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- TECHNICAL NOTES-

Isolation and Characterization of Collagen and Gelatin From Skin of Silver Cheeked Pufferfish *Lagocephalus sceleratus* for Pharmaceutical and Biomedical Applications

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Abstract

Marine species present a rich source of natural bioactive compounds. Collagen and gelatin are one of the most abundant valuable products, which can be an alternative source for pharmaceutical and biomedical applications. Pufferfish species are not consumed due to its toxicity, since containing tetrodotoxin (TTX) in their body. Therefore, it is important to gain economic value for an environmental issue. In the present study, collagen and gelatin were for the first time isolated and characterized from the skin of silver cheeked pufferfish (*Lagocephalus sceleratus*) by characterizing acid-soluble collagen (ASC). The process extraction efficiency for collagen and gelation was found to be a yield of 50.9% and 20.63 %, respectively. The extracted collagen was characterized by proximate analysis, scanning electron microscopy (SEM), fourier transformed infrared spectroscopy (FT-IR), amino acid analysis and SDS-PAGE gel electrophoresis. The mixtures collagen and gelatin extracts were found to be highly pure. The HPCL analysis revealed that there was no detected TTX in the extracted collagen, indicating the biocompatibility of the extracted collagen. This study exhibit the efficiency of this approach and the economic potential of pufferfish skin or other parts to obtain marine collagen with properties suitable for pharmaceutical and biomedical applications.

Keywords:
Collagen, gelatin, pufferfish, *Lagocephalus sceleratus*, silver cheeked pufferfish

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Introduction

Collagen is considered to be one of the most useful biomaterials because it has a wide-ranging of industrial applications (Schmidt et al., 2016). That is a major essential protein in the connective tissue of animal skin and bone and composes about 30% of the total proteins (Ogawa et al., 2003; Li et al., 2013). Collagen has been isolated from the skins of land-based animals, such as cow and pig, and widely used in food, cosmetic, biomedical and pharmaceutical industries (Li et al., 2013). It is used as a vehicle for drugs, proteins, and genes, as well as a substitute for human skin, blood vessels and ligaments (Gómez-Guillén et al., 2011). However, they are no longer preferred because these sources carry many diseases such as transmissible spongiform encephalopathy (TSE), foot and mouth disease (FMD) and avian influenza. There is a strong need to develop new collagen sources as a result.

Recent research has focused on alternative sources for the extraction of collagen, with particular emphasis on fish by-products (Tang et al., 2015). It is extremely interesting to find collagens from the aquatic environment, such as the skins of ocellate pufferfish (Nagai et al., 2002), black drum and sheepshead seabream (Ogawa et al., 2003), brown-backed toadfish (Senaratne et al., 2006), Baltic cod (Sadowska et al., 2003), Nile perch (Muyonga et al., 2004), bigeye snapper (Jongjareonrak et al., 2006), skate (Hwang et al., 2007), grass carp (Zhang et al., 2007) deep-sea redfish (Wang et al., 2007), dusky spine foot, sea chub, eagle ray and stingray (Bae et al., 2008) and brown-banded bamboo shark (Kittiphattanabawon et al., 2010).

Gelatin is a partially hydrolyzed form of collagen, and mostly it has been used as a potential agent in the food and packaging industry. Fish gelatins are normally produced through acid extraction method reported properties typically known for the similar gelatin group. Film-forming applications of gelatin, which is derived by partial hydrolysis of collagen, in the pharmaceutical and food industries include microencapsulation of ingredients and manufacture of tablet and capsule coatings (Jeevithan et al., 2013).

Pufferfishes are marine fish species that are distributed in tropical and subtropical areas of the Atlantic, Indian and Pacific Ocean. Puffers include 28 genera and approximately 184 species all over the world marine waters within the Tetraodontidae family (Farrag et al., 2016), among which at least ten are found in the eastern Mediterranean (Turan et al., 2017). Several large species used for human consumption as delicious food in few countries, particularly in China, Korea, Japan and Taiwan most pufferfish species have not commercial value. The Tetraodontidae family is famous for the occurrence of a powerful toxin in their skin and organs called tetrodotoxin (TTX). TTX is a very potent neurotoxin and one of the strongest marine paralytic toxins (Sato et al., 2008). According to the current European legislative requirements (European Commission, 2004), poisonous fish of the family Tetraodontidae and products derived from them must not be placed on the market. However, not consuming and fishing of this fish causes an increase in their population in the Mediterranean.

Among collagen alternatives, fish provide the best source of raw material because of its high availability, no risk of disease transmission, no-religious barriers and the possibility of higher-yielding collagen. Up to now, little information concerning the characteristics of marine fish skin collagen especially from not commercially important species such as pufferfish has been reported.

