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Extraction and Partial Purification of Guanidine Hydrochloride Soluble Collagen (GSC) and Pepsin Soluble Collagen (PSC) from Marine Bivalve *Anadara granosa* L.

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A B S T R A C T

Though invertebrates constitute about 95% of the animal kingdom, the information about their extracellular matrices, particularly on collagen, is very scanty. Guanidine hydrochloride soluble collagen (GSC) and pepsin soluble collagen (PSC) were extracted from the whole body muscle tissue of a marine bivalve, *Anadara granosa* L. GSC and PSC yields (on dry wt basis) were 1.1 and 7.2%, respectively. SDS-PAGE analysis gave 3 bands with mol wt of 130, 140 and 210 kDa for GSC, while PSC showed only one prominent band with mol wt of 140 kDa and the other two bands (of 130 and 210 kDa) were very light. The FT-IR spectral analysis of the collagen showed 8 peaks for GSC and 11 peaks for PSC, which were within the same range of values as seen in commercial (standard) collagen (human placenta collagen). Thus, it is confirmed that the extracted collagens had triple helical structure, similar to type I collagen. These results further suggest that the collagen from *A. granosa* could be used as a potential alternative source for biomaterials, cosmetics, and other applications.

Introduction

Collagen forms the principal component of intramuscular connective tissues and is known to occur in genetically distinct forms (Bailey *et al.,* 1979). More than 28 types of collagen (i.e., called type I to type XXVIII) have been identified from various animal and human tissues, with each type having a distinctive amino acid sequence and molecular structure to play a unique role in the tissue (Schrieber & Gareis, 2007; Bou-Gharios *et al.,* 2020; Salvatore *et al.,* 2020). In animals and humans, the collagen constitutes more than 30% of the body protein

as skin, bone, and connective tissues and the bones, dermis, tendon, ligaments and cornea of animal body are the specific sites of Type I collagen (Silva *et al.,* 2014) and this Type I contributes more than 70% (Bornstein and Sage, 1980; Gordon and Hahn, 2010). Collagen molecule is formed by three polypeptide strands i.e., α chains and the most common amino acid sequence are Gly-Pro-X and Gly- X-Hyp (Berillis, 2015). Collagen acts as natural substrate for cell adhesion, cell growth and differentiation (Li *et al.,* 2020).

Most common collagen is Type I and has a wide range of applications such as pharmaceuticals, food, biomedical products, and cosmetics due to its cell adhesion properties, biocompatibility, safety, low antigenicity, and biodegradability (Kittiphattanabawon *et al.,* 2005; Pal *et al.,* 2015; Rodrıguez *et al.,* 2017; Li *et al.,* 2020; Wang, 2021). The use of collagen as a biomaterial is currently undergoing a renewal in the tissue engineering field. Collagenous materials from marine sources are potential for tissue engineering applications and also biocompatible than that of collagen from terrestrial origin (Hoyer *et al.,* 2014; Martins *et al.,* 2022). In addition to wound healing properties, collagen used in cosmeceuticals, nutraceuticals and dietary supplements (Rigogliuso *et al.,* 2023).

Collagen is used as one of the crucial biological materials in biomedical applications because of high biocompatibility (Silva *et al.,* 2014; Yang *et al.,* 2014; Subhan *et al.,* 2015) and also potential for tissue engineering applications (Martins *et al.,* 2022). At least one natural bio-constituent is linked with artificial materials used in veterinary medicine (for example implants, medical devices, wound dressings, tables, sutures etc) and this enhance the biocompatibility and speed up the healing process (Parks, 2002). Interstitial collagen's breakdown is important for biological processes like wound healing and tissue remodelling (Brett, 2008). It has a major role in tumor cell spreading or periodontal disease (Khan and Khan, 2013), healing skin damage and slowing down the aging process (Barzkar *et al.,* 2023) hence collage is considered as a promising anti-aging materials (Kapuler *et al.,* 2015; Xu *et al.,* 2021).

