

## STUDY ON SELF-CROSSLINKING OF HYDROGEN PEROXIDE OXIDATING COLLAGEN

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**Abstract.** The utilization of mink waste generated through the industrial process attracted both industry and academia interests. In this study, the use of hydrogen peroxide as an oxidizing agent onto collagen producing self-crosslinking which extracting from mink solid waste was studied by infrared spectrum, fluorescence spectrum and thermal properties. The effect of hydrogen peroxide dosage and reaction temperature on the degree of oxidative self-crosslinking of collagen was analyzed by the changes of molecular structure and thermal stability. It was found that, hydroxide groups on the collagen side-chains can be oxidized to aldehyde groups and carboxyl groups by hydrogen peroxide in alkaline environment. These oxidized groups can crosslink with functional groups on collagen by covalent bond and ionic bond, changing collagen molecular structure and improving thermal stability. When the dosage of hydrogen peroxide was 14.74% and reaction temperature was 40°C ±, the oxidative self-crosslinking of collagen was the strongest. This study provided theoretical basis for the high-value utilization of mink wastes.

### 1 Introduction

Mink is one of the most important products in the international fur trade market with “king of fur” name <sup>1</sup>. In production and the treatment of shabby goods exists the problems of resource utilization of protein waste including hide fiber and wool fiber. Extracting collagen from protein-containing wastes and utilizing it as a resource has become a research hotspot in related fields.

Green and biodegradable oxidants acting on the active groups of collagen molecular chain can form new crosslinking in the system, thus increasing the structural stability of collagen. As a strong oxidant, potassium permanganate has been used in environmental pollution control, tap water treatment and chemical industry <sup>2-5</sup>. However, due to the color of the reaction products, the use of potassium permanganate is limited. Although the price of sodium hypochlorite is low, the chlorine released in the process of oxidation and decomposition will not only cause environmental pollution, but also endanger human health. The oxidation reaction of hydrogen peroxide produces water and oxygen <sup>6</sup>. It is a strong oxidant that meets the ecological requirements and has almost no pollution, it is called “the cleanest” chemical product <sup>7</sup>. At present, it has been widely used in textile, paper-making, food, medicine, electronics and chemical industries <sup>8</sup>. Gong Juxia <sup>9</sup> discussed the effect of hydrogen peroxide treatment on the structure and properties of collagen type I. They believed that hydrogen peroxide played a dual role in crosslinking and degradation of collagen type I. Lu Xingfang <sup>10</sup> studied the effect of hydrogen peroxide on shrinkage temperature and isoelectric point of raw skin during dehairing and the effect of hydrogen peroxide on gelatin. The results showed that the liming skin shrinkage temperature of dehairing by hydrogen peroxide was higher than that of dehairing by sodium sulfide, but the isoelectric point of collagen was lower, moreover, the glass transition temperature of gelatin was increased by hydrogen peroxide. These results indicated that hydrogen peroxide can react with collagen and collagen hydrolysates, but the self-crosslinking of mink collagen by hydrogen peroxide has not been reported. In this paper, the collagen extracted from mink solid waste was taken as the research object and hydrogen peroxide was used as oxidant, discussing the effects of the amount of hydrogen peroxide and reaction temperature on the oxidation and self-crosslinking of collagen from the changes of molecular structure and thermal

stability of collagen by infrared spectroscopy, ultraviolet spectroscopy and differential scanning calorimetry, in order to provide basis for the treatment and resource utilization of the solid waste containing collagen in mink solid waste.

## 2. Experiments and Methods

### 2.1 Materials

Collagen was extracted from mink waste by using pepsin. Glacial acetic acid was purchased from Dasen chemical company (Tianjin, China). Sodium hydroxide was purchased from Xinda chemical company (Shanghai, China). 5 mg/ml collagen solution was prepared dissolving the freeze-dried collagen in 0.01 mol/L HAC.

