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Extraction of Collagen from Mangrove Archeaogastropod *Nerita* (*Dostia*) *crepidularia* Lamarck, 1822

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Abstract: Collagen is the most abundant protein of animal origin, comprising approximately 30% of total animal protein. There are at least 19 variants of collagen, named type I to XIX. Types I, II, III and V are the fibrous collagens. Type I collagen is found in all connective tissue, including bones and skins. It is a heteropolymer of two α 1 chains and one α 2 chain. It consists of one-third glycine, contains no tryptophan or cysteine and is very low in tyrosine and histidine. Guanidine hydrochloride to solubilize the part of collagen referred as GSC and then RS-AL (crude connective tissue fractions) digested with pepsin called PSC were extracted from the tissues of *N. crepidularia*. The GSC and PSC yields (on a dry weight basis) were 0.48% and 1.28% respectively. The FT-IR spectral analysis of the collagen extract from tissues of *N. crepidularia* showed more or less same number of peaks, lying within the same range of values of the commercial collagen (Human placenta collagen) used as a standard. These results suggest that collagen could be obtained effectively from the tissues of *N. crepidularia*

Key words: Collagen · GSC · PSC · FT - IR · *Nerita crepidularia*

INTRODUCTION

Collagen is a fibrous protein found ubiquitously in all multicellular animals. It is a particularly rigid and inextensible extracellular matrix protein that serves as a major constituent of many connective tissues. The characteristic feature of a typical collagen molecule, tropo collagen, is its long, stiff, triple-stranded helix, in which three collagen polypeptide chains are wound around one another in form of a ropelike superhelix. These peptides are extremely rich in proline and glycine, both of which are important for the formation of the collagen-specific helical structure [1-4].

Collagen plays an important role in the formation of tissues and organs and is involved in various cells in terms of their functional expression. Recently, alongside clarification of the biological functions of collagen as an extracellular matrix protein, collagen has been attracting attention as a biomaterial with many unique characteristics such as high tensile strength, low antigenicity, bioresorbability, good biocompatibility, induces coagulation of blood platelets, effects cell differentiation, effects wound healing, control of various characteristics through physical and chemical modifications, moldability, abundant and easily purified [5]. Though the invertebrates comprise approximately 95% of the Animal Kingdom, the information about their collagens and extracellular matrices is scarce. The relative complexity of the invertebrate collagens and the difficulty in their purification and characterization has hindered continued progress in their research. Preliminary studies on invertebrate collagens have been reviewed [6]. Added effort on the analysis of invertebrate connective tissues over two decades has led to the identification of genetically distinct collagen types in a number of species and there has been appreciable success in the isolation and purification of single molecular species of collagen. Hence in the present study, the tissue from the Archaeogastropod, *N. crepidularia*, was chosen to characterize the gastropod collagen.

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MATERIALS AND METHODS

The Archaeogastropod, *N. crepidularia* were collected from the mangroves in Vellar estuary, Parangipettai, Southeast Coast if India (Lat. N 11°29'.45'' Long. 79°46'.028''). The whole tissues were removed from the shell, washed and stored at -20°C for further studies. Procedure of [7] was followed for the extraction of collagen (Guanidine Hydrochloride Soluble Collagen (GSC) and Pepsin Soluble Collagen (PSC) from *N. crepidularia*. All steps were carried out at 4°C.

Fourier Transform - Infra Red Spectrum Analysis: FT-IR spectroscopy of solid samples of standard (Human placenta), GSC and PSC from *N. crepidularia* relied on a Bio-Rad FTIR – 40 model, USA. Sample (10mg) was mixed with 100 mg of dried potassium bromide (KBr) and compressed further to prepare as a salt disc (10mm diameter) for reading the spectrum.

RESULTS

In the present study, both quanidine-soluble collagen and pepsin-soluble collagen were extracted from *N. crepidularia* and further partially characterized.

Yield of Collagen (GSC and PSC): On comparison, the yield of GSC was very low (0.48% on wet weight basis) than that of PSC (1.28% on wet weight basis).

