

PREPARATION AND BIOMEDICAL CHARACTERIZATION OF JELLYFISH (*CHRYSAORA QUINQUECIRRHA*) COLLAGEN FROM SOUTHEAST COAST OF INDIA

SUGANTHI KRISHNAN*, PACHIPPAN PERUMAL

Department of Biotechnology, Periyar University, Salem 63601, Tamil Nadu, India. Email: krishsuganthi@gmail.com.

Received: 19 Apr 2013, Revised and Accepted: 06 Jun 2013

ABSTRACT

Jellyfishes were carefully collected from Parangipettai coastal waters, Southeast coast of India, during post monsoon season. The present research aimed to isolate and characterize the collagens for the first time from marine waters. Collagens (acid-solubilized and pepsin-solubilized collagens) were prepared and partially characterized from jellyfish, *Chrysaora quinquecirrha* whole tissue (WT) and nematocyst suspension (NS). The yield of collagen was 0.48% of GSC and 1.28% of PSC on the basis of lyophilized dry weight. Amino acid content of WT and collagenase digested NS revealed that hydroxyproline and glycine were present in the amounts of 1.8 & 9.2 % and 5.14 & 28.3 % respectively, moreover it was relatively stable at 29.0 °C for 60 min. The highest utilization of collagen is in pharmaceutical applications including production of wound dressings, vitreous implants and as carriers for drug delivery. Moreover, collagen is used for the production of cosmetics as it has a good moisturizing property. Thus the jellyfish tissues and nematocyst suspension will have potential source in biomedical applications, their relevance studies are in progress.

Keywords: Collagen, Pepsin, Drug, Jellyfish, Amino acid.

INTRODUCTION

The marine environment is a rich source of both biological and chemical diversity. This diversity has been the source of unique chemical compounds with the potential for industrial development such as pharmaceutical, cosmetics, nutritional supplements, molecular probes, fine chemical and agrochemicals. In the recent years, a significant number of novel metabolites with potent pharmacological properties have been discovered from the organism [1]. Several well-known chemists and biologists reviewed different aspects of marine natural products. Marine organisms are richly endowed with many enzymes and have unique functions [2]. The study is important from the point of view of obtaining biomedically useful agents for the treatment of diseases and for the better knowledge of the pharmacologically effective substances [3]. The study of natural products which exhibit biological activity derived from plants and animals has been of significant biomedical value, and crude products isolated from the organism have served as a source of drugs and also are starting materials from which useful drugs could be synthesized. Many exciting biologically active substances such as antibacterial, antitumor, antiviral and biologically toxic compounds have been discovered.

Some marine algae, along with codfish, are known to contain collagen protein in them. Collagen matter produced from marine organisms is collectively known as marine collagen protein. Collagen is an important protein in the organisms and the use of collagen in industry for health foods, cosmetics [4] and biomaterials is expanding. Ottani *et al.* [5] 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.

Collagens are often considered as an animal hallmark [6]. They form an abundant structural protein family that is widely represented throughout the tissues of the body. All collagen molecules contain a triple-helical domain, are generally involved in the formation of supra-molecular networks, and are made of three α chains which may or may not be identical [7]. Collagen is a fibrous protein found ubiquitously in all multicellular animals. It is particularly a 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 the form of a rope-like superhelix. These peptides are extremely rich in proline and glycine, both of which are important for the formation of the collagen-specific helical structure [8-11].

Collagen is a natural material of low immunogenicity [12]. Many pharmaceutical applications are known for collagen, e.g. shields, injectable dispersions, sponges and microparticles [13]. Furthermore, collagen is used as a moisturizer in cosmetic creams [14]. Moisturizers are employed for the therapy of so-called 'dry skin'. Lipids work by the principle of occlusion, whereas humectants, such as collagen, attract water in the stratum corneum. In contrast to low molecular weight humectants, such as urea or glycerine, humectants of higher molecular weight, such as collagen, are not absorbed by the stratum corneum but rather stay on the skin surface, where they bind water by hydration and so increase the degree of skin-humidity [15]. Collagen thus represents an excellent film-building polymer in cosmetics. The use of cattle as the main source for collagen has to be reconsidered because of the risks of BSE (bovine spongiform encephalopathy) and TSE (transmissible spongiform encephalopathy). One alternative is the use of collagen or, much safer, collagen from sea animals, such as marine organisms.