The global demand for collagen has been increasing over the years. However, the industrial use of it has been limited to vertebrate collagens, most of which were produced from pig skin (80%), followed by cattle split (15%) and other sources, such as, pig and cattle bones, poultry and fish (5%) (Gelatine, 2009). The vertebrate collagen is the most investigated bio-resources mainly skin, bones and scales of fish wastes (Cherim *et al.,* 2017; Subhan *et al.,* 2015). Therefore, a shift to invertebrate collagen from vertebrate collagen occurred (Lee *et al.,* 2007; Azizur Rahman, 2019; Abirami *et al.,* 2020; Barzkar *et al.,* 2023) since the collagen from jellyfish marine sponges etc., found to have high porosity, interconnected porous structure, high cell viability and no-cytotoxicity (Lee *et al.,* 2007; Pallela *et al.,* 2012). Marine collagen has many benefits over terrestrial sources including its versatility in healing skin damage and slowing down the aging process. Because of difficulties in purification of proteins in bovine and also risk for communicable diseases, the collagen from mammals became less importance (Yemisken *et al.,* 2023) whereas the lack of religious constraints and animal pathogens, the scientists have more attention on collagen from marine invertebrates (Coppola *et al.,* 2020).

The invertebrates comprise approximately 95% of the animal kingdom, the information about their collagens and extracellular matrices is limited. The relative complexity of the invertebrate collagens and the difficulty in their purification and characterization similar to vertebrate collagen has hindered continued progress in their research. The invertebrate collagen and its extraction and purification methodologies as well as biomedical and industrial application have been reviewed (Adams, 1978; Abirami *et al.,* 2020; Ahmed *et al.,* 2020; Wang, 2021). Added effort on the analysis of invertebrate connective tissues over four decades has led to the identification of genetically distinct collagen types in a number of species. And there has been appreciable success in isolation and purification of single molecular species of collagen, in the present study, the whole body tissues of a marine bivalve mollusc, *Anadara granosa* L. was chosen for isolation, charaterization and partial purification of the collagen.

Materials and Methods

Isolation

Bivalve mollusc, *A. granosa* were collected from the Vellar estuary at Porto Novo (Tamil Nadu), Southeast coast of India (Lat. 11°29' N; Long. 79°46' E). The collected animals were immediately stored in an ice box for further use. They were transported immediately to the laboratory and stored at -40°C until use. The whole soft

body tissue was removed from the shell, washed and stored at -20°C for further studies. The whole soft body was homogenized in 5 vol (v/w) of 0.1 *M* NaOH and extracted for 24 h at 5°C. The extraction was done to remove non-collagenous proteins and to prevent the effect of endogenous proteases on collagen, as described by Yoshinaka *et al.,* (1990). The residue, after alkali extraction (RS-AL), was washed thoroughly with distilled water and extracted with 50 m*M* Tris-HCl, *p*H 7.5, containing 4 *M* guanidine hydrochloride (G/HCl) for 24 h at 5°C, as described by Mizuta *et al.,* (1997). The volume of the solvent used in the G/HCl extraction was adjusted to about one half (v/w) that of the initial tissue wt. After centrifugation at 10,000 g for 20 min, the supernatant was dialyzed against distilled water overnight and then against 0.5 *M* acetic acid containing 2*M* NaCl. The resultant precipitate was collected by centrifugation at 10,000 g for 20 min, dialyzed against distilled water and lyophilized. The preparation obtained was referred to as G/HCl-soluble collagen (GSC). After the G/HCl extraction of the RS-AL, the insoluble matter was washed thoroughly with distilled water and digested with porcine pepsin (EC 3.4.23.1; Sigma, USA; crystallized and lyophilized) in 0.5 *M* acetic acid at an enzyme/substrate ratio of 1:20 (w/w) for 48 h at 5°C. After centrifugation at 10,000 g for 20 min, the collagen in the supernatant was used as a pepsin-soluble collagen (PSC) preparation.

Fourier Transform-Infra Red Spectrum Analysis

FT-IR spectroscopy of solid samples of standard (human placenta) (Jackson *et al.,* 1995). GSC and PSC from *A. granosa* were relied on a Bio-Rad FTIR–40 model, USA. The sample (10 mg) was mixed with 100 mg of dried potassium bromide (KBr) and compressed further to prepare as a salt disc (10 mm diam.) for reading the spectrum.

