

Full Length Research Paper

Isolation, characterization and molecular weight determination of collagen from marine sponge *Spirastrella inconstans* (Dendy)

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Collagen is a major structural protein of connective tissues. It can be used as a prosthetic biomaterial applicable to artificial skin, tendon ligaments and development collagen implants. In the present study, an attempt was made to isolate and characterize collagen from the marine sponge, *Spirastrella inconstans*. The total protein content of sponge collagen was relatively high (32%). While determining the molecular weight of crude and purified collagen through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the crude showed three bands (80, 60 and 59 kDa molecular weight) and purified showed only a single band (58 kDa). The structural properties were analyzed by using fourier transform infra red (FT-IR) spectrum and the stability of collagen was also given the single transition peak in differential scanning calorimetry (DSC). The microstructure of sponge collagen showed highly porous and interconnected scaffolds in scanning electron microscopic (SEM) analysis.

Key words: Collagen, *Spirastrella inconstans*, SDS-PAGE, Fourier transform infrared (FT-IR), differential scanning calorimetry (DSC), scanning electron microscopy (SEM).

INTRODUCTION

Collagen is a fibrous protein found ubiquitously in all multicellular animals. It is particularly rigid and inextensible extracellular matrix protein that serves as a major constituent of many connective tissues. It is distributed in skin, bones, cartilage, tendons, ligaments, blood vessels, teeth, cornea and all other organs of vertebrates and constitutes approximately 30% of total

animal protein (Muyonga et al., 2004; Senaratne et al., 2006). The characteristic feature of a typical collagen is long, stiff, triple-stranded helix, in which three collagen polypeptide chains are wrapped around one another in the form of a rope-like upper helix. These peptides are extremely rich in glycine and proline, both are responsible for the formation of the collagen-specific helical structure (Lehninger and Biochemie, 1987; Alberts et al., 1994; Rossler et al., 1995; Zubay et al., 1998). Collagen has a wide range of applications in leather and film industries, pharmaceutical and biomedical fields. The food utilization

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of collagen, in pharmaceutical applications, includes production of wound dressings, and vitreous implants and it also act as carrier in drug delivery. Moreover, collagen is used for the production of cosmetics because it has a good moisturizing property (Swatschek et al., 2002). Collagen is extracted mainly from the skin and bones of ruminant and porcine livestock and poultry (Vollmer and Rosenfield, 1983; Pachence, 1992; Morimura et al., 2002). However, foot-and-mouth disease, bovine spongiform encephalopathy and avian influenza have broken out frequently in recent years (Helcke, 2000; Trevitt and Singh, 2003) and hence the interest in safer sources of collagen including marine animals (fish, jellyfish, marine sponge and squid) and those living in high and cold regions without environmental pollution has greatly increased (Kolodziejska et al., 1999; Nagai et al., 2000; Swatschek et al., 2002; Sadowska et al., 2003). In addition, the collagen extracted from porcine sources cannot be used as a component of some foods, due to religious barriers. Therefore, alternative sources of collagen should be sought. Scientists have found that skin, bone, scale, fin and cartilage of freshwater and marine fish, scallop mantle (Shen et al., 2007), adductor of pearl oyster (Mizuta et al., 2002a) and the muscle layer of the ascidian (Mizuta et al., 2002b) can be used as new sources of collagen.

Furthermore, the collagen in the form of sponge is useful in the treatment of different wounds, such as pressure sores, donor sites, leg ulcers and decubitus ulcers, as it adheres well to wet wounds, absorbs large quantities of tissue exudates, preserve a moist environment, and encourages the formation of new granulation tissue and epithelium on the wound (Chvapil, 1982; Gorham, 1991). Hence, an attempt has been made to isolate, characterize and determine the molecular weight of the collagen from a marine sponge *Spirastrella inconstans*. The structural property of sponge collagen was also studied through FT-IR, DSC and SEM.

MATERIALS AND METHODS

Sample collection

The sponge *S. inconstans* was collected from Devipattinam (Lat. 9° 28' 60" N; Long. 78° 54' 0" E.) at 5-6 meter depth by SCUBA diving along the Southeast coast of India. The sponge was washed with seawater, tap water and then distilled water. The sample was immediately stored in 100% methanol and brought to the laboratory for further study.

