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Extraction and Characterization of Collagen from Rabbit-skins

Keyong Tang, Fang Wang, Xuejing Zheng, Wenpo Feng, Ling Zhang
College of Materials Science & Engineering, Zhengzhou University, Zhengzhou, China, phone: 0086-371-67763216, email: keyongtangzzu@yahoo.com

Abstract: Collagen was extracted from rabbit-skins by the combination of acid swelling and pepsin treatment. A qualitative analysis was carried out by SDS-PAGE. FT-IR and TG/DTG were employed to characterize the physical and chemical properties. The results show that the collagen referred to type I collagen with a complete three-helix structure and there are three apparent weightlessness at before 100°C, 200~450°C, and after 450°C, respectively. After cross-linked by glutaraldehyde, the hydrophilicity and enzyme degradation with I-collagenase in vitro were determined. The results show that, after modification, its water content was 78.7%; its degradation rate dropped from 88.65% to 6.03% ($p < 0.01$). Cytotoxicity of the modification collagen was evaluated by growth suppression technique (MTT chromatometry) against wild type mouse embryonic fibroblasts (MEF-WT). The result shows that the cytotoxicity graduation of the materials from collagen is 1 or 0, nearly no cytotoxicity. This collagen has a potential use in biomedical materials.

Keywords: rabbit-skins; collagen; acid swelling; pepsin treatment; extraction; characterization

1. Introduction

Collagen is a group of natural proteins. In nature, it is found exclusively in animals, especially in the flesh and connective tissues of mammals[1]. It is the main component of connective tissue, and is the most abundant protein in mammal bodies[2]. Around 25%-35% of the protein in the mammal bodies is collagen. Collagen, in the form of fibrils, is usually found in fibrous tissues such as tendon, ligament and skins. It is also abundant in cornea, cartilage, bone, blood vessels, gut, and intervertebral disc. At present, about 29 kinds of collagen has been found with the most commons are type I, type II and type III. It is a structural protein and a extracellular matrix (ECM)[1, 2], playing an important role in the function of cells, tissues and organs[3-6]. Because it is good in biodegradability and biocompatibility, collagen has found a wide application in such fields as medicine, cosmetics, healthy products, and food.

Type I collagen has been widely used in biological medicine. So the extraction of type I collagen from animal body has become one of the focuses attracting the interests of many researchers in recent years. From the reports about the extraction of collagen, we know that the present collagen are mainly from skins and bones of such terrestrial animals as pig [7, 8], cow[9-12], chicken[13], mouse[11] and such marine animals as fish [14, 15], jellyfish [16], squid [17].

Acid [9, 11, 15, 16], salt [12], and enzyme [10, 11] are usually employed in the extraction.

Because of the global environmental pollution, such severe diseases as mad cow disease, foot and mouth disease, and avian flu appear frequently these days. From viewpoints of security and health, special caution should be taken in choosing the raw materials to obtain collagen. Rabbit is an herbivorous animal with a poor resistance to disease. The extent of contamination is much smaller than other animals. The feeding is usually limited. On the other hand, with the continuous improvement of living standards, the demand for rabbit meat is increasing these years and the rabbit skins that need to be processed. Therefore, rabbit skins may be a secure, reliable, economical, and practical source of type I collagen.

In this paper, a new method with the combination of acid method and enzyme method was used to extract collagen from rabbit skins. The collagen was characterized by SDS-PAGE, FT-IR and TG/DTG. The physical and chemical properties of the collagen were studied. After being glutaraldehyde modified, the collagen was the evaluated from viewpoints of water absorption, in vitro enzymatic degradation and cytotoxicity. The results showed that the extracted collagen was type I collagen with a complex triple helix structure. It is good in water absorption, enzyme degradation and biocompatibility. It may find a potential application in biomedical fields.

