Conjugated Film from Reconstruction of Collagen–Soluble Egg Shell Membrane Protein Matrix

Koji TAKAHASHI, Kohkichi SHIRAI
and Makoto HATTORI
Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu-shi 183

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Abstract Soluble egg shell membrane protein (SEP) obtained from egg shell membrane by performic acid oxidation (25°C for 24 h) and pepsin digestion (25°C for 48 h) was used to improve the physical properties of collagen film through reconstructing collagen matrix with SEP. SEP could probably act electrostatically on accelerating molecular rearrangement of collagen in the ratio of about 3 moles SEP : 1 mole collagen, and 1 mole SEP could conjugate with 1 mole collagen to reconstruct matrix. The SEP-conjugated collagen film with a thermally stable and uniform macrostructure and with good flexibility could be obtained from the collagen matrix with SEP by drying it and desalting with ethanol as compared with collagen film.

Key words: Egg shell membrane, Soluble egg shell membrane protein, Collagen, Collagen film, Physical property

Collagen films or membranes are usually used in foods, medicines, and biochemicals because of uniformity in size, physical characteristics and bioadaptability. However, they have some disadvantages. These have relatively high elasticity, undesirable flexibility or softness, as it is known that edible collagen casings have less chewing properties than natural casings due to those great strength. They do not always have a proper denaturation temperature for the drying of wet films from a collagen solution or dough under a hot air stream. The low softness, the first defect, could be prevented by forming collagen films containing non-collagenous substances as a softener. The second defect, low denaturation temperature, could be overcome by increasing intermolecular cohesion of the fibrils. Thus, the addition of a non-collagenous substance interacting with collagen in the collagen solution or dough is probably effective for reconstructing a collagen matrix with desired flexibility and denaturation temperature. This could lead to improved physical properties for collagen films.

For this purpose, soluble substances interacting with collagen are required. As one of such substances, we have reported that pepsin-solubilized elastin could be used as a textural modifier for collagen casings prepared from the reconstruction of collagen–pepsin–solubilized elastin matrix. We have also reported that soluble egg shell membrane protein (SEP) prepared from egg shell membrane by combined treatment of performic acid oxidation and pepsin digestion, accelerated the reconstruction of collagen matrix composed of fibrils with ordered molecular rearrangement and a stable
Collagen-Soluble Egg Shell Membrane Protein Film

These findings suggest that SEP could show effects similar to that of pepsin-solubilized elastin. Our objective was to clarify the action of SEP on the collagen matrix reconstruction and improve the denaturation behavior and mechanical properties of the collagen film through the reconstruction of the collagen-SEP matrix.

**Materials and Methods**

**Preparation of pepsin-solubilized collagen (PSC)**

PSC was prepared from insoluble pigskin collagen according to the procedure of Takahashi and Hattori.

**SEP Preparation**

SEP was prepared from egg shell membrane (QP Corp., Tokyo, Japan) according to the method described previously. In brief, the pulverized egg shell membrane (1g) was treated with 100ml of performic acid (10ml of hydrogen peroxide and 90ml of formic acid) at 25°C for 24 h. After filtering with a glass filter under a vacuum, egg shell membrane was thoroughly washed with water and solubilized in 100ml of 0.5M acetic acid by digesting it with pepsin (Sigma, 3,200U/mg, 10mg) at 25°C for 48 h. The enzymatic reaction was stopped by adding pepstatin (0.2mg). The centrifuged supernatant was dialyzed against water and lyophilized to recover SEP.

**Collagen matrix reconstruction with SEP**

Reconstruction of the collagen matrix with SEP was performed by dissolving PSC in a 0.5 M acetic acid solution at 4°C to give a concentration of about 1mg/ml before adjusting to pH 6.0, 7.0 or 8.0 with 0.5M sodium hydroxide and diluting it to 0.4mg/ml. After degassing, SEP dissolved in the same solvent was mixed with the PSC solution to give the following concentrations: 0.38mg/ml PSC and 0.0095–0.38mg/ml SEP [SEP : PSC = 1 : 40–1 : 1 (w/w)]. This mixed solution was incubated at 30°C to reconstruct the PSC-SEP matrix and the reaction was monitored by the absorbance at 310 nm.

