Process Biochemistry xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Structural characterization, in-vivo acute systemic toxicity assessment and in-vitro intestinal absorption properties of tilapia (*Oreochromis niloticus*) skin acid and pepsin solublilized type I collagen

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ARTICLE INFO

Article history:
Received 21 November 2015
Received in revised form 29 June 2016
Accepted 4 August 2016
Available online xxx

Keywords:
Type I collagens
Denaturation temperature
Ftir
Biocompatibility
Intestinal absorption
Microstructure

ABSTRACT

Acid-soluble (ASC) and pepsin-soluble (PSC) collagens were isolated from tilapia skin and were characterized as type I collagen. The T_d (denaturation temperature) of ASC and PSC was 26.80 and 28.20 °C, respectively. CD (circular dichroism) and FTIR (Fourier transform infrared) spectra of ASC and PSC were slightly different, which confirmed that limited digestion by pepsin disrupted the triple helical structure of collagen. The microstructure depicted a homogeneous, compact, fibrillary, and multilayered sheet-like structure. Notably, no toxicologically effect (survival rate, behavioral activity, respiratory illness, abdominal irritation, eye lid and prolapse) was observed in collagen treated mice. The body weight of the ASC-treated mice (36.1–38.6 g) was significantly higher than that of the PSC-treated mice (32.9–36.7 g). In vitro absorption properties revealed that type I collagen hydrolysates with molecular weights (MWs) ranging from 100 to 12 kDa were effectively absorbed by intestinal villi. Taken together, these results suggest that tilapia type I collagen at the concentrations of 25 and 50 mg/kg bw has no higher risk of chronic toxic effects in mice. Therefore, it was concluded that the administration of tilapia type I collagen had low toxicity and confirmed the in vivo biocompatibility for its wide application in the biomedical industries.

1. Introduction

Type I collagen is the most abundant collagen of the human body which forms large, eosinophilic fibers known as collagen fibers. It is present in scar tissue, the end product when tissue heals by repair, as well as tendons, ligaments and the organic part of bone. Their structure is a right-handed triple superhelical rod consisting of three polypeptide chains. It has widespread applications in the biomedical field because of its excellent biocompatibility and biodegradability [8,9]. Several mammalian type I collagen-based materials such as scaffold, film, and sutures have been commercially available in the biomedical field for the treatment of skin therapy, abdominal wall repair, and blood vessel replacement currently. Many studies have been carried out to investigate the biocompatibility effect of mammalian type I collagen from horse tendons or articular and tracheal cartilage [11,12]. Unfortunately, mammalian collagen have an potential risk of severe infection by containing zoonosis, such as bovine spongiform encephalopathy, avian and swine influenza, and tooth and mouth disease in bovines and pigs. So some other biological materials were studied to reduce this kind of risk.

Marine collagen derived from fish scales, skin, and bone has been widely investigated for its potential application as a human-simulated tissue material and carrier due to its bioactive properties, such as excellent biocompatibility, low antigenicity, and high biodegradability and cell growth potential. Most of all, the use of marine collagen can avoid the risks of the mammalian zoonosis infection completely. In addition, marine collagen can be accepted more easily by many people without religious reason.

Tilapia (*Oreochromis niloticus*) is the most common species of cichlid fish from the tilapiine cichlid tribe. Historically, they have

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http://dx.doi.org/10.1016/j.procbio.2016.08.009
1359-5113/

been of major importance in artisan fishing in Africa and the Middle East, and they are of increasing importance in aquaculture and aquaponics. At present 1.3 million tonnes of Tilapia were cultivated by Chinaper annum, which is the largest tilapia producer in the world. And the US produces 10 thousand tonnes against a consumption of 2.5 million each year. Following the increasing of consumer demand for Tilapia, more and more processing factories were established. However, tremendous processing wastes were discarded for views as being unworthy. This may cause environment problems such as the emission of offensive odors, and resulting in great economic losses to the fishery company. Therefore, enhancing Tilapia utilization efficiency may either reduce the potential pollution threats to the environment or produce more value to increase the benefits to piscatory. At present, the extraction and physicochemical properties of collagen from Tilapia has been widely studied by researchers, and their technique and properties study is underdevelopment as well [5–7,13,14]. However, limited literature is available on the biocompatibility of tilapia type I collagen. And the study of biocompatibility effects of tilapia type I collagen is crucial to the further utilization of Tilapia collagen. In our research, the systemic toxicity effect and intestinal absorption behavior of acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) from tilapia skin were studied, in order to promote its further practical applications in tissue engineering fields.

2. Materials and methods

2.1. Raw materials

Tilapia (*Oreochromis niloticus*) skin was obtained from a private fish processing plant, M/s. Yueqing Ocean Biological Health Care Product Co., Ltd Zhejiang, China, and was used as the raw material for the isolation of ASC and PSC. The residual meat of skins was removed manually and washed with tap water. Then, the skins were cut into small pieces ($0.5 \times 0.5\ cm^2$) prior to the isolation of collagen. The samples were then placed in polyethylene bags and stored at −20 °C until use.