### 2.2 Sample preparation

#### 2.2.1 Effect of NaOH on reaction solution pH

20 ml collagen solution (5 mg/ml) and 2.5 ml 0.1 mol/L NaOH were mixed evenly, placing for 3h at 18°C And then the pH (Acidometer, PHS-3C, Shanghai Precision Science Co., China) and absorbance at 280 nm (Ultraviolet spectrophotometer, UV-2000, Unico, USA) of the solution were measured at different time.

#### 2.2.2 Effect of H<sub>2</sub>O<sub>2</sub> dosage on the self-crosslinking of oxidating collagen

20 ml collagen solution (5 mg/ml) and 2.5 ml 0.1 mol/L NaOH were mixed by gently stirring for 30s, then adding various dosage H<sub>2</sub>O<sub>2</sub> (0.00%, 6.31%, 10.53%, 14.74%, 18.95%, 21.06%) by water bath shaking for 3h at 18°C. The emission and synchronous fluorescence spectra of the solution were measured, then the freeze-dried collagen was tested by Fourier transform spectrometer and Differential scanning calorimetry.

Freeze-drying conditions: pre-freeze for 24h at -18°C, then freeze-dry for 36h at -38°C.

#### 2.2.3 Effect of temperature on the self-crosslinking of oxidating collagen

20 ml collagen solution (5 mg/ml) and 2.5 ml 0.1 mol/L NaOH were mixed by gently stirring for 30 s, then adding 14.74% H<sub>2</sub>O<sub>2</sub> by water bath shaking for 3h at different temperature (20°C 25°C 30°C 35°C 40°C 45°C). The blank sample was prepared by 20 ml collagen solution (5mg/ml). The emission and synchronous fluorescence spectra of the solution were measured, then the freeze-dried collagen was tested by Fourier transform spectrometer and Differential scanning calorimetry. The freeze-drying conditions is the same as 2.2.2.

### 2.3 Fourier transform infrared (FTIR) spectroscopy

FTIR (Fourier transform spectrometer, IRA-1S, Shimadzu, Japan) spectra have been achieved from the samples containing 1 mg collagen in approximately 200 mg potassium bromide with the frequency range from 4000 to 400 cm<sup>-1</sup>, scanning time was 20, resolution rate was 4.

### 2.4 Fluorescence measurements

The measurement was carried out using the Cary Eclipse (Agilent, Australia). Both the excitation and the emission slit openings were set as 5 nm. In the case of the synchronous fluorescence spectra, the initial (excitation) wavelength was set at 280 nm and the wavelength shift ( $\Delta\lambda$ ) was equal to 15 nm

and the scan interval was set between 200~400 nm. The fluorescence emission spectra of collagen, the wavelength was set as 280 nm and the scan interval was set between 290~400 nm.

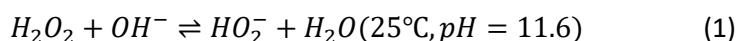
## 2.5 Differential scanning calorimetry (DSC)

The measurement was carried out using the DSC 25 (TA, USA). The samples (4.0~5.0 mg) were weighed accurately into aluminum pans and sealed. Aluminum pans were scanned over a temperature range of 20~200 °C with a heating rate 5 °C/min under N<sub>2</sub> atmosphere.

## 3. Result and Discussion

### 3.1 Effect of NaOH on reaction solution pH

Collagen is oxidized by H<sub>2</sub>O<sub>2</sub> to produce self-crosslinking which took advantage of the oxidizability, H<sub>2</sub>O<sub>2</sub> could easy form HO<sub>2</sub><sup>-</sup> under alkaline conditions <sup>11</sup>.