Isolation of sponge collagen

Collagen was isolated by following the method of Diehl-Seifer et al. (1985). The methanol-conserved sponge materials were washed three times with tap water and distilled water, finally cut into small

pieces and homogenized using homogenizer (Remi, RQ-127A, India). 250 ml of 100 mM Tris-HCl buffer (pH 9.5; 10 mM EDTA; 8 M urea; 100 mM β -mercaptoethanol) were added. The pH of the resulting dark-coloured dispersion was raised from pH 7 to 9 with dilute NaOH. After 24 h of continuous stirring at room temperature, the viscous extract was centrifuged (5000 \times g for 5 min at 2°C) (Remi C- 24 BL, India). The pellet was discarded and collagen was precipitated from the supernatant by adjusting the pH to 4 with glacial CH_3COOH and collected by centrifugation (20,000 \times g, for 30 min at 2°C). The pellet was resuspended in distilled water, centrifuged (20,000 \times g, for 30 min at 2°C) and freeze-dried (Pnequin classic plus, Lark, India).

Purification of crude collagen

The collagen was purified with little modification of the protocol followed by Saravanan et al. (2009) through Sephadex G-50 column chromatography. The column (1.5 \times 50 cm) was eluted with 0.1, 0.2, 0.4 and 0.6 M phosphate buffer saline (pH 7.4) with a flow rate of 0.33 ml/min and the fractions were collected. The active fractions were pooled and loaded in a dialysis membrane and dialyzed against double distilled water at 4°C for 12 h and freeze-dried.

Characterization of sponge collagen

Total protein estimation and its homogeneity

The total protein concentration was estimated by Lowry et al. (1951) method. The molecular weight of the collagen was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and compared with molecular marker - Sigma (Sambrook and Russel, 2001).

FT-IR spectrum analysis

FT-IR spectroscopy of freeze-dried sample of sponge collagen was relied on a Bio-Rad FT-IR – 40, USA. Sample (10 mg) was mixed with 100 mg of dried potassium bromide (KBr) and compressed to prepare as a salt disc (10 mm in diameter) for reading the spectrum further. Spectra were collected between wave numbers of 4000 and 500 cm^{-1} which was compared with standard collagen (Sigma).

DSC measurement

The shrinkage temperature (T_s) of collagen sponge matrix indicating the resistance against thermal denaturation was determined by differential scanning calorimetry (DSC 200 F3 NETZSC H). The sponge collagen sample was taken and hermetically sealed in aluminum pans. It was then heated at the rate of 2°C/ min in the temperature range of 0 to 200°C with an empty aluminum pan as the reference probe. T_s were determined as the onset value of the occurring of endothermic peak and the value of shrinkage enthalpy (ΔH_s) were calculated with respect to the mass of collagen matrix.

SEM analysis

SEM was used to examine the microstructure of the sponge collagen. The collagen sample was cut using a punch and fixed to

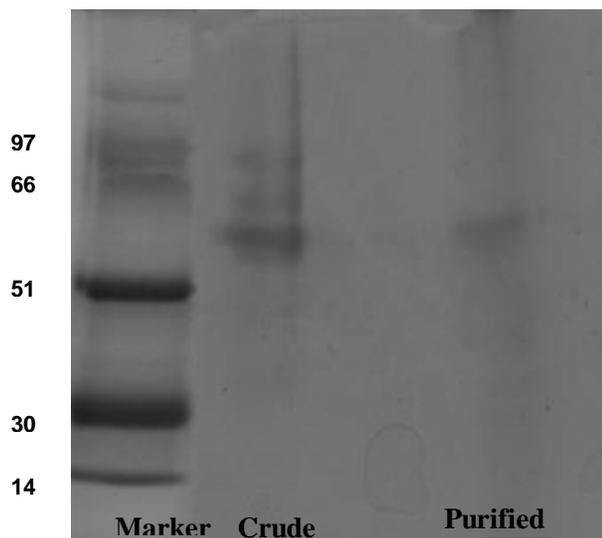


Figure 1. SDS-PAGE of *S. inconstans*.

an adhesive carbon stub. Imaging was carried out using a Tabletop SEM (Hitachi High-Technologies Corp., Japan) operated at 15 kV.

