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Extraction, Purification and Physical Characterization of Collagen from Body wall of Sea cucumber *Bohadschia bivittata*

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ABSTRACT: The body wall of sea cucumber, *Bohadschia bivittata*, was studied with respect to its collagen protein. About 70% of the total body wall protein was accounted for by highly insoluble collagen fibers. Aim of this study was to isolate pepsin-solubilized collagen (PSC) from crude collagen fibres, extracted from body wall of sea cucumber *Bohadschia bivittata*, and also to investigate 67 nm axial repeat *D*-banding of collagen fibrils, which characterizes collagen. Body wall of *Bohadshia bivittata* 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. The ultra structures were analyzed by using Quanta™ Scanning Electron Microscope, Netherlands, under 130 Pa pressure, at a working distance of 6-10 mm at 4.0 to 5.0 kV from magnification 5,000X to 100,000X. Pepsin-solubilized collagen was successfully isolated from disaggregated crude collagen fibres, with 65% yield. SEM analysis of crude collagen displayed a regularly repeated striated pattern, whereas, PSC displayed non-banded sheet like fibers with twisted configuration. In conclusion high yield of Pepsin-solubilized collagen was successfully isolated, and evidence of *D*-periodic bands confirms it as fibrillar collagen. This collagen could be useful as an alternative to mammalian collagen in the nutraceutical and pharmaceutical industries.

Key words: Sea cucumber, *Bohadschia Bivitatta*, Pepsin solubilized collagen (PSC), Scanning electron microscope (SEM).

Introduction

Sea cucumber (*Echinodermata: Holothuroidea*), in wet or dried form, has been used for dietary delicacy and medicinal cure in Asians over many centuries. It has high commercial value, with increasing global production and world trade. In Malaysia, sea cucumber generally known as “Gamat” is a dietary delicacy and a traditional remedy for healing various internal and external wounds. Various bioactive compounds have recently been identified from sea cucumbers, such as, Frondanol A5 from *Cucumaria frondosa* for its chemo preventative effects (Janakiram *et al.*, 2010), Mucopolysaccharides from *Stichopus japonicus* for anticancer effects (Lu *et al.*, 2010), Sulphated polysaccharides from *S. japonicus* for proliferation neural stem/progenitor cells (Zhang *et al.*, 2010), Saponins from *Pentacta quadrangularis* for anti-angiogenic effect (Tian *et al.*, 2005), and Pepsin-Solubilized Collagen (PSC) from *S. japonicas* to improve proliferation of human keratinocytes (Park *et al.*, 2011).

Collagen is an abundant protein in animal tissues and has a wide range of applications in the biomedical, pharmaceutical, cosmetic, and food industries (Kittiphattanabawon *et al.*, 2005). Body wall of sea cucumber (*S. japonicas*) contain about 70% protein consists of highly insoluble collagen fibers (Saito *et al.*, 2002). It is distinct from other proteins in that the molecule comprises three polypeptide chains (*a*-chains), which form a unique triple helical structure. In animal tissue more than 20 genetically distinct collagens exist. Collagen types I, II, III, V and XI (fibrillar collagen) are self-assemble into *D*-periodic cross-striated fibrils (where *D* = 67 nm), the characteristic axial periodicity of collagen, and collectively are the most abundant collagens found in vertebrates. (Lingham-Soliar and Wesley-Smith, 2008; Chapman *et al.*, 1990). Among all fibrillar collagens, type I collagen is the most abundantly found in vertebrates (Kadler *et al.*, 1996).

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 (2003), 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* (Saito *et al.*, 2002), and *Cucumaria frondosa* (Trotter *et al.*, 1995; Liu *et al.*, 2009).

Three known methods of collagen extraction produce, neutral salt-solubilized collagen, acid-solubilized collagen and pepsin-solubilized collagen (Zhang *et al.*, 2007). Many researchers have studied the pepsin-solubilized collagen (PSC) method from different sources, such as from the skin of brownstripe red snapper (Jongjareonrak, 2006), fish waste material (Nagai and Suzuki, 2000), albacore tuna and silver-line grunt skin (Noitup *et al.*, 2005), bone and scale of black drum and sheepshead seabream (Ogawa *et al.*, 2003), Pepsin-Solubilized Collagen (PSC) from *Stichopus japonicus* (Park *et al.*, 2011) though 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 (Sadowska *et al.*, 2003). 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 (Liu *et al.*, 2007).

Thus, animal from marine environment, *Bohadschia" bivittata* was selected for study. It is one of sea cucumber specie live in deep sea water, presented itself with thick body wall, brownish and yellowish exterior (Clouse *et al.*, 2005).

In the present work, the aim of this study was to extract crude collagen and isolate PSC collagen from *Bohadschia bivittata*, and also to find out the evidence for the fibril axial band periodicity that characterizes collagen.

