Oxidative cross-linking of casein by horseradish peroxidase and its impacts on emulsifying properties and the microstructure of acidified gel

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The cross-linking of food proteins is an interesting topic of food science in recent years and served successfully as an approach to modify protein functional properties. In the presented work, horseradish peroxidase (HRP, EC 1.11.1.7) was used to oxidative cross-link casein in presence of H₂O₂. The cross-linking of casein was demonstrated by capillary zone electrophoresis analysis. The central composite design using response surface methodology was used to optimize cross-linking conditions of casein. The optimal cross-linking conditions of casein were as follows: the addition level of HRP was 4.73 µkat·g⁻¹ proteins, temperature was 37°C and reaction time was 2.9 h when casein concentration and pH of reaction medium were fixed at 5% (w/w) and 9.5, respectively. Cross-linked casein was prepared with these optimal conditions and used to analyze its emulsifying activity index, emulsifying stability index and microstructure of acidified gel. The emulsifying activity index and emulsifying stability index of the cross-linked casein were enhanced about 10 and 6% compared to that of casein. The microstructure of acid-induced gel of the cross-linked casein observed by scanning electron microscopy was more compact and uniform than that of casein without cross-linking. Cross-linking of food proteins induced by horseradish peroxidase might serve as an alternative approach to modify functional property of the proteins.

Key words: Casein, horseradish peroxidase, cross-linking, emulsifying property, response surface methodology.

INTRODUCTION

The functional properties of food proteins may be modified by the treatments of specific enzymes. Enzymatic treatments can be carried out under relatively mild conditions and because of the specificity of the reactions, are not likely to lead to the production of toxic products, which might provide with the grant to the safety of final products. Among the several reactions catalyzed by enzymes, some could lead to intra- and inter-molecular cross-linking of proteins (Matheis and Whitaker, 1987). An enzyme that recently has received extensive attention for its ability to cross-link proteins is transglutaminase. Transglutaminase can catalyze the cross-linking reaction between lysine and glutamine residue of protein molecules, which allows structure modification of proteins at molecular level and influences the functional properties of the proteins treated (Motoki and Seguro, 1998; Nielsen, 1995). The cross-linking of casein by transglutaminase had been extensively and deeply studied and some helpful influences on casein functional property had been proved in the past works. It was shown in many research works that cross-linking of casein by transglutaminase led to the improvements in rheological properties, microstructure of chemically acidified gels, syneresis of neutral and acid gels and ethanol stability of casein micelles (Mylärinen et al., 2007; Partanen et al., 2008; Huppertz and de Kruif, 2007). But there also exists a need to study other enzyme systems that are capable of cross-linking side chains of food proteins including casein, because the cross-linking of food proteins induced by these enzymes may be served as an alternative approach and also produces some desired modifications in functional properties of the
proteins treated.

Matheis and Whitaker (1987) had reviewed various enzymatic reactions that might possibly induce cross-linking of proteins and proposed that besides the trans-glutaminase-catalyzed reaction, oxidative cross-linking of food proteins, using various oxidoreductases, should be considered. All amino acids are potential targets of oxidative modification; however, aromatic amino acid residues in proteins are particularly sensitive to oxidation. Tyrosine is the most sensitive residue and the adduct species formed within the aromatic ring are stabilized by delocalization onto neighboring double bonds (Abdelrahim et al., 1997). An important product of protein oxidation is the formation of protein dimer, which is formed by the catalysis of peroxidase (Li and Nicell, 2008) or tyrosinase (Thalmann and Lötzbeyer, 2002). It has been demonstrated that the oxidation of numerous aromatic compounds, such as phenols and aromatic amines, could be accomplished in an aqueous phase using a variety of peroxidases, including tyrosinase, vegetable peroxidases and others (Li and Nicell, 2008). Horseradish peroxidase (HRP, EC 1.11.1.7) represents another interesting biocatalyst that might have potential application in the cross-linking of food proteins. It was found that HRP could induce cross-linking of some proteins in the presence of H$_2$O$_2$ and a low molecular weight hydrogen donor (Stahmann et al., 1977). Enzymatic oxidation of proteins with peroxidase together with H$_2$O$_2$ at basic pH led to an oxidative phenolic coupling of adjacent tyrosine residues to form cross-linked proteins (Aeschbach et al., 1976). Recently, a microbial enzyme Coprinus Cinereus peroxidase was used as an environmentally friendly protein polymerization catalyst to induce polymerization of casein (Steffensen et al., 2008).

