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# Characterisation of acid-soluble and pepsin-solubilised collagen from frog (*Rana nigromaculata*) skin

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ABSTRACT

As the first vertebrates connecting water and land, frogs must have evolved certain specialisations. To find the difference among the collagens from amphibian, fish and mammal animals, acid-soluble collagen (ASC) and pepsin-solubilised collagen (PSC) from frog skin were isolated and characterised. The yield of PSC (19.59%, dry weight) was higher than that of ASC (1.83%, dry weight). The hydroxyproline-to-protein ratio of frog skin was 8.37%, which was higher than that of carp skin ASC (7.83%) but significantly lower than that of calf skin collagen (10.16%), which was in accordance with the living environment of frog. The denaturation temperature of frog skin collagens was approximately 31.5 °C. The SDS-PAGE electrophoresis revealed that ASC and PSC were type I collagens. Fourier transform infrared spectroscopy proved that the ASC and PSC retained their helical structures. The results indicated the properties of frog skin collagen were close to those of skin collagen from freshwater fish. The frog skin collagens can potentially be used in biomaterial and other fields.



## 1. Introduction

Frogs, particularly their legs, are consumed as food by humans in many parts of the world. Originally, they were supplied from local wild populations, but overexploitation led to a diminution in the supply. As a result, frog farming and a global trade in frogs have been developed [1,2]. The main importing countries are France, Belgium, Luxembourg and the United States, and the chief exporters are Indonesia and China [1]. The annual global trade of the American bullfrog *Rana catesbeiana*, which is mostly farmed in China, varies between 1200 and 2400 t [2].

Many researchers have extracted and characterised collagens from marine and freshwater fish [3–6]. Environmental temperature affects collagen stability. Namely, Collagen molecules in solution denature close to the upper limit of the physiological temperature or the maximum body temperature of the animal from which the collagen is extracted [7]. Hence, the thermostability of collagens from deep-sea fish is usually lower than that of collagens from freshwater fish. Collagens from mammals, such as calf and porcine, exhibit higher denaturation temperature ($T_d$) than those from fish. However, few reports were on the collagens from amphibians [8]. Sathya Sai Kumar et al. once utilised collagen from frog (*Rana tigerina* Daudin) skin as a novel substrate in cell culture [9].

Frog resembles freshwater fish in terms of conserving body water effectively. However, the excretory system of frog, similar to that of mammals, comprises two kidneys that remove nitrogenous products from the blood [10]. As the first vertebrates connecting water and land, frogs must have evolved certain specialisations. Accordingly, we intend to explore whether or not the thermostability of collagens from amphibians is between that of collagens from fish and mammals. Research on the properties of frog collagens is crucial to understand the nature of amphibian collagens and identify the influential factors of collagen thermostability. Therefore, this study aims to isolate and characterise acid-soluble collagen (ASC) and pepsin-solubilised collagen (PSC) from the skin of frog (*Rana nigromaculata*).

## 2. Materials and methods

### 2.1. Raw materials

Frogs were manually skinned in biological laboratory in Shangdong Institute of Freshwater Fisheries and then transported in ice to Lianyungang. The samples were washed with chilled tap water, placed in polyethylene bags and then stored at −25 °C until use.

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### 2.2. Chemical reagents

All reagents were of analytical grade. A high-molecular-weight marker kit was purchased from Sigma–Aldrich Chemical Company (St. Louis, Mo., USA).

### 2.3. Proximate analysis of frog skin

The moisture, protein, fat and ash contents of the samples were determined according to the method of AOAC [11]. The hydroxyproline content of the samples was determined using the colorimetric method recommended by the ISO after the material was hydrolysed in 6 M hydrochloric acid for 8 h at 110 °C [12].

### 2.4. Preparation of acid-soluble collagen (ASC) and pepsin-solubilised collagen (PSC)

All preparation procedures for ASC and PSC were performed at 4 °C. ASC was isolated and purified following the method described by Duan et al. [13].

After extracting collagen with 0.5 M acetic acid, the insoluble components were solubilised with 10 vols of 0.5 M acetic acid containing 0.1% (w/v) pepsin (1/10000, Sigma–Aldrich) for 3 d. The solution was centrifuged at 20,000 × *g* for 30 min at 4 °C. The supernatant was salted out by adding NaCl to obtain a final concentration of 2.5 M in the presence of 0.05 M Tris. The resulting precipitate was collected by centrifugation at 20,000 × *g* for 30 min and then dissolved in 5 vols of 0.5 M acetic acid. Salting-out and solubilisation were repeated twice. The resulting solution was dialysed against 0.1 M acetic acid and distilled water for 3 d. The dialysis solution was changed five times every day, and the sample was lyophilised (PSC from frog skin).

