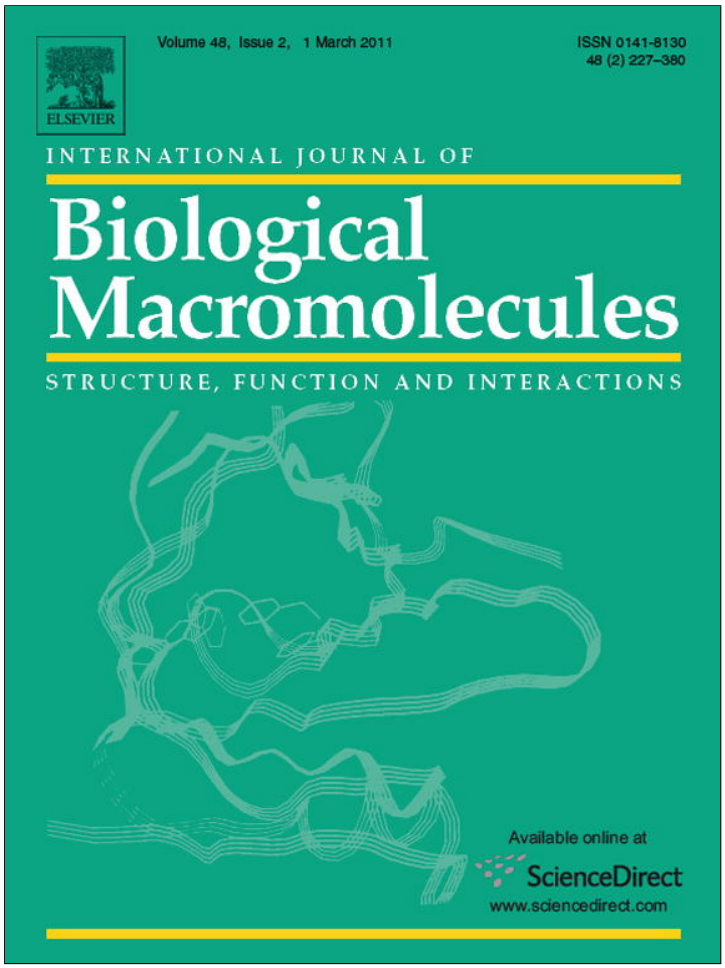

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# Modification of collagen with a natural cross-linker, procyanidin

Lirong He [a], Changdao Mu [b], Jiabo Shi [a], Qian Zhang [a], Bi Shi [a], Wei Lin [a,*]

[a] Department of Biomass and Leather Engineering, National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University, Chengdu, Sichuan 610065, China
[b] Department of Pharmaceutics and Bioengineering, School of Chemical Engineering, Sichuan University, Chengdu, Sichuan 610065, China

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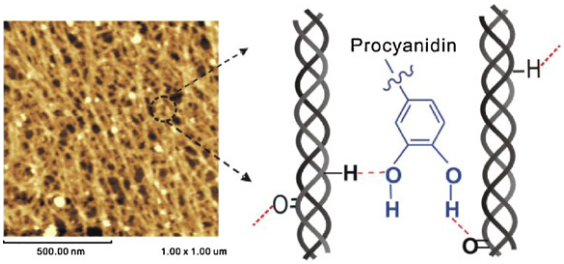
ABSTRACT

We have investigated the modification of collagen with a natural plant polyphenol, procyanidin under acidic conditions. Fourier transform infrared spectroscopy (FTIR) and Atomic force microscopy (AFM) studies demonstrate that the hydrogen bond interactions between collagen and procyanidin does not destroy the triple helix conformation of collagen, and the fibril aggregation occurs because of the cross-linking with procyanidin. The water contact angle (WCA) tests indicate that the hydrophobicity of the procyanidin modified collagen films can be improved. Whereas, the water vapor permeability (WVP) of the films decrease with the increasing procyanidin content due to the formation of denser structure. Moreover, differential scanning calorimetry (DSC) and thermogravimetric (TG) measurements reveal that the collagen/procyanidin films have improved thermal stability in comparison with pure collagen. The present study reveals that procyanidin stabilizes collagen as a cross-linker and preserves its triple helical structure.