Formula (1) shows that strong alkali causes the above equilibrium to move to the right, but excessive pH will accelerate the ineffective decomposition of H<sub>2</sub>O<sub>2</sub>. In order to ensure the self-crosslinking effect of collagen oxidized by H<sub>2</sub>O<sub>2</sub>, it is necessary to determine the pH of solution when adding H<sub>2</sub>O<sub>2</sub>, and the solution pH is regulated by NaOH generally. However, NaOH can hydrolyze collagen, the hydrolysis will not only consume alkali but also produce collagen hydrolysate which will affect the pH of solution and the effect of H<sub>2</sub>O<sub>2</sub>. In this experiment, 2.5 ml NaOH (0.1mol/L) was first added to the collagen solution to make the oxidative self-crosslinking reaction of collagen with H<sub>2</sub>O<sub>2</sub> proceed under alkaline conditions. The change of reaction pH with time is shown in Fig. 1.

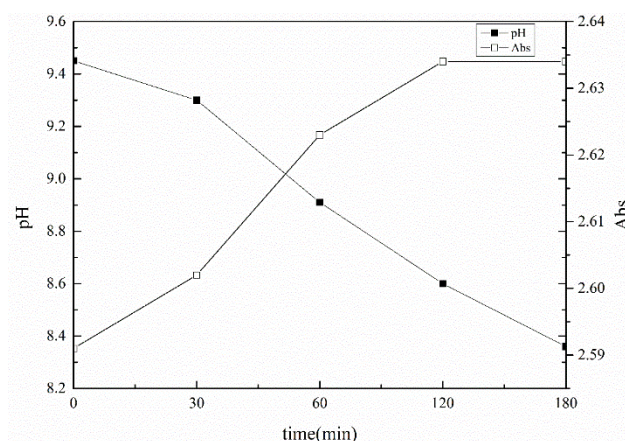


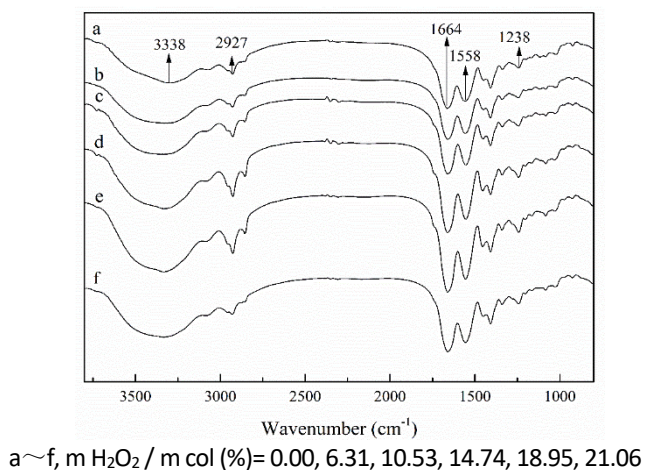
Fig. 1. The relationship between the reaction solution pH and time (2.5 ml NaOH, 18°C)

As can be seen from Fig. 1, with the increase of reaction time, the solution pH decreased but not significant, and remained in the alkaline range within 180 minutes. The initial pH of reaction solution was 9.45, then decreased to 8.36 after 180 minutes. From the absorbance curve of the solution, it can be seen that the hydrolysis of sodium hydroxide breaks some collagen molecular chains and produces polypeptides or amino acids. These exposed amino acids will bind to OH<sup>-</sup> in the solution and H<sup>+</sup> will be increased in the solution. Therefore, the solution pH decreased slowly within 180 minutes.

### 3.2 Effect of H<sub>2</sub>O<sub>2</sub> dosage on the self-crosslinking of oxidating collagen

#### 3.2.1 FTIR spectroscopy

The collagen functional groups can be qualitatively and semi-quantitatively analyzed by infrared spectroscopy to understand the effect of H<sub>2</sub>O<sub>2</sub> on collagen. Infrared Spectrum of oxidized mink collagen with different dosage of H<sub>2</sub>O<sub>2</sub> is shown in Fig. 2.