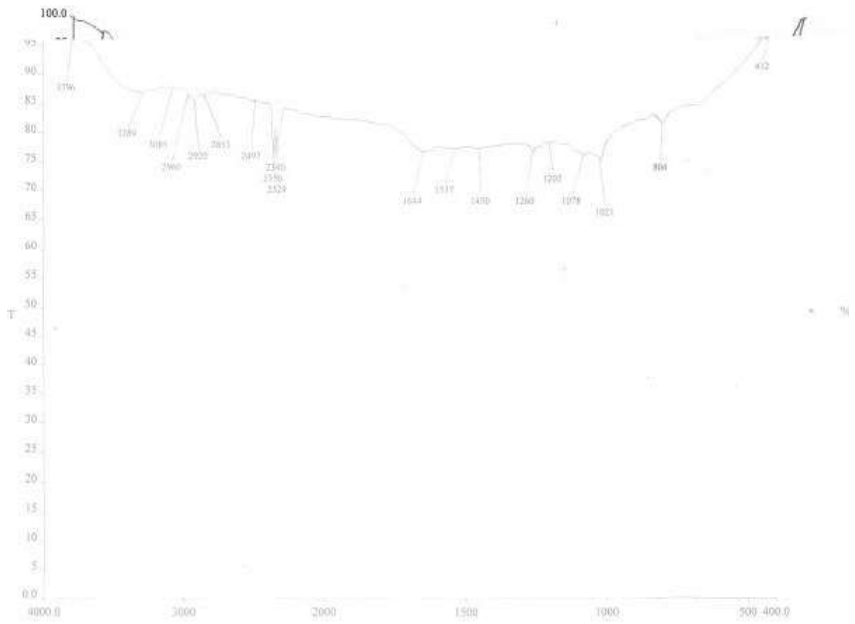

Fig. 1: Showing the FT - IR Spectrum of Standard Collagen

Table 1: Fourier Transform - Infra Red Spectra peak location and assignment for Standard collagen, GSC and PSC

Regions	Standard	GSC	PSC	Assignment
Amide A	3289	3310	3363	NH stretch coupled with hydrogen bond.
Amide B	2920	2922	2927	CH_2 asymmetrical stretch.
-	2853	2851	2853	CH_2 symmetrical stretch
Amide I	1644	1655	1656	C=O stretch / Hydrogen bond coupled with CN stretch
Amide II	1537	1544	1545	NH bend coupled with CN stretch
	1450	-	1458	CH_2 bend
-	-	1403	1451	COO – symmetrical stretch
-	-	-	1340	CH_2 wagging of Proline
Amide III	1260	1235	1241	NH bend coupled with CN stretch
-	1078	1078	1074	C-O stretch
-	1021	1020	1038	C-O Stretch
-	804	831	828	Skeletal stretch
-	-	632	668	Skeletal stretch