In the presented work, the cross-linking of casein catalyzed by HRP was studied. The cross-linking of casein treated with HRP was demonstrated by the analysis of the casein samples with capillary zone electrophoresis (CZE). The degree of cross-linking of casein in the casein samples was also measured by CZE with peak area normalization methodology. Response surface methodology was applied to optimize the reaction conditions of cross-linking of casein, including reaction temperature, time and addition level of HRP. Emulsifying activity index (EAI) and emulsion stability index (ESI) of the cross-linked casein were evaluated and compared with that of casein. The impact of cross-linking on the microstructure of acidified gel of the cross-linked casein was also observed with the technique of scanning electron microscopy.

**MATERIALS AND METHODS**

**Materials and chemicals**

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

**Cross-linking of casein catalyzed by HRP**

Casein solution (5% w/w on protein basis) was prepared by dispersing approximately 5.33 g of casein powder in 100 ml Na$_3$CO$_3$-NaHCO$_3$ buffer (pH 9.5) and keeping at 4°C overnight for rehydration. Casein solution was heated in a water bath at 70°C for 5 min and cooled naturally to ambient temperature (about 20°C). HRP solution (1.0 g·l$^{-1}$ water) was prepared immediately prior to use. After withdrawing a 10 ml sample (zero time sample), the cross-linking reaction was started by addition of 1.0 ml 3% (w/v) H$_2$O$_2$ and 1.0 ml HRP solution to the casein solution (giving approximately 7.3 μkat·g$^{-1}$ proteins) and the reaction mixture was mixed well. The reaction was carried out at ambient temperature with continuous agitation. After 10 ml·samples were withdrawn from reaction mixture after 0.5, 1, 1.5, 2, 2.5 and 3 h, respectively. The HRP in the separated samples was inactivated immediately by heating the samples at 85°C for 10 min. The solution of HRP-treated casein was freeze-dried and then stored at -20°C for further CZE analysis.

**Capillary zone electrophoresis of the cross-linked casein**

The cross-linked casein was subjected to CZE analysis in a capillary electrophoresis system (P/ACE MDQ Capillary Electrophoresis System, Beckman Coulter, Inc. Fullerton, CA, USA) controlled by a System Gold Software Data System version 8.0. The analysis conditions employed were the same as used by Ortega et al (2002). The separations were performed using a fused-silica capillary of 60 cm (50 cm to the detector window) × 75 mm I.D. The running buffer was 14.7 mol·l$^{-1}$ H$_2$PO$_4$, 6 mol·l$^{-1}$ urea and 0.05% hydroxypropylmethyl cellulose (HPMC) and its pH was adjusted to 3.0 with 2 mol·l$^{-1}$ NaOH. Sample buffer (pH 8) consisted of 10 mmol·l$^{-1}$ H$_3$PO$_4$, 8 mol·l$^{-1}$ urea, 10 mmol·l$^{-1}$ DL-dithiothreitol (DTT). Before each injection, the capillary was washed with 0.1 mol·l$^{-1}$ NaOH (5 min), deionized water (5 min), 1 mol·l$^{-1}$ HCl (5 min) and then equilibrated with the running buffer (5 min).

