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Marine Collagen: Extraction and Applications

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ABSTRACT

Collagen is the main protein of the connective tissue and its molecule is formed by three polypeptide strands, named alpha chains. The most common motifs in the amino acid sequence of collagen are Gly-Pro-X and Gly-X-Hyp. Collagen can be extracted from a variety of organisms. The use of cattle as the main source for collagen has been reconsidered because of the bovine spongiform encephalopathy and transmissible spongiform encephalopathy, while porcine origin collagen is increasingly rejected for religious reasons. One alternative is the extraction of collagen from marine sources. Sponges, jellyfishes, squids, octopuses, cuttlefishes and fish offal (bones, skin, scales and fins) can serve as an alternative source of collagen. Nowadays there is a high biotechnological interest of marine collagenous, as witnessed by a wide pattern of applications in biomedicine, food science, and cosmetics.

WHAT IS COLLAGEN?

Collagen is a group of naturally occurring proteins. It is one of the long, fibrous structural proteins whose functions are different from those of globular proteins such as enzymes. It is abundant in most invertebrates and vertebrates [1,2]. It is the main protein of the connective tissue and represents about one-fourth of the total protein content in many animals [3]. The collagen molecule is formed by three polypeptide strands, named alpha chains (Figure 1) and has a molecular mass of about 285 Kd. Each chain possesses the conformation of a left-handed helix. These three helices are twisted together to form a triple helix which is stabilized by hydrogen

bonds. Collagen has high hydroxyproline content and its amino acid composition is quite different from a typical protein. The most common amino acid sequence in collagen are Gly-Pro-X and Gly-X-Hyp, where X is any amino acid other than glycine (Gly), proline (Pro) or hydroxyproline (Hyp). Several reports on invertebrates' collagen have emphasized its morphological and functional characteristics [4-6]. There is a variable amount of covalent cross-linking between the collagen molecule helices (Figure 2). That way well-organized aggregates, such as fibrils, are forming [7]. Collagen fibrils are the aggregation of several subunits (Figure 2), called tropocollagen (approximately 300 nm long and 1.5 nm in diameter). These fibrils are semi-crystalline aggregates of collagen molecules. Collagen fibers are bundles of fibrils. These fibers are a major component of the extracellular matrix that supports most tissues and provides structure to the cells from the outside. Collagen exists in many places throughout the body. So far, 29 types of collagen have been identified and described with types I, II, III, and IV to represent over 90% of the collagen in the body. Type I can be found in many tissues such as skin, liver, bones, aorta and cornea and it is the most abundant collagen type in vertebrates' tissues (about 22% of the total protein). Its a-chain is formed by two a1(I) and one a2 chains ([a1(I)]2a2). Type II and type III are formed by three a-chains of the same type, a1(II)3 and a1(III)3 respectively. Type-II collagen is the basis for articular cartilage and hyaline cartilage. Type III structurally is quite similar with type I. It represents the 5-20% of the total collagen in mammals' tissues such as skin, bones, and aorta. Type-IV collagen chain formation is a1(IV)]2a2(IV) and is found primarily in the basal lamina [8].