Materials and Methods

Prior permission was obtained from the Malaysian Fishery Development off the coastal areas of perhentian island, Terengganu, Malaysia. Two fresh samples of *Bohadschia* spp weighing between 700-800g were handpicked by the divers. Identification of sea cucumber specie was based on the surface morphology and color of *Bohadschia Bivittata* according to Clouse (2005). The body wall of *Bohadschia Bivittata* was dissected free adherent tissues, cut into small pieces (about 2 cm × 2 cm), and stored in small container filled with phosphate buffer saline (PBS). Body wall small pieces were

transported to the lab under ice pack to maintain 4⁰C, and stored under -80⁰C until analysis. The collagen from body wall of *Bohadschia Bivittata* was extracted in the forms of crude and pepsin solubilized collagen (PSC) by method previously described by Cui and Park. (Cui *et al.*, 2007; Park *et al.*, 2011).

Preparation of crude collagen fibrils

All procedures were performed at 4 °C. Body wall small pieces were washed extensively with distilled water. After the samples (100 g wet weight) stir in 1 L of distilled water for 30 min, the water was replaced and the extraction in water was repeated once for 1 h. The water was replaced with 1 L of 4 mM ethylenediaminetetraacetic acid (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 was stirred slowly for 15 mins and the washing steps was repeated twice more. The liquid then replaced with 500 ml of fresh distilled water and stirred for 2 days. The mixture was centrifuged at 9000g speed for 5 min. 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 was repeated. The supernatant was centrifuged at 10,000g for 30 min 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 9000g for 60 min and then the pepsin-solubilized collagen (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 dialysed 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.

Scanning Electron Microscopy (SEM)

Freeze dried crude and PSC collagen sponge were cut into small pieces. An adhesive, double coated conductive carbon tape was applied on the sample holder/stub surface in order to hold the sample in place. Then, the dried samples were coated with a thin layer of gold in a vacuum evaporator (Leica EM SCD50 Sputter Coater, Germany) for viewing on a Quanta™ Scanning Electron Microscope, Netherlands under 130 Pa pressure, using the Everhart-Thornley Detector (ETD), at a working distance of 6-10 mm at 4.0 to 5.0 kV from magnification 5,000X to 100,000X. All procedures were performed in School of Health Sciences, Universiti Sains Malaysia.

Results

Pepsin-solubilized collagen (PSC) was successfully isolated with highest 65% yield from disaggregated crude collagen fibrils, extracted from 100 g bodywall pieces of *Bohadschia Bivittata*. Figure 1 a, and 1 b showed precipitate containing crude collagen fibres and after freeze dried. Figure 2 showed lyophilized pepsin-solubilized collagen (PSC).

SEM analysis of crude collagen from *Bohadshia bivittata*, showed banded structures on collagen fibrils. Fig 3 a and b showed the banded pattern on single fibril of crude collagen by SEM (magnification 100,000X) and bundles of circumference fibrils cross linking each other with dense banded structures (magnification 30,000X). The irregular, wavy collagen fibers arranged singly or in small groups is quite evident in this picture. SEM analysis of PSC showed no banded structures on fibrils. Fig 4 a, showed the SEM analysis of single PSC collagen fibril (magnification 16,000X) with no banded structure on it, whereas Fig. 4 b, showed flat sheets and some flat interconnecting sheet like fibrils (magnification 10,000X).



Fig. 1 (a): Precipitate containing crude collagen fibrils.



Fig. 1 (b): Freeze dried crude collagen.



Fig. 2: Freeze dried PSC collagen

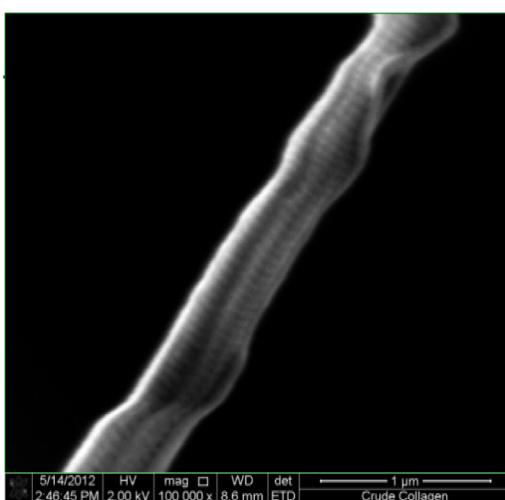


Fig. 3 (a): showing SEM analysis of single crude collagen fibril, showing banded structures on surface.

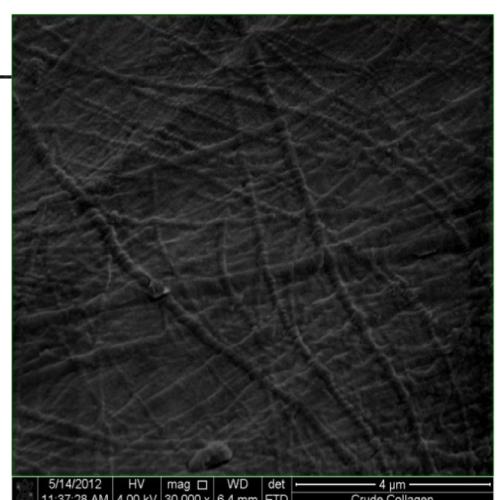


Fig. 3 (b): showing SEM analysis of crude collagen fibril bundles, showing banded structures on surface.