## 1. Introduction

Collagen is nowadays one of the most widely investigated proteins, because it not only represents the main structural protein accounting for approximately one-third of all vertebrate body proteins [1], but also has commercial and industrial significance, as exemplified by traditional leather industry and current biomedical applications [2–4]. Generally, the isolated collagen from skin or tendon exhibits poor thermal stability, mechanical strength and water resistance, due to the destruction of natural cross-linking and assembly structure by neutral salt, acid, alkali, or proteases during the extraction process [5]. Therefore, they are often modified for practical uses by various methods, such as UV-light irradiation, multivalent metal ions, and synthetic aldehyde-based cross-linkers [6,7]. However, the treatment by UV only modifies the surface instead of the bulk of the collagen [8], and the toxic nature of aldehyde compounds reflected in the process of manufacturing and application prohibits their utilization in food and biomaterials [9–11]. Thus, some natural polymers with favorable biocompatibility have been exploited as protein cross-linkers, such as genipin, oxidized alginate and dialdehyde starch [7,12,13].

Procyanidin is a kind of condensed plant polyphenol, ubiquitously found in vegetables and fruits [14]. Due to its free-radical scavenging capacity and high affinity to protein, procyanidin has gained recent interest in dietary supplement and pharmacological applications. And the benefit of antioxidant activity, pharmacological activity and therapeutic potential from procyanidin has been extensively manifested [15,16]. In fact, natural plant polyphenols including procyanidin have long been used to treat animal hide collagen under acidic conditions and confer enhanced stability

* Corresponding author. Tel.: +86 28 85460819; fax: +86 28 85405237.
*E-mail address:* wlin@scu.edu.cn (W. Lin).

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against heat and putrefaction to the resultant leather [17–19]. In practical leather-making, the required amounts of the vegetable materials can be 10–40% (w/w) depend on its affinity for hide substance [20]. The interactions between protein and polyphenol can involve hydrogen bond, covalent linkage, ionic and hydrophobic bonding [21–26]. Nevertheless, the effect of plant polyphenol on the microstructure of collagen, i.e. from triple helixes to fibrils, remains largely unknown. It has been well documented that the most abundant Type I collagen comprises two identical α1(I) chains and a different α2(I) chain. The three α-chains twist together into a unique triple helical molecule of ~300 nm in length and ~1.5 nm in diameter. The quarter staggered arrangement of collagen molecules leads to collagen microfibrils (~40 nm in diameter) and fibrils (100–200 nm in diameter), and the fibrils further assemble into collagen fibres [27]. As increasing use of collagen in biomedical applications, understanding the role of procanidin in the modification of collagen structure and property is helpful to development of new biological cross-linkers and fabrication of novel functionalized biomaterials [2].

In the present work, we have investigated the structure of Type I collagen molecules and microfibrils in the presence of procyanidin by Fourier transform infrared spectroscopy (FTIR) and atomic force microscopy (AFM). The surface hydrophilicity/hydrophobicity, water vapor permeability and thermal stability of the collagen/procyanidin films have also been examined. Our aim is to further explore collagen-polyphenol interactions and understand the structure-property relations of procyanidin modified collagen films.

## 2. Experimental

### 2.1. Materials

The acid soluble collagen used in this work was extracted from the fresh adult bovine Achilles tendon. The details can be found in our previous report [28]. The analysis of the extracted collagen by SDS-PAGE (Bio-Rad Powerpac 300, USA) has been made in our laboratory to verify its purity and structural integrity (Fig. 1) [29]. The isoelectric point for collagen is at pH 7.4 as measured by potentiometric titration method using Nano ZS instrument (Malvern Co., UK). The procyanidin, grape seed extract, was obtained from Tianjing Jian Feng Co. Ltd., China. Widely present procyanidin monomer (catechin or epicatechin) and dimmer are illustratively shown in Fig. 2. The content of oligomer procyanidin was more than 85.0% [30].

### 2.2. Preparation of film

After a 5 mg mL$^{-1}$ aqueous collagen solution was prepared, procyanidin solution with the concentration 0.1–0.4 mg mL$^{-1}$ was incorporated under magnetic stirring. Due to the weak acidic nature of procyanidin and the reasonable solubility of collagen in weak acids, the reaction pH of the mixture was adjusted to 3.0 with acetic acid solution. The final contents for procyanidin were 0%, 2%, 4%, 6% and 8% (w/w on dry collagen mass), respectively. And the collagen concentration was kept at 2.5 mg mL$^{-1}$ for all the samples. Subsequently, the mixture was placed under mild ultrasonic (40 kHz, 120 W) for about 1 min to remove air bubbles and then several drops of ethanol was added to further eliminate air bubbles [31]. The collagen/procyanidin films with thickness around 0.03 mm were formed by casting the solution on a polytetrafluoroethylene (PTFE) plate with a diameter of 15 cm then dried at room temperature for about 1 week.

