Method for Obtaining and Physico-Chemical Characterization of Collagenic Extract of Rhizostoma Pulmo from the Black Sea

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Abstract

Rhizostoma pulmo is a jellyfish from the Black Sea basin that can be a source of natural bioactive compounds with substantial beneficial implications. It is important to use under-exploited marine resources in areas such as pharmaceuticals industry, medicine, cosmetics and dermatology. Marine collagen can be obtained from various sources. Several studies have focused on marine collagen, namely its extraction from alternative sources: fish, invertebrate marine animals such as sea sponges or jellyfish. The novelty is the extraction of marine collagen obtained from jellyfish of the species *Rhizostoma pulmo* found in the Black Sea and along the coast, the physico-chemical characterization, comparison with other types of collagenic extracts from fish and finally the formulation of a pharmaceutical preparation with medical applications.

Keywords: *Rhizostoma pulmo,* jellyfish, marine collagen, bioactive compounds, medical applications.

Introduction

Collagen is the most studied protein, with a wide range of applications, including the pharmaceutical, biomedical and cosmetic industries due to its special characteristics, which are of high biocompatibility, qualitative bioactivity and poor antigenicity [1]. Although there are various and abundant sources of collagen, the existence of various diseases among terrestrial animals is a threat to its use in our daily lives. The research aims to find an alternative that would reveal the many untapped marine sources, such as fish, jellyfish and some marine mammals. A brief description of collagen, its characteristics, sources of marine origin, extraction, collagen peptides and their biological activities, as well as potential applications in various fields, is desired.

Hot water jellyfish (*Rhizostoma pulmo* Macri, 1778). Taxonomic classification: Kingdom of *Animalia*, *Cnidaria*, Class *Scyphozoa*, Subclass *Discomedusae*, Order *Rhizostomeae*, Suborder *Daktyliophorae*, Family *Rhizostomatidae*, Genus *Rhizostoma* [2].

Large jellyfish easily recognizable by the narrow band colored in blue, purple or pink that borders the slightly lobed edge of the umbrella. It is a very common species, numerous in the Black Sea, Mediterranean Sea, Sea of Azov and North Sea. It has a tall bell with a regular diameter of 20-30 cm, with 16 marginal lobes and 8 lobes. The buccal tentacles are overgrown and form a long and voluminous handlebar, fringed at the edges, so the axial length of a specimen can reach 50-60 cm [3].

It is a mycrophage, the primitive mouth being replaced by a pore system. A common species in the Mediterranean, we find it in the Black Sea only in the warm season, brought by hot water currents. Swimmers or those who bathe in the sea water, touching this jellyfish, can get a very unpleasant burn, caused by its bladder cells.

Collagen extraction from different jellyfish species

Collagen is the most abundant protein found in the body. These proteins form fibers that help build and maintain parts of the body, it is also found in the muscles, hair, skin, bones, tendons and ligaments. Researchers have identified at least 28 types of collagen, but those classified as type I, II and III represent 80% to 90% of total collagen [4]. All collagen molecules contain a triple helical domain, are generally involved in the formation of supramolecular networks and are made up of three α chains that may or may not be identical. These α chains contain at least one collagen domain or a triple helix motif characterized by the succession of Gly-Xaa-Yaa triplets where Xaa and Yaa are often Pro and Hyp residues, respectively [5].

Collagen currently has a wide range of applications in various areas of health, namely in cosmetics, the pharmaceutical industry and in healthcare (including plastic surgery, orthopedics, ophthalmology and dentistry).

In non-medical sectors, a notable use of collagen is in the food sector (food processing, as an additive and nutraceuticals), but most often in the form of gelatin, ie in its distorted form.

Fibrillar collagens are the most abundant extracellular proteins, they form a specific family of metazoans. Their structural and physiological properties have been used successfully in the cosmetics, pharmaceutical and food industries. The increase in the number of jellyfish has led us to appreciate this marine life as a natural product for food and medicine. Different species of Mediterranean jellyfish have been tested to investigate the economic potential of their collagens. Different methods of collagen purification (tissues and experimental procedures) were analyzed. The best collagen yield was obtained using the oral arms with predilection for *Rhizostoma pulmo* and the pepsin extraction method (2-10 mg collagen/g wet tissue) see table Nr.1 [6].

Rhizostoma pulmo has been used in other experiments, these drugs are considered harmless to humans and for the abundant source of collage material [6]. The biological properties of *R. pulmo* were compared with mammalian fibrillar collagens in cell cytotoxicity and cell adhesion tests. There were no statistical differences in cytotoxicity ($p > 0.05$) between *R. pulmo* and rat type I collagen [1]. Their structural and physiological properties have been used in the food, cosmetics and pharmaceutical industries.