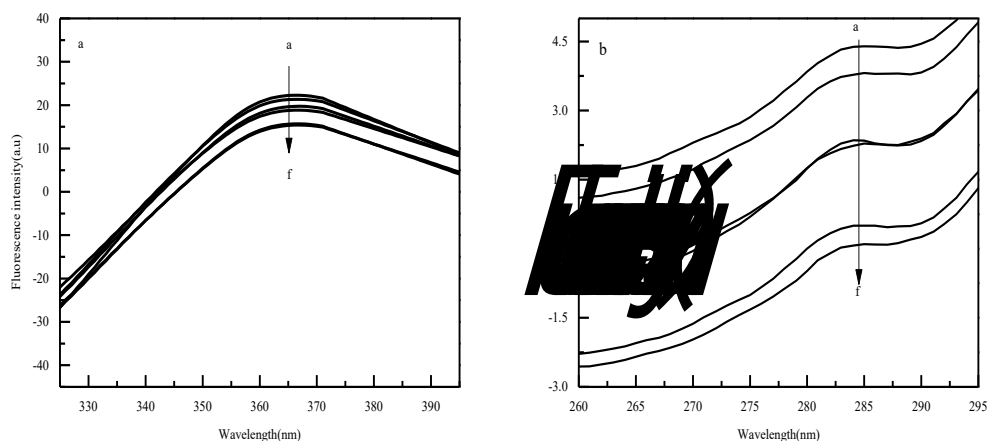
**Fig. 2.** Infrared Spectrum of Oxidized mink collagen with different dosage of H<sub>2</sub>O<sub>2</sub> (18°C, 3h)

As can be seen from Fig. 2, the characteristic absorption peak of collagen amide A band of mink skin before and after oxidation is at 3338 cm<sup>-1</sup>, and it is mainly related to the stretching vibration of hydrogen bond in molecule, and the characteristic absorption peak of amide B band is 2927 cm<sup>-1</sup>. The amide bond is the characteristic functional group for characterizing and analyzing the secondary structure of proteins by infrared spectroscopy<sup>12-13</sup>. The characteristic absorption peaks of amide I band are 1630~1680 cm<sup>-1</sup>, amide I band of mink collagen is at 1664 cm<sup>-1</sup>, which is C=O stretching vibration peak of amide I band and COO<sup>-</sup> antisymmetric contracting vibration peak. 1558 cm<sup>-1</sup> and 1238 cm<sup>-1</sup> are characteristic absorption peaks of amide II band and amide III of mink collagen respectively, amide band II is caused by C-N stretching vibration and heterogeneous N-H bending vibration, while amide band III is mainly caused by in-phase N-H bending vibration and C-N stretching vibration<sup>14</sup>.

It can be seen from the comparison that the absorption area of the characteristic peak (amide band) of mink collagen increased significantly with the increase of the dosage of H<sub>2</sub>O<sub>2</sub> (a~f). This is because hydroxide groups on the collagen side-chains can be oxidized to aldehyde groups and carboxyl groups by hydrogen peroxide in alkaline environment. The oxidation enhanced with the increase of hydrogen peroxide dosage, so the amount of H<sub>2</sub>O<sub>2</sub> increased, and the characteristic absorption peak of mink collagen protein in amide band strengthened.

#### 3.2.2 Fluorescence spectrum

Tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues in protein molecules can absorb and emit fluorescence, so proteins have endogenous fluorescence. Fluorescence spectrum of oxidized mink collagen with different dosage of H<sub>2</sub>O<sub>2</sub> is shown in Fig. 3.



a: a~f, m H<sub>2</sub>O<sub>2</sub> / m col (%) = 6.31, 18.95, 14.74, 21.06, 10.53, 0.00; b: a~f, m H<sub>2</sub>O<sub>2</sub> / m col (%) = 10.53, 0.00, 18.95, 6.31, 14.74, 21.06

