Optimization of a new technology for extraction based on water pressurized with carbon dioxide

Alexandre A. A. Barros[1,2], Ivo M. Aroso[1,2],Tiago H. Silva [1,2] João F. Mano[1,2], Ana Rita C. Duarte[1,2]* and Rui L. Reis[1,2]

[1] 3B's Research Group - Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal

[2] ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

E-mail corresponding author: aduarte@dep.uminho.pt*

ABSTRACT

The objective of this work is to develop and optimize a new technology for extraction of acid soluble compounds, based on water pressurized with carbon dioxide. To prove the concept, we have applied this new process to marine sponges and have successfully obtained collagen/gelatin with properties for application in the biomedical field. Traditionally, marine sponge collagen/gelatin can be isolated after acid, basic or enzymatic treatments processes, all of which require heat or water. Nonetheless, the extraction procedures are very laborious and involve multiple steps, which hinder the scaling up of the processes. The most explored extraction process for this material is typically performed in a two-step process that includes extraction/dissolution in alkaline aqueous media together with a chaotropic agent in high concentration, followed by precipitation under acidic medium. This process is time consuming and requires the use of chemicals and large quantities of water, prompting the need for an alternative process comprising less steps and, ideally, more environmentally friendly. In this sense, we propose a new extraction methodology, under mild operating conditions, in which water is acidified with CO_2 to promote the extraction of collagen/gelatin from different marine sponge species.

INTRODUCTION

Chemical industries have been moving towards the development of innovative processes as the awareness that a sustainable development is becoming mandatory and essential for their competitiveness. Recent progresses in supercritical fluids (SCF) report processes using water in subcritical conditions, i.e., processes that are carried out at temperatures above the boiling point (100ºC and 0.1 MPa) and below the critical point (374ºC and 22.1 MPa) of water. In particular extraction of different active compounds from natural origins has been reported in the literature [1, 2]. A sustainable development relies not only on the development of novel processes but also on the use of alternative sources of raw materials which decrease the dependence from fossil-fuel resources. In this sense, the sea provides a plentiful resource of potential new products for society [3-5]. The current industrial demand for collagen accounts up to 326.000 tons [3] for different fields of application, from alimentary, cosmetics, pharmaceutical to biomedical. The source of 98% of the collagen used has mammalian origin, for instance calf skin and bone, but nowadays these carry a high risk of disease transmission such as bovine spongiform encephalopathy, as well as social and/or religious constraints. Aiming to overcome these drawbacks, alternative sources have been suggested, as is the case of collagen from marine sources [4]. The major objective of this work is hereafter, the exploitation of the possibility of coupling a green extraction process with a natural origin renewable raw-material to obtain marine sponge-origin collagen/gelatin, particularly for biomedical applications. Although marine sponge collagen has unique physico-chemical properties and is as such a promising resource, it is not available in large quantities especially because of the lack of efficient extraction methodologies. The traditional extraction methods used are generally time consuming as they involve several operating steps, have a low selectivity and/or extraction yields. Furthermore, they require the use of large amounts of water, with environment concerns due to the significant volume of eluents to be treated according to straight legislation. Processing industries are does looking forward for the application of alternative processing methodologies, which comply with the green chemistry philosophy. In this work, we propose a new extraction methodology for marine sponge collagen/gelatin, in which water is acidified with carbon dioxide. The proposed process is defined by comprising fewer processing steps and requiring the use of less solvents than the current processes, at the same time responding to the environmental concerns and the industrial challenges of scalability. .

MATERIALS AND METHODS

Materials

Sponge samples of the species *Thymosia sp. and Chondrila nuculla from Porto Fino* collected in Mediterranean Sea and *Chondrosia reniformis* was collected in Israeli coast were kindly provided by Dr.Ronald Osinga (Porifarma), Dr. Martina Milanese (Studio Associato Gaia), and Prof. Micha Ilan (Tel Aviv University) respectively. *Chondrila nuculla* produced by aquaculture in Alassio coast (Mediterranean Sea) was kindly provided by Dr. Martina Milanese (Studio Associato Gaia). The sponges were frozen after collection and were salt leached and freeze dried before processing.

All other chemical reagents were ACS reagent grade and were used as received.

Collagen extraction

Marine sponges were grind in to smaller pieces and 5g of the material were loaded to the high pressure vessel, with 10 ml distilled water. The system was heated at 40ºC and the vessel was pressurized with carbon dioxide at 50 bar, overnight. The extract obtained was filtered with a 0.22 µm filter and frozen. Collagen/gelatine powder was obtained after freeze drying. A schematic representation of high-pressure equipment used is represented in **Figure 1**.