Materials and Methods

The characteristics of collagen are unique, both *in vivo* and in tissue engineering that attempts to attenuate the role of collagen in the extracellular matrix, based on cell cultures that verify the quality and properties of processed collagen.

Due to the fact that there are so many sources from which collagen can be extracted and so many types, it is essential to analyze the characteristics carefully and accurately [7].

There are several techniques that can be used to characterize collagen samples (solid or in solution) addressing morphological, structural and chemical characteristics [8]. With a precise characterization and a better knowledge of the sample, it is easier to obtain a desired biological response or to establish a relationship between the results and the characteristics of the structure of the product obtained.

The aim is to continuously monitor the results, trying to control the characteristics of the sample, resulting in a more predictable and accurate biological response.

The extracting marine collagen from jellyfish

A widely used acid solution for collagen solubilization is also used for the extraction phase, which is also called acid-soluble collagen (AUC), [9].

Skierka and Sadowska [9] tested the influence of various acids on the extraction of collagen from cod skin, including hydrochloric, citric, acetic and lactic acids, of which acetic and lactic acids have been shown to produce higher collagen extraction yields. Unfortunately, the process of collagen extraction normally results in a low yield.

To overcome this impediment, scientists applied an enzymatic treatment using non-collagen-specific proteolytic enzymes to aid the solubilization process, such as trypsin, pancreatin, ficin, bromelain, papain or pepsin, the latter being the most widely used. By applying pepsin, the resulting extract is called Pepsin Soluble Collagen (PSC) or atelo-collagen [10].

This treatment is very useful because it cleaves peptides specifically from the telopeptide region of collagen, which are non-helical ends, and thus, by hydrolysis of non-collagenous proteins, increases the purity of collagen. It results in a much more efficient collagen extraction, as it prepares the sample for solubilization, while reducing the antigenicity caused by telopeptides [11].

For this reason, it is common to use this proteolytic process after AUC extraction, thus obtaining the PSC mentioned above. The antigenicity of collagen is not only derived from its telopeptides, but is also linked to the presence of non-collagenous proteins, cells and cell debris, being the method of NaHO treatment of raw materials[12] important for removing this source of antigenicity. For the recovery stage, the collagen must be precipitated, usually obtained by adding NaCl to a final concentration which can vary between 2.3 M and 2.6 M in Tris-HCl (pH 7.5). The resulting precipitate is collected by centrifugation, dissolved in 0.5 M acetic acid, dialyzed and lyophilized, thereby obtaining a collagen which is soluble in dry acid and soluble in pepsin [13].

Jellyfish collagen is an alternative being an available and efficient source to use as a component of the matrix for tissue engineering, because it has a small amount of impurities. Due to the dry weight of the edible jellyfish, over 40% of it is collagen in an animal in which 95% is water [14].

From jellyfish large part of the body is called the umbrella, which is divided into a major component called the mesoglea and the outer skin: exumbrella and subumbrella collagen is obtained from the mesoglea, following a methodology based on solubilization in acetic acid.

Solution, usually time the extracts are then dialyzed against Na_2HPO_4 solution, resulting in precipitated collagens, which can be separated by centrifugation.

The collagen produced can then be purified by a re-precipitation method: solubilization in acetic acid and precipitation by the addition of solid NaCl. AUC can also be digested with pepsin to obtain atelo-collagen[15].

Purification methods

Jellyfish collagen is an available and viable source to use as a component of the matrix for tissue engineering, as it has a small amount of impurities. One of the methods used to characterize the purity and degradation of collagen is polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE).

By this method - gel electrophoresis, proteins and their fragments can be separated, depending on their size. Protein fragments can be observed by loading protein samples into the small wells of the gel, which, under an applied electric field, pass through the gel matrix according to their size: the smaller ones go further than the large ones, which remain trapped in the net of gel.

Some types of collagen obtained from different sources can be compared to existence data. The type of collagen (I, II) can be identified when the collagen bands are similar. In the case of collagen of the same type, but of different species, in which the amino acid sequence is altered, although the same chain types are present (e.g. α1, α2 and β chains in type I collagen), the position of the bands is slightly altered can be observed [16].

Stomolophus nomurai meleagris jellyfish collagen was prepared by lyophilization and crosslinking with 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide hydrochloride (EDC/NHS) for use in tissues with bioengineering applications [17].

