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# Extraction and characterization of collagen from Antarctic and Sub-Antarctic squid and its potential application in hybrid scaffolds for tissue engineering

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ABSTRACT

Collagen is the most abundant protein found in mammals and it exhibits a low immunogenicity, high biocompatibility and biodegradability when compared with others natural polymers. For this reason, it has been explored for the development of biologically instructive biomaterials with applications for tissue substitution and regeneration. Marine origin collagen has been pursued as an alternative to the more common bovine and porcine origins. This study focused on squid (Teuthoidea: Cephalopoda), particularly the Antarctic squid *Kondakovia longimana* and the Sub-Antarctic squid *Illex argentinus* as potential collagen sources. In this study, collagen has been isolated from the skins of the squids using acid-based and pepsin-based protocols, with the higher yield being obtained from *I. argentinus* in the presence of pepsin. The produced collagen has been characterized in terms of physicochemical properties, evidencing an amino acid profile similar to the one of calf collagen, but exhibiting a less preserved structure, with hydrolyzed portions and a lower melting temperature. Pepsin-soluble collagen isolated from *I. argentinus* was selected for further evaluation of biomedical potential, exploring its incorporation on poly-ε-caprolactone (PCL) 3D printed scaffolds for the development of hybrid scaffolds for tissue engineering, exhibiting hierarchical features.


## 1. Introduction

Natural materials are the focus of extensive research on their application in different areas addressing human well-being, from food sector to cosmetics and medical field, increasing the pressure over natural resources. This is calling the attention of scientists and engineers towards the development of more sustainable processes and the recent exploitation of marine organisms is much more oriented within a circular economy context, in which valorization of byproducts gains a pivotal role [1,2]. Additionally, the Antarctic resources initially studied within the ideal of an unexplored zone of globe devoted to science and peace, are being now the subject for evaluation of valorization strategies with a clear sustainability pattern [3,4]. Indeed, various Parties of the Antarctic Treaty have regularly proposed to assess regulations on bioprospecting in the Antarctic (see www.ats.aq). One of such strategies addresses the isolation of compounds with high added value, to enter the value chain abovementioned, in which collagen has a golden status, due to its favorable properties of non-toxicity, low immunogenicity, biodegradability and biocompatibility [5–8].

Collagen is inserted in the family of fibrous proteins present in multicellular animals and is the most abundant protein in mammals. The main function of collagen is focused on the support and maintenance of structural integrity, providing texture, shape and resilience, and it is also known for having a regulatory role in tissue development [9,10]. About 25% of the human body is composed by this protein, being identified up to today at least 28 genetically different types of collagen, classified based on their organization and supramolecular structures function and distribution in tissues [11–13]. Actually, an epidermal collagen type XXIX has been proposed [14], but the coding gene COL29A1 seems to be identical to COL6A5 and thus its acceptance is under debate [15]. Within marine resources, collagen is commonly isolated from fish skins [16,17] or jellyfish [18], but other sources have

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been also explored, such as sea sponges [19,20], echinoderms [21] and cephalopods [22]. Within this latter group of animals, squids as source of collagen are still poorly explored, but arise as an elegant model for valorization of marine resources in the Antarctic/sub-Antarctic regions due to recent studies on the ecology of squid species from this region of the Globe [23]. Indeed, there are squid species, as *Kondakovia longimana* distributed in Antarctic and sub-Antarctic waters [24], and *Illex argentinus* distributed in sub-Antarctic waters [23,25], allowing a direct comparison between materials obtained from organisms from neighbor regions. Moreover, the latter squid species is largely explored for food purposes [26] and the production of collagen from its skins would represent an attractive way for valorization of by-products.

Collagen is usually obtained at a very low yield through the traditional process, based in acid treatment with organic acids, rendering the designated acid soluble collagen (ASC). However, it is possible to increase the extraction yield with the use of pepsin, which acts on the edge of the polypeptide chains, cutting telopeptides and thus facilitating their solubilization in acidic solutions, with the resulting collagen being denoted as pepsin soluble collagen (PSC) [27].

Considering its favorable features and the central role that collagen assumes in extracellular matrix, it has been widely proposed for biomedical application, namely the types I to V [28,29], with marine origin collagen being consistent with collagen type I (fish skins, scales and bones), type II (jellyfish, fish cartilage) or type IV (sea sponges) [27]. In particular, the use of collagen on the development of biomaterials mimicking extracellular matrix in tissue engineering approaches is a hot topic [30–32]. Collagen scaffolds normally exhibit excellent biocompatibility and tuning of cellular behavior, but lack adequate mechanical properties, from which the combination with synthetic polymers has been proposed [33]. One of the more elegant ways to achieve that combination of enhanced features is the design of hybrid scaffolds, as a way of producing multiscale structures for tissue engineering [34,35]. In this context, the use of 3D printing technology has gained increasing prominence, with the production of an oriented fibrillar structure further modified to achieve a hierarchical architecture [36]. Such modification is intended to affect porosity, surface chemistry and roughness of the scaffolds, together with an increase of cell anchorage points aiming to increase cell seeding efficiency and enhance the performance of the resultant construct [37–39].