SDS-PAGE

The partial purification of GSC and PSC collagens was done by SDS-PAGE using 7.5% polyacrylamide gel as described by the method of Laemmli (1970). The samples (2-5 µg) were electrophoresed along with type I collagen from common carp (*Cyprinus carpio* L.) as standard (mol wt, 100 kDa) (Noda *et al.,* 1975). Gel was stained with Coomassie brilliant blue (CBB) R-250 as described by Fairbanks *et al.,* (1971). The bands were observed under gel documentation system (DigiDoc-IT Imaging System, UVP Ltd., UK) and the mol wt was compared mol wt marker as well as with standard collagen (Sigma, USA). Finally, the mol wt of the GSC and PSC was determined by using Doc-ItLS Acquisition sand Analysis software.

Results and Discussion

In the present study, both GSC and PSC were isolated from *A. granosa* and partially purified. The yield of GSC and PSC was estimated to be 1.1 and 7.2%, respectively on the basis of lyophilized dry wt. Thus, the yield of GSC was very low as compared to PSC. While the yield of skin collagens (on the basis of lyophilized dry weight) of Japanese Sea bass, Chub mackerel and Bullhead shark was about 51.4%, 49.8% and 50.1%, respectively (Nagai and Suzuki, 2000) and for the *Pulmonate gastropods*, *H. pomatia* and *Arion rufus*, the yields of collagen in connective tissues from foot were 51±3.4/28±1.8% and 50±3.3/36±2.4% respectively (Tonar and Marko, 2004). The yield of pepsin-soluble collagen (PSC-SC) extracted from cartilages of Siberian sturgeon, *Acipenser baerii* was 14.69 ± 0.85% on dry weight basis.

The yield of GSC was lower than that of PSC extracted from *A granosa*, it was supported by the finding of Wu *et al.,* (2019), they reported that the yields of GSC and PSC extracted from the whole soft body tissues of surf clam shell, *Coelomactra antiquate* were 0.59 ± 0.03% and 3.78 ± 0.04% (on a dry weight basis) respectively. Comparison with present study, the yield GSC was one time lower and the yield of PSC was two times higher than the report of Wu *et al.,* (2019). This confirmed that the GSC yield was lower compared with PSC yield.

They reported that the yields were very high when compared to that of the present study i.e., the tissues of *A. granosa* reported low yield. But the yields of both PSC and GSC were very low in whole body tissues of *Perna viridis* and Archeaogastropod, *Nerita crepidularia* (the respective yields of *P. viridis* were 0.33% and 0.01%, respectively (Tonar and Marko, 2004) and for *N. crepidularia* were 0.48% and 1.28% (Palpandi *et al.,* 2010). The first 48 h *de novo* synthesis represents 4.52% of the total protein synthesis from cultured mantle cells of nacreous mollusc, *Haliotis* tuberculate (Poncet *et al.,* 2000). Another study conducted by Jayalakshmi *et al.,* (2017) reported that the PSC was nearly two times lower (3.93%) yield in dried skin of *S. pharaonic* as dry weight basis when compared to the present study result.

Collagen synthesis ranged from 0.5% to 5% of the total protein synthesis was found in an *in vitro* study of sea urchin micromeres on 1-5 days culture (Poncet *et al.,* 2000). 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.

In the present investigation, approximately 2–3% of total collagen was solubilized by the extraction method using G/HCl (Poncet *et al.,* 2000). While, about 10-30% of the total collagen could be solubilized from the residue after the G/HCl extraction by the limited pepsin digestion for common octopus (*Octopus vulgaris*) (Mizuta *et al.,* 2003). However, it is also found that PSC exhibited quite a similar pattern to those of the pepsin digest of the GSC, considering the effects G/HCl treatment on collagen, but the same results were found as the PSC from the G/HCl treated RS-AL.

An enhanced yield of the pepsin solubilized collagen accounted by using a disaggregating solution (0.1M Tris–HCl, pH 8.0, containing 0.05 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 i.e., 18.3±2.7% (Yoshinaka *et al.,* 1990). The collagen content may be decreased due to denaturation of protein during the process of methodology and difference in environmental temperature (Rigo *et al.,* 2002).

