-linked casein

Chemical acidified gel of casein or cross-linked casein was prepared according to the method of Koh et al. (2002) with some modifications. Granules of glucono-δ-lactone (GDL) about 0.6 g were added to casein or cross-linked casein solution and mixed thoroughly for 2 min. The acidification was carried out at 40°C. This level of GDL addition was sufficient to induce pH drop to pH 4 in approximately 2 h period.

Casein gel, approximately 1 × 1 × 10 mm, was cut using razor blade from the interior of casein gel block and immediately fixed in 2% (w/w) glutaraldehyde solution for 1 h. The fixed casein gel samples were dehydrated for 15 min in graded ethanol series, which consisted of a 50, 70, 90 and 100 ml/100 ml ethanol solution and tertiary butyl alcohol. All prepared samples were then frozen in liquid nitrogen. The dried gel samples were mounted on aluminum SEM stubs using a carbon-based tape and coated with gold in ES-1010 sputter coater (Hitachi, Japan). The samples were examined in Hitachi S-5700 (Hitachi, Japan) scanning microscope at 5000 × magnifications with accelerating voltage 5 kV.

Statistical analysis

All data were expressed as means ± standard error (SE) from at least three independent trials. The differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA) with Duncan's Multiple Range Test. ANOVA data with a $P < 0.05$ were classified as statistically significant. SPSS 13.0 software and MS Excel 2003 program were used to analyze and report the data.

RESULTS AND DISCUSSION

Cross-linking of casein catalyzed by HRP in the presence of ferulic acid

Casein and HRP-treated casein samples (reaction time was 0.5, 1, 1.5, 2 and 3 h, respectively) were dissolved and subsequently subjected to SDS-PAGE analysis to confirm the occurrence of cross-linking of casein. The analysis results are given in Figure 1, which reveals the different protein compositions in casein and HRP-treated casein samples. The results showed that existences of protein brands with molecular weight larger than 97.4 kDa in HRP-treated casein samples (lanes 1 to 5). This phenomenon did not exist in lane M and indicates the formation of protein polymers as the result of cross-linking of casein. Meanwhile, the disappearance of protein bands (< 31 kDa) was found in cross-linked casein. Image analysis results form densitometer showed that the band color of protein polymers or main casein components become deeper or thinner as reaction time increase, indicating that more protein polymers formed as oxidative cross-linking reaction progressed.

The SDS-PAGE analysis result confirmed that HRP could catalyze the cross-linking reaction between casein molecules under the existence of H_2O_2 and ferulic acid. This was in agreement with the previous finding of other

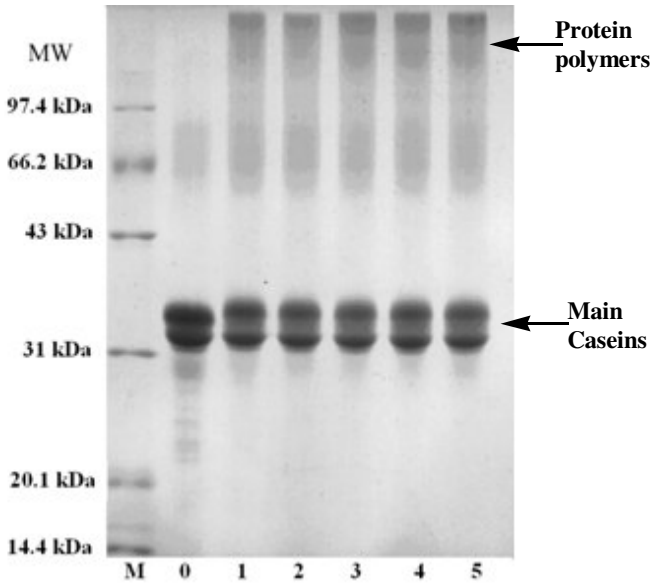

Figure 1. Sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis (SDS-PAGE) of casein and cross-linked casein samples treated with HRP in the presence of ferulic acid. Standard protein markers used and their molecular weights (in kDa) are as follow: egg albumin lysozyme (14.4), trypsin inhibitor (20.1), bovine carbonic anhydrase (31.0), ovalbumin (43.0) bovine serum albumin (66.2) and phosphorylase b (97.4). Lane M, standard protein markers; lane 0, casein; lane 1 to 5, cross-linked casein with reaction time of 0.5 , 1 , 1.5 , 2 and 3 h, respectively.

