International Journal of Biological Macromolecules 87 (2016) 504–513

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Evaluation of alternative sources of collagen fractions from *Loligo vulgaris* squid mantle

Natascia Cozza [a], Walter Bonani [a,b], Antonella Motta [a,b,*], Claudio Migliaresi [a,b]

[a] Department of Industrial Engineering and BIOtech Research Center, University of Trento, Trento, Italy
[b] European Institute of Excellence on Tissue Engineering and Regenerative Medicine and INSTM Research Center, Trento Research Unit, Trento, Italy

ARTICLE INFO

Article history:
Received 30 December 2015
Received in revised form 7 March 2016
Accepted 8 March 2016
Available online 10 March 2016

Keywords:
Collagen
Loligo vulgaris
Mantle
Acid-solubilized collagen (ASC)
Pepsin-solubilized collagen (PSC)
Physico-chemical properties

ABSTRACT

Acid-Solubilized Collagen (ASC) and Pepsin-Solubilized Collagen (PSC) were extracted from the mantle of the common European squid, and were comparatively characterized. ASC and PSC were isolated with an extraction yield of 5.1 and 24.2% (on dry weight basis), respectively. SDS-PAGE showed that the ASC was mostly comprised of α_1- and α_2-chains; while the PSC presented relevant β- and γ-components. GPC analysis confirmed that both the ASC and the PSC consisted of fractions characterized by different molecular weight. Thermal denaturation behavior of ASC and PSC were followed by calorimetric and rheological analyses; denaturation temperature was estimated to be 22 °C for ASC and 21 °C for PSC. Amino acid composition and solubility of collagen were also investigated. Finally, the cytotoxicity of the isolated collagen was evaluated *in vitro* and no cytotoxic activity caused by the collagen extracts was observed. This study demonstrated that squid mantle has potential as an alternative source of collagen-derived materials.

1. Introduction

Collagen material is a biopolymer used in a wide range of applications, in food, cosmetic and pharmaceutical industries [1]. It is frequently used as scaffold also in the field of tissue engineering due to its biocompatibility, biodegradability, low immunogenicity and cell-adhesive properties [2]. In particular, among the fibril-forming collagens, type I and V collagen fibrils have been extensively used as biomaterial for the development of tissue engineering constructs since they contribute to the structural backbone of bone [3].

Nowadays, the main sources of collagen and collagen-derived products, such as gelatin and collagen hydrolysates, are limited to bovine skin and tendon, and porcine dermis [4]. Collagen is however present not only in mammals, but throughout the entire animal kingdom including birds and fishes. Sources, extraction methods and pre-treatments affect the final characteristics of collagen, such as composition, rheological properties, solubility and thermal stability [5] and consequently its biological activity.

In the last years, concerns have been expressed about the use of collagen derived from land-based animals due to the risk of infection and diseases such bovine sponge encephalopathy, transmissible spongiform encephalopathy, foot-and-mouth disease and avian influenza [6,7] and increasing interest has been paid to alternative collagen sources. Marine organisms could be a valuable collagen source being highly available, with no risks of disease transmission [8,9].

Each member of the collagen family is characterized by the repetitions of the proline-rich tripeptide Gly-X-Y involved in the formation of trimeric collagen triple helices [3]. Fibrillar collagens are of two types, acid soluble or pepsin soluble. Neutral salt-extraction and low concentration acid-extraction are the most commonly used methods to isolate collagen from natural tissues [10,11]. Dilute acid solvents are more efficient than neutral salt solutions since they dissociate the intermolecular cross-links of the aldimine type causing the swelling of the fibrillar structures [12]. However, dilute acids will not disassociate less labile cross-links such as keto-imine bonds. Much higher yields can be achieved using proteases, i.e. pepsin that cleaves peptides in the telopeptide region [13]. Extraction and characterization of acid and pepsin-solubilized collagen have been reported for different fish species and fish collagen started to became a potential ingredient for cosmetic, food, pharmaceutical and biomedical applications [14–16]. The amino acid profiles of the two types of collagen may vary, depending on the source, as well as molecular weight and denaturation temperature. During the processing of the material, the presence of covalent cross-links between molecules represents the major impediment to dissolution of collagen from tissues. Therefore, native collagen must be pre-treated before it can be converted into a form suitable for extraction [17]. In addition, as a protein, collagen conforma-

* Corresponding author at: Department of Industrial Engineering and BIOtech Research Center, University of Trento, Via Sommarive 9, 38123 Trento, Italy.
E-mail address: antonella.motta@unitn.it (A. Motta).

http://dx.doi.org/10.1016/j.ijbiomac.2016.03.013
0141-8130/

tions are sensitive to different factors during the extraction process, which can induce the destruction of the native form and consequently cause the loss of specific functions. For example heat can induce the triple helix collapse and the thermal denaturation into gelatin [18].

In view of the large number of potential sources to be studied and the large number of parameters related at the extraction process, the chemical and biological characterization of isolated collagen is necessary and efficient systems of evaluation need to be developed [19].

Loligo vulgaris is a neritic, semipelagic species that occurs abundantly in coastal water from the North Sea to the west coast of Africa [20]. This species is commercially very important. In fact, it is extensively exploited by commercial fisheries during the whole year, with annual catches over 15,000 tons [21]. Due to the good availability, easy and fast storage conditions, there is the possibility to use this species for the extraction of collagen and as a model for the development of a simple and efficient system for study the quality of resulting protein. Each anatomical part of a live organisms has a specific function that can affect the amount of collagen and its degree of aggregation. Squid are fast-growing species and very active during their lifetimes. In particular, their mantle needs to be elastic to perform its propulsive function. Due to its peculiar musculature and the high degree of protein turnover [22], squid mantle presents a promising protein composition and collagen is present in a considerably large amounts inside the tissue (up to about 11% of total protein in the muscle of some squid species, like *Illex argentinus*) [23]. However, at present, there has been no investigation on the collagen extracted from *L. vulgaris*.