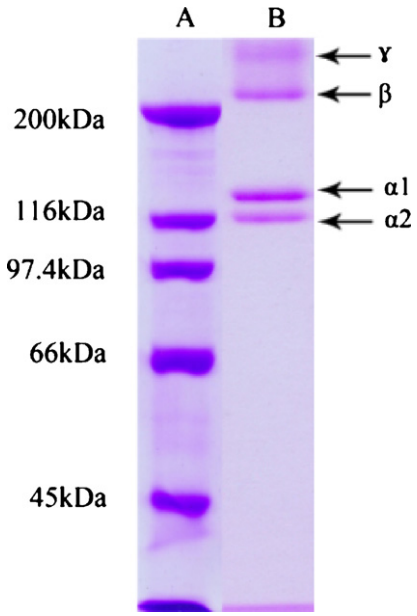


**Fig. 1.** SDS-PAGE analysis of the extracted collagen (B) in comparison with protein markers (A). The protein bands were visualized by staining with 0.1% Coomassie Brilliant Blue R-250.

### 2.3. FTIR spectra measurements

Fourier transform infrared spectroscopy (FTIR) spectra were obtained from the films equilibrated in a desiccator containing silica gel for 24 h at room temperature by a FTIR spectrometer (Nicolet Magna-IR560, USA). All spectra were obtained with a resolution of 4 cm$^{-1}$ in the range of 400–4000 cm$^{-1}$. The spectra plots represent the average of 10 scans.

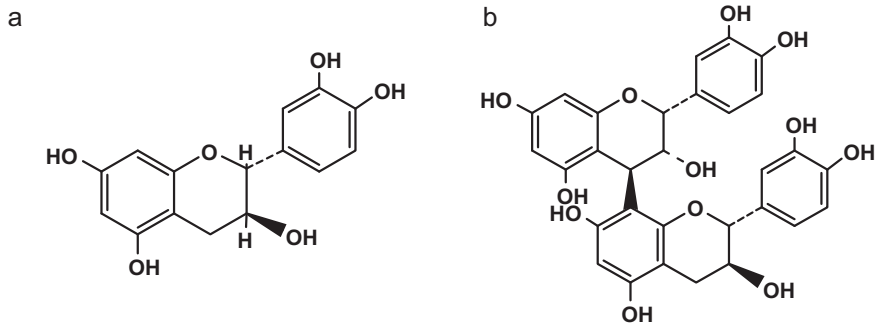


**Fig. 2.** Widely present procyanidin monomer (a) and dimmer (b).

### 2.4. AFM observation

Atomic force microscopy (AFM) was used to determine the topographic change of collagen by introducing procyanidin. Collagen solution (10 μL) with a concentration of $10^{-5}$ (w/w) was dropped onto a fresh mica substrate. Then the procyanidin solution at designated concentration and volume was carefully added onto it. The final weight ratios of procyanidin/collagen were from 0:100 to 8:100. Finally, the samples were dried in a desiccator for 24 h at room temperature before test. The AFM observations were performed on a Dimension 3100 Nanoscope IV equipped with Silicon TESP cantilevers (Shimadzu SPM-9600, Japan) in a non-contact (taping) mode. For each sample, the analyses were made at three different points to confirm the consistency of the observed morphologies.

### 2.5. Water contact angle tests

The hydrophilicity of the film was assessed with a goniometer (dataphysics OCA-H200, Germany) by measuring the surface water contact angle (WCA) at ambient temperature 25 °C, similar to the method reported by Zhang et al. [12]. Uniform drops of distilled water (3 μL per drop) were carefully deposited on the surface of the sample, and the angle between the sample surface and the water drop was measured as a function of time during a period of 5 min. The experimental plot was obtained from average of three positions on each film. The plotted error bars thus indicate the experimental standard deviation from the mean.

