

ISOLATION OF PEPSIN-SOLUBILIZED COLLAGEN (PSC) FROM CRUDE COLLAGEN EXTRACTED FROM BODY WALL OF SEA CUCUMBER (*BOHADSCHIA* SPP).

Y. D SIDDIQUI¹, E. M ARIEF^{1*}, A YUSOFF¹, A. H SUZINA², S.Y ABDULLAH¹

¹School of Dental Sciences, Health Campus, Universiti Sains Malaysia 16150 Kubang Kerian, Kelantan, Malaysia; ²School of Medical Sciences, Health Campus, Universiti Sains Malaysia 16150 Kubang Kerian, Kelantan, Malaysia. Email: erry@kb.usm.my

Received: 23 Mar 2013, Revised and Accepted: 03 May 2013

ABSTRACT

Introduction: Sea cucumber is a marine invertebrate. About 70% of the total body wall protein of sea cucumber is accounted for highly insoluble collagen fibers.

Objectives: The aim of this study was to isolate pepsin-solubilized collagen (PSC) from crude collagen, extracted from body wall of sea cucumber *Bohadschia* spp.

Methods: Body wall of *Bohadshia* spp were cut into small pieces followed by washing with distilled water and then replaced with 4 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M Tris-HCl, pH 8.0, and stirred for 3 days to get precipitated crude collagen fibrils. Disaggregated Insoluble crude collagen fibrils were treated with 0.1 M NaOH and 0.5 M acetic acid containing porcine pepsin to get PSC collagen.

Results: PSC was successfully isolated from disaggregated crude collagen fibres, with 65% yield. According to the electrophoretic pattern, PSC collagen was identified as type I collagen, consisting of three α_1 chains of approximately 138 kDa each.

Conclusion: As high as 60% of PSC was successfully isolated from *Bohadshia* spp and classified as type I collagen. This finding shows the potential use of this collagen as an alternative to mammalian collagen used in the nutraceutical and pharmaceutical industries.

Keywords: Sea cucumber, *Bohadshia* spp, Pepsin solubilized collagen (PSC)

INTRODUCTION

During the past three to four decades many efforts have been committed to isolating numerous biologically active novel compounds from marine sources. Many of such naturally occurring compounds are of immense interest for potential drug development as well as an ingredient of new leads and commercially successful products for various industrial applications, especially, pharmaceuticals, agrochemicals, functional foods and nutraceuticals [1].

Sea cucumbers (*Echinodermata: Holothuroidea*) are one of the potential marine animals with high food and medicinal value. In view of the medicinal potential, modern food and pharmaceutical industry is keenly interested to develop some functional foods and nutraceuticals from different parts of sea cucumbers.

A variety of sea cucumber-derived food and pharmaceutical products are available in South Pacific and Asian markets, including China, Japan, Indonesia and Malaysia [2, 3]. In Asia and the America dry tablets prepared from the body wall of sea cucumbers are consumed as nutraceuticals for physiological benefit. In Malaysia, boiled skin extracts are consumed as a tonic to treat asthma, hypertension, rheumatism and wound cuts and burns [2, 3]. In addition to health medicinal uses, interestingly, there is much demand for sea cucumber as aphrodisiac food to improve sexual performance [2, 3].

Collagen is an abundant protein in animal tissues and has a wide range of applications in the biomedical, pharmaceutical, cosmetic and food industries [4]. It is distinct from other proteins in that the molecule comprises of three polypeptide chains (*a*-chains), which form a unique triple helical structure, which plays a major role in molecular confirmation of collagen [5]. The physical and chemical properties of marine collagen are different from those of mammalian collagen [6]. Body wall of sea cucumber (*S. japonicas*) contains about 70% protein consists of highly insoluble collagen fibers [7]. Commercially processed (dried) sea cucumbers are rich source of crude protein in comparison to most of the sea foods so far in use. According to Chen [8], the fully dried sea cucumber material may contain protein content as high as 83% and is sold as nutraceutical in tabulated or capsulated forms. There is little

information about the collagen of sea cucumber except for few reports on *S. japonicus* [7] and *Cucumaria frondosa* [9,10].

Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods [11]. Pepsin is one of commercially produced enzyme used for protein purification. Three known methods of collagen extraction produce, neutral salt-solubilized collagen, acid-solubilized collagen and PSC [12]. Many researchers have studied the PSC method from different sources, such as from the skin of brownstripe red snapper [13], fish waste material [14], albacore tuna and silver-line grunt skin [15], bone and scale of black drum and sheepshead seabream [16], PSC from *Stichopus japonicus* [17] and obtained higher soluble collagen yield.

Generally, major sources for collagen are the skin and bone of pigs and cows. However, the occurrence of mad cow disease has resulted in anxiety among cattle gelatin users. Additionally, collagen obtained from pig bones cannot be used by many, due to religious constraints [18]. Thus, there is a strong need to develop alternative collagen sources. Marine organisms have been recognized as potential alternative sources, due to their availability, lack of dietary restriction, lack of disease risk, and high collagen yields [19].

Thus, animal from marine environment, *Bohadshia* spp was selected for study. It is one of sea cucumber species lives in deep sea water, presented itself with thick body wall, brownish and yellowish exterior, which resembles *Bohadshia bivittata* [20]. This may be a pioneer study as there is no known report published on the isolation of PSC collagen from crude collagen fibres, extracted from the body wall of sea cucumber *Bohadshia* spp.

In the present work, the aim of this study was to extract crude collagen and isolate PSC collagen from *Bohadshia* spp, as it could be use as an alternative source to mammalian collagen in pharmaceutical and nutraceutical industries.

MATERIALS AND METHODS

Harvesting of Animal

Prior permission was obtained from the Malaysian Fishery Development of the coastal areas of Perhentian Island, Terengganu, Malaysia. Two fresh samples of *Bohadshia* spp weighing between

700-800g were handpicked by the divers. Specie was identified by Professor Ridzwan Ahmed (International Islamic University Malaysia) and also by surface morphology and color of *Bohadschia* spp according to Clouse [20]. The body wall of *Bohadschia* spp was dissected free from adherent tissues, cut into small pieces (about 2 cm × 2 cm), and stored in small container filled with phosphate buffer saline (PBS). The body walls of sea cucumber in small pieces were transported to the lab at 4°C in ice pack, and stored under -80°C until analysis. The collagen from body wall of *Bohadschia* spp was extracted in the forms of crude and PSC by method previously described by Cui and Park [17, 21].

Preparation of crude collagen fibrils

All procedures were performed at 4°C. The pieces of the body wall were washed extensively with distilled water. After the samples (100 g wet weight) were stirred in 1 L of distilled water for 30 minutes, the water was replaced and the extraction in water was repeated once for 1 hour. The water was replaced with 1 L of 4 mM EDTA, 0.1 M Tris-HCl, pH 8.0, and stirred for 3 days. The liquid was decanted and replaced with 1 L of distilled water, in which the samples were stirred slowly for 15 minutes and the washing steps were repeated twice. The liquid then replaced with 500 ml of fresh distilled water and stirred for 2 days. The mixture was centrifuged at 7500g speed for 30 minutes. The supernatant containing free collagen fibrils was collected in beaker, and the pellets were stirred with another 500 ml of distilled water after which the steps were repeated. The supernatant was centrifuged at 7500g for 30 minutes and the precipitate called "crude collagen fibril" was lyophilized using a Christ Freeze Dryer Alpha 1-4 LD (Martin Christ, Osterode am Harz, Germany).