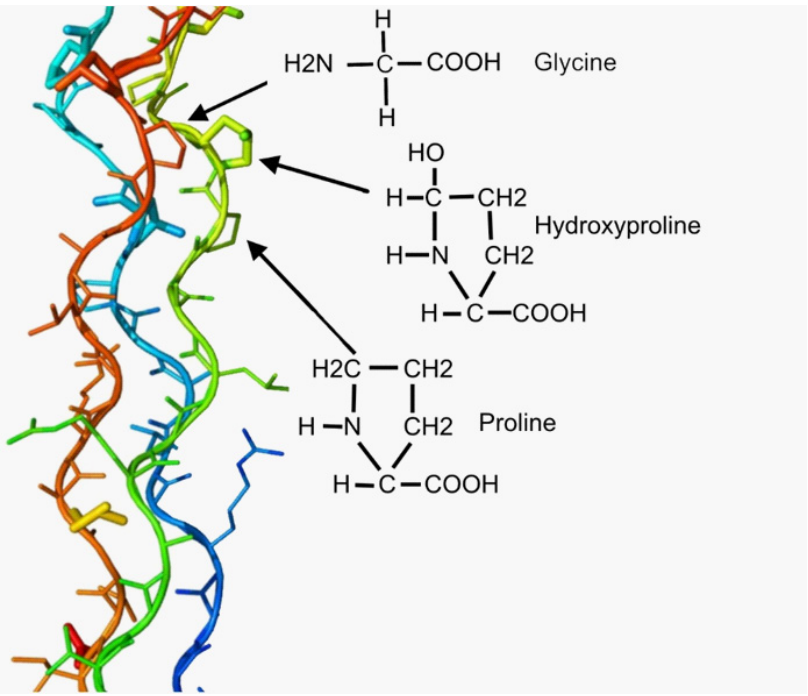

Figure 1: Collagen triple helix formation.

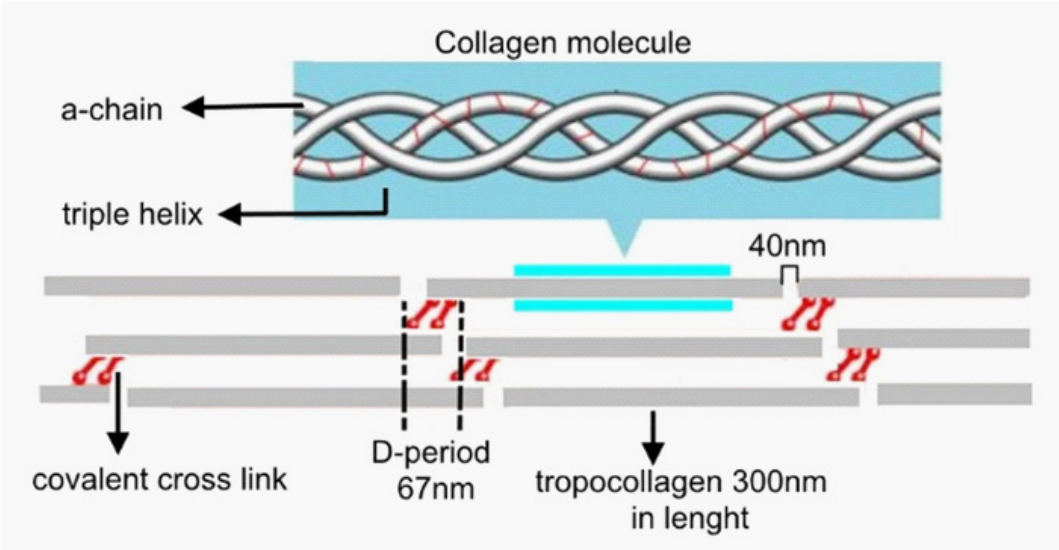

Figure 2: Axial structure of D-periodic collagen fibril.

MARINE COLLAGEN

Collagen can be extracted from various organisms. Preferential sources of collagen are bovine skin and tendon as well as porcine skin. The risks of the bovine spongiform encephalopathy and transmissible spongiform encephalopathy are the main reason that the use of cattle as source for collagen has been reconsidered. Furthermore, porcine origin collagen, is increasingly rejected for religious reasons [9]. One alternative is the collagen from marine sources. Sponges, jellyfishes and fish offal such as bones, skin, scales and fins can serve as an alternative source of collagen. Marine collagens are both fibrillar and nonfibrillar, have lower gelling and melting temperatures than the mammalian collagen but relatively higher viscosities than equivalent bovine forms [10]. Fish collagen is heat sensitive due to labile cross links as compared to mammals. The amino acids proline and hydroxyproline are reduced, compared with mammalian collagen, whereas serine, threonine and methionine are increased in fish collagen (Table I). Large amounts of glycine have also been reported [11]. In particular, the levels of proline and hydroxyproline vary significantly among fish species [12] depends on the environmental temperature in which the fish lives and it affects the thermal stability of the collagens. Most fish collagens have been found to consist two α - chains, which are normally designated as α-1 and α-2 [13,14]. These chain variants have approximately the same molecular weight (95,000 Da). The existence of collagen in marine as well as freshwater sponges was first proved electron microscopically in 1980s [15]. Boute [16] with genomic and complementary DNA studies showed that proteinaceous fibrous materials from sponge contain the classic collagenous Gly-X-Y motif, where hydroxyproline (Hyp) occupies any one of the positions in the triplet motif, other than that of glycine (Gly). Studies have proven, by characterizing cDNA and genomic clones, that in sponges there are at least two gene families.