### 2.6. WVP tests

The water vapor permeability (WVP) of the film was measured according to the ASTM E96-00 gravimetric method with some modifications. The thicknesses of the collagen films were measured using a caliper micrometer (No. 7326, Mitutoyo Mfg. Co. Ltd., Japan). The average thickness was obtained from measuring 5 to 7 random positions of the film. Films were cut into rotundity with a diameter of 3 cm and firmly fixed onto the opening of cells containing dry silica gel (≈50.0 g). Subsequently, the cells were placed in a desiccator with distilled water at 25 °C until the weight change of the cells became constant. The WVP was calculated by $WVP = (m_2 - m_1)\cdot d/(S\cdot P\cdot t)$, where $m_2 - m_1$ is the maximum weight gain of the film during the process, $d$ is the average thickness of the film, $S$ is the permeation area (28.26 $cm^2$ herein), $P$ is the vapor pressure difference for the atmosphere between the silica gel and pure water (3159 Pa at 25 °C), $t$ is the time for the weight of the sample to achieve balance [32]. For each film, the measurements were performed at three different areas and the WVP values used for plot were calculated from averages of the three. The experimental error is thus obtained by calculating the standard deviation from the mean.

### 2.7. DSC measurements

To examine the thermal stability of the collagen films, differential scanning calorimetry (DSC) analysis was conducted on Netzsch DSC 200 PC in the temperature ranged from 10 to 150 °C with a constant heating rate of 5 °C/min under a nitrogen flow. The sample ≈3 mg was sealed in an aluminum pan with an empty aluminum pan as the reference.

### 2.8. TG measurements

Thermal weight loss of the collagen films was determined by a thermogravimetric analyzer (Netzsch TG 209F1, Germany) from 35 to 800 °C at a heating rate of 20 °C/min under nitrogen atmosphere.

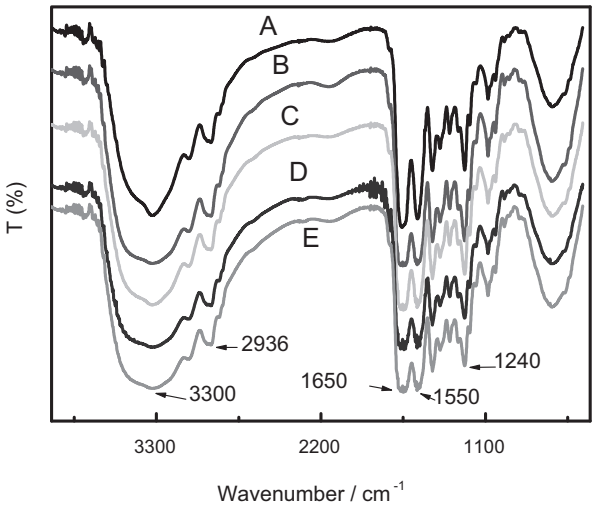


**Fig. 3.** FTIR spectra of collagen/procyanidin films with different contents of procyanidin (A: 0%, B: 2%, C: 4%, D: 6%, E: 8%).

For each sample, about 4 mg specimen was taken for the analysis and the flow rate of the nitrogen was 35 $cm^3$/min.

## 3. Results and discussion

### 3.1. Influence of procyanidin on the structure of collagen

The FTIR spectra of protein molecules can be correlated directly to their backbone conformation. Fig. 3 shows FTIR spectra of pure collagen and collagen/procyanidin films. As revealed before, the intact collagen has a special triple helix conformation which is characterized by its feature amide bands in IR spectra [33]. The amide A and B bands at 3350 $cm^{-1}$ and 3087 $cm^{-1}$, respectively, are mainly associated with the stretching vibrations of N–H groups. The amide I band at 1650 $cm^{-1}$ is dominantly attributed to the stretching vibrations of peptide C=O groups. And the amide II absorbance at 1550 $cm^{-1}$ arises from the N–H bending vibrations coupled to C–N stretching vibrations. The Amide III centered at 1240 $cm^{-1}$ is assigned to the C–N stretching and N–H bending vibrations from amide linkages, as well as wagging vibrations of $CH_2$ groups in the glycine backbone and proline side chains [34]. Fig. 3 indicates that the positions of these major amide bands do not change with the increasing procyanidin content, particularly the amide I band related to collagen triple helix [35] maintains at the same position and intensity; but only the amide A, I and II bands are broaden to some degree. The former suggests that the collagen triple helix in the films can be preserved after the introduction of procyanidin [13]. And the latter can involve the hydrogen bond interactions between procyanidin and collagen [34]. Some researchers observed the shift of the amide I band from 1651 to 1641 $cm^{-1}$ after adsorption of proanthocyanidin onto collagen fibrous matrix, indicating hydrogen bonding interaction [36]. It is generally accepted that the hydrogen bonding plays a dominant role in the stabilization of collagen by plant polyphenol under acidic conditions, and relatively minor differences in the dipole character of the individual phenolic substances appear to influence their binding to collagen. The side chain hydroxyl, carboxyl, amino and amide groups of collagen provide the potential interacting sites for the formation of hydrogen bonds with the phenolic hydroxyl groups of procyanidin.