Isolation of pepsin-solubilized collagen (PSC)

The "crude collagen fibril" was stirred in with 20 volumes (v/w) of 0.1 M NaOH for 3 days in order to remove non-collagenous materials effectively and to exclude the effect of endogenous proteases on collagen. The residue after alkali extraction was thoroughly rinsed with distilled water and then stirred with 10 volumes (v/w) of 0.5 M acetic acid containing porcine pepsin (Sigma Chemical Co., USA) at an enzyme/substrate ratio of 1:100 (w/w). After digestion for 3 days, the suspension was then centrifuged at 7500g for 60 minutes and then the PSC in the supernatant salted out by adding NaCl to a final concentration of 0.8 M. The resultant precipitate collected by low speed centrifugation was dissolved in 0.5 M acetic acid and dialyzed against 0.02 mol/l Na₂HPO₄ (pH 8.0) to inactivate pepsin. After several changes of 0.02 mol/l Na₂HPO₄, the precipitate was collected by low speed centrifugation and then dissolved in 0.5 M acetic acid, dialysed against 0.1 M acetic acid for 2 days and lyophilized.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as previously described by Laemmli [22], using a discontinuous Tris-HCl/glycine buffer system with 7.5% resolving gel and 4% stacking gel. The collagen samples were



Fig. 1 (A): It shows live sea cucumber *Bohadshia* spp acquire diffused spicules with brownish colour spots on skin resemble this specie with *Bohadschia Bivittata*

dissolved in a sample buffer (0.06 M Tris-HCl, pH 6.8, containing 2% SDS, 25% glycerol, 0.1% bromophenol blue) and then boiled for 3 min. Electrophoresis was conducted using the Mini PROTEAN 3 Cell (Bio-Rad Laboratories Inc., Richmond, CA) at 120 V. After electrophoresis, gels were stained for 30 minutes with 0.1% Coomassie brilliant blue R-250 solution followed by destaining in a solution containing distilled water, methanol, and acetic acid at a ratio of 8:1:1 (v/v/v). SDS, glycerol, bromophenol blue, Coomassie brilliant blue R-250, and SDS-PAGE standards were purchased from Bio-Rad Laboratories [10, 23].

RESULTS AND DISCUSSION

PSC was successfully isolated with highest 65% yield from disaggregated crude collagen fibrils, extracted from 100 g body wall pieces of *Bohadschia* spp. Figure 1 shows live sea cucumber *Bohadschia* spp and its body wall, whereas figure 2 (a) and 2 (b) shows steps of crude collagen preparation. Figure 2 (c) and 2 (d) shows precipitate containing crude collagen fibres and after freeze dried respectively. Figure 3 shows lyophilized PSC.

Figure 4 shows SDS-PAGE patterns of PSC collagen from the body wall of sea cucumber *Bohadschia* spp that has eletrophoretic pattern of type 1 collagen consisting of major component α_1 of approximately 138 kDa and small amount of β dimmers. The SDS-PAGE patterns (α_1 and β dimer) of the PSC from *Bohadschia* spp were similar to those reported for collagens from other sea cucumber species (*Cucumaria frondosa* and *Parastichopus californicus*) [9, 10].

Currently, the vast majority of collagen for research and commercial use are fabricated from animal tissue derivatives. Extraction from animal tissues often involves one of the following standard techniques [24] i.e. pepsin digestion: to release soluble monomeric atelocollagen that is devoid of terminal telopeptides [25]. The other technique on acid solubilization: to liberate monomeric tropocollagen with telopeptides intact [26].

Matsumura [27] originally showed that whole collagen fibrils could be isolated from sea cucumbers and starfish by exposure of tissues to a disaggregating solution containing 0.5 M NaCl, 0.2 M 2-mercaptoethanol, 0.05 M EDTA, 0.1 M Tris HCl, pH 8.0. Mastumara also reported that EDTA was unnecessary for the disaggregation of holothuroid dermis. In the present study, it was found that tissues begin to disaggregate in the EDTA solution. This result was consistent with Trotter and Cui [9, 21]. Incubation of sea cucumber body wall sequentially in water, EDTA, and water, extracted disaggregated crude collagen fibres. The method of exposure of sea cucumber body wall to water following chelation of divalent cations suggested the electrostatic interactions to be important in the maintenance of tissue integrity.