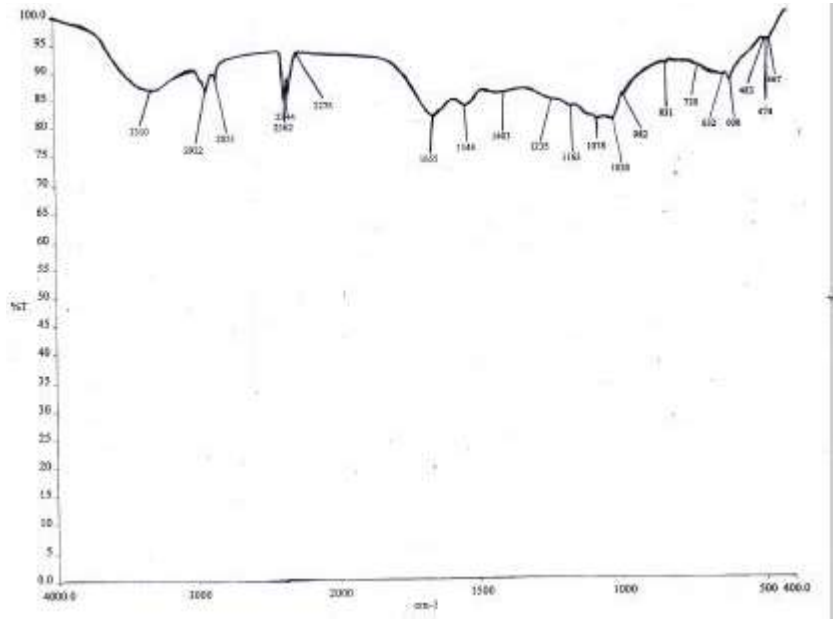

Fig. 2: Showing the FT - IR Spectrum of Guanidine Soluble Collagen (GSC) of *N. crepidularia*

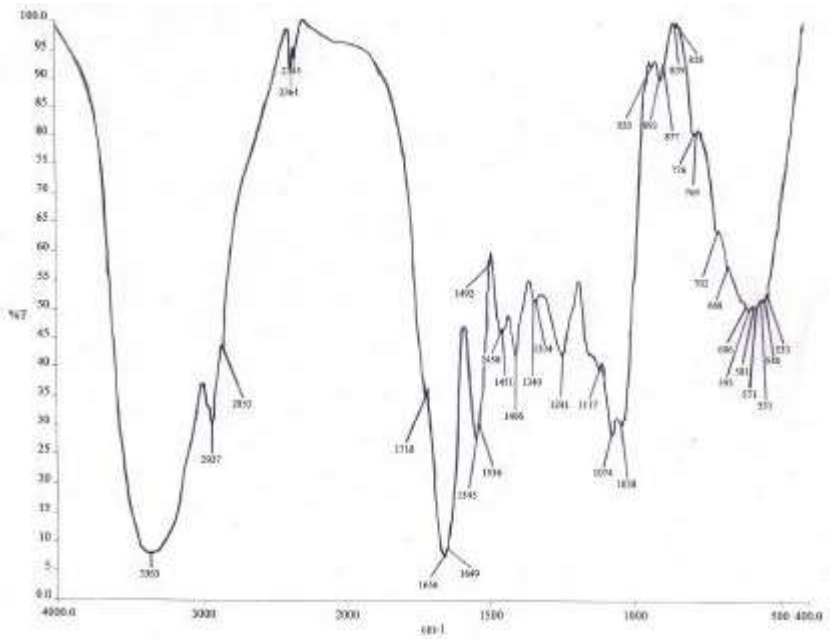

Fig. 3: Showing the FT - IR Spectrum of Pepsin Soluble Collagen (PSC) of *N.crepidularia*

FT-IR Spectral Analysis: The FT -IR spectrum of standard collagen showed 10 major peaks (Fig. 1.); whereas the FT – IR spectrum of the both (GSC and PSC) depicted 11 and 13 peaks (Fig. 2, 3 and Table 1) respectively. The wave length details and their corresponding chemical structures are given in Table 1.

DISCUSSION

Ottani *et al*. [8]' defined "collagen" as a large (and growing) family of related proteins, sharing some common traits but also exhibiting wide differences and fulfilling various functional roles in different connective tissues. Generally, vertebrate collagens and gelatins

have low polysaccharide contents. The cuticle of *Ascaris lumbricoides* [9] is similar in this respect, but collagens from earthworm cuticle (*Lumbricus* sp.) [10] and the cuverian tubules of *Holuthuria forkali* [9] contain large amounts of polysaccharide. There is insufficient evidence available to show that the collagen found in snail from which they were derived.