Collagen is an important protein in the organisms, and the use of collagen in industry for health foods, cosmetics, and biomaterials is expanding. At present, because of the outbreak of bovine spongiform encephalopathy (BSE) and the foot-and-mouth disease (FMD), the main sources of collagen in many fields are limited to those of bovine or porcine dermis, so more and more studies turn to find safe sources of collagen. There have been many reports of collagen in aquatic animals. Collagen possesses a major advantage in being biodegradable, biocompatible, easily available and highly versatile. However, since collagen is a protein, it remains difficult to sterilize without alterations to its structure. The review presents a comprehensive overview of the various applications of collagen-based biomaterials developed for tissue engineering, aimed at providing a functional material for use in regenerative medicine from the laboratory bench to the patient bedside [16]. Collagen is the most abundant animal protein polymer representing nearly 30% of total protein in the animal body. It is the main structural element of bones, cartilage, skin, tendons, ligaments, blood vessels, teeth, cornea and all other organs of vertebrates.

Collagen can be extracted from various sources considering that it is one of the most abundant proteins on earth. It can be extracted from almost every living animal, even including alligators [17] and kangaroos [18]. Marine life forms are also a considerable source of collagen, which can be extracted from sponges [19-23]. These collagens are widely used in the industry, but less for research and clinical usage. All these collagen sources are worth investigating considering that collagen properties differ from one animal to another [24].

Wound dressings that are also delivery systems represent an interesting application for collagen based applications. Recent studies have shown the feasibility and more importantly the benefits of implants delivering antibiotics [25-26]. The delivery properties of collagen-based biomaterials also display great potential for ulcer treatment and abdominal wall defect reconstruction [27-29].

The industrial use of collagen has been expanded and now includes foods, cosmetics and biomedical materials. Although there are many studies about collagen in marine vertebrates and invertebrates, the main sources of industrial collagen are limited to those from bovine and pig skins. Among marine invertebrates, some jellyfish are used in Chinese food owing to their unique textures. There is possibility to use the jellyfish (sea nettle) for the extraction of collagen. Therefore, the aim of this study was to isolate and characterize collagen from the jellyfish *Chrysaora quinquecirrha*. Particularly, however, little information is available on jellyfish collagen. In this paper, we report the preparation and thermal properties of collagens obtained from the same. These have potential use as alternatives to mammalian collagen in foods, cosmetics and biomedical materials.

MATERIALS AND METHODS

Sample

Jellyfish, *C. quinquecirrha*, were collected from Parangipettai coastal waters during post monsoon season, Southeast coast of India, cooled in ice and transported to the laboratory. The whole tissues were excised, washed with distilled water and extracted with 0.1M NaOH. The insoluble substances was lyophilized and stored at -20 °C until use.

Extraction of Acid- and Pepsin-Solubilized Collagens (ASC and PSC)

All the preparative procedures were performed at 4°C. Jellyfish tissues were extracted with 0.5 M acetic acid for 2 days, and the extracts were centrifuged at 10,000× g for 30 min. The residues were re-extracted with the same solution for 1 day, and these extracts were centrifuged under the same conditions. The supernatants were combined and salted out by adding NaCl to a final concentration of 0.7 M. The precipitated collagens were separated by centrifugation at 10,000× g for 30 min and redissolved in 0.5 M acetic acid to precipitate with NaCl again. The resultant precipitates were dialyzed against distilled water and lyophilized. The residues from the acetic acid extraction were suspended in 0.5 M acetic acid and digested with 0.5% (w/v) pepsin (Sigma p7000) for 72 h. The extraction steps of PSC were the same as the extraction of ASC method [30-31].

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli [32] using 5% stacking gel and 7.5% resolving gel. The samples were dissolved in 0.6 M Tris- HCl buffer (pH 6.8) which contained 25% (v/v) glycerine, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, and 0.1% (w/v) Bromophenol blue. After electrophoresis, gels were visualized with Coomassie Brilliant Blue R-250.

Amino Acid Analysis

Whole tentacle (WT) and nematocyst suspensions (NS) of *Chrysaora quinquecirrha* were collected and prepared according to previously described methods [33]. Samples of WT were hydrolyzed with 6 N HCl in an autoclave for 3 hr and applied to an amino acid analyzer. Specimens of NS were incubated with collagenase for 18 hr at 37°C [34]. The resulting solutions were placed in a cellulose bag and dialyzed overnight at 4°C against distilled water. Both the bag contents and the solution outside the bag were then lyophilized. These lyophilates were hydrolyzed as above and a measured the amount of each separately applied to an amino acid analyzer and the hydrolysates were analyzed by an amino acid analyzer HP1100 (Agilent, USA).