In the present study PSC collagen was isolated with maximum of 65% yield on (dry weight basis), which is higher than the PSC collagen isolated from the body wall of sea cucumber (*Stichopus japonicas*) which was 26.6% [17] and from the body wall of giant red sea cucumber 20% [10] as shown in table 1.



Fig. 1 (B): It shows the body wall of sea cucumber *Bohadschia* spp.



Fig. 2 (A): It shows the cutting of sea cucumber body wall into small pieces and weighing them.



Fig. 2 (B): It shows the stirring of body wall pieces in 1 L of 4 mM EDTA (EDTA), 0.1 M Tris-HCl, pH 8.0.

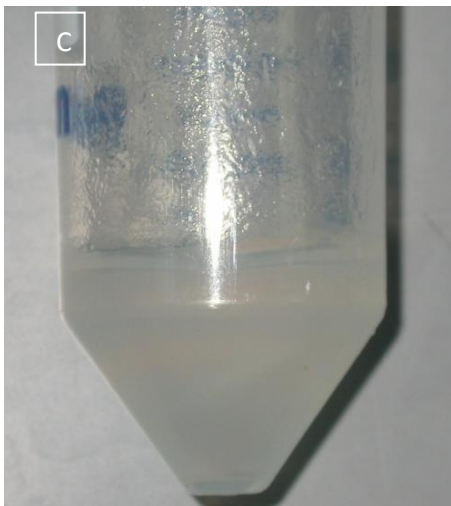


Fig. 2 (C): It shows the precipitate containing crude collagen fibrils.

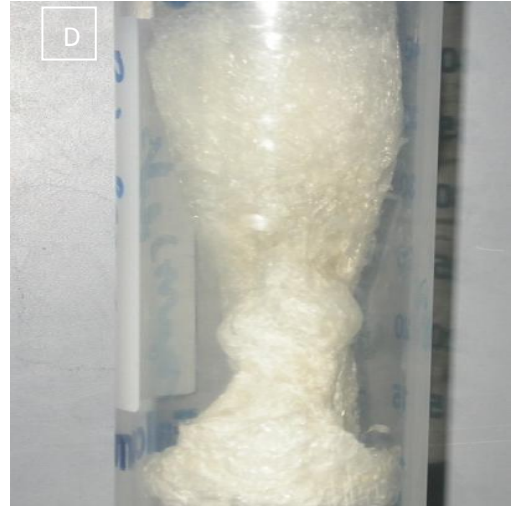


Fig. 2 (D): It shows freeze dried crude collagen.

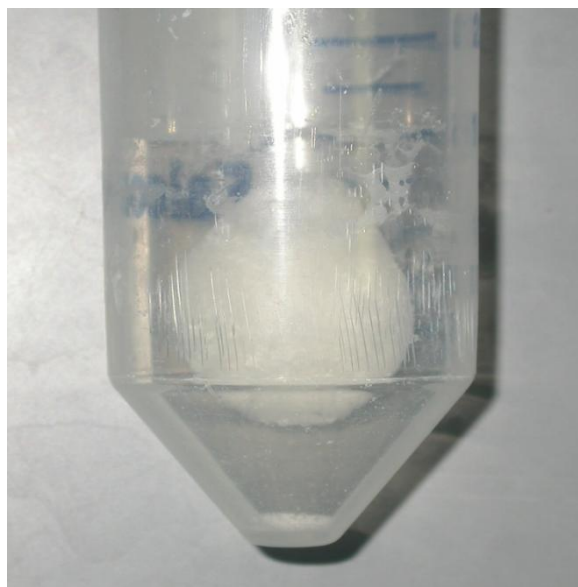


Fig. 3: It shows freeze dried PSC collagen sample.