Jeong and his collaborators [18] developed scaffolds collagenous extracts from jellyfish and poly (d, l-lactide-co-glycolide) (PLGA), by lyophilization and electrospinning, proposing their application on vascular grafts.

Addad has proposed the use of EDC-NHS crosslinked jellyfish collagen in various reports as a replacement for bovine or human collagen in biomedical applications [6]. More recently, porous scaffolds composed of refibrillated collagen, previously extracted from *Rhopilema esculentum* jellyfish, exhibited elastic behavior and were able to support hMSC culture, with overexpression of chondrogen markers under chondrogen stimulation, suggesting the use of cartilage tissue engineering structures [19].

Collagen extracted from other jellyfish species has also been proposed for later use in various applications, such as those extracted from *Cyanea nozakii* [20].

Results and Discussions

Marine collagen sources are known to have low antigenicity, *in vivo* studies should be considered before performing their use in engineering and tissue regeneration applications to verify the feasibility of using selected collagen in human implants.

In order to use alternative sources of collagen, all potential commercial alternatives to collagen must be considered. Collagen cannot be easily and advantageously replaced with other molecules, in their existing or developing compositions, it is necessary to verify whether the proposed options are economically viable and have similar biocompatibility profiles, physico-chemical and biological properties. Collagen also poses some risks due to its human or animal origins.

Table Nr.1 Extraction yields of collagen from different species of jellyfish expressed mg/g

Species	**Collagen extracions(mg/g)**	**Organ**	**Number ofanimals used**
Rhizostoma pulmo	0.83 to 3.15	umbrella	(3 animals)
Rhizostoma pulmo	2.61 to 10.3	oral arms	(5 animals)
Cotylorhiza tuberculata	0.453	umbrella	(1 animal)
Cotylorhiza tuberculata	1.94	oral arms	(1 animal)
Pelagia noctiluca	0.074	whole body	(1 animal)

Aurelia aurita	0.0079	whole body	(1 animal)

The primary biomedical applications of collagen were in biomaterials, especially as drug and gene carriers, tissue engineering, absorbable surgical suture, osteogenic and bone filling materials, hemostatic agents, immobilization of therapeutic enzymes, and burn/wound cover dressings [21].

Analysis of the antioxidant activity (AA) of protein compounds in *R. pulmo* demonstrated based on the extraction and characterization of membrane fractionated proteins, both soluble and insoluble, the latter being digested by sequential enzymatic hydrolysis with pepsin and collagenase. All jellyfish proteins showed significant antioxidant activity, from low molecular weight (MW) proteins correlated with higher antioxidant activity [22].

Recent major outbreaks of communicable diseases, such as prion disease, bovine spongiform encephalopathy (mad cow disease) or Creutzfeld-Jacob disease, have severely affected bovine and human health care products and have been banned in some cases (eg human by-products), for operations on the spinal cord and brain).

Collagen from marine sources such as that extracted from jellyfish avoids major problems arising from cultural practices and religious beliefs, which may limit the use of bovine and porcine products by some consumers and in certain parts of the world.

Properties of collagen

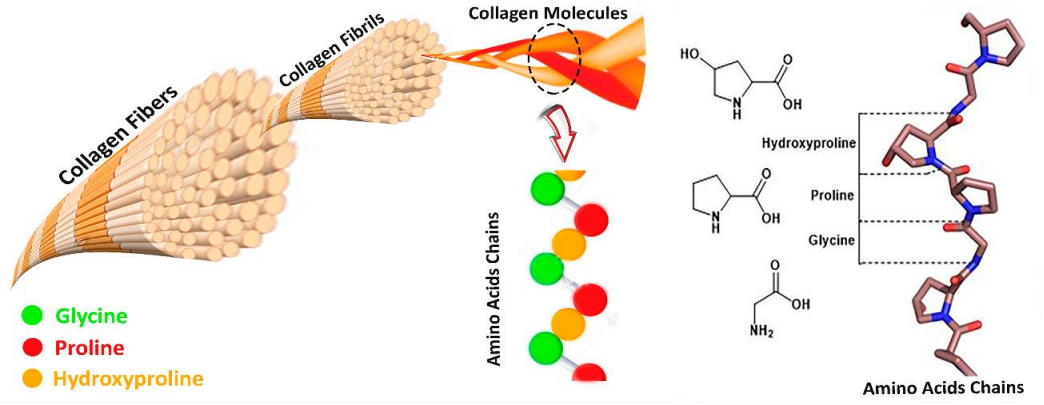

Figure 1. (**a**) Approximate content of collagen in different tissues; (**b**) Structure of collagen fibers, fibrils, triple helices of alpha chains and amino acid residues, 4-hydroxyproline (Hyp), glycine (Gly), and proline. **(c)** Amino acids chains structure of collagen. [https://www.elsevier.com/es-es/connect/medicina/colagenos-tipos-composicion-distribucion-tejidos].

