Collagen-Carboxymethyl Dextran Conjugate without Platelet Aggregation

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Abstract Pepsin solubilized collagen-carboxymethyl dextran (PSC-CMD) conjugate was prepared to improve the function of collagen by using cyanogen bromide. The conjugation between PSC and CMD was confirmed by SDS-PAGE, isoelectric focusing and size-exclusion chromatography. The molar ratio of the conjugate was PSC : CMD = 1 : 2.3. The isoelectric point was shifted from 9.0 to 5.5 by conjugation. Denaturation temperature of the conjugate molecule was 42.2°C, which is 1.9°C higher than that of PSC. The PSC-CMD conjugate maintained self-assembling ability but showed a very slow aggregation as compared with that of PSC. Reconstructed matrix of the conjugate had lower denaturation temperature and random molecular rearrangement structure as compared with PSC matrix. The platelet aggregation inducing ability, analyzed by spectroscopy disappeared.

Key words: Collagen, Platelet aggregation, Conjugation

Collagen is a major component of extracellular matrix. Collagen comprises approximately 40% of whole body proteins and, especially, type I collagen accounts for 90% of body collagen. Collagen provides many biological phenomena, such as scaffold for cell attachment, supporting differentiation and migration, and maintaining cell function and morphology shape, as well as excellent physical strength and molding properties. Collagen has been widely used in foods, medicals and biochemicals, such as edible sausage casing, artificial skin and cell culture material, respectively. However, collagen is known as platelet aggregation inducer, resulting in blood coagulation. The collagen-platelet aggregation interaction is mediated by von Willebrand factor (vWF) and platelet membrane glycoprotein, which belongs to integrin family. Collagen has tri-peptide sequence Arg-Gly-Asp, which is a recognition site of integrin family. In the application to medical material such as artificial vein, it is thus necessary to inhibit the platelet aggregation. The platelet aggregation, induced by collagen, was improved by fixation of heparin and protamine to collagenous tissues and by succinylation of collagen, which means the endowment of carboxyl group to collagen molecules. The gradual release of the fixed heparin from collagenous tissue brings to the platelet aggregation on the exposed collagenous tissue in time, suggesting that the stable inhibition of the platelet aggregation could be achieved by preventing its release. Since acidic polysaccharide has large exclusion space and polyanionic charge as compared with succinylation, it is thought that the platelet aggregation could be stably inhibited by conjugating covalently collagen and acidic polysaccharide having carboxyl group. Recently, we have reported that the conjugation with carboxymethyl dextran (CMD) could be successfully applied to improve functionalities of whey protein without the essential structural change of the protein moiety. This report aimed to prepare a novel collagen-CMD
conjugate to maintain matrix reconstructability and inhibit platelet aggregation.

Materials and Methods

Materials

Dextran T10 (molecular weight, 10kDa) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Cyanogen bromide was purchased from WAKO Pure Chemical Industries, Ltd (Osaka, Japan).

Preparation of pepsin solubilized collagen (PSC)

Insoluble pigskin collagen cubes prepared according to the method of Takahashi et al. were pulverized with a POLYTRON homogenizer (Kinematica, Switzerland). Pepsin solubilized collagen was prepared from the insoluble pig skin collagen by the following procedure. Ten milligrams of pepsin (Sigma, 3200 U/mg) was added to 200 ml of 0.5 M acetic acid containing 1 g of pulverized insoluble collagen and the reaction mixture was incubated at 25°C for 24 h with gentle stirring. The reaction mixture was centrifuged at 16,900 × g for 40 min to remove insoluble collagen. PSC was obtained from this supernatant. PSC was added with 20 g of sodium chloride, then recovered by centrifugation at 1,080 × g for 30 min and the precipitate was repeatedly re-dissolved in 200 ml of 0.05 M acetic acid for 3 times. Finally, PSC was dialyzed against 0.05 M acetic acid, lyophilized and kept at −20°C until use.