Some studies on collagen reveal 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 crosslink between molecules. Collagen is insoluble in organic solvents (Piez, 1985). In some tissues and the skin of young animals, cross-linking 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). In this study, acetic acid was used for the extraction of collagens since the extraction of fresh and even the negligible crosslinked collagen molecules present in the outer skin mussel (Friess, 1998). In the present investigation, the yield of GSC and PSC from the tissue of *A. granosa* was 1.1 and 7.2% respectively, which was higher than the yield of collagen prepared without RS-AL treatment in Kuruma prawn *Panaeus japonicas* (Yoshinaka *et al.,* 1990). Moreover, the solubility of GSC was increased when compared with previous studies.

Further the FT-IR spectrum of standard collagen showed 10 major peaks (Fig. 1); whereas the spectrum of the both GSC and PSC extracted from *A. granosa* depicted 8 and 11 peaks (Figs. 2 & 3), respectively. A study investigated by Krishnamoorthi *et al.,* (2017) reported 14 peaks of PSC type similar to the standard collagen which noticed 14 peaks. The wavelength details and their corresponding chemical structures are given in Table 1.

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 (Doyle *et al.,* 1975). The skin collagen of young Nile perch and it showed that the amide region 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 than that of the adult Nile perch skin collagen were at 3458 cm^{-1}, 2926 cm^{-1}, 1654 cm^{-1}, 1555 cm^{-1} and 1238 cm^{-1}, respectively (Muyonga *et al.,* 2004).

In the present investigation, the main bands in *A. granosa* tissues were observed correspondingly in the amide regions of A, I, III at 3332 cm^{-1}, 1667 cm^{-1} and 1246 cm^{-1} in GSC, respectively and in the amide regions of A, B, I, II and III at 3296 cm^{-1}, 2924 cm^{-1}, 2874 cm^{-1} 1654 cm^{-1}, 1534 cm^{-1} and 1237 cm^{-1} in PSC, respectively. In the present study, no main band was observed in the amide B region. While the main bands in *N. crepidularia* tissues were observed in the amide regions of A, B, I, II and 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 (Palpandi *et al.,* 2010). Thus we can confirm that triple helical structure is present in *A. granosa* (Zhao *et al.,* 2018).

Table.1 Details of FT-IR spectrum of standard collagen (Laemmli, 1970), guanidine-soluble collagen (GSC) and pepsin-soluble collagen (PSC) extracted from *A. granosa*

Regions	Standard (cm^{-1})	GSC (cm^{-1})	PSC (cm^{-1})	Assignment
Amide A	3289	3332	3296	**NH stretch coupled with hydrogen bond.**
Amide B	2920	-	2924	**CH_2 asymmetrical stretch.**
	2853	-	2874	**CH_2 symmetrical stretch**
Amide I	1644	1667	1654	**C=O stretch/hydrogen bond coupled with CN stretch**
Amide II	1537	-	1534	**NH bond coupled with CN stretch**
	1450	1470	1458	**CH_2 bond**
Amide III	1260	1246	1237	**NH bond coupled with CN stretch**
	1078	1072	1078	**C-O stretch**
	1021	1025	1024	**C-O stretch**
	804	773	860	**Skeletal stretch**
	-	**640**	**643**	**Skeletal stretch**

Figure.1 Showing the FT-IR spectrum of standard collagen (human placenta)