Determination of Denaturation Temperature

The denaturation temperature was measured according the method of Nagai and Suzuki. [35]. Ten mL of the 0.03% collagen solution in

0.1 M acetic acid were used for the viscosity measurement, and measurement was done using an Ostwald viscometer. The thermal determination curve was obtained by measuring solution viscosity at several temperatures from 10°C to 60°C; the temperature was raised stepwise and maintained for 30 minutes at each point. The denaturation temperature (Td) was determined as the temperature at which the change in viscosity was half completed.

RESULTS AND DISCUSSION

Jellyfish species were collected from Parangipettai coast and frozen after being collected, and isolated the tissues, nematocysts from the jellyfish tentacles by using sterile sea water. This technique is now frequently used [36].

In order to estimate the potential source of collagen, we have tested different collagen extraction procedures from whole tissue (WT) and nematocyst suspension(NS). The extraction yields of acid-soluble collagen (ASC) and pepsinized (PSC) collagens from whole tissue and nematocyst suspension were 0.48 and 1.2% respectively. The highest yields were obtained with the acid-soluble extraction method, and when the extraction was carried out on whole tissues. In this study, the jellyfish collagens were extracted by 0.5 M acetic acid and pepsin. This collagen had abundant amino composition with moderate stability. Nematocyst suspension (NS) also contain a collagen-like material. It has been previously reported that this protein shows the peculiar characteristic of being partially solubilized by disulfide-reducing agents [37]. It was found that a large amount of collagen was obtained from rhizostomous jellyfish mesogloea, although the yield was lower than that from edible jellyfish exumbrella collagen [38].

Nagai et al. [39] 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 [40]. Compared to this, the tissues of *C. quinquecirrha* reported low yield of about 0.48% of GSC and 1.28% of PSC against the total collagen content on wet weight basis.

Amit Kumar [41] 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 [42]. TA

The collagen samples were analysed using SDS-PAGE, the molecular weight ranging from 15-105 kDa, revealed the presence of medium sized proteins. This could be categorised as (i) Medium sized Cytolytic Actinoporins (~ 20kDa), (ii) Cardio stimulatory proteins (~ 28kDa) and (iii) Cytolysin with or without Phospholipase (~ 40kDa) in both crude and fractionated proteins. Lucia Cariella et al.[43] isolated and partially characterized Rhizolysin, a high molecular weight protein with hemolytic activity from the jellyfish *Rhizostoma pulma*. It had a molecular weight of approximately 260 kDa. Burnett and Long [44] compared a method for preparing jellyfish nematocyst suspension from sea nettle *Chrysaora quinquecirrha* fishing tentacles at the beachside. Endean [45] isolated two myotoxins (600 kDa and 150 kDa), a hemotoxin (70kDa) and a neurotoxin fraction (150 kDa) by the use of Sephadex G-200 chromatography. Rottini et al [46] reported the purification and properties of a cytolytic toxin and a hemolytic toxin from the jellyfish *Carybdea marsupialis*.

Eight per cent of the NS dry weight was found to be protein. The colorimetric results indicated that 5.4 per cent of the total protein was hydroxyproline. According to preliminary findings [38] suggested that hydroxyproline and hydroxylysine, which are characteristic of collagen. Amino acid content is shown as residues per 1000 total residues of WT revealed that hydroxyproline and glycine were present in the amounts of 1.8 and 9.2 per cent respectively. The collagenase digested NS contained hydroxyproline and glycine in respective concentrations of 5.14 and 28.2 per cent (Table 1).