2. Experimental

2.1 Materials and apparatus

Rabbit was commercially available. Pepsin, Type I collagenase and MTT (methyl thiazolyl tetrazolium, methyl thiazolyl tetrazolium) were from Sigma. Protein Marker was made by the TaKaRa Biotechnology Co., Ltd., Dalian, China. DMEM, Dulbecco's minimum essential medium, was from Gibco Company. Fetal calf serum, FCS, was purchased from Hangzhou Evergreen Biological Engineering Materials Co., Ltd., China. Acetone, acetic acid, NaCl, and Na_2S were all of analytical grade and made by Tianjin Dongli District Chemical Reagent Factory. KCl and $Na_2HPO_4 \cdot 12H_2O$, Analytical Pure, were from Shantou Jinsha Chemical Co., Ltd., China. Ca $(OH)_2$, analytical grade, was from No. 3 Chemical Reagent Plants, Tianjin, China. NaOH, analytical grade, was from Yantai Chemical Co. Ltd., China. EDTA (ethylenediamine tetraacetic acid), Analytically Pure, was from Tianjin Chemical Reagent Co., Ltd. China. KH2PO4, Analytically Pure, was from Suzhou Chemical Reagent Factory, Anhui, China. The 50% glutaraldehyde, Analytically Pure, was made by Damao Chemical Reagent Factory, Tianjin, China. DMSO (dimethyl sulphoxide), analytically pure, was made by No.1 Chemical Reagent Factory, Tianjin, China. SDS (sodium dodecylsulphate), analytically pure, was from Kermel Chemical Reagent Co. Ltd., Tianjin, China. Polyethylene glycol 20000, chemically pure, was from Kermel Chemical Reagent Co. Ltd., Tianjin, China.

Electrophoresis Cell (DYCZ-24DN) and electrophoresis power (DYY-8C) were from Beijing Liuyi Instrument Factory, China. Microhardness tester (MHV 2000) was from Shanghai Hugonggaofeng Co., Ltd. China. SANS materials testing machine was from MTS SYSTEMS Company, China. Air/CO_2 humidified incubator (MCO-15AC) was from SANYO,

Japan. Multiwell microplate reader (Mode 680) was from Bio-Rad, USA. Scanning electron micrograph (SEM, quantum 200) was from FEI Company, Holland. Nicolet 200 Flourier transform infrared spectrometer (FT-IR) was from Thermo Nicolet Company, USA. X–ray diffractometer (PW-1710) was from Philips Company, Holland.

2.2 Extraction and purification of collagen

The collagen was extracted from rabbit skins using a method similar to the conventional procedure mentioned in references. In brief, the procedure involves washing, de-hairing, and mincing.

Pretreatment: The rabbit skins were weighed and the weight was used as the basis for determining the amount of the chemicals in the subsequent process. After being washed thoroughly, the rabbit skins were soaked in a NaCl solution containing 10% NaCl and 2000% water of the rabbit skins in weight fro an overnight. The subcutaneous fat layer was removed by hand and then, dehairing was processed. The dehairing agent was a paste containing 5% Na2S and some Ca(OH)2. After being applied to the flesh side of skin at room temperature for 4h, the de-hairing was finished. The skins was washed and cut into grain size. Some acetone was used to degrease the skins for 6~8h. After being naturally dried, the samples were stired at 4 ºC for subsequent uses

Extraction of collagen: The pre-treated rabbit skins were washed with distilled water and soaked in 10 volumes of 0.5mol/L acetic acid at 4 ºC for 96h. Some samples were homogenated in distilled water. The homogenated mixture was added 0.5mol/L acetic acid solution containing pepsin. After being mixed well, the mixture was ready for enzymatic hydrolysis at 4 ºC for 72h, occasional agitation was neede..

The above mixture was centrifuged at 20 000g and 4 ºC for 30min (the same below) and the pH of the supernatant was adjusted to about 8 with 10% NaOH. NaCl was added to a concentration of 2.5mol/L for salting out. After being centrifuged, the supernatant was discarded. The precipitation was dissolved in a solution of acetic acid and the pH was adjusted to 8.0. NaCl was added to 2.5mol/L at 4 ºC for the second salting out. After being centrifuged, the supernatant was discarded. The precipitation was briefly washed with distilled water for desalination and dissolved in 0.5mol/L acetic acid. 0.01mol/L acetic acid was used for dialysis. After being concentrated with PEG and vacuum freeze-dried, the samples were stored at 4 ºC for subsequent study.

2.3 Preparation of the collagen films

The freeze-dried collagen was dissolved in 0.5mol/L acetic acid to yield a 1% of collagen solution. The solution was divided into two. One was added 0.25% glutaraldehyde [18, 19] at 4 ºC to be cross linked for 24h and the other was used as a control. The collagen solutions were poured in a six-well plate, 5mL for each well. The collagen films were obtained when the collagen solution was freeze-dried.