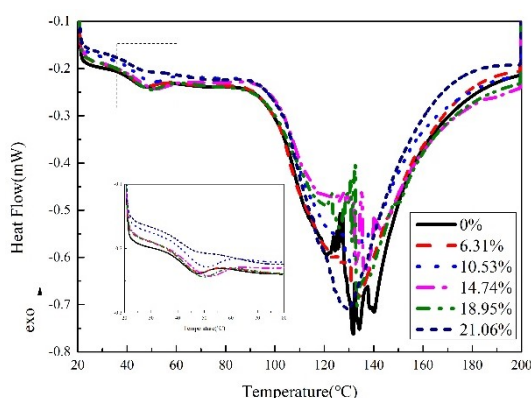
**Fig. 3.** Emission fluorescence spectra (a) and synchronous fluorescence spectra (b) of Oxidized mink collagen with different dosage of H<sub>2</sub>O<sub>2</sub> (18°C 3h)

When the excitation wavelength is 280 nm, tryptophan and tyrosine are the main factors for the intrinsic fluorescence of proteins<sup>15</sup>. Because the mink collagen does not contain tryptophan, so the intrinsic fluorescence of collagen solution is mainly produced by tyrosine at 280 nm. The intrinsic fluorescence of proteins decreases the fluorescence signals intensity due to the interaction with solvents or solute molecules. This phenomenon is called fluorescence quenching which is a common phenomenon in protein fluorescence analysis. Fig. 3 (a) shows that when the excitation wavelength is at 280 nm, the absorption peak of mink collagen is about 365 nm. When the amount of H<sub>2</sub>O<sub>2</sub> increased from 0.00% to 21.06%, the fluorescence intensity of mink collagen from high to low was 6.31%, 18.95%, 14.74%, 21.06%, 10.53%, 0.00%.

Synchronous fluorescence spectroscopy can be used to study the polarity around protein amino acid residues and the change of protein conformation. When  $\Delta\lambda=15$  nm, the change of microenvironment around tyrosine residues can be obtained<sup>16</sup>. Fig. 3 (b) shows that the characteristic absorption peak of collagen is 287 nm when the dosage of H<sub>2</sub>O<sub>2</sub> is 0% while the characteristic absorption peak of collagen is 285 nm when the dosage of H<sub>2</sub>O<sub>2</sub> is increased from 6.31% to 14.74%. It is suggested that the interaction between H<sub>2</sub>O<sub>2</sub> and collagen molecules can slightly reduce the microenvironment polarity of tyrosine. When the dosage of H<sub>2</sub>O<sub>2</sub> increased from 18.95% to 21.06%, the absorption peak shifted from 284 nm to 285 nm, and the polarity of tyrosine microenvironment increased. This is because when the amount of H<sub>2</sub>O<sub>2</sub> is less than 14.74%, H<sub>2</sub>O<sub>2</sub> acts on collagen molecules oxidizing hydroxyl groups on collagen side chains to aldehyde or carboxyl groups, these aldehyde or carboxyl groups bind with active groups in the form of covalent bonds or ionic bonds, embedding tyrosine residues and destroying the conformation of microenvironment. When the dosage was more than 18.95%, the oxidative hydrolysis of collagen by H<sub>2</sub>O<sub>2</sub> increased which further broke the peptide chain and shortened the molecular chain, resulting in more exposure of tyrosine residues, thus increasing the polarity of the microenvironment.

### 3.2.3 DSC thermogram

Collagen molecules are composed of three helices. When collagen is heated, its secondary, tertiary and quaternary structure will be destroyed and degenerated. When the temperature continues to rise, collagen will undergo thermal degradation, collagen peptide chain will break, the primary structure will be destroyed, and decomposed into gelatin. The thermal denaturation temperature and thermal degradation temperature of collagen can be characterized by DSC. DSC curves of Oxidized mink collagen with different dosage of H<sub>2</sub>O<sub>2</sub> is shown in Fig. 4.