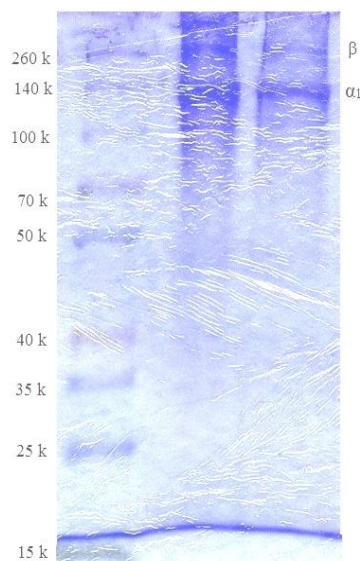


Fig. 4: SDSPAGE pattern of PSC collagen Lane 1: Protein marker; Lane 2 and 3: Pepsin – Soluble collagen.

Table 1: Shows the PSC collagen yield (on dry weight basis) from different species of sea cucumber using 100 g of body wall as starting material.

Sea cucumber species	Source	% PSC Yield	Author
Stichopus japonicus	Body wall	20.8	[10].
Stichopus japonicus	Body wall	26.6	[17].
Bohadschia spp	Body wall	65	Our study

We assumed, high yield of PSC collagen from the body wall of *Bohadschia* spp could be due to thick body wall of this species, which might contain high amount of collagen protein. Wen., *et al* [28] studied the chemical composition and nutritional quality of eight common sea cucumbers (*Stichopus hermanni*, *Thelenotaananas*, *Thelenota anax*, *Holothuria fuscogilva*, *Holothuria fuscopunctata*, *Actinopyga mauritiana*, *Actinopyga caerulea* and *Bohadschia argus*), among these species crude protein content was the highest in *B. argus* (62.1%), which is one of *Bohadschia* spp. Crude collagen fibril (Tropocollagen) at its both ends surrounded by telopeptide C and N, which makes collagen less soluble under acidic condition. Such cross linkages could be removed by pepsin, which produce a formation of atelocollagen (without telopeptide) without changing the integrity of triple helix [10]. In our study porcine pepsin was used, due to unavailability of pepsin from other sources. PSC collagen is purified and solubilized form of crude collagen. Collagen from body wall could not be solubilized by limited pepsin digestion at all. This is probably due to the occurrence of glycosaminoglycan and other non-collagenous material, which is widely distributed between collagen fiber bundles and between collagen fibres [29]. On the other hand they were completely dispersed into fibrils by treatment with the disaggregating solution to give an extremely viscous suspension. After treatment with 0.1 M NaOH, these disaggregated fibrils were found to be completely solubilized by pepsin digestion under vigorous stirring to form a highly viscous solution and the solubilized collagen was easily isolated by selective precipitation with 0.8 M NaCl. The effect of pepsin on solubilization of body wall collagen was strongly dependent on the degree of stirring, under gentle stirring about 90% of the disaggregated fibril remain intact [7]. Collagen yield by using PSC method was higher (20.8%) than collagen yield by using acid solubilized collagen method 3.4% [10]. The differences in yields suggest that interchain cross-linkages exist in the telopeptide region of the collagen, which makes the collagen less soluble under an acidic condition. Therefore, increased yield of collagen from skin of giant red sea cucumber was observed using pepsin digestion procedures [10]. During collagen purification it is required to eliminate the antigenic components of the protein, represented by the telopeptide fragments regions of collagen type I. Such purification that is more efficient after treatment with pepsin

[30]. In commercial usage atelocollagen (without telopeptides) is preferred due to the associated cross-species antigenicity of the p-determinant located in the telopeptides of animal-derived collagen [31]. However, PSC collagen extracted from animal sources presents only a small degree of antigenicity, and is therefore considered acceptable for tissue engineering in humans [30].