Even type IV collagen was demonstrated in the homoscleromorph sponge *Pseudocorticium jarrei* by cDNA and genomic DNA studies [16,17].

Table 1: Amino Acid (%) composition of collagen from different sources.

Amino acid	Sturgeon swim-bladder collagen [11]	Lung fish *Neoceratodus* skin collagen [11]	Sponge *Chondrosia reniformis* Collagen [18]	Sponge *Hyalonema sieboldin* collagen [19]	Ox-hide collagen [20]
Ala	11.6	11.7	7.7	6.2	9.5
Gly	27.7	24.0	18.9	24.5	27.2
Val	2.31	2.56	5.5	2.9	3.4
Leu	2.55	3.37	6.2	4.3	-
Ile	1.65	1.64	5	3.8	5.6
Pro	12.8	14.8	5.4	6.5	15.1
Phe	2.53	2.60	3.2	2.2	2.5
Tyr	0.46	0.19	2.1	1.2	1.0
Ser	5.8	4.71	4.2	4.7	3.37
Thr	3.79	3.18	4.7	5.6	2.28
Cys	.	-	0.3	0.2	-
Met	1.43	0.59	1.4	0.4	0.8
Arg	10.0	9.1	4.9	4.8	8.59
His	0.83	0.80	1.1	1.6	0.74
Lys	3.46	3.63	2.8	2.0	4.47
Asx	6.9	6.6	9.5	10.7	6.3
Glx	11.4	11.9	10.3	9.3	11.3
H-LPro	11.8	9.8	2.4	6.9	14.0
L-HLys	1.9	0.88	4.3	1.6	1.1

ISOLATION OF COLLAGEN

There are three major methods for collagen extraction producing neutral salt solubilized collagen, acid solubilized collagen and pepsin solubilized collagen [21]. Freshly synthesized and negligibly crosslinked collagen molecules are extracted by neutral salt solutions [22]. The extracted material is purified by dialysis, precipitation, and centrifugation. Dilute acidic solvents, such as citrate buffer, 0.5 M acetic acid, or hydrochloric acid (pH 2–3) are more efficient than neutral salt solutions. Collagen from bone, cartilage, or material from older animals contain higher percentages of keto-imine bonds and have a lower solubility in dilute acid solvents [23]. Much higher yields compared with acidic extraction can be achieved by taking advantage of the fact that the collagen triple-helix is relatively resistant to proteases, i.e pepsin or chymotrypsin below approximately 20˚C [24].

Fish Collagen

Waste materials, such as skin, bones, fins and scales are generated in large amounts 50-70%

during fish processing [25]. These waste materials are very rich in collagen and have received increasing attention as collagen sources. Many extraction methods have been described for fish waste materials collagen (Figure 3).