In case of the matrix reconstruction at pH 7.0, after incubating for 24 hr, the reaction mixture was centrifuged at 5,000 rpm for 30 min, and washed with 0.05 M acetate buffer at pH 7.0 and water three times, respectively, prior to lyophilization and amino acid analysis. Amount of the reconstructed matrix and SEP content of the matrix were evaluated by determining PSC and SEP contents in the PSC-SEP matrix, respectively. SEP and PSC contents were determined from cysteic acid content and glycine content corrected by glycine content of SEP, respectively.

**Preparation of SEP-conjugated PSC film (PSC-SEP film)**

The PSC–SEP matrix was reconstructed on an acrylic resin tray (10×10×0.5cm³) at 33°C for 24 hr [solvent pH, 7.0; PSC conc., 0.3%; SEP : PSC, 1 : 40–1 : 1 (w/w)] and dried under air stream at 33°C for 24 hr. The PSC-SEP film was obtained by desalting with ethanol and drying again as described above.

**Differential scanning calorimetry (DSC)**

DSC for PSC–SEP film was performed to evaluate the thermal denaturation using a DSC apparatus (Seiko SSC-5020 DSC-100, Japan) as described by Takahashi et al.

**Measurement of mechanical property**

Mechanical property of the PSC–SEP film was measured in air (dry condition) and water (wet condition) using a thermal mechanometer (Seiko SSC-5020 TMA/SS 100, Japan) as described previously. In the measurement under the dry condition, sample (2w×1l×0.15 or 0.20mm³) was chucked on the probe and extended at a loading rate of 50g/min at 25°C in order to obtain the stress-strain curve. In the measurement under the wet condition, the chucked sample (2w×1l×0.15 or 0.20mm³) was held entirely in water at 25°C for 15 min and extended at a loading rate of 5 g/min in water.

**Analytical methods**

Amino acid composition of SEP and PSC was determined as described previously. Protein
concentration, total saccharides, uronic acids and hexosamines were determined by the microbuiret method$^6$, the phenol–sulfuric acid method$^2$, the sulfuric acid–carbazole method$^1$ and an improved Blix's method$^4$, respectively. Isoelectric point (pl) and molecular weight of SEP were estimated by isoelectric focusing and size–exclusion chromatography, respectively, as described previously$^{11}$.

**Results and Discussion**

**Action of SEP on collagen matrix reconstruction**

SEP obtained from egg shell membrane by performic acid–pepsin treatment had peculiar structure with very high contents of acidic amino acids (339 residues/1,000 residues) containing cysteic acid (106 residues/1,000 residues), small amount (1.5% as glucose) of saccharides containing uronic acids (0.28% as galacturonic acid) and hexosamines (0.79% as glucosamine), pl 4.5 and molecular weight 22,000 as characterized in the previous paper$^{11}$.

SEP has been recognized to be important for the orderly assembly of collagen molecules with a stable macrostructure$^{11}$. Since the SEP-conjugated PSC film was prepared from the PSC–SEP matrix in this study, the action of SEP on the matrix reconstruction from PSC solution was investigated by varying the mixed ratio of SEP.

The absorbance/incubation time curves for mixed solutions of SEP and PSC [SEP : PSC = 1 : 40–1 : 1 (w/w)] showed no lag phase of the collagen matrix reconstruction (Fig. 1). With increasing in SEP, the rising of the curve increased remarkably, suggesting that the velocity of the matrix reconstruction depended on the amount of SEP mixed. Thus, the time required for the maximum velocity of the matrix reconstruction ($T_{\text{max}}$) was recorded as the point of the highest slope on the curve and the accelerating degree of SEP was expressed as the ratio of $T_{\text{max}}$ with SEP to that (4.5 min) of the control. The amount of the reconstructed matrix and SEP content were also evaluated by determining PSC and SEP contents in the PSC–SEP matrix, respectively after the matrix reconstruction reaction. Then, the ratio of $T_{\text{max}}$, the reconstructed matrix (%) and the SEP content (SEP mole/PSC mole) of the matrix were plotted against the molar ratio of mixed SEP (Fig. 2). The ratio of $T_{\text{max}}$ suddenly decreased with the increase in the molar ratio of mixed SEP, indicative of the rapid acceleration of molecular assembly, and reached its stationary state at about the mixed molar ratio 3 : 1 (SEP : PSC). This means that the interacting points of collagen molecule with SEP were probably saturated by the mixed molar ratio 3 : 1. The reconstructed matrix of the control was 68%. In the presence of SEP, it linearly increased and reached the stationary state (96%) at about the mixed molar ratio 3 : 1, corresponding to the effect on the velocity of molecular rearrangement of collagen described above. It has been previously shown that the SEP mixed in the medium was well incorporated into the matrix$^{11}$. The SEP content of the matrix linearly increased with the increase in SEP and turned to a gradual increase at the mixed molar ratio 2–3 : 1 (SEP : PSC). The saturated SEP content was thus
Collagen-Soluble Egg Shell Membrane Protein Film