All experiments were carried out in the cationic mode (anode at the inlet and cathode at the outlet). The sample introduction was achieved by pressure injection for 5 s at 3.4 kPa. During sample analysis, a constant voltage was applied and the separation temperature was kept at 25°C with circulating coolant surrounding the capillary. The detection was carried out at 214 nm (data collection rate 5 Hz). The capillary was rinsed sequentially between successive electrophoretic runs with deionized water (2 min) and a solution of NaOH whose concentration was 0.01 mol·l$^{-1}$ (2 min) and equilibrated with the running buffer (5 min). Between runs, the capillary was purged for 5 min with running buffer. The first electropherogram in a series was always discarded.

For the dissociation of casein, all analysis samples were dissolved in sample buffer and left at least 1 h at ambient temperature before filtration (0.45 μm) and analysis. The concentration of casein standard and the cross-linked casein in analysis samples was fixed at 5 mg·ml$^{-1}$.

**Experimental design and reaction condition optimization**

Response surface methodology (RSM) was employed to determine the effect of combined treatments of temperature(°C, $X_1$), reaction time(h, $X_2$) and addition level of HRP (μkat·g$^{-1}$ proteins, $X_3$) on the cross-linking of casein. The optimal experiment was carried out according to a central composite face-centered design. The ranges
and levels of the variables investigated in our work are given in Table 1. The quadratic model for predicting the optimal point was expressed according to the following equation (1).

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ij} x_i x_j + \sum_{i<j}^{k} \beta_{ij} x_i x_j + \epsilon
\]  

(1)

Where \( Y \) was the dependent variable (degree of cross-linking of casein, \%) to be modeled, \( \beta \), \( \beta_i \), \( \beta_{ij} \), and \( \epsilon \) were regression coefficients of the model and \( X \) was the coded level of the independent variables. The regression equation above was optimized for optimal values using Design Expert Software (Version 7.0). The statistical significance of the second order model equation was determined by F-value and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2.

The complete design consisted of twenty experimental points, including six replications of the center points and triplicate experiments were performed at all design points in randomized order. Calculations and graphics for experimental results were also performed by Design Expert Software (Version 7.0), keeping one variable at its central level. The combination of different optimized variables, which yielded the highest Y value, was determined in an attempt to verify the validity of the model. Subsequently, additional three experiments were conducted to verify the validity of the statistical experimental strategies and the cross-linked casein prepared was used for EAI and ESI evaluation later.

**Emulsifying activity index and emulsion stability index of the cross-linked casein**

The emulsifying properties of casein and the cross-linked casein samples were evaluated by a turbidimetric method (Pearce and Winsel, 1978) to obtain their emulsifying activity index and emulsion stability index. To prepare emulsions, 25.0 mL of refined soy bean oil and 75.0 mL of casein or the cross-linked casein solution (0.01, 0.02, 0.03 and 0.04% on protein basis respectively) in 0.2 M NaOH was used. The emulsions were immediately transferred into a 250 ml capacity glass beaker. Aliquots of freshly prepared emulsion were taken 0.5 cm from the bottom of the beaker and dispersed into 5 ml of 0.1% sodium dodecylsulfate (SDS) solution. The absorbance of these samples was measured at 500 nm against 0.1% SDS solution as described above. EAI (m²·g⁻¹) and ESI (%) of casein or the cross-linked casein were calculated by using equation (2) and (3). Each EAI and ESI evaluation was carried out triplicate.

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

(2)

\[
ESI(\%) = \frac{A_{10}}{A_0} \times 100
\]  

(3)

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

**The microstructure of acidified gel of the cross-linked casein**

Acidified gel of casein or the cross-linked casein was prepared with a reference method (Koh et al., 2002) with some modifications. Granules of glucono-δ-lactone (GDL) about 0.6 g were added to casein or the 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 gel block and immediately fixed in 2% glutaraldehyde solution for 1 h. The fixed gel samples were dehydrated in a graded ethanol series. This consisted of 15 min in each of a 50, 70, 90, 100, 100, 100 ml/100 ml ethanol solution and tertiary butyl alcohol. All 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.