Meanwhile, besides unchanged amide I band as indicated above, the IR absorption ratios of Amide III to 1450 $cm^{-1}$, denoted as $A_{III}/A_{1454}$ thereafter, are also considered to be a measure of preser-

**Table 1**
FTIR absorption ratios of $A_{III}/A_{1450}$ for collagen/procyanidin films with different contents of procyanidin.

| Procyanidin/collagen (w/w) | $A_{III}/A_{1450}$ |
|---|---|
| 0% | 1.0 |
| 2% | 0.99 |
| 4% | 0.99 |
| 6% | 0.98 |
| 8% | 0.97 |

vation of integrity of collagen triple helixes [37,38]. Table 1 shows that the ratios for collagen/procyanidin films slightly decrease from ≈1.0 for pure collagen to 0.97 at most, indicating the triple helix conformation is not destructed by procyanidin, since the ratio for the denatured collagen, gelatin is ≈0.6 [37,38]. Note that at the molecular level of native collagen structure, the hydrogen bond relevant to water is crucial for stabilizing the triple helix structure [39,40]. Here, it is conceivable that the procyanidin molecules can displace the water and thus create new hydrogen bond interactions with collagen. As a result, the helix structure of collagen is preserved. In other words, procyanidin molecules act as cross-linkers but without destroying the backbone structure of collagen. The schematic illustration is shown in Fig. 4.

Fig. 5 shows the AFM morphological changes of collagen induced by procyanidin. The pure type I collagen (Fig. 5a) exhibit typical fibrillar structure on the mica substrate where many curved molecules or microfibrils lie and are overlapped with one another, similar to previous reports [41,42]. While in the presence of procyanidin (Fig. 5b and c for 2 and 8% procyanidin, respectively), the entangled fibrous network topographies are observed for the first time, indicating the occurrence of aggregation. The similar aggregation is also observed at 4 and 6% procyanidian (data not shown). And as the procyanidin content increases, the conformational heterogeneity of collagen appears due to the procyanidin cross-linking. The AFM images directly reveal the effect of polyphenol on collagen structure at the microfibril level.