Table1: Amino acid distribution in invertebrate collagen Whole tentacle and Collagenase digestible NSMS

Amino Acid	No. No of Residues per 1000 total residues		
	Invertebrate range*	Whole Tentacle	Collagenase - digestible NS
Glycine	286-324	92	83
Glutamic acid	77-110	148	84
Leucine	118-37 1111\	53	37
Phenylalanine	15-12	261	15
Amide nitrogen	44-102	137	60
Arginine	21-57	40	38
Isoleucine	13-24	57	51
Alanine	56-114	52	61
Valine	13-34	42	28
Threonine	18-52	37	29
Serine	23-105	43	39
Lysine	9-37	84	36
Hydroxyproline	24-165	18	8
Histidine	0-8.4	16	8
Tyrosine	0-11	0-19	15
Methionine	0-12	0-11	8
Proline	13-28	0-66	90
Hydroxylysine	0-30	0-1	0-2
Cysteine	0-6	0-2	0-3

* (Gross (1963))

Sadowska *et al.* [47] also observed that the share of collagen in total protein was considerably determined on the basis of hydroxyproline in samples. In this study, collagen obtained from the jellyfish could be possessed properties typical of type I collagen. The amino acid analyses of our collagenase-digested preparations strongly fulfil one of the major criteria listed above and support the hypothesis that the nematocyst is a complicated structure containing a significant amount of hydroxyproline and other amino acids bound together in a collagen-like structure.

The denaturation temperature was determined in *Chrysaora* collagen showed 29°C in 60 minutes. These values were similar to those obtained from other marine organisms such as, muscles of carp (32.5°C), eel (30.2°C), common mackerel (26.9°C), saury (24.0°C), chum salmon (20.6 °C) and skins of carp (31.7°C), eel (29.3 °C), common mackerel (26.1°C), edible jellyfish exumbrella (26.0 °C) [38]. It is known that collagen stability is correlated with environmental and body temperature [48].

However, isolation and characterization study of Scyphozoan jelly fish *C. quinquecirrha* has not yet been undertaken so far in Indian coastal waters. Hence it is obvious that the collagen based materials produced from the *C. quinquecirrha* extracts could solved the supply problem of raw materials for future benefits mainly in the medical and industrial applications.

CONCLUSION

ICollagen has great medicinal promise because it is the essential building material of cell tissue, cartilage, teeth and bone. The collagen was extracted from *Chrysaora* jellyfish, by limited pepsin digestion and characterized. It was found that typical quantity of collagen was obtained, although the yield was lower than that from edible jellyfish exumbrella collagen [38]. If we can use these collagens properly according to their characteristics, the industrial use of jellyfish collagen could be extended in various fields. Collagen-based biomaterials are of the utmost importance for biomedical aspects and regenerative medicine. Because of its superior biocompatibility and low immunogenicity, collagen is still the protein of choice for biomaterials preparation. It is planned to analyze the *Chrysaora* jellyfish collagen in chemical detail and to compare some of its properties, particularly its subunit composition, most present research is aimed at the optimization of collagen-based biomaterials for medical applications by enhancing mechanical strength, biodegradability or delivery characteristics. Considering the bioavailability of jellyfish collagen and its biological properties, this marine material is a good candidate for replacing bovine or human collagen in selected biomedical applications.

ACKNOWLEDGEMENT

The authors are thankful to the authorities of the Periyar University for providing facilities and to the UGC-New Delhi, (Grant number: F.4-2/2006(BSR)/13-482/20119BSR) for financial support to the first author through Dr. D.S. Kothari Post Doctoral Fellowship Scheme.