Preparation of carboxymethyl dextran (CMD)

Carboxymethyl dextran was prepared according to the method of Hattori et al. In brief, dextran (10 g) was dissolved in 40 ml of 15% monochloroacetic acid solution containing 3.5 g sodium hydroxide and incubated at 40°C for 48 h. The reaction was stopped by addition of acetic acid (3 ml). CMD was obtained after dialysis against distilled water and lyophilization. The degree of modification was measured by hydrochloride-methanol titration. The degree of modification value was 25 carboxyl group residues per one dextran molecule.

Preparation of PSC-CMD conjugate

Pepsin solubilized collagen-carboxymethyl dextran conjugate was prepared by the modified method of Marshall and Rabinowitz. 1.25% CMD solution (40 ml) was adjusted at pH 10.7 with 0.5 M sodium hydroxide. CMD was activated by addition of cyanogen bromide (125 mg) with gentle stirring. The solution was maintained at pH 10.7 with 0.5 M sodium hydroxide during the activating reaction. After 30 min cyanogen bromide (125 mg) was added. The activating reaction was stopped by adjusting to pH 9.0 with 0.1 M hydrochloric acid after 1 h. The reaction mixture was dialyzed against distilled water at 4°C for 2 h, which was adjusted at pH 9.0 with 1 M Na₂CO₃. By addition of 0.25% PSC solution (20 ml), the PSC and CMD were covalently cross-linked through incubation at 4°C for 12 h, and pH was maintained at 9.0 during the reaction with 1 M Na₂CO₃. The cross-linking reaction was stopped by addition of 10% glycine solution (4 ml), and stirred at 4°C for 2 h. PSC-CMD conjugate was added with 8 g of sodium chloride, then centrifuged at 1,080 × g for 40 min and the precipitate was repeatedly re-dissolved in 40 ml of 0.05 M acetic acid 3 times to remove un-reacted CMD. Finally, the PSC-CMD conjugate was dialyzed against the same solution, lyophilized and kept at 20°C until use.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Pepsin solubilized collagen-carboxymethyl dextran conjugate was heated in electrophoretic buffer containing 3% SDS and 3.2 M urea at 100°C for 5 min and electrophoresed by the modified method of Leammli, using 5% polyacrylamide gel. Protein bands were stained with Coomassie Brilliant Blue (CBB) R-250, and saccharide bands were stained by periodic acid-shiff stain method (PAS). The coincidental migration band, stained with CBB and PAS as described above, was taken as evidence of the conjugate.

Isoelectric focusing

Isoelectric focusing of PSC-CMD conjugate was done by the method of Awdh et al., using 4.2% polyacrylamide gel. Protein bands were stained with CBB G-250.

Size-exclusion chromatography (SEC)

Size-exclusion chromatography was done using HPLC system (Tosoh, Tokyo, Japan) and TSKgel G 5000PWXL (7.8 ID × 300 mm) (Tosoh, Tokyo, Japan)
equilibrated with 0.067 M phosphate buffer at pH 8.0. The PSC-CMD conjugate was dissolved in same buffer to give a protein concentration of 100 μg/100 μl and applied to the column after denaturation at 80°C for 15 min, and eluted at flow rate 0.5 ml/min. Absorbance was monitored at 230 nm.

**Ion-exchange chromatography (IEC).**

Ion-exchange chromatography was done by using DEAE Toyopearl 650S column (2.2 ID × 20 cm) (Tosoh, Tokyo, Japan) equilibrated with 0.05 M Tris-HCl buffer at pH 7.0 containing 2 M urea. The PSC-CMD conjugate (10 mg/100 ml) was applied to the column and eluted by a linear gradient elution of 0 to 1 M NaCl in the same buffer at a flow rate 0.5 ml/min.

Protein and polysaccharide were detected by absorbance at 230 nm and phenol-sulfuric acid method,

**Collagen matrix reconstruction**

Reconstruction of the collagen matrix (collagen gel with three-demential network structure) was performed according to the procedure of Nomura et al. The PSC-CMD conjugate was dissolved in 0.05 M acetic acid at 4°C to give a collagen concentration of about 0.45 mg/ml and adjusted to pH 7.0 with 0.5 M NaOH. The solution was centrifuged at 38,800 × g for 30 min to remove any insoluble fraction. After the collagen concentration was adjusted to 0.4 mg/ml and degassed, the solution (3 ml) was incubated at 37°C to reconstruct the matrix and the reaction was monitored by absorbance at 310 nm.