Characterization of sponge collagen/gelatin

Collagen/gelatin extracts were analysed using different analytical techniques. The morphology of the powder obtained from the different extractions was observed by scanning electron microscope (SEM). The isoelectric point of the different collagen/gelatin extracts was determined by titration with NaHO and HCl. The quantification of collagen on the extracts was performed using Sircol Assay kit (Life Science Assays, UK), a specific colorimetric method for the determination of collagen/gelatin. The molecular weight of the extracts was determined by gel permeation chromatography (GPC-SEC). The extracts were also characterized by circular dichroism (CD), infrared-spectroscopy (FTIR), UV-Vis spectroscopy and differential scanning calorimetry. In vitro cytotoxicity studies of the extracts were carried out in accordance with ISO/EN 10,993.

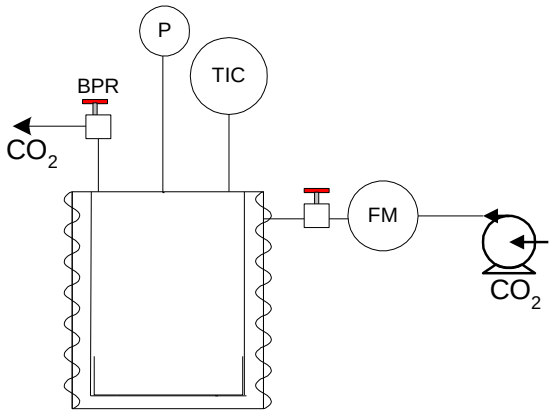

Figure 1. *Schematic representation of extraction equipment for carbon dioxide acidified water.BPR – back pressure regulator, P – pressure transducer, TIC, temperature controller, FM, flow meter.*

RESULTS

Marine origin collagen/gelatin has been reported to be a great promise but so far there has been a gap between the scientific interest and the industrial application of this source of collagen/gelatin. In the case of collagen from marine sponge, with peculiar properties, this is even clearer due the absence of high yield extraction processes. To overcome this, new, simple technologies, for the isolation and purification of this highly valuable biopolymer should be developed. In this work, a new extraction methodology, under mild operating conditions, in which water is acidified with carbon dioxide to promote the extraction of collagen/gelatin was tested for different marine sponge species.

The extraction process herein described comprises a single step, reducing chemicals and water consumption. The acidification of the aqueous solutions by carbon dioxide induces the extraction of collagen/gelatine, which was recovered as a powder from the aqueous solution after freeze-drying. The physico-chemical characterization of the recovered products have shown that the proposed extraction process allows higher extraction yields (~15%) than the ones described in the literature, while at the same time maintaining the fundamental material characteristics. *Kreuter et al*. and *Swatschek et al.* [6, 7], report the extraction of collagen from marine sponges with lower recovery yields and involve several operating steps. In the present study, the lowest yield was obtained for the *Chondrilla nuculla (Alassio)* sponge (~9%) and the highest yield was obtained from *Chondrilla nuculla (Porto Fino)* sponge (~17.3%). While *Chondrilla nucula* from Porto Fino is a wild sponge, *Chondrilla nuculla* from Alassio were obtained from aquaculture experiments. Further studies are required in order to understand the differences obtained in the extraction yield for this sponge from different proveniences, in particular to address the effectiveness of aquaculture methodology regarding sustainable production. Additionally, the quantification of the collagen content in each extract revealed that the extracts recovered from *Thymosia sp.* and *Chondrilla nuculla (Porto Fino)* have a high value of collagen, near 82 %.

The morphology of the powders obtained was observed by scanning electron microscopy. The images are presented in **Figure 2**.

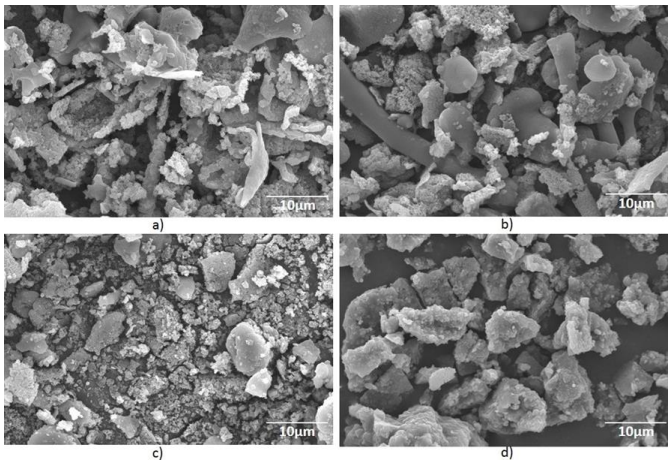

Figure 2. *SEM micrographs of the powder obtained from a) Chondrilla nuculla (Alassio), b) Chondrilla nuculla (Porto Fino), C) Chondrosia reniformis and d) Thymosia sp.*