2.4 Characterization of the collagen

SDS-PAGE: The SDS-PAGE analysis was conducted according to references [13, 15, 20]. The separating gel was 8% and the stacking gel was 5%. Coomassie brilliant blue R-250 was used for staining.

FTIP analysis: Fourier transforming infrared spectroscopy (FT-IR) was used in the study. FT-IR spectrum of the lyophilized collagen samples was obtained from discs containing 2.0 mg sample in about 100 mg of potassium bromide (KBr). The spectrum was obtained using a FT-IR from 4000 to 400 cm−1at data acquisition rate of 2 cm−1 per point.

Thermal stability: The thermal analyzer form was employed for the collagen sample with nitrogen as the protection gas. The temperature was from 20 ºC to 600 ºC at the heating rate of 5 ºC/min. The thermal degradation temperature was determined form the TG/DTG curves.

Collagenase degradation: For both the freeze-dried collagen and cross-linked collagen, samples were exactly weighed, noted as W0, and placed in a 0.1mol/L Tris-HCl (pH = 7.4) solution containing 200IU collagenase at 37 ºC. After the samples were shaked at the speed of 20rpm for 4h, the digestion was ended with the addition of 100μL of 50mmol/LEDTA. The reaction mixtures were centrifugal at 500g, 4 ºC for 15min, rinsed with triple-distilled water rinse 3 times, 10min each time. After being freeze-dried, was samples were weighed again and noted as W1. the collagease degradation rate was determined as[21, 22]:

$$\text{Collagease degradation rate}=\frac{W_0-W_1}{W_0}\times 100\% \quad (1)$$

The SPSS10.0 software was used for the statistics, t test of the independent sample with the result of $p<0.05$, indicating a statistical significance.

2.5 Hygroscopicity.

Hydroscopicity was evaluated according to the water content when the samples were in the wet state. The collagen samples were exactly weighed, noted as W0. after being immersed in 10 times of its weight of deionized water for 24h, the samples were removed, and centrifuged at 500g for 3 min to remove surface water. The samples were then weighed again and noted as W1 to calculate water content.

$$\text{Water content}=\frac{W_1-W_0}{W_1}\times 100\%。 \quad (2)$$

Acute toxicity: The acute toxicity evaluation of biocompatibility is one of the most important properties for biomaterials. The national standard methods of China, GB/T16886.5-2003/ISO 10993-5: 1999[23], GB/T16175-1996[24] and related method [25] were used to study the acute toxicity of collagen. The methods are all of cell growth inhibition assay, which is the MTT assay.

Preparation of extracts: A certain amount of collagen was dipped in 70% alcohol for an instant sterilization, ultraviolet radiated on the sterilized bench, and dried. The dried samples were immersed in serum-free DMEM medium containing 100,000 U/L of penicillin and streptomycin, respectively. The ratio of collagen to the extraction medium (DMEM medium)

was 0.2g/mL. The mixture was placed in a incubator at 37 ºC for 24h to obtain the extract. The exact was added with serum to 10% and stored at 4 ºC for subsequent use.

Cell Culturing: The MEF-WT cells (wild-type, mouse embryonic fibroblasts) were normally stored for use. In the cell incubator at 37 ºC and 5% CO2, the cells were cultured in DMEM medium. The medium contained 10% fetal bovine serum, 4.5g/L glucose, and 25mg/mL ascorbic acid. The liquid was changed every two days.

Cell activity: 200μL of 5 000/mL cell suspension was added in a 96-well plate. After being cultures for 1 day, the samples were further cultured in collagen extracts for 2, 4, and 7 days, respectively. The normal medium was then used again with 20μL MTT. The MTT was a PBS solution containing 0.5% MTT. After cultured for 4h, 200μL of DMSO was added and the mixture was shaked for dissolution for 10min. The OD was obtained at 570nm using microplate reader. The normally cultured sample was the normal control (negative control), and the medium supplemented with phenol solution (64g/L) was the positive control. The relative growth rate of cell (RGR) was calculated as [24, 25] :