Fig. 2. Relation between mixed ratio of SEP and $T_{max}$ ratio, reconstructed matrix or SEP content.
The mixed ratio of SEP, the molar ratio of SEP to PSC in the reaction medium; reconstructed matrix, w/w%; SEP content, molar ratio of SEP to PSC in the reconstructed matrix.

thought to be about 1 mole SEP per 1 mole PSC. These results suggest that SEP could act on the molecular rearrangement of collagen in the ratio of about 3 moles SEP : 1 mole PSC, and that 1 mole SEP could conjugate with 1 mole collagen to reconstruct the matrix. It is thus considered that the excess SEP and PSC could be included in the network of the matrix and would be in existence as a kind of fillers in the SEP-conjugated PSC film, when the reconstructed matrix was dried without washing to eliminate the excess SEP and PSC.

The effect of pH on the reconstruction of PSC-SEP matrix was investigated (Fig. 3). At any pH, the matrix was rapidly reconstructed with SEP as compared with the control, and with the decrease in pH, reconstruction was accelerated as indicated by the difference between the PSC–SEP matrix reconstruction curve and the control at each pH. In particular, at pH 6.0, the control could not reconstruct the matrix, while in the presence of SEP, matrix was rapidly reconstructed ($T_{max}$, 2.1 min), indicating reconstruction of the matrix over a wide pH range. Since the pIs of PSC and SEP were about 9 and 4.5, respectively, the decrease in pH from 8.0 to 6.0 resulted in increase in the positive charge of PSC. Therefore, the primary driving force for the matrix reconstruction was probably the electrostatic interaction.

Features of SEP-conjugated PSC film (PSC-SEP film)
The control film was colorless and transparent, while the transparency of the SEP-conjugated PSC film decreased with the increase in SEP content and the PSC-SEP films at the ratio of 1:4 and 1:1 were colored pale yellow due to the color of SEP itself. Since SEP was insoluble in ethanol for desalting, SEP mixed in the medium was thought to be included in the film without any loss. The thickness of PSC-SEP films was 0.15 mm except for the film (0.20 mm) at the ratio of 1:1. The control film was insoluble in 40-fold water at 30°C for 24 hr, while the PSC-SEP film was partially dissolved with the increase in SEP and the solubility of the PSC-SEP film at the ratio of 1:1 showed 16%,
suggesting a practically stable film. If the dissolved substance was SEP instead of PSC, about 30% of SEP in that film is estimated to be in a free or mobile state. Thus, immobile SEP is calculated to be about 70% of the mixed SEP. However, since the amount of SEP combined with collagen (incorporated SEP) was estimated to be about 10% of the mixed SEP from the result of SEP content of the corresponding PSC–SEP matrix, about 60% of the mixed SEP was considered to be immobilized or combined with collagen during drying to form film.

**Thermal denaturation of PSC–SEP film**

Thermal denaturation of the control film occurred in a region of about 42–56°C and with two endothermic peaks around 48°C and 53°C (Fig. 4), indicating that there were two different components in the thermal stability. Since the control matrix showed a relatively sharp peak around 48°C (Fig. 4), the peak component around 53°C was thought to be formed by drying. Dehydration by drying could enhance the amount of contact among molecules, microfibrils and fibers. During drying, some organization such as the orderly assembly may occur in some parts in the control film, resulting in the increase in the intermolecular cohesion. The DSC curve for the PSC–SEP film at the ratio of 1 : 40 practically showed one endothermic peak around 54°C unlike the control film. An increase in SEP resulted in the disappearance of the low temperature peak and the very sharp transition (about 3°C) of the high temperature peak, while increases in the ratio above 1 : 1, the denaturation temperature slightly decreased. This was thought to be caused by the collagen matrix reconstruction with somewhat unstable microstructure due to too rapid molecular assembly shown in Fig. 1. Since any PSC–SEP matrix corresponding to each PSC–SEP film indicated two endothermic peaks around 48°C and 53°C (Fig. 4), the organization resulting in the increase in the intermolecular cohesion was thought to occur in the whole matrix by drying in case of the matrix with SEP. Thus, the PSC–SEP film with a stable and uniform macrostructure could be obtained from the matrix reconstructed with SEP as compared with the control film.