The typical fibrillar structure of collagen was not observed for any of the samples. However, our results are in agreement of nodular collagen, extracted from eel fish, has been reported in the literature in a work by *Veeruraj*, [3]. Chemical characterization is however of outmost importance to determine the nature of the extracted compounds. For this different techniques were used.

The extracted collagen/gelatin was characterized by circular dichroism and compared to commercial gelatin and collagen type IV and I.

CD spectra for collagen controls are composed of two peaks, a positive around 221nm and one negative around 192nm, a characteristic profile of the collagen triple helix. However, for gelatin and for the extracted material for *Chondrilla nuculla (Alassio), Chondrilla nuculla(Porto Fino) and Chondrosia reniformis* the positive peak around 220 nm is absent, suggesting the existence of random coils [8]. In the case of *Thymosia sp.* the CD spectra is not conclusive due the presence of other peaks that may suggest presence of others compounds.

The FTIR spectrum for the extracts from *Chondrilla nuculla (Alassio), Chondrilla (Porto Fino), Chondrosia reniformis and Thymosia sp.* is composed of several bands that can be attributed to amide B (2950 cm^{-1} and 2932 cm^{-1}), amide I (1644 cm^{-1}), amide II (1523 cm^{-1}) and amide III (1239 cm^{-1}). The FTIR spectra suggests that the chemical structure of the extract obtained is from collagen/gelatin [3].

The isoelectric point was determined after titration of collagen/gelatin extracts with NaHO and HCl. The initial pH of the extracts solutions was basic, approximately around 9.5. All the samples present a similar behaviour, showing an isoelectric point around pH 8. The titration curves show a flattening area at pH 6 that eventually levels off at pH 2. The behaviour of the extracts present however a second critical point noted at pH 5, common to the four samples. *Highberger*, in 1939, reports the existence of two critical points in the pH mobility of collagen and gelatin, correspondent to a pH of 4.7 and 7.7, respectively [9]. Our observations are in agreement with the finding herein referenced and may suggest the presence of a mixture between collagen and gelatin in the extracts.

The molecular weight for the extracted collagen/gelatin was determined, by GPC-SEC, to be 112.77 (12.60) KDa for *Chondrilla nuculla (Porto Fino)*, 110.59 (8.75) KDa for *Chondrilla nuculla (Alassio)*, 208.92 (14.96) KDa for *Chondrosia reniformis* and 155.40 (20.42) KDa for *Thymosia sp*. within the values typically found for collagen molecules (100-200KDa). The thermal degradation temperature, was evaluated by differential scanning calorimetry and was determined to be between 30ºC and 45ºC, which is close to that of calf skin collagen (40.8ºC) [10].

In vitro citotoxicity results showed that collagen/gelatin extracted from *Chondrosia reniformis* and *Chondrilla nuculla* does not compromise the metabolic activity of the L929 cells. The exception was for the extract from *Thymosia Sp*. Even though 82% of the obtained extract was quantified has being collagen, there may be some cytotoxic compounds present in the extract. This is consistent with the results obtained from the CD spectrum that suggested the presence of other compounds. Further purification steps would then be, in this case, required to overcome the toxicity observed.

The extraction process here described has been successfully applied to obtain collagen/gelatin from marine sponges, with high applicability in the biomedical field, while at the same time, reducing the number of processing steps, chemicals and time. Moreover, the simplicity of the proposed extraction process makes on believe in its applicability to other collagen/gelatin sources, furthermore increasing its industrial potential. In fact, its success can be even more pronounced in sources such as fish skins, from which collagen is extracted mostly by acidic treatments. This new extraction process represents a step forward for the valorisation of the potential of marine resources.

CONCLUSION

A new process using acidified water with high pressure carbon dioxide was developed and applied to the extraction of collagen/gelatin from marine sponges. The extracted material was confirmed to be a mixture of collagen and gelatin using different physical and chemical analysis techniques. Cytotoxicity behaviour confirmed that the collagen/gelatin obtained is a promising material for biomedical applications. The extraction of sponge-origin collagen/gelatin with the proposed methodology was successfully achieved.

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