**Differential scanning calorimetry (DSC)**

The reconstructed matrix, performed in the same condition of collagen matrix reconstruction, was collected by centrifugation at 1,080 × g for 40 min. The matrix (1 mg) was sealed in a cell and DSC was performed using a DSC apparatus (Seiko SSC-5020 DSC 100, Japan) described by Takahashi et al. Denaturation parameters, onset temp ($T_o$), peak temp ($T_p$) and conclusion temp ($T_c$) were determined from the DSC curve.

**Scanning electron microscopy (SEM)**

Scanning electron microscopy for the reconstructed matrix was done by the procedure of Takahashi and Hattori. The matrix, reconstructed for 24 h according to the condition described above, was washed to remove free conjugates with the fresh buffer by centrifuging at 1,080 × g for 40 min three times. The supernatant was removed after every washing. The washed matrix was fixed with 0.05 M acetate buffer at pH 7.0 containing 2% glutaraldehyde at 4°C for 12 h, washed thoroughly with distilled water and dehydrated in ethanol with stepwise concentrations of 50, 70, 95 and 99.5%. After critical point drying with carbon dioxide and Pt coating with an ion spatter, the matrix was examined by a JSM-6300 scanning electron microscope (JEOL, Tokyo, Japan) at an accelerated voltage of 5 kV.

**Platelet aggregation assay**

The platelet aggregation assay was done by the modified method of Born using Simadzu UV160 spectrophotometer (Kyoto, Japan) with a heating apparatus and a stirrer. Platelet rich plasma (PRP) was prepared from healthy volunteer's peripheral blood by centrifuging at 226 × g for 10 min, platelet poor plasma (PPP) was prepared from the remaining plasma by centrifuging at 2,000 × g for 10 min. The PRP was diluted with PPP to $3 \times 10^6$ cells/ml. This platelet suspension (3 ml) and PPP were placed in the sample and reference cuvettes, respectively. The platelet suspension was pre-incubated at 37°C for 3 min with stirring at 400 rpm. The PSC-CMD conjugate was dissolved in phosphate buffered saline (Ca$^{2+}$, Mg$^{2+}$ free) (PBS(-)) at collagen concentration of 200 μg/ml and a 0.3 ml aliquot was added to the platelet suspension. The platelet aggregation was measured by recording the decrease of absorbance at 600 nm. Stirring was maintained during the aggregation assay.

**Results and Discussion**

**Chemical features of PSC-CMD conjugate**

The chemical features of the conjugate are shown in Table 1. The yield of the conjugate was 68%. The molar ratio of PSC to CMD in the conjugate, determined by absorbance at 230 nm and phenol-sulfuric acid method was 1 : 2.3. The coincidence of protein and saccharide bands on SDS-PAGE pattern and the shift of isoelectric focusing point from 9.0 of PSC to 5.5 confirmed the conjugation of PSC and CMD. The SEC pattern for the conjugate also showed that...
Table 1. Chemical features of the PSC-CMD conjugate

<table>
<thead>
<tr>
<th></th>
<th>PSC-CMD conjugate</th>
<th>PSC</th>
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<tbody>
<tr>
<td>PSC : CMD (mole)</td>
<td>1 : 2.3</td>
<td></td>
</tr>
<tr>
<td>Isoelectric pointb</td>
<td>5.5</td>
<td>9.0</td>
</tr>
<tr>
<td>Denaturation temp. (°C)c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T₀</td>
<td>38.8</td>
<td>37.3</td>
</tr>
<tr>
<td>Tₑ</td>
<td>42.2</td>
<td>40.3</td>
</tr>
<tr>
<td>Tₚ</td>
<td>46.3</td>
<td>43.5</td>
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</table>

a Determined by absorbance at 230nm and phenol-sulfuric acid method, respectively.
b Evaluated by isoelectric focusing.
c Evaluated by DSC.