Future scope of *Bohadschia* spp collagen

Collagen from the body wall of sea cucumber *Bohadschia* spp could replace mammalian collagen due to its: abundance of collagen protein in its body wall, high collagen yields, lack of dietary restrictions and lack of transmissible diseases which is one of drawback of mammalian collagen, i.e bovine spongiform encephalopathy (mad cow disease), ovine and caprine scrapie, and other zoonoses for collagen products of bovine origin and other animal sources as well [32]. Thus collagen obtained from *Bohadschia* spp could be used as potent biomaterial and utilized in pharmaceutical and nutraceutical industries.

Crude form of collagen could be utilized in cosmetic products for the nourishment of skin as major portion of skin composed of type 1 collagen. It could be used in food industries and pharmaceutical industries in the gelatin and tabulated form.

PSC collagen due to its better solubility could be used in field of tissue engineering. It could be used as barrier membrane and as a scaffold in many areas such as: Maxillofacial surgeries, Periodontology, Orthopedics and Rheumatology practice.

It may promote osteogenic cell differentiation and proliferation which will offer new avenue for the treatment of bone defects, i.e., promoter in fracture healing, bone induction agent for incorporation in cement composites used in skeletal reconstruction and joint replacement to further stimulate osteogenesis and cytotoxic effect on bone tumor. This requires further research.

CONCLUSION

PSC isolated from body wall of sea cucumber *Bohadschia* spp exhibits higher yield than other species of sea cucumber and

classified as type I collagen with molecular composition (α_1)₃. This collagen could be alternative source to mammalian collagen and have potential uses in pharmaceutical and nutraceuticals industries in addition to its future scope in the field of tissue engineering.

ACKNOWLEDGMENT

This work is supported under USM short-term grant No. 304/PPSG/6139040. The authors gratefully acknowledge the support from Malaysian Fishery Development of the coastal areas of Perhentian Island, Terengganu.