Collagen and gelatin have been recently used in the preparation of biodegradable biomedical products such as tablets, shields, films, sheets, microspheres and scaffolds, etc.

Therefore, the aim of this study is to isolate and characterize collagen from commercially invaluable and unexploited pufferfish silver cheeked pufferfish (*Lagocephalus sceleratus*).

The silver cheeked pufferfish (*Lagocephalus sceleratus*) was caught from Iskenderun Bay by using trawler. All the samples were put in plastic bags individually and frozen at -20 °C till they were transported to the laboratory. The skins were manually removed with a lancet and washed with cold distilled water. The clean skins were cut into small pieces (1 x 1 cm^2) using a lancet (Figure 1). All the prepared samples were stored at -20ºC prior to collagen extraction.

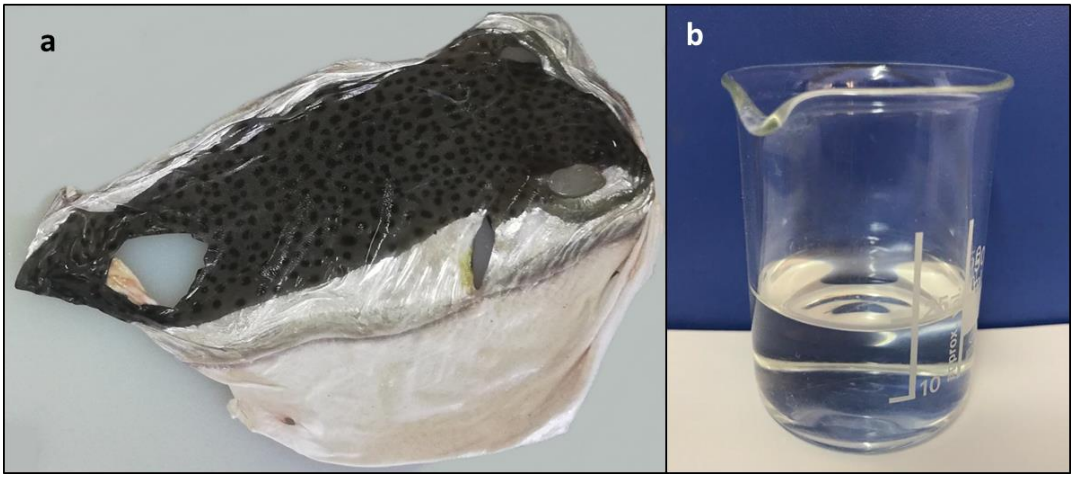

Figure 1. a: Peeled skin of silver cheeked pufferfish (*Lagocephalus sceleratus*) b: Colorless jelly-like structure of collagen extracted from *L. sceleratus* skin.

Acid soluble collagen (ASC) was extracted using the method of Nagai & Suzuki, (2000) and Li et al., (2013) with slight modification. All processes were carried out at +4ºC with continuous stirring. To remove noncollagenous proteins, the prepared skin was mixed with NaOH. The mixture was continuously stirred for 2 days at +4ºC. The pretreated skins were defatted with 10% butyl alcohol. Defatted skin was washed with cold water, followed by soaking in acetic acid. The collagen was precipitated by adding NaCl to a final concentration of 2.6 M. The resultant precipitate was collected by centrifuging and dissolved in a minimum volume of acetic acid and successively dialyzed against 0.1 M acetic acid for 2 days and distilled water for 2 days with a change of solution once per 12 h.

Moisture, ash, fat, protein contents and amino acid analyses of all the collagen samples were determined according to Antoine et al., (1999). This method is based on that of Joseph and Marsden (1986) with several modifications. The HPLC column was used without a guard column. A Spectroflow 980 programmable fluorescence detector fitted with a 5 µL flow cell was used with an excitation monochromator setting at 330 nm and an emission cutoff filter of 418 nm. Other detector settings are 0.1 PMT signal, 10% zero offsets, 1.0 s response (rise time units), and 10-3 A full-scale output range. Data analysis was carried out using the SAS general linear models procedure.

Electrophoretic patterns were measured according to the method of Laemmli (1970) with a slight modification, using 10 % resolving gel and 5 % stacking gel. After electrophoresis, the gel was stained with silver nitrate. High molecular weight protein markers were used to estimate the molecular weight of proteins. Calfskin type I collagen (CSC) (Sigma Aldrich) was used as a standard. The infrared spectra of all the collagen samples were recorded in potassium bromide (KBr) disks with a Fourier transform IR spectrophotometer. One milligram of the dry sample was mixed with 100 mg of dry KBr, and the mixture was pressed into a disk for spectrum recording. The spectra with automatic signal gain were collected in 32. Collagen was redissolved in 0.5 M acetic acid at a concentration of 5% (w/v), followed by dialyzing against distilled water. The morphologies of the electrospun fibres and membrane were observed under a scanning electron microscope (SEM).