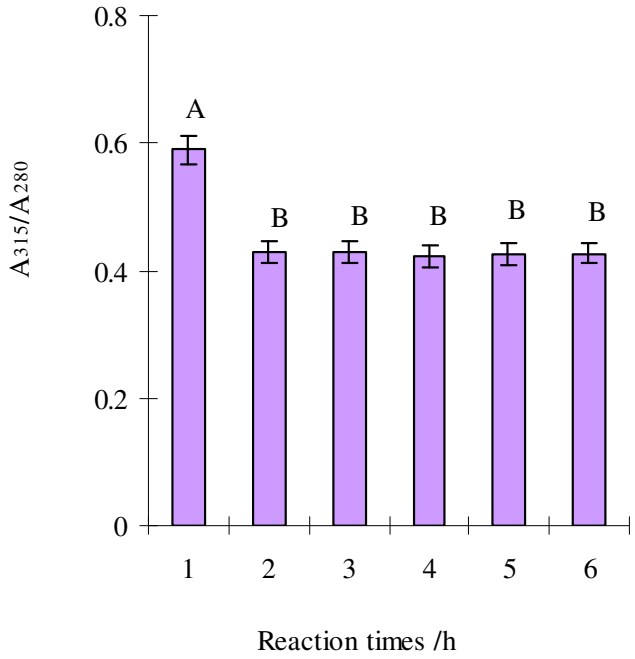

Figure 2. The changes of UV ratios (A_{315}/A_{280}) of the cross-linked reaction mixture at different reaction times. The number of trial times was three. The UV ratio of cross-linked casein with reaction time of 1 h is saved as control. Different capital letters above columns indicates that one-way ANOVA of means are significantly different ($P < 0.05$).

research works that showed that phenolic acids could be applied to cross-link soy protein isolate (Ou et al., 2005) and gelatin (Strauss and Gibson, 2004; Cao et al., 2007).

The ratio of UV absorbance at 315 nm to the absorbance at 280 nm of reaction mixture also gave some support indirectly to the analysis results of SDS-PAGE. Ferulic acid has a maximum absorbance at 315 nm and proteins have a maximum absorbance at 280 nm. As shown in Figure 2, the ratio of UV absorbance (A_{315}/A_{280}) of reaction mixture decreased as cross-linking reaction began and then kept stable. It implied that the content of ferulic acid decreased during early stage of cross-linking reaction. The disappearance of ferulic acid was caused by the incorporation of ferulic acid molecules to casein molecules as the result of cross-linking. According to the proposed reaction mechanism (Strauss and Gibson, 2004), when casein are cross-linked in the presence of HRP, H_2O_2 and ferulic acid, a ferulic acid molecule acts as a mediator (in the form of ferulic acid quinone) to cross-link two casein molecules in a mode of “hand by hand”. Therefore the cross-linking of casein will lead to the decrease of ferulic acid in reaction mixture.

Emulsifying activity index and emulsifying stability index of cross-linked casein

Emulsifying activity index (EAI) of casein or cross-linked casein prepared with the reaction conditions listed in Table 1, was evaluated with emulsions prepared at six casein concentrations and the results are shown in Figure 3. Totally, the evaluation results showed that the emulsifying activity index of casein and cross-linked casein were changed with the increase in protein concentration and emulsifying activity index of cross-linked casein was improved compared to that of casein. Cross-linked casein had the highest emulsifying activity index (1.42 $m^2 \cdot g^{-1}$ proteins) at protein concentration of 0.03% (w/w) while casein had the highest emulsifying activity index (1.35 $m^2 \cdot g^{-1}$ proteins) at protein concentration of 0.02% (w/w). Emulsifying stability index (ESI) of casein and cross-linked casein were also evaluated at six protein concentrations and are shown in Figure 4. Emulsifying stability index of cross-linked casein was higher than that of casein at same protein concentration. The results indicated that the cross-linking of casein catalyzed by HRP in the presence of hydrogen peroxide and ferulic acid led to the improvement in emulsion stability.