**Fig. 4.** DSC curves of Oxidized mink collagen with different dosage of  $H_2O_2$  (18°C, 3h)

As can be seen from Fig. 4, there were two obvious endothermic peaks on DSC curves of mink collagen before and after oxidation with  $H_2O_2$ . When the amount of  $H_2O_2$  increased from 0% to 14.74%, the peak value of thermal denaturation temperature gradually moved to the right, and when the amount of  $H_2O_2$  was more than 14.74%, the peak value moved to the left. The effect of  $H_2O_2$  on thermal stability of mink collagen is shown in Table 1.

**Table 1.** The effect of c on thermal stability of mink collagen (18°C, 3h).

	$H_2O_2$ (%)					
	0.00	6.31	10.5	14.7	18.9	21.0
			3	4	5	6
Denatured temperature (°C)	46.91	47.27	49.4	51.2	48.7	46.8
Degradation temperature (°C)	131.7	133.8	130.	136.	133.	129.
Heat content (J/g)	490.7	482.1	421.	406.	441.	443.
	3	3	18	24	90	73

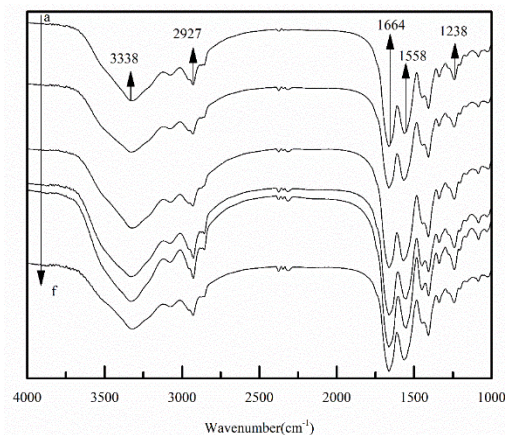
The thermal stability of collagen is provided by the polar interaction between polar groups on the molecular chain and the hydrogen bond formed<sup>17</sup>. Table 1 shows that with the increase of  $H_2O_2$  dosage (0%~21.06%), the thermal denaturation temperature and degradation temperature of collagen increases first and then decreases, reaching the maximum when the dosage is 14.74%. It is suggested that  $H_2O_2$  at higher concentration can oxidize hydroxyl groups of collagen side chains to aldehyde or carboxyl groups, and bind with active groups in molecular chains by covalent or ionic bonds. Covalent bond energy is much larger than hydrogen bond and polar bond energy<sup>17</sup>, and it was proved that  $H_2O_2$  oxidation had cross-linking effect and improved the thermal stability of collagen. When the amount of  $H_2O_2$  was more than 14.74%, the oxidative hydrolysis of collagen peptide chains dominated, resulting in the decrease of the thermal denaturation temperature of collagen. In conclusion, under alkaline conditions, when the amount of  $H_2O_2$  was 14.74%, the self-crosslinking effect of oxidized mink collagen was the best.

### 3.3 Effect of temperature on the self-crosslinking of oxidating collagen

#### 3.3.1 FTIR spectroscopy

Infrared Spectrum of Oxidized mink collagen with different reaction temperature is shown in Fig. 5. As can be seen from Fig. 5, when the reaction temperature was increased from 20°C to 25°C the peak shape of the characteristic absorption peak of collagen in infrared spectra remained almost unchanged. When the reaction temperature increased from 30°C to 40°C the absorption peaks of

collagen at  $3338\text{ cm}^{-1}$ ,  $2927\text{ cm}^{-1}$  and  $1664\text{ cm}^{-1}$  increased, while the other peaks did not change significantly, the absorption peaks of collagen at the above three points were weakened at  $45^\circ\text{C}$ . The results showed that the increase of appropriate temperature was beneficial to the oxidation of collagen by hydrogen peroxide, and increased the content of aldehyde or carboxyl groups in the collagen molecular chain. When the temperature is too high ( $>40^\circ\text{C}$ ), hydrogen peroxide decomposes excessively, which reduces the oxidation.