Collagen is abundant in most invertebrates as well vertebrates [6]. It makes up about one fourth of the protein content in multicellular animals [11]. Several reports on invertebrate collagen emphasized its morphological and functional characteristics [12]. Much of the earlier work on invertebrate collagens was oriented towards a study of its distribution in various groups and the results were based mainly on X – ray diffraction analyses. However, chemical analyses of the collagen were made on *Lumbricus*, *Allolobophora*, byssus threads of *Mytilus edulis* and the sponging fibrils by [10-13]. Some interesting results also appeared on the cuticle of *Ascaris*, *Holothuria*, *Helix*, *Metridium*, *Physalia* and *Thyone*. The amino acid composition of the collagens of the Lobster, Blue crab, Octopus, Squid, Pearl Oyster and Abalone was studied by Kimura *et al*. [14]. From the available literature, it can be stated that collagenous connective tissue seems to occur throughout the animal kingdom even in arthropods which have an extracellular system dominated by chitinous skeleton. Another interesting feature of the invertebrate collagens is the presence of large proportions of sugar which cannot be degraded of as they can be with vertebrates. Of the invertebrate collagens and gelatins reviewed by Watson [10] only the collagen derived from the cuverian tubules of the sea cucumber had been found to contain hydroxylysyl residues (4.7 per 1000 total residues) [15]. However, detected hydroxylysine in all the invertebrate gelatins that they studied, *Metridium*, *Phyaalia* and spongin B having 25, 30 and 24 hydroxylysyl residues per 1000 residues respectively, the highest yet reported for any collagen or gelatin.

In multicellular organisms, ECM is composed mainly of collagens and proteoglycans. In Mollusca, the presence of collagen molecules has been demonstrated for several years, in particular, mussel byssus collagen from bivalves, squid skin collagen, or abalone *Haliotis* muscle collagen [16-21]. In the same way, PGs appear to be widely distributed in invertebrates and chondroitin sulfate as well as heparan sulfate has been detected in molluscs [22-28]. The shell growth begins with the secretion by the mantle cells of an ECM, which is most responsible for later events leading to the mineralization process [29].

In gastropod species, the foot muscle of the abalone, *Haliotis discus*, was reported to show the seasonal change of collagen content, corresponding well to meat toughness [18]. In the earlier [30] examined and quantified collagen synthesis by cultured mantle cells. During the first 48 hours, de novo synthesis represents 4.52% of the total protein synthesis from nacreous mollusc, *Haliotis tuberculata*. In invertebrates, an in vitro study focusing on collagen synthesis has been conducted in sea urchin micromeres [31-33]. On days 1–5 of culture, collagen synthesis ranged from 0.5% to 5% of the total protein synthesis [32]. The crude connective tissue fractions (RS – AL) from the tissues contained mucous material and were very insoluble in 0.5M acetic acid, so the RS – AL was treated with G/HCl solution to remove mucous material and solubilize part of the collagen. The G/HCl soluble protein was effectively salted out by dialyzing against 0.5 M acetic acid containing 2 M NaCl and consisted mainly of collagenous material. Approximately 2 – 3% of total collagen was solubilized by this extraction method. On the other hand, about 10-30% of the total collagen could be solubilized from the residue, after the G/HCl extraction, by the limited pepsin digestion for all the species examined [34]. However it is also found that PSC also exhibited quite a similar pattern to those of the pepsin digest of the GS collagen, considering the effects G/HCl treatment on collagen, but the same results were found as the PS collagen from the G/HCl treated RS-AL. Mizuta *et al*. [7] reported an enhanced yield of the pepsin solubilized collagen by using a disaggregating solution (0.1M Tris – Hcl, pH 8.0, containing 0.05 M EDTA, 0.5 M EDTA, 0.5 M NaCl and 0.2 M – mercaptoehanol) and found 0.67% of collagen from the wet tissue of oyster *Crassostrea gigas*, the yield of PSC when RS – AL treated with this solution was 30.9±2.4% of total collagen, which was significantly higher than that without treatment (18.3±2.7%).

Splits of the skins of invertebrates and vertebrates such as cattle and pig as well as vertebrates bones are the main sources of collagen used in the food, pharmaceutical, cosmetic and leather industries [35-39]. The main drawback in use of cattle and pig source is the infective agent that can be transferred from animals into human beings. Additionally, the collagen obtained from pig bones cannot be used as a component of some food item for religious reasons. This paved the way for the strong need to develop alternative collagen sources.