Fig. 1. The SEC patterns of PSC-CMD conjugate.
The arrowheads express each elution points of α and β chain of collagen subunit, and free CMD molecule. HPLC conditions: column, TSKgel G 5000PWXL (7.8×300 mm); mobile phase, 0.067 M phosphate buffer at pH 8.0; flow rate, 0.5 ml/min; Sample denatured at 80°C for 15 min was applied and protein (-----) and saccharide (------) were detected by absorbance at 230 nm and phenol-sulfuric acid method, respectively.

heavy collagen subunit chains above α chain had saccharide, which was colored by the phenol-sulfuric acid method (Fig. 1). From these results, it was therefore demonstrated that PSC and CMD was covalently cross-linked to each other. However, SEC pattern showed a small peak at 11.5 ml, which had smaller molecular weight than that of α chain. This indicates that the conjugate had small amounts of collagen peptide due to cyanogen bromide treatment.

Fig. 2. DEAE chromatographic pattern of the PSC-CMD conjugate.
The PSC-CMD conjugate (10 mg/100 ml) was dissolved in 0.05 M Tris-HCl buffer containing 2 M urea at pH 7.0, applied to DEAE Toyopearl 650S column (Tosoh, 2.2×20 cm) equilibrated with the same buffer, and eluted by a linear gradient elution of 0 to 1 M NaCl in the same buffer at flow rate 0.5 ml/min. The protein (■) and CMD (○) were detected by absorbance at 230 nm and phenol-sulfuric acid method, respectively. The marked fractions (220-320 ml) indicated by arrowhead were recovered as the purified PSC-CMD conjugate.

The conjugate was purified by ion-exchange chromatography (IEC) to remove the peptide. The elution pattern showed the fractions having protein and saccharide at the elution volume of 220-320 ml (Fig. 2), monitored at 230 nm and phenol-sulfuric acid method, respectively. These fractions were recovered and lyophilized as the purified PSC-CMD conjugate without collagen peptide. Recovery of the purified conjugate was 21.3%. The conjugate easily dissolved in 0.05 M acetic acid, 0.067 M phosphate buffer at pH 8.0 and phosphate buffered saline, but not in distilled water. Thermal denaturation of the PSC suspension in distilled water, which was evaluated by DSC, occurred in the region of about 37-44°C, while the conjugate of about 39-46°C (Table 1). This indicates the improvement of the thermal stability (approximately 2°C) of collagen molecule by conjugating with CMD.

Matrix reconstructability of PSC-CMD conjugate

The progress of the collagen matrix reconstruction can be evaluated as the absorbance/incubation time curve (Fig. 3, Panel A), including three steps corresponding to the lag phase, growth phase and
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Fig. 3. Collagen matrix reconstruction curves. Sample ; (------), PSC/CMD conjugate ; (……..), PSC ; (———), PSC/CMD mixture. Conditions for matrix reconstruction: collagen concentration, 0.4 mg/ml; solvent, 0.05 M acetate buffer at pH 7.0; temperature, 37°C; ratio of PSC/CMD mixture = 1: 2.3 mole; detection, absorbance at 310 nm. (A), absorbance/incubation time curves; (B), differential curves for the data in panel (A).

Table 2. Effect of conjugation of CMD on collagen matrix reconstruction

<table>
<thead>
<tr>
<th></th>
<th>lag time (sec)</th>
<th>T_max* (sec)</th>
<th>Reconstructed matrix (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSC</td>
<td>188</td>
<td>340</td>
<td>53</td>
</tr>
<tr>
<td>PSC/CMD mixture</td>
<td>n.d.*</td>
<td>157</td>
<td>69</td>
</tr>
<tr>
<td>PSC-CMD conjugate</td>
<td>325</td>
<td>514</td>
<td>51</td>
</tr>
</tbody>
</table>

*a Incubation time at the maximum velocity point for collagen matrix reconstruction.