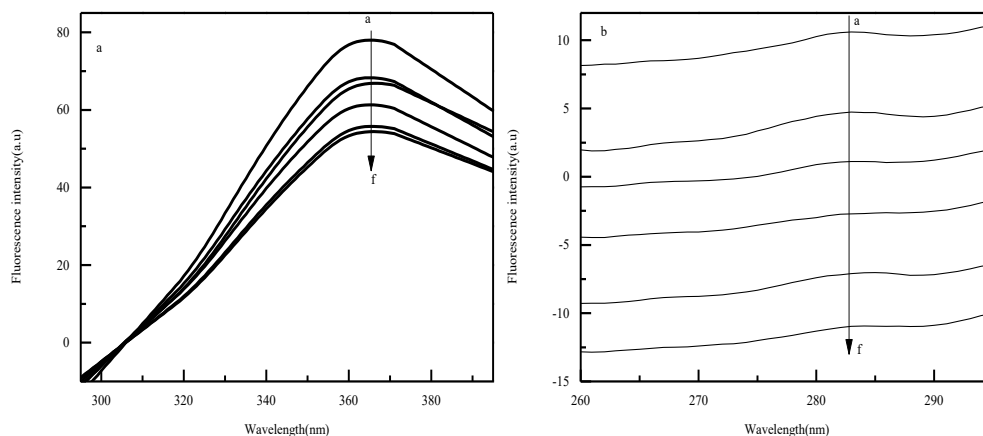


a~f,  $T(^{\circ}\text{C}) = 20, 25, 30, 35, 40, 45$

**Fig. 5.** Infrared Spectrum of Oxidized mink collagen with different reaction temperature (14.74%  $\text{H}_2\text{O}_2$ , 3h)

### 3.3.2 Fluorescence spectrum

Infrared Spectrum of Oxidized mink collagen with different reaction temperature is shown in Fig. 6. As can be seen from Fig. 6, when the excitation wavelength was 280 nm, the absorption peak of collagen protein appeared at 362 nm. The fluorescence intensity decreases with the increase of reaction temperature. It is suggested that the reaction of collagen self-crosslinking with hydrogen peroxide is extremely sensitive to temperature. Appropriate increase of temperature ( $35^\circ\text{C}$ ) is beneficial for hydrogen peroxide to oxidize hydroxyl groups in collagen molecular chains to aldehyde or carboxyl groups, and bond with covalent or ionic bonds of active groups. Tyrosine is encapsulated resulting in a reduction of fluorescent sources quantity. The absorption peaks of collagen synchronous fluorescence spectra remained almost unchanged at different reaction temperatures.

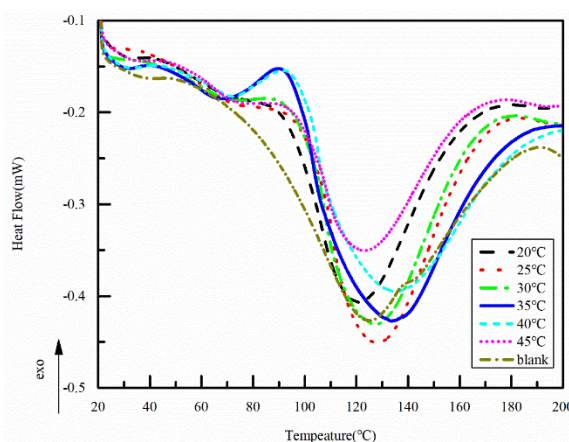


a: a~f,  $T(^{\circ}\text{C}) = 30, 20, 40, 25, 45, 35$ ; b: a~f,  $T(^{\circ}\text{C}) = 30, 20, 45, 35, 25, 40$

**Fig. 6.** Emission fluorescence spectra (a) and synchronous fluorescence spectra (b) of Oxidized mink collagen with different reaction temperature (14.74%  $\text{H}_2\text{O}_2$ , 3h)

### 3.3.3 DSC thermogram

DSC curves of oxidized mink collagen with different reaction temperature is shown in Fig. 7.