REFERENCES

1. Kijjoa A, Swangwong P. Drugs and cosmetics from the sea, *Mar Drugs* 2004; 2: 73-82.
2. Sadowska, M, Kolodziejaska I, Niecikowska C. Isolation of collagen from the skins of Baltic cod (*Gadus morhua*). *Journal of Food Chemistry* 2003; 81: 257-262.
3. Newman D, Craig GM. Marine natural products and related compounds in clinical and advanced preclinical trials. *Journal of Natural Products* 2004; 67: 1216 - 1238.
4. Zhang Z, Li G, Shi B. Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. *J. Soc. Leath. Techn. Chem.* 1985;90: 23-28.
5. Miura S, Kimura S. Jellyfish mesogloea collagen. Characterization of molecules as $\alpha 1\alpha 2\alpha 3$ heterotrimer. *J. Biol. Chem* 2005; 260, 15352-15356.
6. Exposito, JY, Cluzel C, Garrone R Lethias C. Evolution of collagens. *Anat. Rec.* 2002; 268: 302-317.
7. Ricard-Blum S, Ruggiero F, van der Rest M. The collagen super family. *Top. Curr. Chem.* 2005; 247:35-84.
8. Lehninger A L, 1987. Biochemie, VCH Verlagsgesellschaft mbH, Weinheim, Germany pp: 107 - 108.
9. Alberts B, Johnson A, Lewis J, Raff M, Roberts K. Molecular Biology of the Cell. 2002; Garland Science: New York, USA.
10. Rossler B, Kreuter J Scherer D. Collagen microparticles: preparation and properties. *J. Microencapsul.* 1995; 12:49-57.
11. Zubay, GL. Biochemistry. WCB Wm. C. Brown Publishers, 1998. US pp: 90 - 92.
12. Deyl Z, Miksik I. Comparison of different electrokinetic separation modes applicable to a model peptide mixture (collagen type I and III CNBr fragments). *J. Chromatogr. B Biomed. Sci. Appl.* 2000; 745:251-260.
13. Leitinger, B Hohenester E. Mammalian collagen receptors. *Matrix Biol.* 2007; 26:146-155.
14. Ramshaw JA, Peng Y, Glattauer V, Werkmeister JA. Collagens as biomaterials. *J. Mater. Sci. Mater. Med.* 2009; 20 (1): S3-S8.
15. Margaron Y, Bostan L, Exposito JY, Malbouyres M, Trunfio-Sfarghiu, Berthier A, Lethias , Y Tenascin X. Increases the stiffness of collagen gels without affecting fibrillogenesis. *Biophys. Chem.* 2010;147: 87-91.