The physical and chemical properties of collagen from cattle skins are considerably not the same as from those of invertebrate skins. Therefore the methods used for isolation of collagen from cattle skins are not effective enough for the isolation of collagen from fish, cuttlefish, squid skins etc. There are only a few works dealing with the practical utilization of connective tissue offal of marine vertebrates and invertebrates [40-46] Other works, concerning collagen of marine animals focus mainly on cognitive aspects, such as collagen content in tissues, its genetic types, amino-acid composition, extent of intra and intermolecular cross linking, susceptibility to endo and exogenous enzymes and thermal stability of collagen Nagai and Suzuki [47]. Isolated about 2% of ASC and 35% of PSC from the skin of *S. lycidas* on dry weight basis. From the skin of Japanese Sea bass, Chub mackerel and Bullhead shark, the yield of collagens was very high and the values were about 51.4%, 49.8% and 50.1% respectively on the basis of lyophilized dry weight [44]. Compared to this the tissues of *N. crepidularia* reported low yield of about 0.48% of GSC and 1.28% of PSC against the total collagen content on wet weight basis. Tonar and Marko, [48] studied the yield of 51±3.4/28±1.8 and 50±3.3/36±2.4 of collagen connective tissue, from foot of pulmonate gastropods *H. pomatia* and *Arion rufus*. Amith Kumar Chaturvedi [49] studied the yield of PSC and GSC from whole body tissues of *Perna viridis* was found to be 0.33% and 0.01% respectively. The collagen content may be decreased due to denaturation of protein during the process of methodology and difference in environmental temperature [50].

The collagen content in the skin of Baltic cod (*Gadus morhua*) was 21.5% on the wet weight basis and about 71.2% on the dry weight basis [51] both the collagen and non-collagen protein content in cod skins depends upon the fishing season. During starvation albumins and globulins are degraded and the collagen content in skins increases [52-54] extracted 60% of PSC and only 12% of ASC from the cartilage of *S. officinalis* on wet weight basis whereas, in the same animal. Sivakumar and Chandrakasan [55] estimated the yield of ASC and PSC as 5.52±1.3 mg/g and 27.6±3.07 mg/g from the cranial cartilage and cornea. In *O. vulgaris*, [34] extracted 1.4% and 1.9% of collagen from the arm and mantle muscles and the protein content was also reported as 9.1% and 14.0% respectively. But in the present study 0.48% of GSC and 1.28% of PSC (Wet weight basis) was extracted from the N. *crepidularia*. Sadowska *et al*. [51] studied and established that the gross composition of skin of cod caught in the same season in different years is almost constant and the collagen content in the skin amounts an average to 21.5% in the wet weight and 71.2% in the dry weight. It was also absorbed that the share of collagen in total protein was considerably determined on the basis of hydroxyproline in samples. Further in the skin of cod the non-collagen proteins, peptides and amino acids were estimated as 4.9% and 16.3% respectively on a wet and dry weight basis. Some studies on collagen reveals that the collagen represents the chief structural protein accounting for approximately 30% of all vertebrate body protein. The major impediment in the dissociation of collagen type I from tissue is the presence of covalent crosslinks between molecules. Collagen is insoluble in organic solvents [56]. In some tissues, notably in skins of young animals, crosslinking is sufficiently low to extract a few percent under appropriate conditions. The most commonly used solvents are dilute acetic acid or neutral salt solution (0.8M NaCl). The acetic acid is used to extract fresh and negligible crosslinked collagen molecules present in the outer skin of the animal. The extracted material is purified by precipitation, centrifugation and dialysis [57]. In the present study also, acetic acid was used for the extraction.