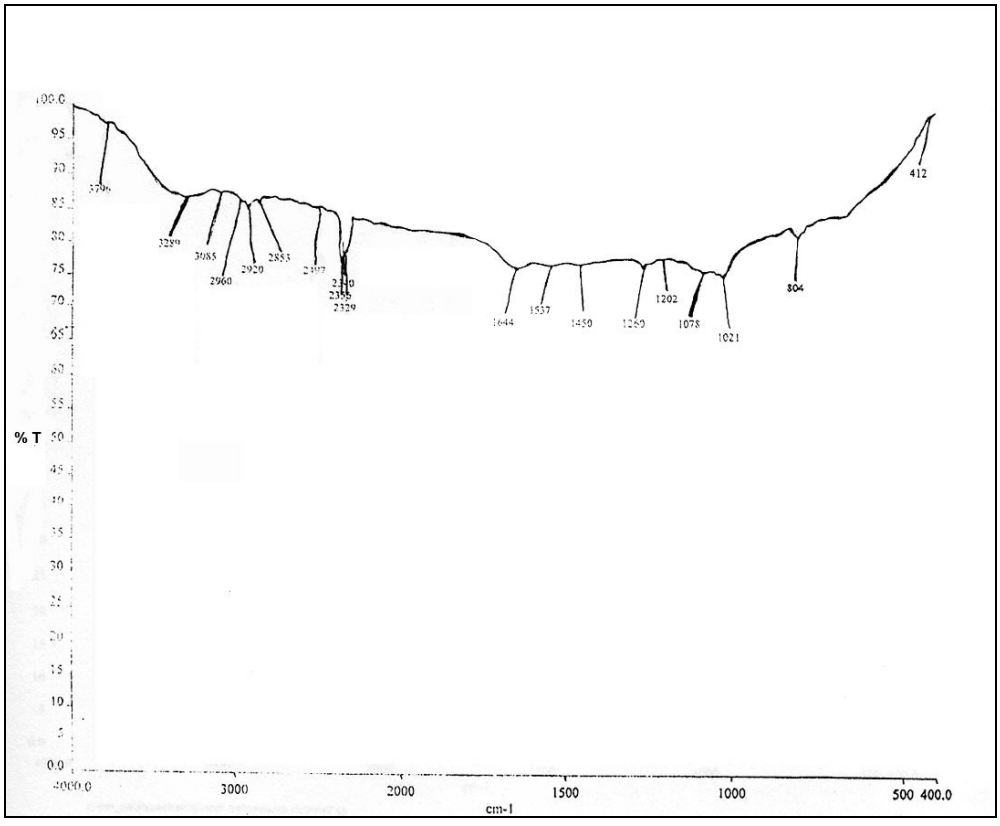


Figure.2 Showing the FT-IR spectrum of GSC (guanidine hydrochloride-soluble collagen) of *A. granosa*

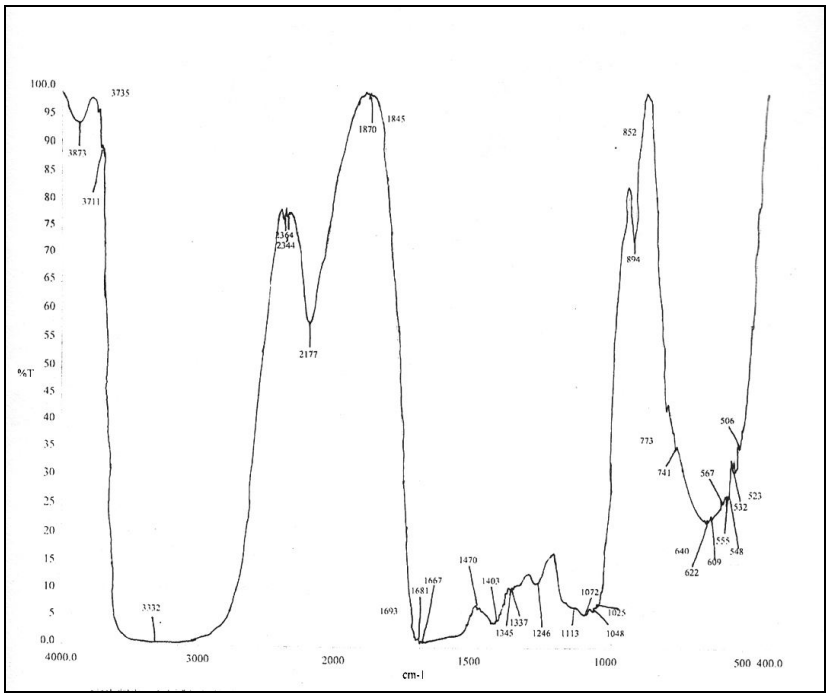


Figure.3 Showing the FT-IR spectrum of PSC (pepsin soluble collagen) of *A. granosa*