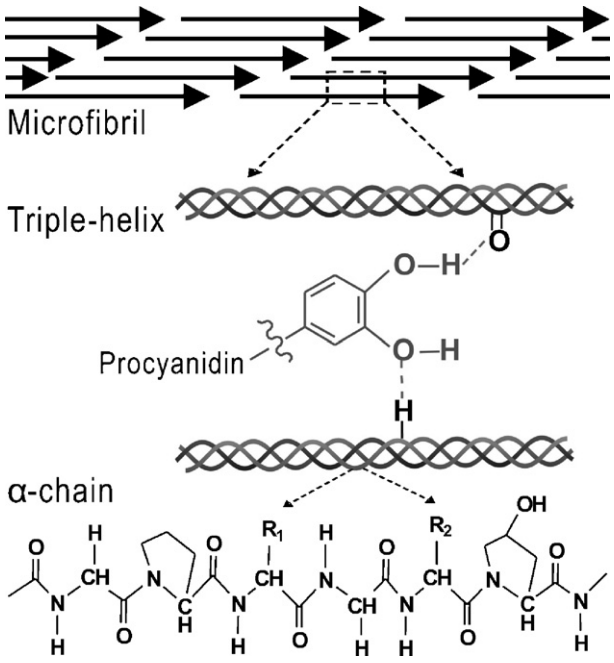


**Fig. 4.** Schematic illustration showing the reaction of procyanidin and collagen by creating new hydrogen bonds.

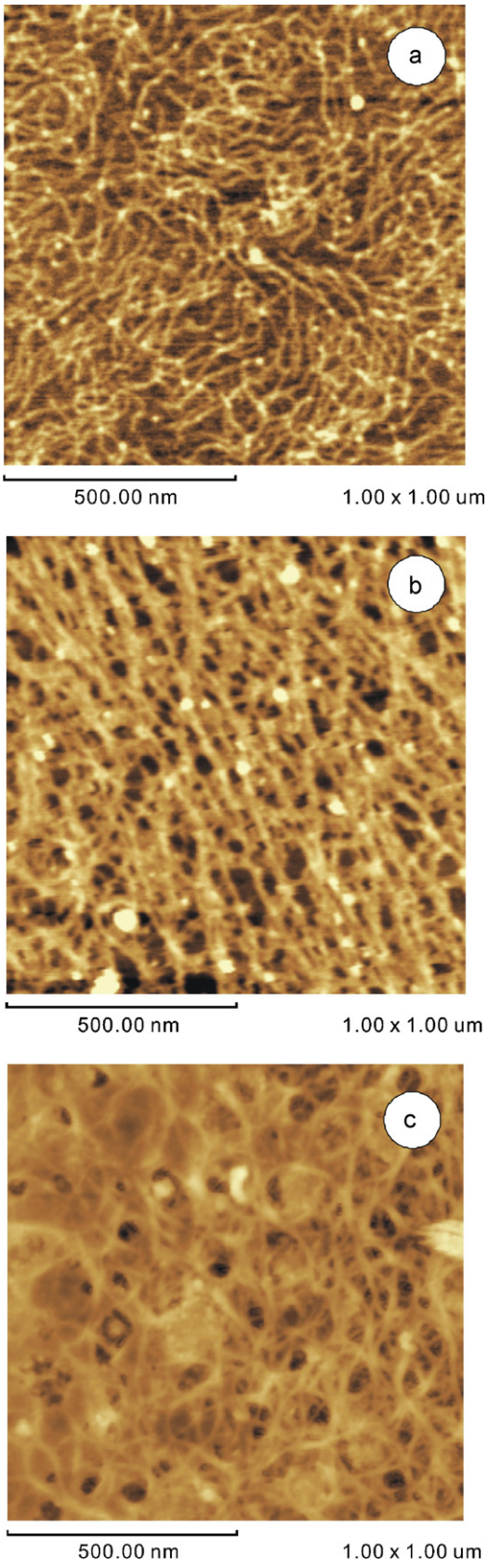


**Fig. 5.** AFM images of collagen on mica substrate in the presence of procyanidin. The ratios of procyanidin to collagen (10 μg mL$^{-1}$) are (a) 0:100, (b) 2:100, (c) 8:100.

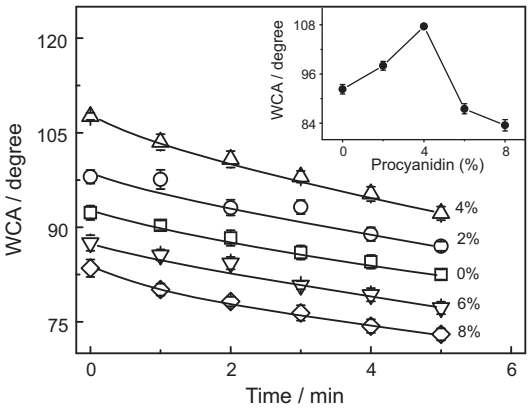


**Fig. 6.** Water contact angle (WCA) of collagen/procyanidin films as a function of time. The inset shows the procyanidin content dependence of WCA at initial time. Symbols denote experimental points and solid lines just serve to guide the eye.