stationary phase^14,20,21^ The time required for the maximum velocity of the matrix reconstruction (T_max) corresponding to the second step, was given by the peak top on the differential curve of the absorbance/ incubation time curve (Fig. 3, Panel B). The T_max of PSC was 340 sec (Table 2). However, Tmax of the conjugate increased 1.5 times that of the PSC. This indicated that the velocity of the matrix reconstruction was much reduced by the conjugation. On the other hand, only the mixture of PSC and CMD containing the same ratio as those of the conjugate was reduced to about 1/2 of PSC, indicating the marked acceleration of the molecular rearrangement of collagen probably due to the electrostatic interaction between collagen molecule and CMD. It was thus suggested that the large T_max of the conjugate was caused by the steric hindrance of the conjugated CMD. The amount of the reconstructed matrix was estimated by measuring the concentration of collagen in the centrifuged supernatant after matrix reconstruction. The reconstructed matrix for the conjugate and PSC were 51% and 53%, respectively, while for the PSC/CMD mixture was 69%. From these results, it was considered that the conjugate had very low velocity of the molecular rearrangement, while finally having had the matrix reconstructability similar to that of PSC.

Features of PSC-CMD conjugate matrix

Scanning electron micrographs for the reconstructed matrices are shown in Fig. 4. The PSC matrix had twisted fibrils and well-developed network structure without any regular constriction. Mean diameter of PSC fibrils was 75 ± 14 nm (expressed as mean ± SD, n = 100). In the case of PSC/ CMD mixture, the reconstructed matrix had predominant thick fibrils (120 ± 37 nm) with banding pattern at regular intervals about 70 nm. Since fibrils with the regular matrix reconstruction shows a periodic cross-striated structure by transmission electron microscopy, indicative of ordered molecular rearrangement like the native type^{20}, this microscopic observation suggests that the reconstructed PSC fibrils had random rearrangement of collagen molecule, while the reconstructed fibrils of the PSC/CMD mixture had the orderly rearrangement like native type. On the other hand, the conjugate matrix showed slightly twisted and well-developed network structure without any regular reconstruction, while having had significantly thick fibrils (106 ± 22 nm) as compared with that of the PSC matrix. The conjugate fibrils were thus thought to have random molecular rearrangement. Thermal denaturation of the matrices occurred in a region of about 41-56°C (Table 3), which was about 4-10°C higher than those of PSC (Table 1) and the conjugate described above. The T_p of the conjugate matrix was 43.9°C, which was 5.2°C lower than that of PSC matrix. The conjugated
Fig. 4. Scanning electron micrographs of collagen matrices.
Collagen matrix was reconstructed in 0.05 M acetate buffer pH 7.0 at 30°C for 24 h. After fixing and critical point drying, the observation was done at an accelerate voltage of 5 kV by JEOL JSM-6300 SEM. Bars in micrographs indicates 100 nm.

Table 3. Denaturation temperature of reconstructed matrices

<table>
<thead>
<tr>
<th>Matrix</th>
<th>$T_o^a$</th>
<th>$T_p^b$</th>
<th>$T_c^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSC</td>
<td>43.3</td>
<td>49.1</td>
<td>52.2</td>
</tr>
<tr>
<td>PSC/CMD mixture</td>
<td>48.0</td>
<td>50.4</td>
<td>56.3</td>
</tr>
<tr>
<td>PSC-CMD conjugate</td>
<td>41.2</td>
<td>43.9</td>
<td>47.5</td>
</tr>
</tbody>
</table>

$^a$ Onset temperature; $^b$ peak temperature; $^c$ conclusion temperature.

Conditions: DSC, SEIKO SSC5020 DSC 100; sample weight, 1 mg; reference, water; heating rate, 2 deg/min; cell, Ag; atmosphere, He at 40 ml/min.

CMD thus lowered the intermolecular cohesion of collagen fibrils due to the electrostatic repulsion and the steric hindrance. From these results, it can be said that the conjugate could reconstruct the matrix composed of well-developed and thick fibrils but less thermally stable with random molecular rearrangement.