RESULTS

Total protein and collagen content

In *S. inconstans*, the total protein content corresponds to 32% and the yield of collagen was found to be 0.16%. The sponge collagen was examined by SDS-PAGE, using a 12% resolving gel and the molecular weight of purified collagen from *S. inconstans* was recorded as 58 kDa (Figure 1). In the present study, the isolated crude collagen showed three bands with 80, 60 and 59 kDa molecular weight; whereas the purified collagen recorded only a single band with 58 kDa molecular weight in SDS-PAGE.

FT-IR spectral analysis

The FT-IR spectrum of sponge collagen recorded 11 peaks (Figure 2) whereas standard collagen represented 17 peaks (Figure 3). The spectral peak assignment of sponge collagen and standard collagen is given in Table 1. From the FT-IR spectrum, amide A band was found at 3400 cm^{-1} which showed that the NH groups are involved in the hydrogen bonds. The amide B band was found at 2360 cm^{-1} which was the asymmetrical stretch CH_2 . The amide I band was observed at 1647 cm^{-1} , which was the absorption band of the C=O stretching associated with the secondary structure of the protein. The amide II and amide III absorption was between 1161 and 1471 cm^{-1} .

Therefore, the FT-IR spectrum clearly showed the helical arrangements of the collagen from sponge *S. inconstans*.

DSC measurement

According to the thermal denaturation curve, the denaturation temperature of sponge collagen was found at 70°C (Figure 4). The result shows that the sponge (*S. inconstans*) collagen withstood up to 70°C .

SEM analysis

SEM was performed to characterize the micro-architecture of marine sponges. The analysis of sponge collagen under lower magnification revealed that it was highly porous, interconnected with scaffolds and their surface was rough and uneven and some rod-like spicule structure appeared (Figure 5a, b, c and d). At higher magnification, the particles exhibited a honey-comb-like structure of spongin fibres with a size of the pores on the surface ranging from 2 to $10\text{ }\mu\text{m}$ (Figure 5 e, f, g and h). The fibres of the scaffold contained spicules which were attached or embedded within the fibrous network in a mixture of orientation.

DISCUSSION

Sponges (Porifera) represent the lowest and simplest metazoan phylum still present today. The marine sponge *S. inconstans* could be found ubiquitously in the Bay of Bengal, India and contains a lot of collagen. It is a characteristic feature of collagen from sponges, which is composed of a fibrous organic network (collagen), a non-structural ground substance of a glycoprotein nature and inorganic skeletal components. Isolation of sponge collagen is a new era. Limited studies are available in collagen from sponge because of its low solubility. Nagai et al. (2001) obtained 2% of acid soluble collagen (ASC) and 35% of pepsin soluble collagen (PSC) from the skin of *Sepia lydicus*, which is higher than that of the present observation. Nagai and Suzuki (2000) reported the yield of 5.2% of ASC on a dry weight basis and 50% of PSC from skin of paper nautilus *Argonauta argo*, on the basis of wet weight. The values are high as compared to collagen from *S. inconstans*. This variation in the amount of collagen may be due to the concentration of acetic acid used and reduced solubility of collagen in the extraction solvent.

Mizuta et al. (2003) reported 1.4 and 1.9% of collagen in arm and mantle muscles of *Octopus vulgaris*, which is higher than that of the present study (0.16% collagen). This difference may be due to more skin content of