**RESULTS AND DISCUSSION**

**Cross-linking of casein catalyzed by HRP**

Casein and some HRP-treated casein samples (reaction time was 1, 2 or 3 h, respectively) were solved in water and subsequently subjected to CZE analysis to confirm the occurrence of cross-linking in casein. The analysis results are presented in Figure 1.

Ortega et al. (2002) had applied CZE technique to separate bovine caseins; \( \alpha_1 \)-casein, \( \beta \)-casein, \( \kappa \)-casein and various phosphorylation states of the \( \alpha_1 \)-casein and \( \alpha_2 \)-casein, as well as some genetic variants of \( \beta \)-casein (A’, A” and B) were well separated. In our work, casein

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**Table 1. Independent variables and their levels used in central composite design for oxidative cross-linking of casein by HRP and H₂O₂.**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Coded levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature, ( X_1 ) (°C)</td>
<td>-1.682</td>
</tr>
<tr>
<td>Reaction time, ( X_2 ) (h)</td>
<td>-1</td>
</tr>
<tr>
<td>Addition level of HRP, ( X_3 ) (µkat·g⁻¹ proteins)</td>
<td>0.636</td>
</tr>
</tbody>
</table>

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Addition level of HRP, \( X_3 \) (µkat·g⁻¹ proteins) and reaction time, \( X_2 \) (h) and temperature, \( X_1 \) (°C) were optimized between -1.682 and +1.682 considering 6 center points and triplicate experiments (11 points). The quadratic model for predicting the optimal point was expressed according to the following equation (1).
and selected HRP-treated casein samples were subjected to CZE analysis with Ortega’s method. Six peaks were found in untreated casein sample and identified as the main casein components $\alpha_s$-, $\alpha_s^0$-, $\kappa$-, $\beta_B$-, $\beta_{A1}$-, $\beta_{A2}$- casein, which are labeled as peak 1 to peak 6 (Figure 1a). A new peak, labeled peak 7, was found behind peak 6 ($\beta_{A2}$-casein) in the HRP-treated casein samples, which might be the polymer of the monomeric forms of the casein (Figures 1b-d). Moreover, it was also found that the decrease of monomeric forms of main casein components occurred clearly, for the heights of the peaks of casein appeared to be lower as cross-linking reaction progressed, implying the cross-linking of casein and formation of casein polymers. The high resolution of CZE technique allowed the monitoring of changes of casein components. Therefore, we applied this technique to measure the degree of cross-linking of casein in the HRP-treated casein sample with peak area normalization method.

Optimization of the cross-linking conditions of casein

The central composite design (CCD) was applied to evaluate the optimal temperature ($X_1$), reaction time ($X_2$) and addition level of HRP ($X_3$) (independent variables) for the cross-linking of casein (dependent variable) and the degree of cross-linking of casein obtained from CZE analysis was used as response ($Y$). The levels of the variables for the CCD experiments were selected based on the previous experimental results (unpublished results). Tables 2 and 3 give ANOVA results of response surface design and the significance of the regression coefficients of the model respectively. The results indicated that the addition level of HRP had the highest significance ($P = 0.0012$) on the cross-linking of casein (Table 2). In statistical analysis, the second order polynomial equation for the cross-linking of casein was obtained as below (equation 4). The fitting of the model was calculated to be 0.9374 by the coefficient of determination $R^2$, indicating that 93.74% of the variability in the response could be explained by the model. The statistical significance of the second order model equation was evaluated by the F-test analysis of variance, which revealed that this regression was statistically significant at 88.1% of confidence level (Table 3).

$$Y = -31.994 + 1.736X_1 + 1.436X_2 + 2.134X_3 - 0.001X_1X_2 - 0.012X_1X_3 + 0.043X_2X_3 - 0.023X_1^2 - 0.275X_2^2 - 0.191X_3^2$$