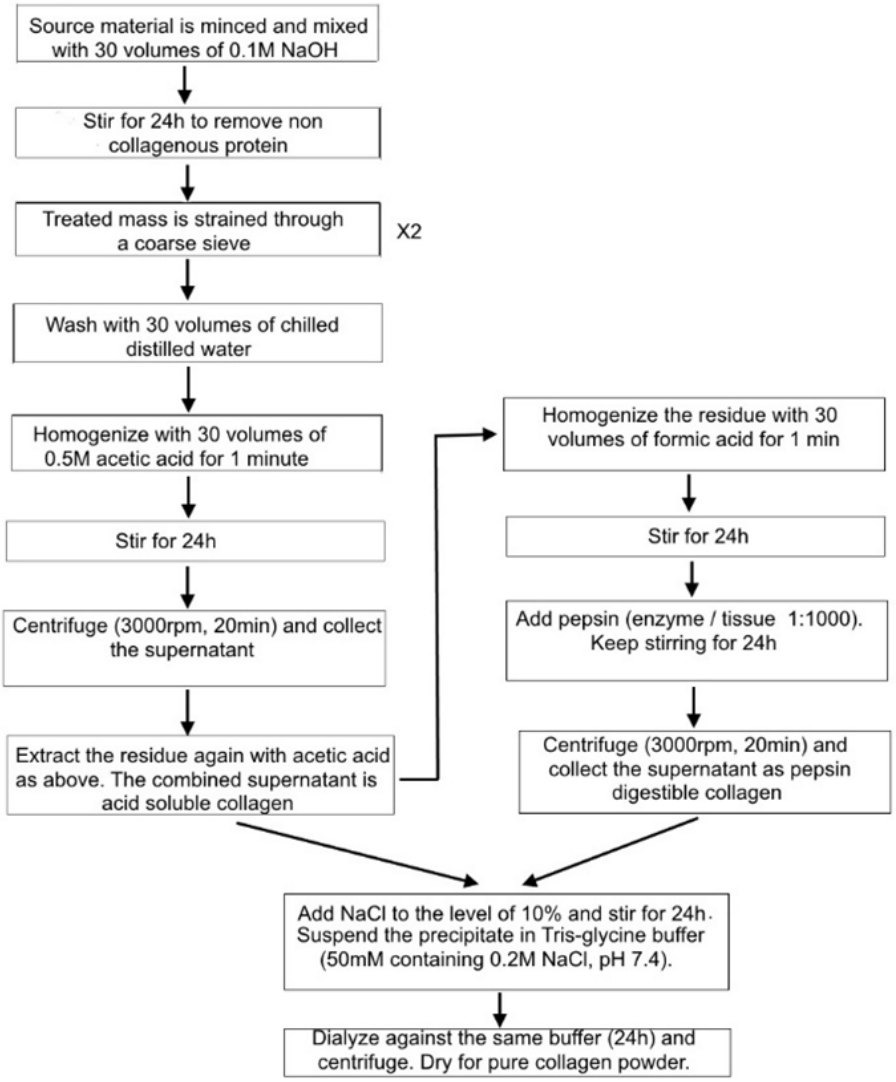

Figure 3: A method to isolate fish skin collagen [26].

According to Sadowska [27] with the use of a one-stage 24h extraction of Baltic cod skins with acetic or citric acid, about 20% of the total collagen can be extracted (skins of Baltic cod contain about 21.5% collagen). After three 24h extractions of whole skins with citric acid, pH=2.2 (0.5 M solution), 85% of collagen can be separated. So, if the collagen content in skins is 21.5%, about 182 kg of collagen can be produced from 1000 kg of cod skins [27]. Nagai and Suzuki [28] extracted acid solubilized collagen from the skins of Japanese sea-bass, chub mackerel and bullhead shark and lyophilized the final product. The yields of these collagens were very high

and were about 51.4% for Japanese sea-bass, 49.8% for chub mackerel and 50.1% for bullhead shark respectively, on the basis of lyophilized dry weight. Japanese sea-bass and bullhead shark collagens were shown to comprise at least two different a chains, a1 and a2, with the amount of the a2 chain to be very small. Chub mackerel collagen showed only a single a1 chain.