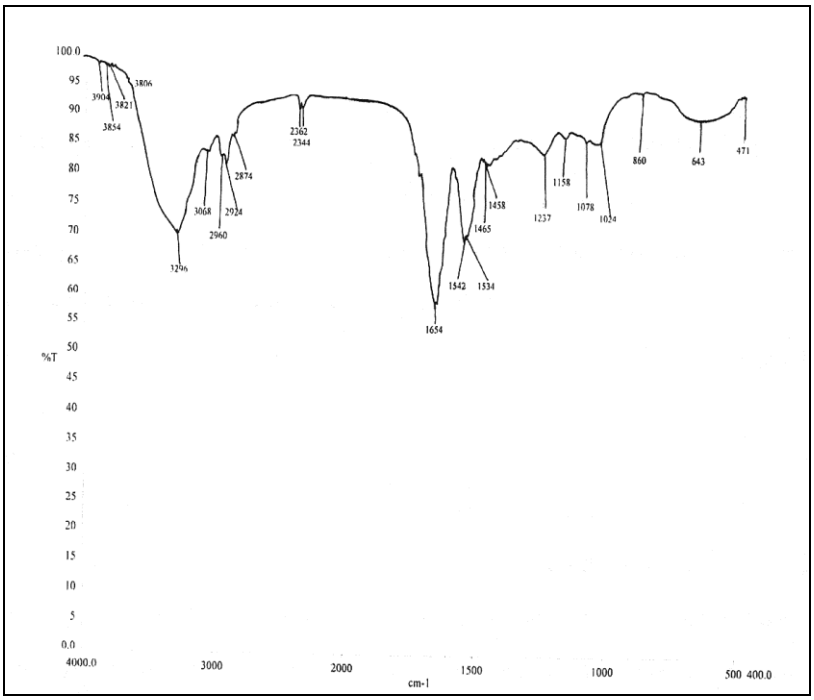
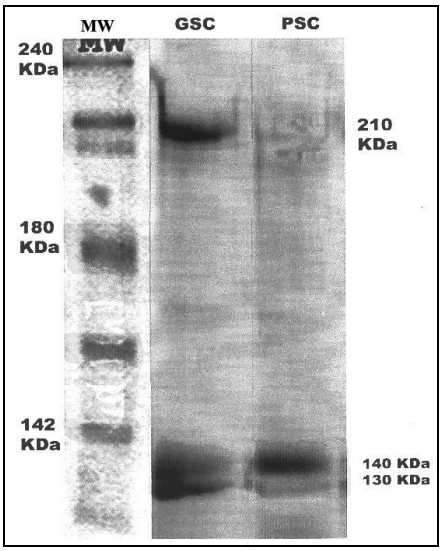


Figure.4 SDS-PAGE analysis (7.5% gel) of *A. granosa* collagen: MW, Mol wt standard (100 kDa) showing six bands of type I collagen from *C. carpio* (lane 1); GSC, Guanidine hydrochloride-soluble Collagen (lane 2); PSC, Pepsin-soluble Collagen (lane 3) (Lane-3).



The amide-A band is associated with the N-H stretching frequency. According to Doyle *et al.,* (1975), a free N-H stretching vibration occurs in the range of 3400-3440 cm^{-1} and when the NH group is a peptide which 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 (Wang *et al.,* 2008).

Similarly, the amide-A band of Pepsin Soluble Collagen (PSC) from the skin of *S. pharaonis* showed the -NH-stretching at 3423 cm^{-1} (Krishnamoorthi *et al.,* 2017). Comparing to this, in the present investigation also, the amide A band of GSC and PSC are located at 3332 cm^{-1} and 3296 cm^{-1}, respectively. It also indicates the involvement of more NH groups in hydrogen bonding of GSC and PSC.

The amide B band of skin of Walleye Pollock (*Theragra chalcogramma*) collagen was found at 3080 cm^{-1}. Similarly, in this study also, the amide B band was located at 2924 cm^{-1} of PSC only which is related to the asymmetrical stretch of CH_2 (Muyonga *et al.,* 2004) and amide I band position was observed at 1655 cm^{-1} and 1656 cm^{-1} (GSC and PSC, respectively), which is the absorption band of C=O stretching and is associated with the secondary structure of the protein (Liu *et al.,* 2007). Li *et al.,* (2013) also reported that the amide B band of PSC-SC was found at position of 2965.38 cm^{-1}, which is associated with the asymmetrical stretch of CH_2.

Similarly, the major absorption bands of PSC extracted from cartilages of Siberian sturgeon, *Acipenser baerii* were in amide band regions such as the peak of amide A (PSC-SC 3405.92 cm^{-1}), amide B (PSC-SC 2965.38 cm^{-1}), amide I (PSC-SC 1655.09 cm^{-1}), amide II (PSC-SC 1547.04 cm^{-1}), and amide III (PSC SC 1243.98 cm−1) (Luo *et al.,* 2018).