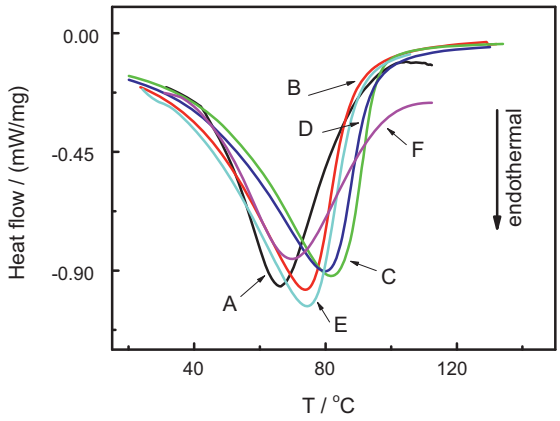


**Fig. 8.** DSC thermographs of collagen/procyanidin films with different contents of procyanidin (A: 0%, B: 2%, C: 4%, D: 6%, E: 8%, F: 100%). The corresponding $T_d$ is determined to be 65.5 °C, 73.6 °C, 82.3 °C, 80.8 °C, 74.3 °C and 70.0 °C, respectively.

### 3.2. Hydrophobic and hydrophilic properties of collagen/procyanidin films

In order to explore the correlation between the microstructure of procyanidin modified collagen and its surface property, the hydrophobic-hydrophilic properties of collagen/procyanidin films have been examined by water contact angle (WCA) tests as a function of time (Fig. 6). Generally, a high WCA indicates hydrophobicity, whereas a low angle means hydrophilicity. It can be seen that the addition of procyanidin causes the initial WCA to increase ≈15°. The facts indicate that the hydrogen bonding formation leads the cross-linking of collagen and procyanidin, and in turn the hydrophobicity of the film is improved. Further increasing the content of procyanidin (above 4%) leads WCA to decrease instead, as shown in the inset, which is likely due to the incomplete reactivity of excessive procyanidin in the films and the hydrophilic nature of procyanidin. Note that the WCA for the pure collagen film (≈92°) is about 10° higher than the gelatin film in the literature [11], indicating the structural integrity of the collagen used in the present study. Fig. 6 also indicates that the dependence of WCA on time displays coincident decreasing trend for all the samples. This could be attributed to the surface loosen texture of the film resulted from procyanidin induced aggregation, as reflected in the AFM images.

Fig. 7 shows the water vapor permeability (WVP) of the films. The property relates to the network structure and available hydrophilic groups on the channel surface. Unlike contact angle tests (Fig. 6), in which there is an inflection point at ~4% of the cross-linker, the measured WVP decreases continuously with the

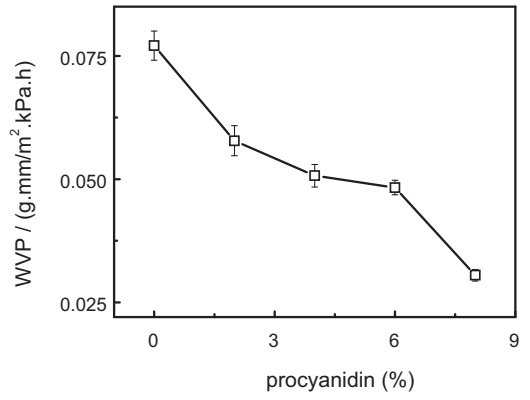


**Fig. 7.** Water vapor permeability (WVP) of collagen/procyanidin films with different contents of procyanidin. Symbols denote experimental points and solid lines just serve to guide the eye.

increasing procyanidin content. This suggests that the procyanidin cross-linking results in a denser structure and it therefore prevents moisture permeation even under the excess procyanidin. The result is similar to the collagen gels cross-linked by dialdehyde starch [13].

### 3.3. Thermal stability of the collagen in the presence of procyanidin

The thermal stability of collagen in the presence of procyanidin was assessed by DSC analysis. Fig. 8 shows the DSC thermographs of collagen and collagen/procyanidin films with different content of procyanidin. The endothermic peak is known to be associated with the transformation from triple helical to random coil of collagen [43], and the peak value is normally assigned to the denaturation temperature ($T_d$). The change of $T_d$ shows the similar trend as previous WCA results for procyanidin modified collagen films, i.e. first increase and then decrease with increasing procyanidin content. Similarly, the cross-linking and incomplete reactivity of procyanidin can explain the change. Note that the content of the collagen is constant in all the collagen/procyanidin films, and the pure procyanidin gives its $T_d$ at 70.0 °C. Therefore, it is understandable that the excessive procyanidin (in the case of 6 and 8%) causes the decrease of the measured $T_d$ (80.8 and 74.3 °C, accordingly) instead of staying at the same level as 4% (82.3 °C). Besides, the higher $T_d$ of collagen/procyanidin composites than each individual indicates that their interaction is not a simple physical effect. It should be noticed that the improvement in $T_d$ is favorable for maintaining the triple helix structure of collagen which is important for the biocompatibility and mechanical properties of collagen-based materials [13].