(3)

$$RGR = \frac{\text{OD value of experimental group}}{\text{OD value of normal control}} \times 100\%$$

Six grades of cytotoxicity was used to evaluate the cytotoxicity of the samples as shown in Table 1, where both grade 0 and grade 1 are qualified

Table 1 Classification Standard of

RGR，%	Cytotoxicity Grade
≥100	0 级
75~99	1 级
50~74	2 级
25~49	3 级
1~24	4 级
0	5 级

3. Results and discussion

3.1 Electrophoresis of collagen

Collagen is composed of a triple helix structure with two α1 chains and one α2 chain. In the electrophoresis pattern in Figure 1, the β band above 200KDa was the polymer of the spiral

chain of collagen. The α1 and α2 bands are attributed to the collagen α1 chain and α2 chain [13, 20, 26]; No apparent band appeared below the α band. These results suggested that the extracted collagen was pure and kept the basic structure of collagen I. No hydrolysis took place in the extraction to yield small molecules.

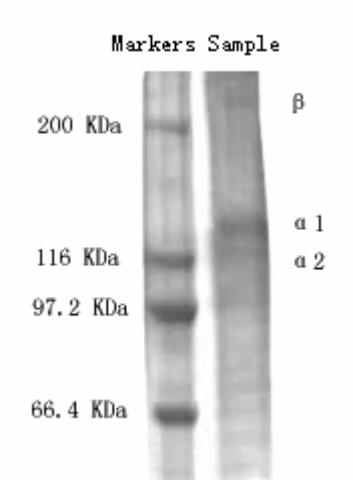

Figure 1 Electrophoretic patterns of the extracted collagen sample

3.2 FT-IR analysis

The FT-IR spectra of the collagen sample were shown in Figure 2. In Figure 2, the bands at 3410cm-1 and 2930cm-1 were denoted to Amide A and Amide B, which are the expanding vibration peaks of. The band at 1640cm-1 was the expanding vibration peak of C=O in Amide I, and the band at 1543cm-1 was the bending vibration peak of N-H in Amide II. The peaks at 1401cm-1 and 1237cm-1 are the Symmetrical expansion vibration peaks of –COO and C-N in Amide III [27-29].

The band at 1451cm-1 was assigned to the vibration of tetrahydropyrrole of proline and hydroxyproline in collagen, indicating that the extracted collagen sample keeps the complete three-helixed structure.[30] The Absorption strength ratio of the peaks 1237 to 1451cm-1 was 1.09, which falls between 1.0-1.1, indication a complete secondary structure of collagen[31, 32]. The ratio for modified collagen is usually 0.6. The existence of the peak at 1640cm-1 also suggests a complete three-helixed structure. [33, 34] Therefore, the extracted collagen keeps a complete spatial conformation

3.3 Thermal stability

The TG/DTG curves of the collagen sample were shown in Figure 3. From Figure 3, It can be seen that there are three significant weight loss stages when the collagen samples were heated. The first one occurred at 100 ºC with a weight loss of around 8%, which corresponds to the desorption process of the physically adsorbed water in the collagen sample. The second stage appears at the temperature range from 200 ºC to 450 ºC with the weight loss is about 60%. It should be the thermal oxidative decomposition of collagen. The weight loss after 450

°C may be the procedure of carbonation by the thermal degradation. The results are consistent with that by Roveri [35] .