TTX extraction was according to the method of Yotsu et al., (1987) with a minor modification. Then the evaporation was carried out using an evaporator and the residue dissolved in 1 mL of methanol was transferred to the vials for analysis. The LC-MS-MS system consisting of an Agilent 6460 triple quadrupole mass spectrometer coupled to an Agilent 1200 HPLC system was used. The presence of TTX in fish skin collagen was investigated. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by using Duncan's Multiple Range Test (Steel & Torrie, 1980). For pair comparison, the T-test was used. Statistical analysis was performed using the Statistical Package for Social Sciences SPSS (Pallant & Manual, 2010)

Collagen and gelatin were isolated from the skin of *L. sceleratus* yield of 50.9 % and 20.63 %, respectively. The yield of the isolated collagen after salt precipitation at neutral pH was 50.9 % on the basis of lyophilized dry weight. The yield of collagen of silver cheeked pufferfish was higher or closer than the collagen yield obtained from other marine fish skins, bones (Nagai and Suzuki, 2000), fins (Nagai, 2004) and other marine organisms such as jellyfish, cuttlefish, etc. (Nagai et al., 2000, 2001; Nagai and Suzuki, 2002).

According to the proximate composition results, the silver cheeked pufferfish skin was found to have fairly high moisture content (75.1%). The present result could be explained by the fact that collagen molecule in the skin was most likely cross-linked by covalent bonds through the condensation of aldehyde groups at the telopeptide region as well as the intermolecular cross-linking, leading to a decrease in the solubility of collagen (Foegeding et al., 1996; Zhang et al., 2007).

The protein contents of skin collagen *Lagocephalus sceleratus* was 2.45 %. By the extraction process, the bulk of the protein was obtained; meanwhile, the great mass of water was excluded with the help of freeze-drying.

Glycine concentration in the amino acid composition of examined collagen was 35.1 %. Glycine is the most dominant amino acid in collagen pronounced as Type 1 described by Muyonga et al. (2004), and all members of the collagen family are characterized by domains with repetitions of the proline-rich tripeptides. Glycine is involved in the formation of the triple helix, except for the first 14 amino acid residues from the N-terminus and the first 10 amino acid residues from the C-terminus of the collagen molecules (Foegeding et al., 1996). The other highest contents of other amino acids found in the silver cheeked pufferfish skin collagen was imino acid (17.2 %), alanine

(15.01 %), hydroxyproline (7.2 %), glutamic acid (6.8 %) and proline (5.6 %) in descending order. The detected imino acid (17.2 %) content of in the silver cheeked pufferfish skin collagen was lower than those of porcine skin collagen reported by Ikoma et al. (2003). Generally, imino acid content in fish collagen was lower than those in mammalian collagen (Foegeding et al., 1996).

The collagen extracted from the silver cheeked pufferfish skin consisted of chains (a_1 and a_2), and the band intensities of a_1-chains were approximately 2-fold that is higher than those of a_2 chains. High molecular weight components, particularly b components, small amounts of g components, and other cross-linked molecules with higher molecular weight were also observed in the skin collagen. In higher vertebrates, type I collagen is widely known as the major fibrillar collagen that is found in most organs and also was the first collagen to be identified (Saito et al., 2001). The kinds of skin collagen were mainly composed of type I (Foegeding et al., 1996) which were also found in other fishes (Nagai et al., 2002; Nagai & Suzuki, 2000; Muyonga et al., 2004).

Two different analysis method was used in order to determine TTX level in the obtained collagen from the silver cheeked pufferfish *L. sceleratus*. TTX was not determined in the obtained skin collagen with LC/MS/MS method. The TTX level of collagen is below the detection limit.

In conclusion, the collagen was for the first time successfully isolated from the skins of silver cheeked pufferfish *L. sceleratus* which showed high solubilities in the acidic pH ranges. However, the solubility's were decreased in the presence of NaCl at concentrations above 2%. Therefore, the use of skin wastes of pufferfish is an economic alternative source to the cattle and porcine collagen for industrial purposes. This study revealed the efficiency of the used approach and the economic potential of pufferfish skin or other parts to obtain marine collagen/gelatin with properties suitable for pharmaceutical and biomedical applications.

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