Figure 1. Capillary electrophoresis analysis results of casein (A) and the cross-linked casein prepared at 1, 2 and 3 h respectively (B, C and D). The peaks in electropherogram of casein or the cross-linked casein samples were identified as: 1, $\alpha_s$-casein; 2, $\alpha_s^0$-casein; 3, $\kappa$-casein; 4, $\beta_B$-casein; 5, $\beta_{A1}$-casein; 6, $\beta_{A2}$-casein; 7, polymer of casein.
levels of temperature, reaction time and addition level of HRP for the maximal cross-linking of casein were 37°C, 2.9 h and 4.73 µkat·g⁻¹ proteins, respectively. To confirm this result, three experiments were conducted independently at optimal conditions. The actual result for the degree of cross-linking of casein was 6.90% (mean of three experimental results), which demonstrated good correlation between predicted and actual result and verified the validity of the model.

Emulsifying activity index and emulsion stability index of the cross-linked casein

EAI of casein or the cross-linked casein was measured with emulsions prepared at four protein concentrations and the evaluation results are given in Figure 3a. Although the EAI of casein and the cross-linked casein both decreased with the increase in protein concentrations (from 2.07 m²·g⁻¹ to 1.28 m²·g⁻¹ for casein, or from 2.25 m²·g⁻¹ to 1.35 m²·g⁻¹ for the cross-linked casein), EAI of the cross-linked casein was about 10% higher than that of casein at same protein concentration. ESI of casein and the cross-linked casein were also measured at four protein concentrations, as shown in Figure 3b. ESI of the cross-linked casein was increased as protein concentration increased and also about 6% higher than that of casein at same protein concentration. The evaluation results indicated that the cross-linking of casein led to the improvement in emulsion activity and emulsion stability of casein.

Although published studies were not found to study the effect of HRP-treatment on the emulsifying properties of food proteins, some related works might give support and confirmed that transglutaminase treatment of food proteins could affect their emulsifying properties in different behaviors. Motoki et al (1984) studied the polymerization of several food proteins (αsl-casein, κ-casein, or soybean 7S and 11S globulin) by transglutaminase and the results showed that the emulsifying activity of polymerized αsl-casein was higher than that of the native protein in the range of pH 4~6. Meanwhile, Liu and Damodaran (1999) had reported a decrease in the EAI but increase in the emulsion stability of β-casein treated with transglutaminase. Contrary to these results, Siu et al. (2002) reported that a decrease in the EAI or the ESI of oat globulin

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### Table 2. ANOVA response for linear, quadratic and interactive effects of variables used in the model for oxidative cross-linking of casein by HRP and H₂O₂.

<table>
<thead>
<tr>
<th>Model term</th>
<th>Coefficient estimated</th>
<th>P-Value</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-31.994</td>
<td>0.6882</td>
<td>–</td>
</tr>
<tr>
<td>X₁</td>
<td>1.736</td>
<td></td>
<td></td>
</tr>
<tr>
<td>X₂</td>
<td>1.436</td>
<td>0.2660</td>
<td>–</td>
</tr>
<tr>
<td>X₃</td>
<td>2.134</td>
<td>0.0012</td>
<td>**</td>
</tr>
<tr>
<td>X₁²</td>
<td>-0.023</td>
<td>&lt;0.0001</td>
<td>**</td>
</tr>
<tr>
<td>X₂²</td>
<td>-0.275</td>
<td>0.0005</td>
<td>**</td>
</tr>
<tr>
<td>X₃²</td>
<td>-0.191</td>
<td>&lt;0.0001</td>
<td>**</td>
</tr>
<tr>
<td>X₁⋅X₁</td>
<td>-0.001</td>
<td>0.9290</td>
<td>–</td>
</tr>
<tr>
<td>X₁⋅X₂</td>
<td>-0.012</td>
<td>0.3246</td>
<td>–</td>
</tr>
<tr>
<td>X₁⋅X₃</td>
<td>0.043</td>
<td>0.4551</td>
<td>–</td>
</tr>
</tbody>
</table>

a X₁, temperature(°C); X₂, reaction time (h); X₃, addition level of HRP (µkat·g⁻¹ proteins).

b –, not significant; **, P < 0.01.