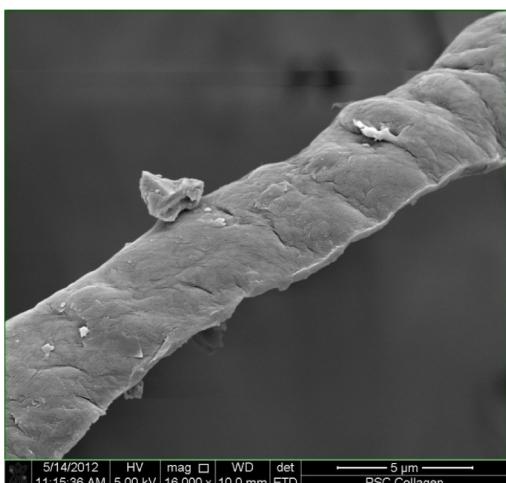


Fig. 4 (c): showing SEM analysis of single PSC collagen fibril showing no banded structures.

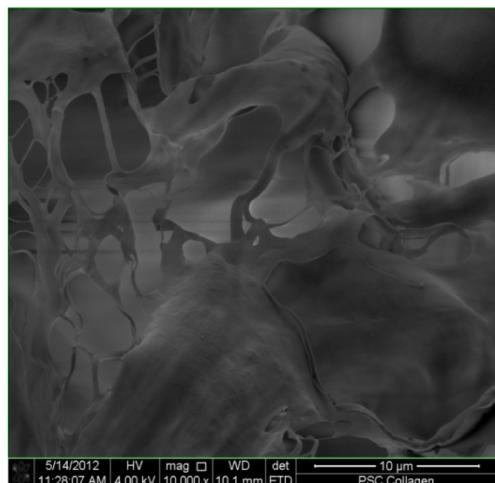


Fig. 4 (d): showing SEM analysis of multiple PSC collagen fibril showing no banded structures.

Discussions

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 (Walton *et al.*, 2010), i.e. pepsin digestion, which is to release soluble monomeric atelocollagen that is devoid of terminal telopeptides (George *et al.*, 2008). The other technique on acid solubilization is to liberate monomeric tropocollagen with telopeptides intact (Gross and Kirk, 1958).

Matsumura *et al.* (1973) 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 present study, it was found that tissues begin to disaggregate in the EDTA solution, this result was consistent with Trotter *et al.*, 1995 and Cui *et al.*, 2007. 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 a maximum of 65% yield on (dry weight basis), which is higher than the PSC collagen isolated from the bodywall of sea

cucumber (*Stichopus japonicas*) which was 26.6% (Park *et al.*, 2011) and from the bodywall of giant red sea cucumber 20% (Liu *et al.*, 2009). The 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* (2010) studied the chemical composition and nutritional quality of eight common sea cucumbers (*Stichopus herrmanni*, *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* sp. 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 (Liu *et al.*, 2009). 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 (Kariya *et al.*, 1990). On the other hand, it 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 (Saito *et al.*, 2002). Collagen yield by using pepsin-solubilized (PSC) method was higher (20.8%) than collagen yield by using acid solubilized collagen (ASC) method 3.4% (Liu *et al.*, 2009). The difference in yields suggests 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 (Liu *et al.*, 2009). 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 (Xiong, 2008). 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 (Lynn *et al.*, 2004). However, PSC collagen extracted from animal sources presents only a small degree of antigenicity, and is therefore considered acceptable for tissue engineering in humans (Xiong, 2008).

The interactions between the collagen molecules result in the characteristic quarter repeat of 67nm (*D*-bands) and in a complex cross-striation banding (Cui *et al.*, 2007b), this banding pattern is found in fibrillar collagen, types I, II, III, V and XI self-assemble into *D*-periodic cross-striated fibrils, and collectively are the most abundant collagens found in vertebrates (Kadler *et al.*, 1996). In fibrils prepared for electron microscopy, dehydration leads to lowered (and rather more variable) measured values of *D*, usually in the range 55-65 nm (Chapman *et al.*, 1990). The result of crude collagen was compared with the transmission electron microscope (TEM) picture of collagen from the dermis of sea cucumber, *Cucumaria frondosa* (Trotter *et al.*, 1995). In our study, the same banded structure on collagen fibril was seen in *Bohadshia bivittata* as in *Cucumaria frondosa*. The non-banded collagen structures seen in PSC may be because of limited pepsin digestion which may wash out banded structures. Exposure of collagen solution to pepsin results in removal of C and N telopeptides, assembly being limited to the formation of small non-banded fibrous aggregates (Kadler *et al.*, 1996).

Conclusion

Pepsin-solubilized collagen isolated from body wall of sea cucumber *Bohadschia bivittata* exhibits higher yield than other species of sea cucumber from previous studies. Crude collagen fibril from sea cucumber, *Bohadschia bivittata* showed the typical banded structure, characteristic feature of fibrillar collagen. Collagen in the PSC form showed non-banded fibrils, which could be attributed to the pepsin digestion resulting in washing out of the banded pattern of collagen fibrils. Crude and PSC collagen from *Bohadschia bivittata* 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.

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