16. Remi Parenteau B, Robert Gauvin R François B. Collagen-Based Biomaterials for Tissue Engineering Applications. *Materials* 2010; 3: 1863-1887.
17. Wood A, Ogawa M, Portier M, Schexnayder M, Shirley M, Losso JN. Biochemical properties of alligator (*Alligator mississippiensis*) bone collagen. *Comp. Biochem. Physiol. Biochem. Mol. Biol.* 2008;151:246-249.
18. Johnson KA, Rogers G, Roe SC, Howlett CR, Clayton M, Milthorpe MKSchindhelm K. Nitrous acid pretreatment of tendon xenografts cross-linked with glutaraldehyde and sterilized with gamma irradiation. *Biomaterials* 1999; 20: 1003-1015.
19. Exposito JY, Cluzel C, Garrone R, Lethias C. Evolution of collagens. *Anat. Rec.* 2002;268: 302-316.
20. Exposito JY, Cluzel C, Garrone R, Lethias C, Garrone Q. Short chain collagens in sponges are encoded by a family of closely related genes. *J. Biol. Chem.* 1999; 266: 21923-21928.
21. Subramanian A, Lin HY. Crosslinked chitosan: its physical properties and the effects of matrix stiffness on chondrocyte cell morphology and proliferation. *J. Biomed. Mater. Res.* 2008; 75A:742-753.
22. Sugiura, H, Yunoki S, Kondo E, Ikoma T, Tanaka J, Yasuda K. *In vivo* biological responses and bioresorption of tilapia scale collagen as a potential biomaterial. *J Biomater Sci Polym.* 2009; Ed. 20, 1353-1368.
23. Song E, Yeon Kim, Chun T, Byun HJN, Lee Y.M. Collagen scaffolds derived from a marine source and their biocompatibility. *Biomaterials* 2006;27: 2951-2961.
24. Lin YK, Liu D.C. Comparison of physical-chemical properties of type I collagen from different species. *Food Chem*, 2006;99:244-251.
25. Adhirajan N, Shanmugasundaram N, Shanmuganatha S, Babu M. Functionally modified gelatin microspheres impregnated collagen scaffold as novel wound dressing to attenuate the proteases and bacterial growth. *Eur. J. Pharm. Sci.* 200;36:235-245.
26. Sripriya R, Kumar MS, Ahmed MR, Sehgal PK. Collagen bilayer dressing with ciprofloxacin, an effective system for infected wound healing. *J. Biomater. Sci. Polym. Ed.* 2007;18: 335-351.
27. Liyanage SH, Purohit GS, Frye JN, Giordano P. abdominal wall reconstruction with a Permacol implant. *J. Plast. Reconstr. Aesthet. Surg* 2006; *Anterior* 59: 553-555.
28. Ansaloni L, Catena F, Gagliardi S, Gazzotti F, D'Alessandro L, Pinna AD. Hernia repair with porcine small-intestinal submucosa. *Hernia* 2007; 11: 321-326.
29. Bellows CF, Jian W, McHale M.K, Cardenas D, West JL, Lerner SP, Amiel GE. Blood vessel matrix: A new alternative for abdominal wall reconstruction. *Hernia* 2008;12: 351-358.
30. Zhuang Y Hou, L, Zhao H, Zhang X, Li BF. Effects of collagen and collagen hydrolysate from jellyfish (*Rhopilema esculentum*) on mice skin photoaging induced by UV irradiation. *Journal of Food Science* 2009a ;74: H183-H189.
31. Zhuang Y, Zhao L, Li BF. Optimization of antioxidant activity by response surface methodology in hydrolysates of jellyfish (*Rhopilema esculentum*) umbrella collagen. *Journal of Zhejiang University.* 2009b. *Science B*, 10, 572-579.
32. Laemmli UK. "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4," *Nature* 1970; 227(5259): 680-685.
33. Burnett JW, Stone JH, Pierce H, Cargod G, Layne H, Sutton JS. A physical and chemical study of sea nettle nematocysts and their toxin. *J. invest. Derm.* 1968; 51:330-336.
34. Fugimoto D, Adams E. Intraspecies composition difference in collagen from cuticle and body of *Ascaris* and *Lumbricus*. *Biochem. biophys.* 1964; *Res. Commun.* 17:437-442
35. Nagai T, Worawattanamateekul WN, Suzuki T, Na-kamura T, Ito T. "Isolation and Characterization of Collagen from Rhizostomous Jellyfish (*Rhopilema Asa- mushi*)," *Food Chemistry* 2000; 70(2): 205-208.
36. Bloom D, Burnett JW, Alderslade P. Partial purification of box jellyfish *Chironex fleckeri* nematocyst venom isolated at the beachside. *Toxicon* 1998; 36: 1075 - 1085.
37. Goldner R, Burnett JW, Stone J, Dilaimy MS. The chemical composition of sea nettle nematocysts. *Proc. Soc. exp. Biol. Med.* 1969;131:1386-1388.
38. Nagai, T., Ogawa, T., Nakamura, T., Ito, T., Nakagawa, H., Fujiki, K., Nakao, M and T. Yano, 1999. Collagen of edible jellyfish exumbrella. *Journal of the Science of Food and Agriculture*, 79, 855-858.
39. Nagai, T., Yamashita, E., Taniguchi, K., Kanamori, N and N. Suzuki, 2001. Isolation and characterisation of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*). *Food Chemistry*, 72, 425-429.
40. Nagai, T., and N. Suzuki, 2000. Isolation of collagen from fish waste materials - skin, bone and fins. *Food Chem* 68(3): 277-281.
41. Amit Kumar, C., 2008. Isolation and partial characterization of collagen from the whole body tissues of *Perna viridis* (Linnaeus, 1758). M.Sc. dissertation, Annamalai University, India pp. 1 - 44.
42. Rigo, C., D.J.Hartmann and A. Bairat, 2002. Electrophoretic and immunochemical study of collagens from *Sepia officinalis* cartilage. *Biochem. Et. Biophys. Acta.* 1572:77 - 84.
43. Anderluh, G., and P. Macek, 2002. Cytolytic peptide and protein toxins from sea anemones (Anthozoa: Actiniaria). *Toxicon*, 40: 111-124.
44. Burnett JW, Long-Rowe KO. Sea nettle *Chrysaora quinquecirrha* lethal factor: Purification by recycling on m-aminophenyl boronic acid acrylic beads. *Toxicon* 1994; 32(4): 467- 478.
45. Edean, R, Monks SA, Cameron AM. Toxins from the box-jelly fish *Chironex fleckeri*. *Toxicon*;1993. 31(4): 397 - 410.
46. Rottini G, Gusmani L, Parovel E, Avian M, Patriarca P. Purification and properties of acetylolytic toxin in venom of the jellyfish *Carybdea marsupialis*. *Toxicon* 1990;33:315 - 326.
47. Sadowska M, Kolodziejska I, Niecikowska, C. Isolation of collagen from the skins of Baltic cod (*Gadus morhua*). *Journal of Food Chemistry* 2003;81: 257-262
48. Rigby BJ. Amino-acid composition and thermal stability of the skin collagen of the antarctic ice fish. *Nature* 1968;219: 166-167.