Elegant and attractive alternative sources of collagen have been developed and proposed, using recombinant expression techniques. This allowed the expression of several types of human collagen in bioreactor-based eukaryotic systems and their subsequent isolation and purification [23]. Collagen-producing marine species have a distinct advantage in having a lower risk of transmitting infectious agents to humans and are considered safe for cultural and religious use.

It is anticipated that further structural stabilization of such marine collagens may be required by chemical processes, resulting in higher denaturation temperatures and increased resistance to enzyme degradation [24]. Marine collagen will provide new opportunities in health, diversification and reduction of safety risks and cultural and religious concerns, through the development of techniques for the production of collagen from alternative sources.

Medical, cosmetic, skin care and other collagen applications

Collagen is known for its biological action, with a great potential to be used in various fields such as dermatology, cosmetics, beauty. The current trend is to look for safe, high quality and reasonably priced ingredients. Marine proteins, and especially marine collagens, are nowadays found to be excellent functional ingredients for the cosmetics and pharmaceutical industries [25].

Due to water solubility, safety, biocompatibility, biodegradability, and easy extractability, as well as low immunogenicity, marine collagen has attracted scientific consideration for biomaterial applications [25]. On the other hand, due to the enormous amount of marine waste by-product such as fish skins, bones, scales, cartilage, and heads, marine-based collagen has been used in various biomaterial applications such as bone tissue engineering, skin tissue engineering and regeneration, cartilage tissue engineering, wound dressing, drug delivery, etc.[26]

The properties of collagen lead to the development of creams, gels, serums with moisturizing action, anti-aging, anti-wrinkle or protection against UV radiation.

For the cosmetics industry, marine collagen was obtained from cold-water fish skins, such as cod, haddock and salmon. In addition, it is also produced from fish scales, by decalcification and enzymatic hydrolysis [25].

Jellyfish collagen has demonstrated comparable structural properties and stability compared to mammalian collagen. Jellyfish collagen also showed comparable immunogenic responses (platelet and leukocyte activation/cell death) and cytokine release profile compared to mammalian collagen *in vitro*. Studies highlight the potential of jellyfish collagen as a safe and biocompatible biomaterial for both osteoarthritis repair and subsequent applications of regenerative medicine [26].

Another property of marine collagen used is that it heals wounds from various traumas (burns, grafts, ulcers, etc.), and collagen-based materials are used specifically to prevent moisture and heat loss from damaged tissue, while providing a microbial

infiltration barrier [27]. Collagen extracts have also been used in pharmaceutical preparations, for example, collagen barriers in ophthalmology, mini-pellets and tablets for protein delivery, gel formulations in combination with liposomes as a control material for transdermal delivery and nanoparticles for gene delivery [28].

Conclusions

Collagens are known for their biological action, with great potential for use in the biomedical, pharmaceutical and cosmetic fields. The future lies in finding viable alternative sources of safe, high-quality, low-cost raw materials.

Collagen is the most studied protein, with a wide range of applications due to its special characteristics, which have a high biocompatibility, good bioactivity and poor antigenicity. Marine proteins, obtained from various fish, jellyfish, sponges or other marine life are excellent functional products for the cosmetics industry, but also for the formulation of new pharmaceutical preparations for dermatological use.

It has been shown that the marine collagen source has applications in healing wounds resulting from various traumas (burns, ulcers, bedsores), the collagen-based materials being used mainly to prevent moisture and heat loss from the damaged tissue, while providing a barrier to microbial infiltration. Jellyfish collagen also has structural, stability properties comparable to that extracted from mammals.

The collagen extract also showed comparable immunogenic responses (platelet and leukocyte activation/cell death) and cytokine release profile compared to mammalian collagen *in vitro*. Studies highlight the potential of jellyfish collagen as a safe and biocompatible biomaterial for both osteoarthritis repair and subsequent applications of regenerative medicine.

Collagen preparations are also used in drug delivery systems, for example, collagen shields in ophthalmology, mini-pellets and tablets for protein delivery, gel formulations in combination with liposomes as a control material for transdermal delivery and nanoparticles for eyelash delivery. The marine collagen extracted from *Rhizostoma pulmo* jellyfish will offer new and real opportunities, being an alternative source, especially in the field of health due to the diversification and reduction of safety risks as well as pre-existing cultural and religious concerns over "standard" sources.

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