Motoki et al. (1984) reported that the polymerization of several food proteins (α_{s1}- and κ-casein, or soybean 7S and 11S globulin) by transglutaminase led to the emulsifying activity of polymerized α_{s1}-casein higher than that of the native protein in the range of pH 4~6. Liu and Damodaran (1999) reported a decrease in the EAI but increase in the emulsion stability of β-casein treated with transglutaminase. Our result shared similarity to Motoki’s

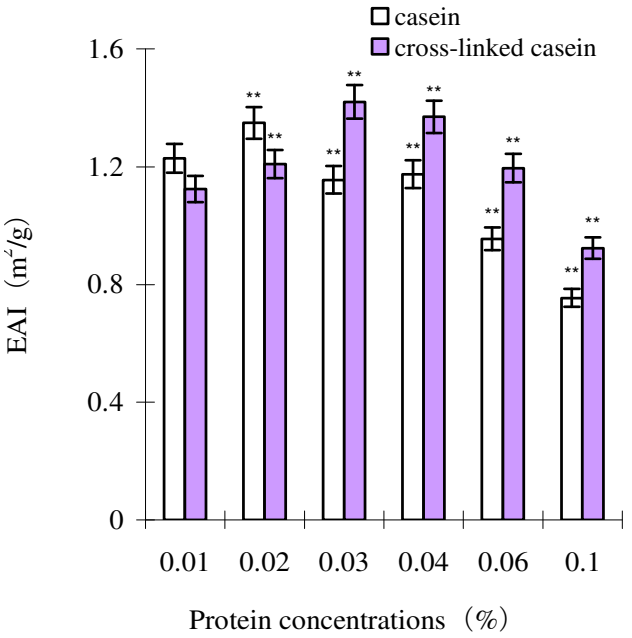

Figure 3. Emulsifying activity indexes of casein and cross-linked casein evaluated at six protein concentrations. Each evaluation was carried out triplicate and the results are reported as mean± standard error. The EAI of casein or cross-linked casein at 0.01% (w/w) are saved as control. Two star marks above columns indicate that one-way ANOVA of means are significantly different (P<0.01).

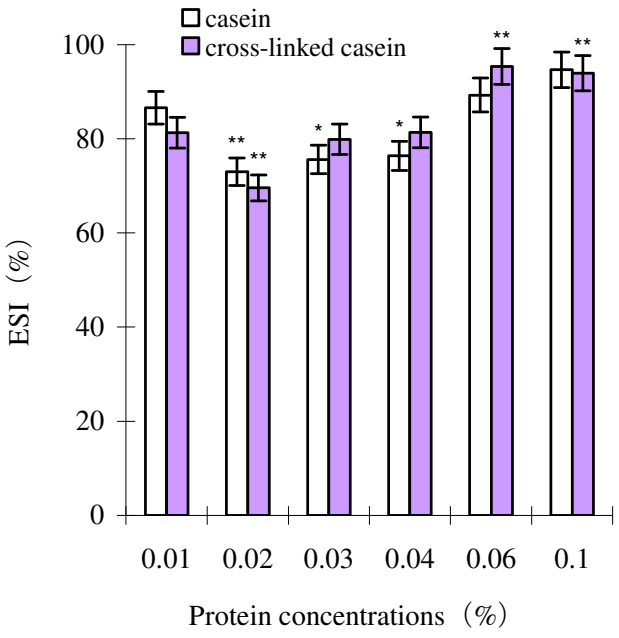

Figure 4. Emulsifying stability indexes of casein and cross-linked casein evaluated at six protein concentrations. Each evaluation was carried out triplicate and the results are reported as mean± standard error. The ESI of casein or cross-linked casein at 0.01% (w/w) are saved as control. One and two star marks above columns indicate that one-way ANOVA of means are significantly different (P < 0.05 and P < 0.01).

or Liu and Damodaran's result that cross-linking of casein by HRP in the presence of hydrogen peroxide and ferulic acid also led to the improvement in EAI or ESI of cross-linked casein.

Microstructure of acidified gel of cross-linked casein

The microstructures of acidified gels of casein and cross-linked casein observed under scanning electron microscopy are shown in Figure 5.