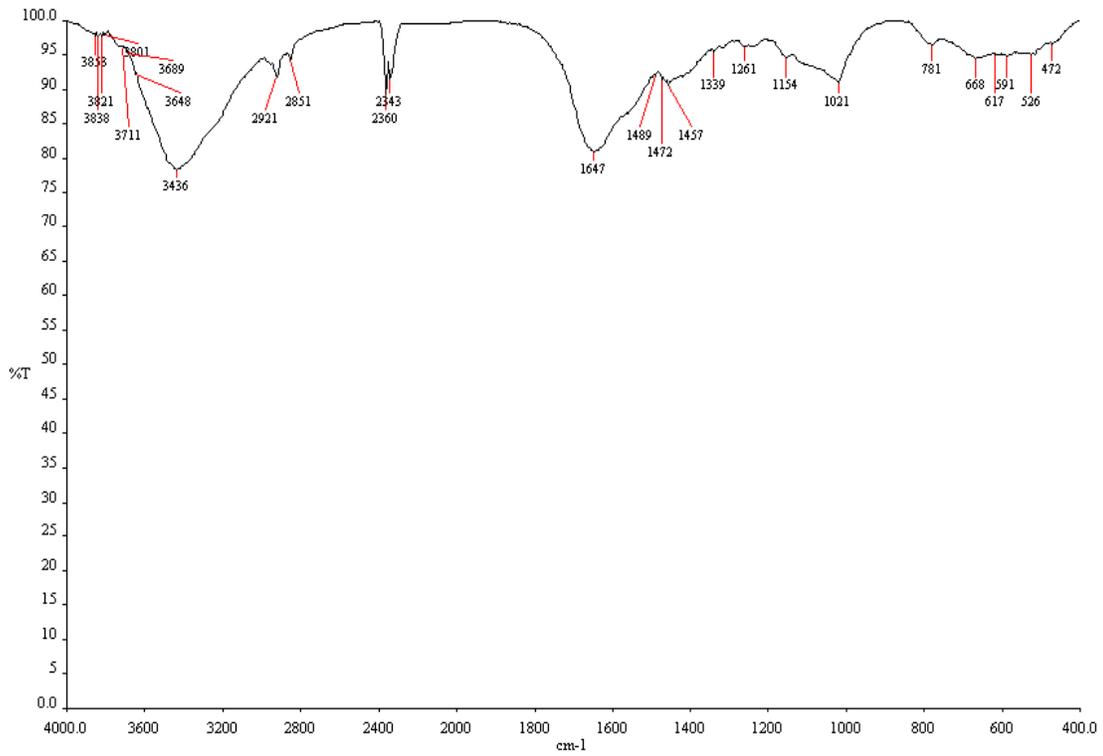


Figure 2. FT-IR spectrum of standard collagen.