The curves of weight loss and first derivate related to the rate of weight loss (DTG) for the collagen/procyanidin films with different procyanidin content are shown in Fig. 9a and b. Two different steps can be distinguished in the thermograms for collagen and collagen/procyanidin films. The first one from 35 to 150 °C is related to the breakage of inter- and intra-molecular hydrogen bonds accompanied by gradual loss of water [43]; and for pure collagen, it is indicative of the destruction of the triple helices. The second one in the range of 200–500 °C is associated with the decomposition of the collagen chains. As can be seen from Table 2, most of weight losses for collagen and the composite films occur in this heating process. It is obvious that the residual weights of collagen in the presence of procyanidin have more than 20% increases, showing the thermal denaturation and degradation is hindered by the cross-linking. The similar effect has been reported in the hyaluronic acid modified collagen using EDC/NHS as cross-linkers [43]. The results

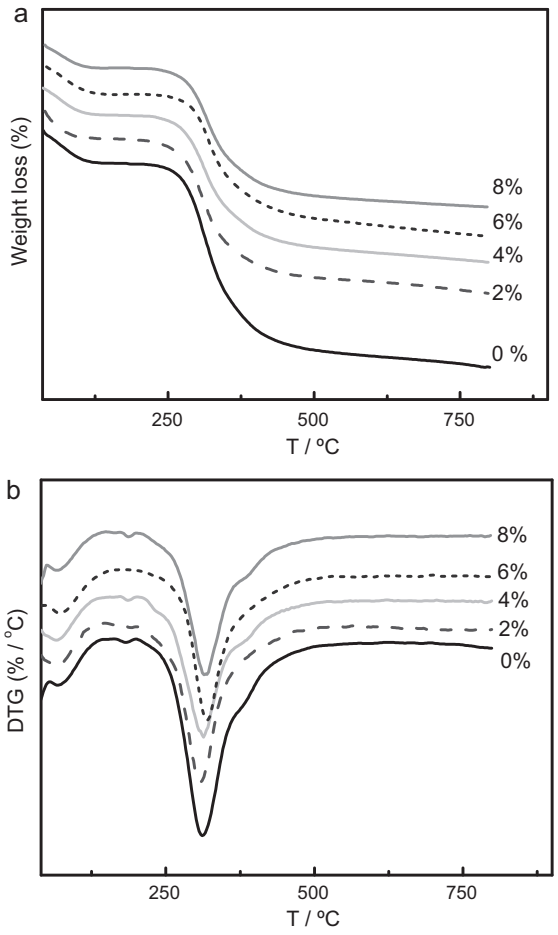


**Fig. 9.** (a) Weight loss and (b) DTG thermograms of collagen/procyanidin films with different contents of procyanidin.

**Table 2**
Weight losses at each defined process and residual weights of collagen/procyanidin films with different contents of procyanidin (w/w %) determined by TG analysis.

| Procyanidin/ collagen (w/w) | Weight loss (%) at 35–150 °C | Weight loss (%) at 200–800 °C | Residual weight (%) |
|---|---|---|---|
| 0% | 14.0 | 76.0 | 10.0 |
| 2% | 12.0 | 57.7 | 30.3 |
| 4% | 11.2 | 57.1 | 31.7 |
| 6% | 11.1 | 54.7 | 34.2 |
| 8% | 10.0 | 55.3 | 34.7 |

further prove the interactions between collagen and procyanidin promote the structure thermostability of collagen/procyanidin films.

## 4. Conclusion

The studies on the structure and property of collagen in the presence of procyanidin can lead to the following conclusions. The hydrogen bond interaction is mainly responsible for the stabilization of collagen by procyanidin. The procyanidin treatment does not destroy the triple helix conformation of collagen, but induces the aggregation of collagen microfibrils. In comparison with pure collagen, the hydrophobicity of the collagen/procyanidin films can be improved with less amount of procyanidin. While the water vapor permeability of the films decrease with increasing procyanidin because the cross-linkings of procyanidin and collagen lead to denser network structure. The procyanidin modified collagen also exhibits improved thermal stability. Our results are of significance for the development of new cross-linkers and design of collagen biomaterials.

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