**Fig. 7.** DSC curves of oxidized mink collagen with different reaction temperature (14.74% H<sub>2</sub>O<sub>2</sub>, 3h)

As can be seen from Fig. 7, the self-crosslinking samples of collagen peroxide showed obvious endothermic peaks at different reaction temperatures. There are two endothermic peaks on the DSC curve of collagen when the temperature is 35°C and 40°C. The effect of denatured temperature on thermal stability of oxidized mink collagen is shown in Table 2.

**Table 2.** The effect of denatured temperature on thermal stability of oxidized mink collagen (14.74% H<sub>2</sub>O<sub>2</sub>, 3h).

	Reaction temperature (°C)						Blank
	20	25	30	35	40	45	
Denatured temperature (°C)	-	-	-	65.89	71.25	-	-
Degradation temperature (°C)	119.9	126.7	126.2	132.6	133.4	121.2	123.4
Heat content (J/g)	273.7	294.8	285.9	296.5	285.7	249.3	306.5

Note: The blank sample was untreated collagen, i.e. without NaOH and H<sub>2</sub>O<sub>2</sub>.

As can be seen from Table 2, the denaturation temperatures of collagen at 35°C and 40°C were 65.89°C and 71.25°C respectively. With the increase of reaction temperature, the thermal degradation temperature increases first and then decreases, reaching the maximum at 40°C (133.43°C), indicating that the peptide chain of collagen molecules breaks and the amino acid residues are destroyed. This is because when hydrogen peroxide acts on collagen molecules, hydroxyl groups are oxidized to aldehyde or carboxyl groups, which bind to the active groups on the molecular chain by covalent bonds or ionic bonds, thus increasing the thermal stability of collagen. Increasing the temperature properly can decompose hydrogen peroxide into more hydrogen peroxide radicals (H<sub>2</sub>O), promoting the oxidation self-crosslinking reaction and enhancing the stability of the peptide chains. When the reaction temperature is higher than 40°C hydrogen peroxide produces more ineffective decomposition and the oxidation decreases. In conclusion, the self-crosslinking effect of collagen peroxide is better when the reaction temperature is 40°C.

The thermal degradation temperature of collagen treated with hydrogen peroxide was higher than that of blank sample (123.47°C) when the reaction temperature was raised from 25°C to 40°C. The low reaction temperature (20°C), the weak oxidation of collagen side chain hydroxyl by hydrogen peroxide, and the hydrolysis of collagen to a certain extent, resulting in the thermal

degradation temperature of collagen which is oxidized at 20°C is lower than that of blank samples. The higher the reaction temperature (> 40°C), the more ineffective decomposition of hydrogen peroxide was produced, and the hydrolysis of collagen molecular chain itself was intensified, the main chain structure was destroyed. Therefore, the thermal degradation temperature of collagen oxidized at 45°C is lower than that of blank samples.

#### 4. Conclusion

The self-crosslinking mechanism of hydrogen peroxide collagen oxidating was studied by analyzing the mink collagen thermal properties, infrared spectrum and fluorescence spectrum. Infrared spectrum showed that hydroxide groups on the collagen side-chains can be oxidized to aldehyde groups and carboxyl groups by hydrogen peroxide in alkaline environment; Fluorescence spectrum showed that hydrogen peroxide changes protein conformation and tyrosine microenvironment; The change of thermal stability of collagen proved that hydrogen peroxide can make new crosslinks between collagen molecular chains and increase the thermal denaturation temperature of collagen. Under alkaline conditions, when the dosage of hydrogen peroxide was 14.74% and reaction temperature was 40°C, the oxidative self-crosslinking of collagen was the strongest.

#### Acknowledgement

This work was financially supported by the National Natural Science Foundation of China (No.21606139).

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