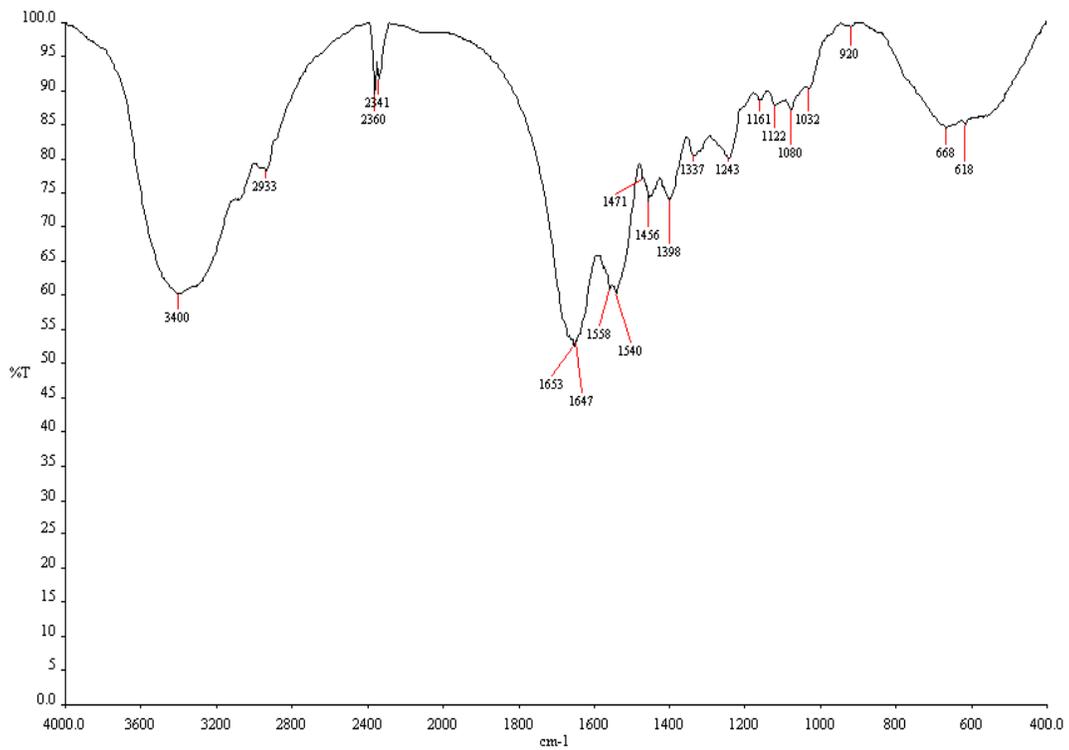
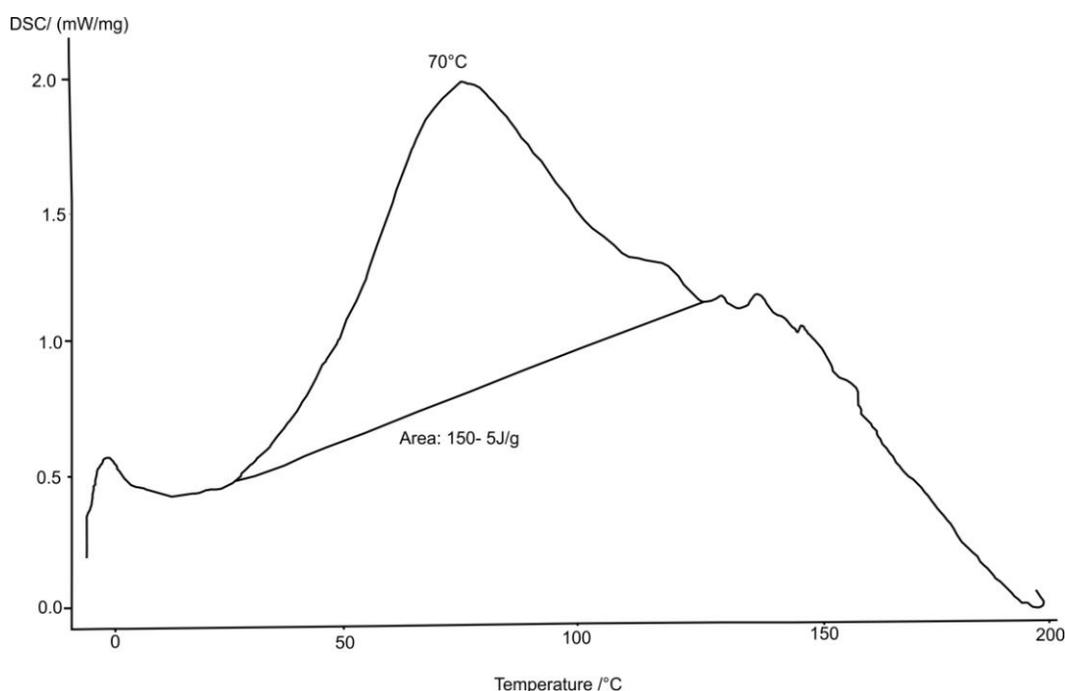


Figure 3. FT-IR spectrum of purified collagen from sponge *S. inconstans*.

Table 1. The FT-IR spectral peak location and assignment sample and standard collagen.

Regions	Standard	Sponge collagen	Assignment
Amide A	3436	3400	NH stretch coupled with hydrogen bond
Amide B	2360	2360	CH ₂ asymmetrical stretch
-	2343	2341	CH ₂ asymmetrical stretch
Amide I	1647	1647	C=O stretch/ hydrogen bond coupled with CN stretch
Amide II	1472	1471	NH bend coupled with CN stretch
-	1457	1456	CH ₂ bend
Amide III	1154	1161	NH bend coupled with CN stretch

**Figure 4.** DSC thermogram of sponge collagen.

cephalopod than sponge which is in good agreement with the result of Mizut a et al. (2003) who 9.1 and 14% of total protein content present in the arm and mantle of *Octopus vulgaris*, respectively. The total protein content of *S. inconstans* was found (34%) to be high when compared to the protein content of *O. vulgaris*. The crude protein content of brown backed toadfish (*Lagocephalus groveri*) skin on dry weight basis was 90.3% (Senaratne et al., 2006). However, the crude protein content on wet weight basis of brown backed toadfish skin was higher than those contained in *S. inconstans*.