It can be seen from Figure 5a that the network of casein gel was characterized as a loose structure with big, irregular holes. However, a more compact, homogeneous structure and small pores existed between protein granules in acidified gel of cross-linked casein (Figure 5b), which indicates the microstructural improvement induced by the cross-linking of casein. Cross-linking casein led to the inter-molecular cross-links of casein molecules and had a helpful effect on the formation of acidified gels with uniform structure. The impacts of cross-linking of food proteins with other enzymes on the gel microstructure were also found in other research works. Recent studies had indicated that transglutaminase treatment had led to the microstructure of tofu, soy protein isolate and chicken meat more continuous and uniform coral-like structure (Tang, 2007; Trespalacios and Pla, 2007; Gan et al., 2008). Our result was consistent with these findings.

Conclusion

Casein was subjected to oxidative cross-linking catalyzed by horseradish peroxidase (HRP) in the presence of H_2O_2 and a cross-linker ferulic acid. The analysis results from SDS-PAGE confirmed the occurrence of cross-linking of casein, for protein bands with molecular weight less than 31 kDa disappeared and protein polymers with molecular weight large than 97.4 kDa were found in electrophoresis profiles of cross-linked casein. The analysis results from UV absorbance also supported the occurrence of cross-linking of casein as the content of ferulic acid decreased during early stage of cross-linking of casein, which was the result of the incorporation of ferulic acid to casein molecules. Oxidative cross-linked casein was prepared at casein concentration of 5% (w/w), HRP addition level of 3 $\mu kat \cdot g^{-1}$ proteins, ferulic acid of 6 $mmol \cdot L^{-1}$, reaction temperature of 37°C, 3% (w/w) H_2O_2 addition of 1 ml and reaction time of 3 h. Evaluation results showed that the emulsifying activity index or emulsifying stability index of cross-linked casein prepared was enhanced compared to that of untreated casein, implying that oxidative cross-linking of casein was helpful to improve its emulsifying properties. Microstructure of acid-induced gel of cross-linked casein observed under scanning electron microscopy showed a more compact and uniform characteristics than that of untreated casein, which also implied

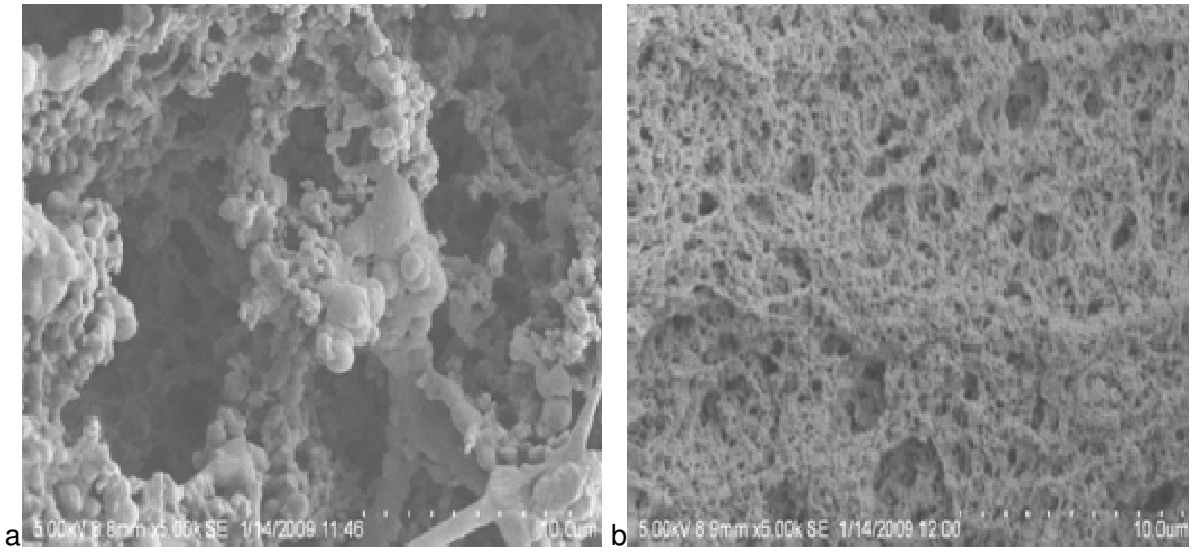

Figure 5. Microstructures of chemical acidified gels prepared from casein (a) and cross-linked casein (b) observed under scanning electron microscopy at 5000×.

that oxidative cross-linking of casein by HRP in the presence of hydrogen peroxide and ferulic acid also had beneficial impact on its gel structure.

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