Calcified tissues is another material than fish collagen can be extracted from. The extraction method for the calcified tissues is quite different than the ones for the skin, because these tissues have to be decalcified after the removing of the non-collagen proteins. According to Nagai and Suzuki [28], bone collagen can be prepared from skipjack, Japanese sea-bass, ayu, yellow sea bream and horse mackerel. The bones were decalcified with EDTA and were solubilized with acetic acid. The yields of collagens, on the basis of lyophilized dry weight, were very high: 42.3% for skipjack tuna, 40.7% for Japanese sea bass, 53.6% for ayu, 40.1% for yellow sea bream and 43.5% for horse mackerel. The submit structures were quite different and were as follows: Japanese sea bass; (α1)2α2, horse mackerel; (α1)3 and ayu; α1α2α3. These results suggest a wide distribution of molecular forms in fish bone collagen. Duan [29] working with carps' bone reported a very low yield 1.06% of collagen extraction and suggested that there are different structures of bone collagens between freshwater fish and marine fish. Collagen type I was successfully isolated from the scale of Rohu and Catla with yield of 5 wt% [30]. Deep-sea redfish (*Sebastes mentella*) were also used for collagen extraction from their bones and scales as well. The amounts of protein recovered from bones and scales were 25.4% (wt) and 53.6% (wt) respectively [31]. As in type I collagen from bovine Achilles tendon, the collagens from deep-sea redfish comprised of at least two different a-chains, a1 and a2. So, collagens from the scale and bone of deep-sea redfish might be type I collagen mainly, with slight differences in amino acid composition of a-chains [31]. Ikoma [32] extracted type I collagens from fish scales of *Pagrus major* and *Oreochromis niloticas* as a possible underutilized resource for medical materials. The total yields of soluble and pepsin soluble collagen were about 2 wt.%. Collagens (acid-soluble and pepsin-soluble) isolated from the bones and scales of black drum (*Pogonia cromis*) and sheepshead seabream (*Archosargus probatocephalus*) were characterized as typical type-I [33]. Very high yields were reported by Nagai [34] for collagen extraction from scales of sardine *Sardinops melanostictus*, red sea bream *Pagrus major* and Japanese sea bass *Lateolabrax japonicas.* On a dry weight basis; sardine 50.9%, red sea bream 37.5% and Japanese sea bass 41.0%, respectively. These scale collagens were heterotrimers with a chain composition of two α1 and one α2*.* Fins are also used for collagen extraction. Acid soluble and acid insoluble collagen from the caudal fin of Japanese sea-bass had a yield of 5.2% and 36.4% respectively [28]. These collagens showed only a single a-band, a1. It seemed that, although these collagens contained an a2 chain, there was little of it.

Sponge Collagen

Marine sponges can be an alternative source for collagen extraction. These animals, the most primitive of multicellular animals (Metazoa), are anatomically simple, a mass of cells formed of a porous skeleton made of organic (collagen fibres and/or spongin) and inorganic (spicules)

components [35]. The fibrillar collagen is a huge component of the organic stuff in sponges. The nonfibrillar collagen (called spongin) encoded in sponges, is a short-chain molecule that shares features with basement membrane collagen, type IV [36,37].

Collagen was isolated from the sponge *Geodia cydonium* with a yield of 1.7 % in the absence of denaturing agents. It had the typical amino acid composition and was associated with the carbohydrates galactose and glucose [38]. Approximately 30% glycine, 6% proline, 8% hydroxyproline and 1% hydroxylysine. Sponge species of the family Superiditae are found to be rich in collagen [39]. Collagens type I and IV were isolated and partially characterized from the marine demosponge, *Ircinia fusca* [35]. After prior estimation of hydroxyproline, total collagen of *I. fusca* was calculated to be 123.68±6.18 mg/g dry sponge wt. In addition, the differential extractions and consequent quantification of the *Irciniid* collagens evidenced the presence of higher insoluble form of collagen (31.71±1.59 mg/g dry sponge wt), than salt soluble and acid soluble collagens (20.69±1.03 and 17.38±0.87 mg/g dry sponge wt, respectively), which would be due to the possession of higher collagenous, irciniid filaments by *I. fusca* [35]. Marine sponge *Chondrosia reniformis* Nardo was also used for collagen extraction with a yield 30% [40]. In agreement with earlier studies, sponge collagen was insoluble in dilute acid mediums. The amino acids composition of *Chondrosia* is similar with *Spongia* and *Ircinia* [41]. The collagen of *Chondrosia* has higher aspartic acid and phenylalanine content and lower glutamic acid content. The percentage of glycosylated hydroxylysine is the same in *Chondrosia* and in *Ircinia* collagens, but the percentage of the total lysine hydroxylated is much higher in *Chondrosia* collagen [41]. According to Heinemann [42] fourier transform infrared reflection-absorption spectroscopy of the purified sponge collagen of *Chondrosia reniformis* showed remarkable analogy of peak positions and intensities with the spectra of fibrillar calf skin type I collagen, despite the diverse phylogenetic and evolutionary origin.