Therefore, in the present work, first acid-solubilized collagen (ASC) and then pepsin-solubilized collagen (PSC) from squid mantle (*L. Vulgaris*) were isolated and characterized in order to provide a parallel comparison of these collagens. A complete characterization of the physico-chemical properties of the resulting proteins was performed, exploiting different techniques. The extracted collagen was characterized using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Fourier Transform Infrared Spectroscopy (FTIR) and Gel Permeation Chromatography (GPC). Amino acid composition and solubility of collagen were also evaluated. Denaturation temperature was measured by viscosity change and confirmed with thermal analyses using Differential Scanning Calorimetry (DSC). No cytotoxic effect of isolated collagens was observed after the extraction process.

2. Materials and methods

2.1. Materials

Acetic acid, Sodium hydroxide (NaOH), Sodium chloride (NaCl), Bovine collagen type I, Pepsin, Disodium hydrogen phosphate (Na_2HPO_4) and high molecular weight markers were purchased from Sigma-Aldrich (St. Louis, MO, USA).

All the chemicals and reagents were of analytical grade and were used without further purifications.

2.2. Squid mantle preliminary treatment

Squid *L. vulgaris* caught in the northern Adriatic Sea was purchased at Trento local market and kept in ice using a solid/ice ratio of 1:2 (w/w). Squid was washed with iced tap water (0–2 °C), then skin and tentacles were discarded. The squid mantle was cut into small pieces ($0.5 \times 0.5\ cm^{-1}$). Squid mantle fragments were soaked in 0.1 M NaOH for 2 days to remove non-collagenous proteins, and then washed with DI water for 1 day. All the procedures were carried out at a temperature lower than 4 °C.

2.3. Isolation of acid- and pepsin-solubilized collagen fractions

2.3.1. Extraction of acid-solubilized collagen

Acid-solubilized collagen fraction (hereinafter ASC) was extracted according to [24] with some modifications. Briefly, pretreated mantle fragments were finely minced and then treated with a 0.5 M acetic acid solution for 3 days under continuous stirring. Extraction was carried out at a temperature of 4 °C. The mixture was later centrifuged at 80,000*g* for 2 h to pellet non-solubilized collagen fractions. Solid residues underwent a second extraction process under the same conditions. The filtrates obtained in the two processes were later mixed and collagenous molecules were precipitated by adding NaCl to a final concentration of 0.9 M. Precipitated proteins were recovered by centrifugation at 20,000*g* for 30 min at 4 °C and re-dissolved in a minimum volume of 0.5 M acetic acid. The solution was dialyzed against 0.1 M acetic acid for 2 days and then against DI water for 1 day in a Slide-A-Lyzer Cassette (MWCO 3500 Da from Pierce, Rockford, Illinois, USA). The resulting dialysate was freeze-dried to obtain the ASC fraction.

2.3.2. Extraction of pepsin-solubilized collagen

After ASC extraction, the remaining insoluble collagen was washed with DI water and treated with 0.5 M acetic acid with 0.1% (w/v) pepsin for 3 days at 4 °C under continuous stirring. The mixture was centrifuged at 80,000*g* for 2 h to remove residues and dissolved collagen molecules in the supernatant were salted-out by addition of NaCl to a final concentration of 0.9 M. Precipitate proteins were then separated by centrifugation at 20,000*g* for 30 min at 4 °C, dissolved in 0.5 M acetic acid and dialyzed against 0.02 M Na_2HPO_4 solution for 1 day to inactivate pepsin and against 0.1 M acetic acid for 2 days. Finally, the solution was dialyzed against DI water for 1 day, and the resulting dialysates were freeze-dried to obtain the pepsin-solubilized collagen fraction (hereinafter PSC).

2.4. Characterization of extracted collagen fractions

2.4.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

All SDS-PAGE kit and reagents were purchased from Invitrogen (Carlsbad, CA, USA). First, the lyophilized collagen powders were re-dissolved in NuPAGE® LDS Sample Buffer at a concentration of 0.5 mg/ml and incubated at 70 °C for 10 min. Samples (15 μg protein) were analyzed by one-dimentional SDS-PAGE, with a XCell4 SureLock™ Midi-Cell (Carlsband, CA, USA), using NuPAGE® Tris-Acetate SDS Running Buffer, with a constant voltage of 150 V. Acrylamide SDS-PAGE NuPAGE® Novex Tris-Acetate Gels (3–8% gradient) were used for electrophoresis. The acrylamide gels were stained using a Coomassie stain (Imperial Protein Stain). Gel separation patterns were digitalized by a GEL LOGIC 200 (Kodak Scientific Imaging Systems, Rochester, NY, USA) imaging system. SeeBlue® Plus2 Pre-Standard was used as Molecular Weight (MW) reference.

2.4.2. Gel permeation chromatography (GPC)

Gel permeation chromatography (GPC) analysis of the isolated collagens was conducted with a Shodex SB-805HQ column (Shodex OH pak®, Showa Denko, Munich, Germany). Freeze-dried ASC and PSC samples were re-dissolved in 0.5 M acetic acid to obtain a protein concentration of 0.5 mg/ml. The obtained collagen solutions were dialyzed against DI water, using a cellulose membrane (MWCO 3500 Da from Pierce, Rockford, Illinoys, USA). The chromatography system was operated with a flow rate of 1 ml/min and elution was detected with a Jasco UV-1570 detector set (Jasco, Bouguenais, France) at 224 nm. Calibration curve was obtained with Low/High Molecular Weight Gel Filtration Calibration Kit (GE Healthcare Europe, Freiburg, Germany).