REFERENCES

- Venugopal, V., Marine products for healthcare: functional and bioactive nutraceutical compounds from the ocean. CRC press 2008; 13.
- Mehmet, A.H., et al., Proximate composition and fatty acid profile of three different fresh and dried commercial sea cucumbers from Turkey. International Journal of Food Science & Technology, 2011; 46 (3): 500-508.
- Fredalina, B.D., et al., Fatty acid compositions in local sea cucumber, *Stichopus chloronotus*, for wound healing. Gen Pharmacol, 1999; 33(4): 337-40.
- Kittiphattanabawon, P., et al., Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*). Food Chemistry, 2005; 89(3): 363-372.
- Kadler, K.E., et al., Collagen fibril formation. Biochemical Journal, 1996; 316 (1): 1.
- Nishimura, T., A. Hattori, and K. Takahashi, Structural weakening of intramuscular connective tissue during conditioning of beef. Meat science, 1995; 39(1): 127-133.
- Saito, M., et al., Collagen as the major edible component of sea cucumber (*Stichopus japonicus*). Journal of Food Science, 2002; 67(4): 1319-1322.
- Chen, J., Overview of sea cucumber farming and sea ranching practices in China. SPC beche-de-mer Information Bulletin, 2003; 18: 18-23.
- Trotter, J.A., et al., Covalent composition of collagen fibrils from the dermis of the sea cucumber, *Cucumaria frondosa*, a tissue with mutable mechanical properties. Comparative Biochemistry and Physiology Part A: Physiology, 1995; 112 (3-4): 463-478.
- Liu, Z., A.C.M. Oliveira, and Y.C. Su, Purification and characterization of pepsin-solubilized collagen from skin and connective tissue of giant red sea cucumber (*Parastichopus californicus*). Journal of Agricultural and Food Chemistry, 2009; 58(2): 1270-1274.
- Sundarand, W.A., Isolation, purification and medium optimization of lipase enzyme producing strains of *aspergillus niger* isolated from natural sources. International Journal of Pharmacy and Pharmaceutical Sciences, 2013; 5 (2).
- Zhang, Y., et al., Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (*Ctenopharyngodon idella*). Food Chemistry, 2007; 103(3): 906-912.
- Jongjareonrak, A., Characterization and functional properties of collagen and gelatin from bigeye snapper (*Priacanthus macracanthus*) and brown stripe Red Snapper (*Lutjanus vitta*) skins. Prince of Songkla University, 2006; 155.
- Nagai, T. and N. Suzuki, Isolation of collagen from fish waste material skin, bone and fins. Food Chemistry, 2000; 68(3): 277-281.
- Noitup, P., W. Garnjanagoonchorn, and M.T. Morrissey, Fish skin type I collagen. Journal of aquatic food product technology, 2005; 14(1): 17-28.
- Ogawa, M., et al., Biochemical properties of black drum and sheepshead seabream skin collagen. Journal of Agricultural and Food Chemistry, 2003; 51(27): 8088-8092.
- Park, S.Y., et al., Pepsin-solubilized collagen (PSC) from Red Sea cucumber (*Stichopus japonicus*) regulates cell cycle and the fibronectin synthesis in HaCat cell migration. Food Chemistry, 2011; 1132.
- Sadowska, M., I. Kolodziejska, and C. Niecikowska, Isolation of collagen from the skins of Baltic cod (*Gadus morhua*). Food Chemistry, 2003; 81(2): 257-262.
- Liu, H.Y., D. Li, and S.D. Guo, Studies on collagen from the skin of channel catfish (*Ictalurus punctatus*). Food Chemistry, 2007; 101(2): 621-625.
- Clouse, R., D. Janies, and A.M. Kerr, Resurrection of *Bohadschia bivittata* from *B. marmorata* (Holothuroidea: Holothuriidae) based on behavioral, morphological, and mitochondrial DNA evidence. Zoology, 2005; 108(1): 27-39.
- Cui, F., et al., Characterization and subunit composition of collagen from the body wall of sea cucumber *Stichopus japonicus*. Food Chemistry, 2007; 100(3): 1120-1125.
- Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 1970; 227(5259): 680-685.
- Bama, P., et al., Extraction of collagen from cat fish (*Tachysurus maculatus*) by pepsin digestion and preparation and characterization of collagen chitosan sheet. Int J Pharm Pharm Sci, 2013; 2(4): 133-137.
- Walton, R.S., D.D. Brand, and J.T. Czernuszka, Influence of telopeptides, fibrils and crosslinking on physicochemical properties of Type I collagen films. Journal of Materials Science, Materials in Medicine, 2010; 21(2): 451-461.
- George, J., J. Onodera, and T. Miyata, Biodegradable honeycomb collagen scaffold for dermal tissue engineering. Journal of Biomedical Materials Research Part A, 2008; 87(4): 1103-1111.
- Gross, J. and D. Kirk, The heat precipitation of collagen from neutral salt solutions: some rate-regulating factors. Journal of Biological Chemistry, 1958; 233(2): 355.
- Matsumura, T., M. Shinmei, and Y. Nagai, Disaggregation of connective tissue: preparation of fibrous components from sea cucumber body wall and calf skin. Journal of Biochemistry, 1973; 73(1): 155-162.
- Wen, J., C. Hu, and S. Fan, Chemical composition and nutritional quality of sea cucumbers. Journal of the Science of Food and Agriculture, 2010; 90(14): 2469-2474.
- Kariya, Y., et al., Glycosaminoglycan involved in the cation-induced change of body wall structure of sea cucumber *Stichopus japonicus*. Connective Tissue Research, 1990; 25(2): 149-159.
- Xiong, X., New insights into structure and function of type I collagen. 2008.
- Lynn, A., I. Yannas, and W. Bonfield, Antigenicity and immunogenicity of collagen. Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2004; 71(2): 343-354.
- Jongjareonrak, A., et al., Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of Brownstripe red snapper (*Lutjanus vitta*). Food Chemistry, 2005; 93(3): 475-484.