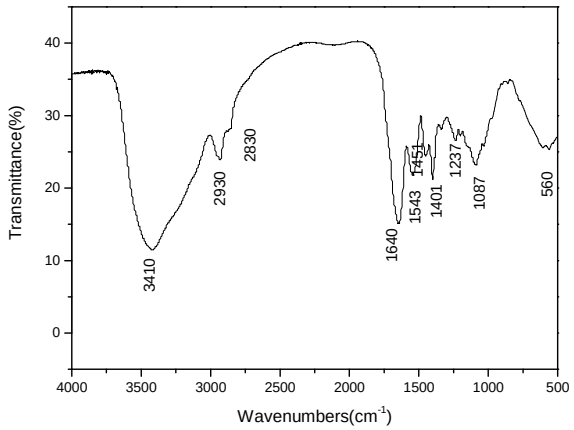

Figure 2 FT-IR spectra of the extracted collagen sample

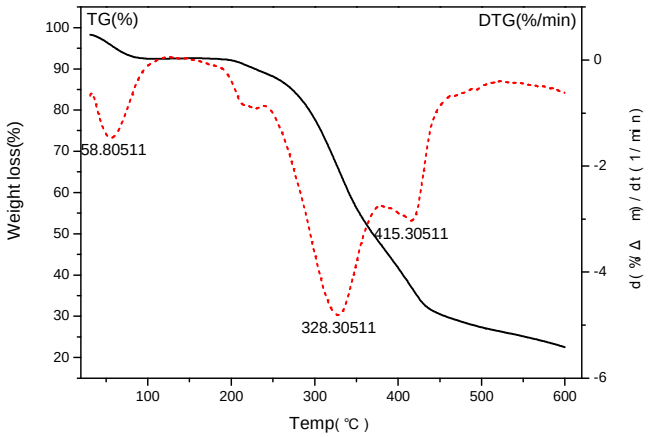

Figure 3 TG–DTG curves of the extracted collagen sample

3.4 Hygroscopicity

In the evaluation of biological materials, water absorption is one of the most important indicators. Only when the biomaterials have good water adsorption and water retention can they provide a necessary micro-environment for the cell to survive and growth.

Around 70% of the body in weight is water. So biomaterials for medicine engineering should be good in hydrophilic. In the present paper, the water content in the cross-linked collagen was more than 78.7%. It is to say that the collagen has a good hydrophilic property, even after glutaraldehyde crosslinking. As biomedical materials, good water absorption is of great significance to ensure the exchange of matters.

3.5 Enzyme degradation

In tissue engineering, the extracellular matrix in vitro (ECMs) is very important. The porous scaffold in vitro should be able to provide a necessary surface mechanical support for cell adhesion and regeneration. Besides, a matched degradation rate for the regeneration of tissues or organs is also needed. Therefore, in vitro enzymatic degradation is an important basis for evaluation the stability of biomaterials. The in vitro enzymatic degradation stability of collagen by collagenase was evaluated, and the results were shown in Table 2.

Table 2 shows that the in vitro enzyme degradation rate of the collagen was decreased to 6.03% from 88.65% by glutaraldehyde cross-linking with a significant difference ($p < 0.01$). It was suggested that the collagen has good enzyme stability after crosslinking.

Table 2 Enzyme Degradation of the Collagen Samples

Groups	Degradation rate(%)	SD(%)
Collagen(a)	88.65	0.11
Collagen(b)*	6.03	0.38

(a)-- non-crosslinked sample; (b)-- crosslinked with GA; SD--standard deviation
*p<0.01

3.6 Acute toxicity

In the study, it was found that both cells of the normal control group and experimental group grew on the wall normally with a elongated spindle shape and translucent cytoplasm. The measured OD values were shown in Table 3.

The cell relative growth rate was more than 75% or 100%. According to the grading standard of GB/T16175-1996[24], it was evaluated that the collagen may be classified as grade o or grade 1, suggesting that it has no or only minor cytotoxicity. It is satisfactory for biomedical engineering materials.

Table 3 OD of MEF-WT cell cultured for 2, 4, and 7 days

Group	OD		
	2d	4d	7d
Normal control	0.1058(100%)**	0.4200(100%)**	0.3857(100%)**
Positive Control	0.0335(31.7%)	0.1110(26.4%)	0.1580(41.0%)
Collagen(a*)	0.0820(77.5%)	0.3693(87.9%)	0.4400(114.1%)
Collagen(b*)	0.1017(96.1%)	0.3530(84.0%)	0.4597(119.2%)

* a and b are the experimental, a--100% extraction, b—50% extraction
** Control, the values were used to obtain the relative cell growth ratio (%) for other groups

Conclusions

A method with the combination of acid and enzyme was used to prepare collagen and the collagen was the type I collagen with a a stable triple-helix structure. When being heated, the

extracted collagen will undergo three weight-loss stages: before 100 ºC, 200 ~ 450 ºC, and after 450 ºC, respectively. After being crosslinked with 0.25% glutaraldehyde, the collagen has good water absorption, stability against enzymatic degradation and biocompatibility. The extracted collagen is suitable for biomedical materials.

Acknowledgements

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