In this work, collagen has been extracted from *K. longimana* and from *I. argentinus* and characterized by Fourier transform infrared spectroscopy (FTIR) and amino acid analysis to assess their chemical features, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to address their purity and identify collagen type and differential scanning calorimetry (DSC) to evaluate melting temperature. Selected collagen samples have been further incorporated into 3D printed poly-ε-caprolactone (PCL) scaffolds, with evaluation of morphological and mechanical properties, stability in aqueous media and in vitro compatibility with L929 cells.

## 2. Material and methodologies

### 2.1. Materials

Individuals from *K. longimana* squid species were collected in the Antarctic (Scotia Sea) during an expedition of the British Antarctic Survey in 2013 and kept frozen at −20 °C. After thawing, skins and muscle were separated and used as raw material for collagen isolation. Skins from *I. argentinus* squid species, captured from the Patagonian shelf (sub-Antarctic waters), resulting from processing of squids for food products were kindly provided by Dr. Julio Maroto (Fundación CETMAR, Vigo, Spain). Poly-ε-caprolactone (with $M_w$ 70,000 to 90,000), was purchased to Sigma-Aldrich. All other reagents were of analytical grade and used as received.

### 2.2. Extraction of ASC and PSC from K. longimana and I. argentinus squids biomass

Collagen was extracted from squid materials according to a methodology adapted from [22,40]. All processes were carried out at 4 °C. Squid skins and muscles were soaked in 0.1 M NaOH (VWR International) solution for 6 h (changed every 2 h), with magnetic stirring, to remove non-collagenous proteins. When using skins, pigments were removed by treatment with 1% $H_2O_2$ solution for 12 h. Skins and muscles were then washed abundantly with distilled water to obtain a pH close to neutral and further soaked in 0.5 M acetic acid (VWR International) solution, for 72 h with magnetic stirring. The mixture was centrifuged at 9000*g* for 25 min and the supernatant (with ASC) was collected and kept at 4 °C. The precipitate was re-extracted in 0.5 M acetic acid solution with 3.3 mg of pepsin A (Sigma-Aldrich) per gram of raw material, during 72 h, with magnetic stirring, followed by centrifugation at 9000*g* for 25 min. The supernatant (with PSC) was collected and kept at 4 °C. The ASC and PSC were further dialyzed against 0.1 M acetic acid solution for 12 h, with solution changed every 2 h, frozen at −80 °C and freeze-dried.

The extraction of collagen was performed in triplicate for each squid material for assessment of reproducibility regarding the extraction yield.

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

The infrared spectra of collagen samples were obtained in KBr pellets using a Shimadzu- IR Prestige 21 spectrometer in the spectral region of 4000–800 $cm^{-1}$ with resolution of 2 $cm^{-1}$ and taking the average of 32 scans.

### 2.4. Differential scanning calorimetry (DSC) analysis

Thermal profiles of ASC and PSC samples were assessed by DSC (Q100 Thermal Analysis), from 0 to 80 °C, at a heating rate of 1 °C $min^{-1}$ to assess melting temperature. An empty capsule was used as reference and the presented values are an average of three independent measures. Values of melting temperature were determined as the average of three independent measurements for each sample.

### 2.5. Amino acid analysis

Amino acid analysis was performed at *Centro de Investigaciones Biológicas* of the Spanish National Research Council (CSIC), in Madrid (Spain). Collagen samples were firstly completely hydrolyzed and further separated using an ion exchange column. After post-column derivatization by ninhydrin, the samples were analyzed at two wavelengths (440 and 570 nm), using a Biochrome 30 (Biochrome Ltd., Cambridge, U.K.). An internal standard of norleucine was used for quantitative analysis. Three independent measurements for each sample were made for the quantification of the average amino acid contents.

### 2.6. Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a 7.5% separating acrylamide gel and a 3% stacking acrylamide gel, with a voltage of 60 V for 20 min and for an hour with a constant voltage of 144 V. The samples were applied into the wells and electrophoresed in Bio-Rad Miniprotean 3 cell vertical electrophoresis tank. Protein bands were stained for 35 min with staining solution (0.1% Comassie Brilliant Blue R-250, from BioRad) and distained with two distaining solutions (containing distilled water, methanol and acetic acid).