The absorption between the 1246 cm^{-1} and 1237 cm^{-1} (Amide III) and, 1470 cm^{-1} and 1458 cm^{-1} (Amide II) (GSC and PSC respectively) wavelength demonstrated the existence of helical structure. In PSC, Amide I band consists of a higher proportion of the component at 1654 cm^{-1}.

This band is linked to the extent of intermolecular interactions in collagen and collagen-like peptides (Paschalis *et al.,* 2001). The other considerable difference was the lower intensity of the component with a peak at 1667 cm^{-1} in GSC. This component has been attributed to random coils (Darby and Creighton, 1993) suggesting a lower extent of unwinding of the triple helix in the GSC. The CH_2 asymmetrical and symmetrical stretching vibrations of amide B regions and amide II band were not

found in the GSC of *A. granosa* but found in the standard collagen and PSC of *A. granosa*. These two amide B region and amide II region was found in the collagen from the skin of both adult and young Nile prech (Muyonga *et al.,* 2004).

Figure 4 shows the SDS-PAGE pattern of partially purified collagens compared to Type I collagen from common carp (*Cyprinus carpio* L.). In the present study, GSC showed three prominent bands corresponding approximately to the molecular weight of 130, 140 and 210 kDa and PSC showed only one prominent band with 140 kDa. But the remaining bands with 130 kDa and 210 kDa stained very lightly.

Later study showed that three bands of PSC were reported with the molecular weight of 110 (β1), 108 (α1) and 102 (α2) kDa (Krishnamoorthi *et al.,* 2017) and noticed prominent band for β1 and lights bands in α1 and α2 regions. Similar bands pattern noticed in SDS-PAGE analysis of PSC from skin and connective tissues of giant red sea cucumber *Parastichopus californicus* in which the major component in α1 of approximately 140 kDa and in β chains, a small amount of dimers found (Liu *et al.,* 2010). The SDS gel profile of ASC extracted from outer skin of cuttlefish *Sepiella inermis* showed three bands with mol wt of 86, 63 and 58 kDa (Vairamani *et al.,* 2012).

The most collagens from aquatic animal's by-products like skins, bone, scales etc., found to have type I collagen which composed of two different α-chains such as α1 and α2 with a α1-chain/α2 chain band intensity ratio of approximate 2:1 (Kaewdang *et al.,* 2014; Krishnamoorthi *et al.,* 2017; Tziveleka *et al.,* 2017; Abdollahi *et al.,* 2018; Wang *et al.,* 2018; Luo *et al.,* 2018). The presence of α1- and α2-chains and also the previous studies (Kittiphattanabawon *et al.,* 2010; Wang *et al.,* 2018) confirmed that one type of collagen from the whole body tissues of *A. granosa* is type I collagen ([α1(I)]2α2(I)). The GSC and PSC extracted from pearl oyster (*Pinctada fucata*) and *Crassostrea gigas* exhibited relatively a similar form (Mizuta *et al.,* 2002, 2004). The FTIR spectra were consistent with the results of SDS–PAGE. It seemed, therefore, PSC retained more intermolecular cross-links during solubilisation with acetic acid but the triple helical structure normally held together by intra-molecular hydrogen bonds (Paschalis *et al.,* 2001) was extensively destroyed.

FTIR results confirmed the existence of helical arrangements of the two collagens. So from the results of present study, it could be concluded that the collagen from *A. granosa* may be used as a potential source as an alternative sources of biomaterials, cosmetic, and other applications. Further studies using NMR and GC–MS could bring out more details about complete structure of GSC and PSC from the bivalve *A. granosa*.

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Author Contributions

C. M. Ramakritinan: Conceptualization, Supervision, Methodology, Validation, writing-reviewing. Javle Shrirang Ramdas Nilima: Investigation, Formal analysis. V. Dinesh Kumar: Investigation, Formal analysis. S. Vairamani: Investigation, Formal Analysis, Writing-Reviewing. A. Shanmugam: Conceptualization, Supervision, Methodology, Validation, Formal Analysis, Writing - Original Draft, Funding

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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