### 2.5. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

Electrophoresis was conducted on 7.5% gels by using the method described by Laemmli [14]. High-molecular-weight markers (Sigma–Aldrich) were used to estimate the molecular weights of the proteins.

### 2.6. Amino acid analysis and UV–vis spectra

Frog skin ASC was hydrolysed in 6 M HCl at 110 °C for 24 h without oxygen [15]. The hydrolysates were investigated using a Hitachi 835-50 amino acid analyser. The UV–vis absorption spectra of the collagen were recorded by a Shimadzu spectrophotometer (Model UV-754). ASC samples at 1 mg were dissolved in 2 mL of 0.5 M acetic acid, and the collagen solutions were centrifuged at 5000 × *g* for 10 min at 4 °C. The absorbance of the clarified samples was determined at different wavelengths (from 190 nm to 450 nm) to obtain the UV–vis spectra of each sample.

### 2.7. Determination of denaturation temperature

$T_d$ was measured according to the method described by Duan et al. [13]. $T_d$ was determined as the temperature at which the change in viscosity was half completed.

### 2.8. Peptide mapping

The peptide profiles of fish skin collagens were mapped according to the method of Laemmli [14]. High-molecular-weight markers (Sigma Chemical Co., St. Louis, Mo., USA) were used to estimate the molecular weight of the proteins.

### 2.9. Fourier transform infrared (FTIR) spectroscopy (FTIR)

The FTIR spectra of ASC and PSC from frog skin were recorded on a TENSOR-27 FTIR system (Bruker Optics, Inc., Massachusetts, USA).

### 2.10. Inductively coupled plasma mass spectrometry (ICP-MS)

Inductively coupled plasma mass spectrometry (Bruker ICP-MS M90) (Bruker Optics, Inc., Massachusetts, USA) was used to determine the content of Pb, Hg, and As. The collagens were dissolved in distilled water by heating at 35 °C, followed by cooling at ambient temperature. The ICP-MS parameters were as follows: applied coolant gas flow rate of 18 L/min; auxiliary gas flow of 1.0 L/min; carrier gas flow rate of 0.26 L/min; sampling depth of 6.5 mm and power of 1.4 kW.

## 3. Results and discussion

### 3.1. Proximate composition of frog skin

Table 1 shows the proximate analyses of frog skin. The moisture, protein, fat and ash contents of frog skin were 73.99%, 24.30%, 0.54% and 1.81%, respectively. The compositions of frog skin were similar to those of silver carp and cod skins [6].

Accordingly, the hydroxyproline-to-protein ratios of frog and calf skins were 8.73% and 10.16%, respectively. The hydroxyproline contents of carp and cod skins were 7.69% and 6.77%, respectively [6]. Hydroxyproline stabilises the triple-stranded collagen helix because of its hydrogen bonding ability through its —OH group [16]. Hydroxyproline contents of the skins increases in the order of cod, silver carp and frog, which are deep-sea fish, freshwater fish and amphibian, respectively. This finding further proved that hydroxyproline content is dependent on species, living environment and body temperature [17].

### 3.2. Preparation of ASC and PSC

The yields of ASC and PSC from frog skin were 1.83% and 19.59%, respectively, on a dry weight basis. The yield of PSC from frog skin was higher by more than 10 times than that of ASC. Fish collagens are usually extracted using acidic solutions without the aid of pepsin. The yields of ASC from Japanese sea bass, chub mackerel and bullhead shark were approximately 51.4%, 49.8% and 50.1%, respectively (dry weight) [18]. However, the frog skin collagens exhibited a low solubility in 0.5 M acetic acid; thus, pepsin proteolysis was performed. It suggests that the telopeptide region of the collagen molecule is highly cross-linked [19]. The extractability of PSC significantly improved with the addition of pepsin. Pepsin can cleave the cross-linked regions at the telopeptide can be cleaved without damaging the integrity of the triple helix [20].

### 3.3. Denaturation temperature

The transition curves of the $T_d$ of the frog skin collagens are shown in Fig. 1. The collagens were dissolved in 0.1 M acetic acid, and the $T_d$ was determined by measuring viscosity. The $T_d$ of the collagens was defined as the temperature at which the change in viscosity was half completed. The collagens were heated at different temperatures. The relative viscosities of ASC and PSC were almost stable from 5 °C to 15 °C and then slightly decreased at 15 °C to 25 °C. The viscosity rapidly decreased from 25 °C, and the fractional changes of relative viscosity were almost zero at 36 °C for the two collagens. The transition curves of ASC and PSC were similar, and their $T_d$ values were approximately 31.5 °C.