Lee et al. (2007) found that the molecular weight of collagen from pig skin (150 to 205 kDa) is high when compared to sponge collagen (58 kDa). The molecular weight of yak bone collagen and its molecular weight

distribution ranged from about 25.3 to 11.7 kDa (Li et al., 2009). Although, much is now known about mammalian adhesion molecules, invertebrate and non-mammalian animal models of cell adhesion have been the focus of cell adhesion research since the turn of the century. An understanding of the events and the evolutionary forces that drive change among adhesion molecules can lead to a better understanding of mammalian adhesion molecules and their mechanisms of action. For example, purified extracellular matrix proteins have been recently studied from a number of non-mammalian or invertebrate species to acquire insight into the functions and evolution of these kinds of molecules (Erickson, 1993). The sponge extracellular matrix is composed of collagen fibrils, proteoglycans, and perhaps structural proteins. It is

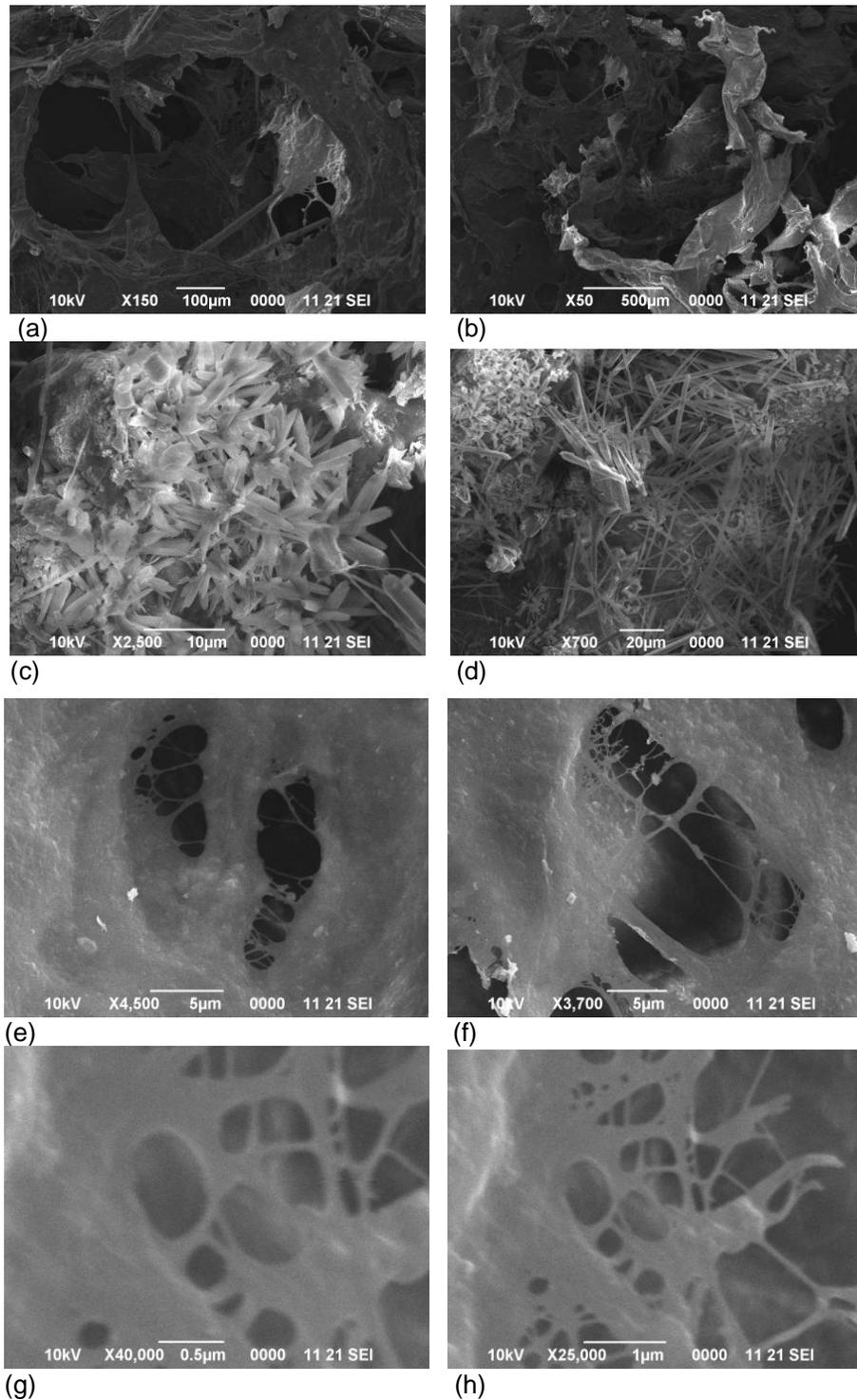


Figure 5. SEM images of collagen isolated from *S. inconstans*.

possible that homologues of the mammalian structural extracellular matrix proteins such as laminin, fibronectin,

vitronectin or fibrinogen that bind to both cell surface receptors, collagen and extracellular matrix proteoglycans

exist in sponges (Martin and Timpl, 1987).

FT-IR investigation shows the existence of helical arrangement of collagen, when compared to acid soluble collagen from walleye pollock skin. The amide A band of walleye pollock skin collagen was found at 3328 cm^{-1} , which shows that there were NH groups involved in hydrogen bonds. The amide B band of collagen was found at 3080 cm^{-1} , which is related to asymmetrical stretch of CH_2 (Muyonga et al., 2004) as well as amide I band and amide III were observed 1648 and 1236 cm^{-1} , respectively (Liu et al., 2007). The pig collagen FT-IR shows amide band at 3337 cm^{-1} . In the present study, the band 3342 cm^{-1} suggesting the presence of hydrogen bond and the amide I band position of pig collagen was found at the range of 1650 to 1655 cm^{-1} , similarly sponge collagen shows 1663 cm^{-1} . The overall FT-IR spectra result shows band at the helical arrangement and their functional were more or less matching with walleye pollock skin collagen and pig collagen species.