Other Marine Source Collagen

Jellyfish is a prominent source for marine collagen extraction. Jellyfish has the potential to become a significant source of collagen because its collagen content is more than 60% [43]. Many investigations are concerned with collagen from jellyfish species, such as *Rhopilema asamushi*, *Stomolophus meleagris*, *Catostylus tagi* and *Rhizostoma pulmo* and report high collagen recovery rates [44-46]. Amino acid analyses revealed a composition similar to vertebrate collagen with, however, a lower content of hydroxyproline, which leads to relatively low denaturation temperatures between 26 and 29.9 °C. Some of the jellyfish collagens are comparable to vertebrate collagen IV or V [47,48] and some show a unique structure with a fourth a-chain [43]. Jellyfish collagen of *S. meleagris* that is similar to vertebrate collagen type II [49]. The yield of jellyfish collagen extraction is about 35.2% on the basis of the lyophilized dry weight [43].

Cuttlefish outer skins could be another material for collagen extraction. Acid solubilized collagen and pepsin solubilized collagen were isolated from the outer skin waste material of

cuttlefish *Sepia lycidas* with the yields to be 2% and 35% respectively on the basis of the lyophilized dry weight [50]. In the way of making more effective use of underutilized fisheries resources, collagen was prepared from the octopus *Callistoctopus arakawai* arm [51]. The arm was only slightly solubilized in acetic acid but on digestion with 10% pepsin (w/v), pepsin-solubilized collagen was successfully produced. The yields of acid-solubilized collagen and pepsin-solubilized collagen, on the basis of lyophilized dry weight, were about 10.4% and 62.9%, respectively. It is suggested that this collagen is a heterotrimer with a chain composition of α1α2α3. Kołodziejska [52] proposed high yield acid soluble collagen isolation from squid *Illex argentinus* whole skins (53% of the collagen contained in the skins could be extracted). Collagens, acid-solubilized and pepsin-solubilized, were also prepared from diamondback squid (*Thysanoteuthis rhombus*). The yield of acid-solubilized collagen was very low, about 1.3% on a dry weight basis while the yield of pepsin-solubilized collagen was very high, about 35.6% on a dry weight basis [53].

MARINE COLLAGEN APPLICATIONS

Thus far, the industrial use of collagen has mainly been limited to vertebrate collagen. 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 possibility of higher yielding collagen. Recent studies sparked a high biotechnological interest of collagenous extracts from fishes and marine sponges, as witnessed by a wide pattern of applications in biomedicine, food science, and cosmetics. The fibrils of sponge *Ircinia fusca* have a band periodicity of 67 nm with a 300 nm and height of 20 nm, which resembles the type I human collagen [54]. A similar ultrastructure and organization of collagen has also another marine sponge, *Chondrosia reniformis* Nardo [42], while Boute [16] reported the existence of collagen type IV in sponges. This suggest that purified collagens of marine sponges are identical to the human types I and IV and may be safe alternatives to the potential harmful bovine originated collagens.

Marine collagen are being presented as excellent ingredients for the cosmetic industry [55-57]. It has anti-aging and anti-wrinkling factors, it can be used for the development of creams or gels with high moisturizing action and it seems to protect against the UV radiation [58]. Collagen-based materials has being used to prevent moisture and heat loss from wounded tissue, while providing as well as microbial infiltration barrier [55,56]. A possible application of the *Chondrosia reniformis* extracted collagen as a moisturizer in cosmetic preparations was investigated by Swatschek [40] using non-invasive in vivo measurement techniques. That study clearly demonstrated that conventional collagen can be substituted by marine collagen. One of the most popular facial rejuvenation technics are the injectable fillers. Implantable collagen hydrogels have been examined as agents for delivery chemotherapeutic agents [54]. Liquid collagen supplement has been isolated from marine origin and it proved to deliver collagen through the upper layer of the skin deep to the lower layers of the human epidermis [54]. Development of collagen shields in ophthalmology, gel formulation in combination with liposomes as controlling material for

transdermal delivery, mini-pellets and tablets for protein delivery and nanoparticles for gene delivery are some other application of the marine collagen [40,55,56,59-61].