2.2. Isolation of tilapia skin collagen

2.2.1. Pretreatment

The skin pieces of tilapia were mixed with 0.1 M NaOH at a sample-to-solution ratio of 1:10 (w/v) to remove the non-collagenous proteins for 3 days and the alkali solution was changed every day. Then, the samples were washed with tap water, until a neutral pH of washing water was obtained. In order to remove the fat content, the sample was treated with 10% butyl alcohol at a sample-to-solvent ratio of 1:10 (w/v) for 24 h, washed with distilled water repeatedly, and used for further processing.

2.2.2. Isolation of ASC and PSC

Type I collagen was isolated from tilapia skin according to our earlier method [6]. All the preparation procedures were performed at 4 °C. For the isolation of ASC, the pretreated skin was soaked in 0.5 M acetic acid (1:6, w/v) for 4 days with continuous shaking, and the extracts were centrifuged at 20,000 × *g* for 30 min at 4 °C. The supernatant was collected and salted out by adding 2 M NaCl. Then, the precipitates was redissolved in a minimum volume of 0.5 M acetic acid and dialyzed against distilled water until a neutral pH was obtained. The dialyzed sample was lyophilized using a lyophilizer (LabconcoFreezone 2.5, Kansas City, MO, USA) and further referred to as ASC. For the isolation of PSC, the pretreated skin was soaked in 0.5 M acetic acid containing 1% pepsin (1:6, w/v), and then the abovementioned procedure was followed.

2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The protein pattern was analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [15] with modifications. Briefly, the purified collagens were dissolved in 5% SDS, kept in a water bath at 60 °C for 20 min, and centrifuged at 1890 × *g* for 3 min. The supernatant was mixed with a same volume of sample buffer(pH 6.8) containing 1 M Tris-HCl, 1% 2-mercaptoethanol, 40% sucrose, 20% glycerol, 0.02% bromophenol blue, and 1% SDS. The mixture was loaded onto a polyacrylamide gel, composed of 10% separating gel and 4% stacking gel and was subjected to electrophoresis at a constant current of 50 mA. After electrophoresis, the gels were fixed with a mixture of 5:1 methanol: acetic acid composed of 83.3 ml and 16.7 ml methanol and acetic acid, respectively for 1 h. This was followed by staining with 0.5 g Coomassie blue R-250 in 150 ml methanol and 50 ml acetic acid for 30 min. Finally, the gels were destained with a mixture of 300 ml methanol and 100 ml acetic acid.

2.4. Differential scanning calorimetry

Differential scanning calorimetry (DSC) was conducted following the method of Rochdiet al. [16]. The samples were rehydrated with distilled water at a solid-to-solution ratio of 1:10 (w/v). DSC was performed using a differential scanning calorimeter (Model-DSC822e, Mettler-Toledo GmbH, Switzerland). The temperature calibration was conducted using an indium standard. Samples were weighed in aluminum pans and sealed. Subsequently, the samples were scanned at 5 °C/min from 20 to120 °C using ice water as the cooling medium. An empty pan was used as the reference. The denaturation and melting temperatures were estimated from the DSC thermogram.

2.5. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of the samples were obtained using a Nicolet 6700 FTIR spectrometer (Thermo fisher Scientific Inc., Waltham, MA, U.S.A.) equipped with a DLaTGS detector. The samples (5 mg) were mixed with dried KBr (100 mg), ground in a mortar and pestle, and subjected to a pressure of approximately 5×10^6 Pa in an evacuated die to produce a 13 × 1-mm clear transparent disk. The absorption intensity of the peaks was calculated using the baseline method. The resultant spectra were analyzed using the ORIGIN 8.0 software (Thermo Nicolet, USA).

2.6. Circular dichroism

The molecular conformation of collagen was assessed using a circular dichroism (CD) spectropolarimeter (Jasco J-810, Shanghai, China) according to the method of Cao et al. [17]. Briefly, samples were dissolved in 0.1 M acetic acid to obtain a final concentration of 0.1 mg/ml and were stirred for 6 h. Then, the sample solutions were placed in a quartz cell with a path length of 10 mm. The spectra were recorded from the wavelength range of 190–300 nm at 25 °C. The CD spectra of the collagens were obtained after subtracting the acetic acid spectrum.

2.7. Scanning electron microscopy

The morphological characteristics of the collagens extraction were visualized by SEM-S4800 (Hitachi, Tokyo, Japan). The collagen samples were mounted on the sample holder of a standard scanning electron microscope and fixed. The sample holder was used to prepare 20-s glow-discharge carbon support adhesive films coated with gold ions using an auto fine coater. The samples were then

Please cite this article in press as: J. Zhang, et al., Structural characterization, in-vivo acute systemic toxicity assessment and in-vitro intestinal absorption properties of tilapia (*Oreochromis niloticus*) skin acid and pepsin solublilized type I collagen, Process Biochem (2016), http://dx.doi.org/10.1016/j.procbio.2016.08.009