**Platelet aggregation assay**

Collagen is known as a platelet aggregation inducer. The method of Born\(^2\) was applied to analyze collagen-platelet interaction, *in vitro*. Upon addition of PSC, the absorbance of the platelet suspension at 600 nm decreased with the incubation time, indicating the...
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**Table 4. Effect of the CMD conjugation on the platelet aggregation**

<table>
<thead>
<tr>
<th></th>
<th>lag time&lt;sup&gt;a&lt;/sup&gt; (sec)</th>
<th>(A_V&lt;sup&gt;b&lt;/sup&gt;&lt;/span&gt; ((\Delta A/\text{sec}))</th>
<th>(A_I&lt;sup&gt;c&lt;/sup&gt;&lt;/span&gt; ((\Delta A))</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSC</td>
<td>562</td>
<td>3.4 \times 10^{-3}</td>
<td>0.414</td>
</tr>
<tr>
<td>PSC/CMD mixture</td>
<td>500</td>
<td>4.3 \times 10^{-3}</td>
<td>0.066</td>
</tr>
<tr>
<td>PSC-CMD conjugate</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
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</table>

<sup>a</sup> Onset time of a platelet aggregation curve.  
<sup>b</sup> (Starting Abs. – Abs. at \(T_{\text{max}}\)) /\(T_{\text{max}}\), where \(T_{\text{max}}\) is the time required for the maximum aggregation velocity.  
<sup>c</sup> The difference in absorbance at 600 nm before and after aggregation.  
<sup>d</sup> Not determined.

The platelet aggregation curves. Conditions for platelet aggregation: sample, PSC, PSC-CMD conjugate and PSC/CMD mixture (PSC : CMD=1:2.3 mole); solvent, PBS (-); amount of sample solution, 0.3 ml; temperature, 37°C; stirring 400 rpm; platelet suspension, 3.0 \times 10^6 cells/ml (3 ml); detection, absorbance at 600 nm.

**Fig. 5.** The platelet aggregation curves. From this absorbance/incubation time curve (a platelet aggregation curve), lag time, aggregation velocity \(A_V\) and aggregation intensity \(A_I\) were obtained (Table 4) as the characteristics for platelet aggregation behavior by the following manner. The lag time, \(A_V\) and \(A_I\) were defined as the onset time of aggregation, the slope of the tangent at the time \(T_{\text{max}}\) required for the maximum aggregation velocity, that is (starting Abs–Abs at \(T_{\text{max}}\))/(\(T_{\text{max}}\)), and the difference in the absorbance before and after aggregation, respectively. The platelet immediately aggregated by addition of ADP solution (1 mM), confirming that the used platelet had normal aggregation activity. The PSC induced typical platelet aggregation at 562 sec, while CMD and PBS(-) induced no aggregation. The PSC/CMD mixture induced strong aggregation, and lag time, \(A_V\) and \(A_I\) were 0.9, 1.3 and 1.5 times that of PSC, respectively. However, in the case of the conjugate, the aggregation did not occur during this assay. As main factors of platelet aggregation with collagen, the aggregation degree of collagen molecule, the orderly structure of the reconstructed fibril and the collagen network structure were indicated<sup>11,23</sup>. Since the PSC/CMD mixture could reconstruct the matrix composed of fibrils having orderly rearrangement and strong intermolecular cohesion as described above, the strong platelet aggregation with the PSC/CMD mixture was thought to be caused by acceleration of the matrix reconstruction with added CMD. On the other hand, the conjugate has no platelet aggregation inducing ability, in spite of the maintenance of self-assembling ability. The platelet has membrane glycoprotein (GP) IIb-IIIa, which recognize the Arg-Gly-Asp sequence of collagen<sup>17</sup>). This recognition mechanism works as a trigger for the aggregation. It is thus considered that the conjugated CMD could inhibit the interaction between the vWF and/or receptor on the platelet membrane and collagen.

**Concluding remarks**

In this study, we prepared PSC-CMD conjugate using cyanogen bromide. The conjugate has molar ratio of PSC : CMD=1 : 2.3 and 1.9°C higher denaturation temperature than that of PSC, indicating higher intramolecular stability. The conjugate still maintained self-assembling ability, and the reconstructed matrix showed a decrease in the denaturation temperature, which means low intermolecular stability. The interaction between the conjugate and platelet disappeared. These findings are important in the application of collagen as a biomaterial.

**References**