### Table 3. ANOVA for response surface quadratic model obtained for oxidative cross-linking of casein by HRP and H₂O₂.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>32.24</td>
<td>9</td>
<td>3.58</td>
<td>16.63</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Residual</td>
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<td>10</td>
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<tr>
<td>Pure error</td>
<td>0.11</td>
<td>5</td>
<td>0.022</td>
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<td></td>
</tr>
<tr>
<td>Corrected total</td>
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<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R Square</td>
<td>0.9374</td>
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<td></td>
</tr>
<tr>
<td>Adjusted R Square</td>
<td>0.8810</td>
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<td></td>
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<tr>
<td>Coefficient of variation</td>
<td>9.09</td>
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</tr>
</tbody>
</table>
Figure 2. The response surface plots for the optimization of the cross-linking of casein: (a) addition level of HRP and reaction time (temperature at the central of its level), (b) addition level of HRP and temperature (reaction time at the central of its level), (c) temperature and reaction time (addition level of HRP at the central of its level).

Figure 3. Emulsifying activity index (EAI) (a) and emulsion stability index (ESI) (b) of casein and the cross-linked casein evaluated at different protein concentrations. Each evaluation was carried out triplicate.

treated with transglutaminase. Our result shared similarity to Motoki’s or Liu and Damodaran’s result that oxidative cross-linking of casein would lead to the improvement in EAI or ESI of casein.

The microstructure of acidified gel of the cross-linked casein

The microstructure of acidified gel of casein or the cross-linked casein was observed with scanning electron microscopy and results are shown in Figure 4. The acidified gel was prepared by acidification of casein or the cross-linked casein with GDL. The network of casein gel was characterized as a loose structure with big and irregular holes (Figure 4a). However, a more compact, homogeneous structure and small pores were formed...
between protein granules in acidified gel of the cross-linked casein (Figure 4b), indicating that microstructural improvement occurred. These microstructural improvements were possibly due to the more inter- and intra-molecular interactions in the acidified gel of the cross-links of casein. Recent studies also had indicated that transglutaminase treatment had led to the microstructure of soy protein isolate and chicken meat more continuous and uniform coral-like structure (Tang, 2007; Trespalacios and Pla, 2007; Gan et al., 2008). The result in our study was consistent with these findings, showing that cross-linking of casein catalyzed by HRP had the ability to form acidified gel with better microstructure.

Figure 4. The microstructures of the acidified gels prepared from casein (a) and the cross-linked casein (b) observed with scanning electron microscopy at 5000 ×.

Conclusion

Casein was oxidative cross-linked by HRP in presence of H$_2$O$_2$ and the results from CZE analysis confirmed the occurrence of cross-linking. When some HRP-treated casein samples were analyzed by CZE, a new peak was found in the protein profiles and the peak height of main casein components appeared lower. The cross-linking conditions of casein catalyzed by HRP were optimized with response surface methodology. When casein concentration and pH of reaction medium were fixed at 5% (w/w) and 9.5 respectively, the optimal conditions were that the addition level of HRP was 4.73 µkat-g$^{-1}$ proteins, temperature 37°C and reaction time 2.9 h. Using these conditions, the cross-linked casein with degree of cross-linking about 6.90% could be prepared. Analysis results showed that the emulsifying activity index or emulsion stability index of the cross-linked casein prepared was enhanced about 10 or 6% compared to that of untreated casein, implying that oxidative cross-linking of casein could give beneficial impact to its emulsifying properties. The microstructure of acid-induced gel of the cross-linked casein observed by scanning electron microscopy was more compact and uniform than that of casein, which also showed that oxidative cross-linking of casein could improve microstructure of acidified gel. The results from our study show the potential application of HRP to modify the property of food proteins.

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