**Mechanical property of PSC–SEP film**

Each film showed a convex stress–strain curve under the dry condition, but a concave curve under the wet condition (Fig. 5). This indicates that the fibrils in film can slip more easily in water than in air. The PSC–SEP film, especially, the film at the ratio of 1 : 1 indicated low flexibility under the dry condition as com-

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**Fig. 4.** DSC curves for PSC–SEP films. Figures in the panel, the weight ratio of SEP composition in film. PSC–SEP films were prepared from the reconstructed matrices containing the same SEP compositions by air-drying and desalting with ethanol. Conditions for DSC: DSC, Seiko SSC-5020 DSC 100; sample weight, 0.7 mg; reference, water; heating rate, 2 deg/min; cell, Ag; atmosphere, He at 40 ml/min.
Collagen-Soluble Egg Shell Membrane Protein Film

Fig. 5. Stress-strain curves for PSC-SEP films. Environment: (a), in air (dry condition); (b), in water (wet condition). ——, PSC-SEP film; -----, control film. Figures in the panel, the weight ratio of SEP composition in film (SEP : PSC). Conditions for mechanometry: thermal mechanometer, Seiko SSC-5020 TMA/SS 100; sample size, (a) 2×2×0.15 or 0.20 mm³, (b) 2×1×0.15 or 0.20 mm³; loading rate, (a) 50 g/min, (b) 5 g/min; temperature, 25°C.

pared with the control film, while under the wet condition, very high flexibility. For example, the initial Young's modulus for the control film was 6.9×10⁶ Pa under the wet condition, while for the PSC-SEP films at the ratio of 1:10 and 1:1 were only 86% and 52% that of the control film, respectively. Elongation at the stress of 0.5×10⁷ Pa also indicated 41% for the PSC-SEP film at the ratio of 1:1 and 27% for the control film. Since SEP in the fibrils and the fibril network well absorbed water and increased in free motion, it was thought to loosen network structure and extend easily as compared with the control film. Egg shell membrane is reported to be available for the recovery of burns⁷ and the sorption ability of various metal ions⁸. It is thus expected that PSC-SEP film could obtain these physiological and chemical properties.

Conclusion

Soluble egg shell membrane protein (SEP) obtained from egg shell membrane by performic acid oxidation (25°C for 24 hr) and pepsin digestion (25°C for 48 hr) could probably act electrostatically on accelerating molecular rearrangement of collagen in the ratio of about 3 moles SEP : 1 mole collagen, and 1 mole SEP could conjugate with 1 mole collagen to reconstruct matrix. The SEP-conjugated PSC film with a thermally stable and uniform macrostructure and with good flexibility could be obtained from collagen matrix with SEP by air-drying and desalting with ethanol as compared with collagen film. These findings provide a new possibility for improving physical properties of collagen film.

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コラーゲン-可溶性卵殻膜タンパク質マトリックス
再構築からの複合フィルム

高橋幸資・白井幸吉・服部 誠
東京農工大学農学部、府中市 183

卵殻膜を過酸酸化（25℃で24時間）およびペプシン消化（25℃で48時間）して得た可溶性卵殻膜タンパク質（SEP）を、SEPによるコラーゲンマトリックス再構築することを通じて、コラーゲンフィルムの物性を改良するために用いた。SEPは、おそらくコラーゲン1分子にSEP3分子の割合で静電的に作用してコラーゲンの分子再配列を促進し、SEP1分子がコラーゲン1分子と結合してマトリックス再構築しうると考えられた。SEP結合コラーゲンフィルムは、SEPによるコラーゲンマトリックスを乾燥し、エタノールで脱塩して得られ、コラーゲンフィルムと比較して、熱的に安定で一様なマクロ構造を持ち、良好な柔軟性を持っていた。

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