Collagen is the most commonly used biomaterial in tissue engineering. Collagen extracted from higher vertebrates is usually more cross-linked and has higher denaturation temperature. That makes it less elastic and well toned. Collagen from fishes is less cross-linked and its solubility is much higher than others [62]. Marine collagen in general is found to be about 60% purer than bovine collagen and much more safer [54]. Collagen-based scaffolds for tissue engineering applications can be prepared from jellyfish extracted collagen. Song [63] generated porous scaffolds by freeze-drying and subsequent chemical cross-linking of acid solubilized jellyfish collagen. Biocompatibility (attachment of human fibroblast and immune response after implantation of the scaffolds *in vivo*) found to be similar to the others collagen sources. Tubular porous scaffolds from marine collagen reinforced with poly(lactic-co-glycolic) acid fibers were developed by freeze-drying and electrospinning techniques [64,65]. These constructs were cultivated to test the influence of electrospinning parameters on cell adhesion and proliferation. Another type of porous scaffold was established by Lee [66], combining jellyfish collagen and hyaluronic acid. Hoyer [67] manufactured porous 3-D jellyfish collagen scaffolds with an interconnected pore structure by freeze-drying and subsequent chemical cross-linking. It was a cytocompatible matrix with the potential to support and maintain chondrogenic stimulation of human mesenchymal stem cells. Pallela [68] developed a novel marine sponge (*I. fusca*) collagenous composite Chi-HAp-MSCol scaffold that will have potential prospects in the field of bone tissue engineering. Based on properties such as good thermal stability, interconnected porosity, and in vitro cell proliferation, a new way for successful development of new generation bone repairing and augmentation devices is opened. The use of collagen extracts from *C. reniformis* was also proposed in the form of nanoparticles as penetration enhancers for the transdermal delivery of 17 beta-estradiolhemihydrate in hormone therapy [60] and as enteric coating for gastroresistant delayed-release tablets [61]. Shark derived collagen is another potential source to fulfill the human collagen needs as food supplement [69]. Shark skin collagen is easy to prepare, and represents a possible resource for use on an industrial scale. By giving an irreversibly hydrolyzed form of shark collagen (gelatin) to ovariectomized rats, the bone mineral density of bone epiphysis was increased. That fact indicates that, shark gelatin would be useful for as a food supplement for treating osteoporosis [69].

Collagen is also important to the food processing industry. Edible films and coatings are a unique category of packaging materials, differing from other bio-based packaging materials or from conventional packaging. They are formed from edible ingredients, such as collagen. Such films prepared from mammalian collagen have found a number of uses in the area of drug release agents in the field of medicine. O'Sullivan [70] with acetic acid extraction successfully recovered collagen from fish skins and this collagen was subsequently used to produce collagen films, demonstrated its potential as a film-forming ingredient. In a later study, the acid-soluble collagen

from Alaska pollack surimi refiner discharge, having a thermal denaturation temperature slightly higher than that for Alaska pollack skin, was proposed as a potentially functional food ingredient [71]. Soluble form (acid-soluble or pepsin-soluble) marine collagen may also be used as emulsifier. Collagen gained in acid-soluble form from surimi refiner discharges had higher emulsifying activity (EA) than both acid-soluble collagen from skin and the commercial emulsifier, Tween-80 [72]. Emulsifying capacity is an extremely important functional property in food processing, and it has been studied extensively in such food systems as myofibrillar proteins. Emulsifying capacity (EC) of collagenous material from the muscle and skin of hake (*Merluccius merluccius* L.) and trout (*Salmo irideus* Gibb) have been demonstrated by Montero and Borderías [73]. Expressed in terms of the quantity of soluble protein, EC can be regarded as higher in the collagenous material from the hake than in that from the trout, and higher in the muscle connective tissue than in the dermal connective tissue.

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