The regions of amides I, II and III are known to be directly related with the shape of a polypeptide. Amide A band (3400-3440 cm^{-1}) is related to N-H stretching vibrations. Amide I band (1600-1660 cm^{-1}) is associated with stretching vibrations of carbonyl groups in peptides, being the most important factor in investigating the secondary structure of a protein. Amide II (~ 1550 cm^{-1}) is associated with NH bonding and CN stretching. Amide III (1320 – 1220 cm^{-1}) is related to CN stretching and NH and it is involved with the triple helical structure of collagen [58-60]. Muyonga *et al*., [60] studied the skin collagen of young Nile perch showed the amide regions bands of A, B, I, II and III were observed at the wavelengths of 3434 cm^{-1}, 2924 cm^{-1}, 1650 cm^{-1}, 1542 cm^{-1} and 1235 cm^{-1} respectively and that of the adult Nile perch skin collagen were at 3458 cm^{-1}, 2926 cm^{-1}, 1654 cm^{-1}, 1555 $cm^{-1}$and 1238cm^{-1}, respectively. Correspondingly in *N. crepidularia* tissues, the present study also with the main bands were observed in the amide regions of A, B, I, II, III at 3310 cm^{-1}, 2922 cm^{-1}, 1655 cm^{-1}, 1544 cm^{-1} and 1235cm^{-1} in GSC respectively and 3363 cm^{-1}, 2927 cm^{-1}, 1656 cm^{-1} , 1545 $cm^{-1}$and 1241 cm^{-1} in PSC respectively. Plepis *et al*. [61] observed the band ratio between 1240 cm^{-1} (amide III) and 1454 cm^{-1} and confirmed that the triple helical structure is present in skin (SKC), scale (SCC) and bone (BOC) collagen of *Sebastes mentella* like that in this study the band ratio were observed between 1241cm^{-1} (amide III) and 1458cm^{-1} in PSC by which we can confirm that triple helical structure were present in *N. crepidularia.*

The amide A band is associated with the N-H stretching frequency. According to Doyle *et al*. [62] a free N-H stretching vibration occurs in the range of 3400 - 3440cm^{-1} and when the NH group is a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies. The amide A band of skin collagen of *S. mentella* was at 3425 cm^{-1}, while those of scale and bone were at 3296 cm^{-1} and 3300 cm^{-1} respectively, which indicate that more NH groups of scale and bone were involved in hydrogen bonding than in skin [63]. Comparing to this in the present investigation also the amide A band of GSC and PSC are located at 3310 cm^{-1} and 3363cm^{-1} respectively. It also indicates the involvement of more NH groups in hydrogen bonding of GSC and PSC.

The peaks of amide I and amide II of PSC (1655 cm^{-1} and 1548 cm^{-1} respectively) were at a higher frequency than those of ASC (1644 cm^{-1}). These indicated PSC had a higher degree of molecular order than ASC, since the shift of these peaks to higher frequencies was associated with an increase in the molecular order [64]. It was found that the amide I band, with characteristic frequencies in the range from 1600 to 1700 cm^{-1} was mainly associated with the stretching vibrations of the carbonyl groups (C=O bond) along the polypeptide backbone [64] and was a sensitive marker of the peptide secondary structure [59]. The amide B band of skin of walleye pollock (*Theragra chalcogramma*) collagen was found at 3080 cm^{-1}. Similarly in the present study also the amide B band was located at 2922 cm^{-1} and 2927 cm^{-1} of GSC and PSC respectively, which is related to the asymmetrical stretch of CH_2 [60]. [65] observed amide I band position was observed at 1655 and 1656cm^{-1} (GSC and PSC respectively), which is the absorption band of C=O stretching and is associated with the secondary structure of the protein. The absorption between the 1235 and 1241 cm^{-1} (amide III) and 1403 and 1451 cm^{-1} (GSC and PSC respectively) wave length demonstrated the existence of helical structure. In PSC amide I band to consist of a higher proportion of the component at 1655 cm^{-1}. This band is linked to the extent of intermolecular interactions in collagen and collagen-like peptides [62, 66, 67] The other considerable difference was the lower intensity of the component with a peak at 1655 cm^{-1} in GSC. This component has been attributed to random coils [66] suggesting a lower extent of unwinding of the triple helix in the GSC. It seemed, therefore, PSC retained more intermolecular cross-links during solubilisation with acetic acid but the triple helical structure, normally held together by intramolecular hydrogen bonds [68] was extensively destroyed. So from the results of present study, it could be concluded that *N. crepidularia* may be used as a potential source for collagen. Further studies using NMR and GC – MS could bring out more details about complete structure of GSC and PSC from the mangrove Archaeogastropod *N. crepidularia*

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