The collagen thermal stability is usually characterized by the denaturation temperature (Td) in solution and the shrinkage temperature (Ts) of the fiber. The thermal denaturation profile of sponge collagen has provided useful clues to the thermal stability of collagen. DSC is a well-developed analytical method for the measurement of transitions in polymers and usually used to investigate the thermal stability of collagen. DSC has been used extensively as a sensitive technique to quantify the addition of covalent cross-links and reductions in triple-helical content (Mentink and Hendriks, 2002; Nagai et al., 1999). When hydrated collagen is heated, the crystalline triple helix is transformed into amorphous randomly coiled peptide chains that results in shrinkage of the collagen fibre formed (Nagai et al., 1999; Hormann and Schlebusch, 1971). The thermal denaturation curve of acid-soluble collagen from walleye pollock skin (Td) of collagen was 24.6°C ; lower by about 12°C than that of collagen from porcine skin (Nagai et al., 1999). In the present study, thermal stability of the sponge collagen was found at 70°C , when compared to walleye pollock and porcine collagen and also had more amino acids residues. Denaturation temperature is known to increase with increasing amounts of amino acids residues. The thermal denaturation of collagen is related to the thermal stability of collagen by amino acid content and hydroxproline content (Nagai et al., 1999).

The commercially available collagen sponge (Insat) consists of thick sheets and fibres. Xiu et al. (2009) reported that the lyophilized *Sipunculida* collagen had a loose porous structure and they found that the pore size of the collagen was increased due to the water content. Lin et al. (2011) found that the SEM revealed that the sponge skeleton possessed a collagenous fibrous network consisting of interconnecting channels and a

porous structure that support cellular adhesion, aggregation and growth. In the present study, the SEM images of sponge collagen demonstrated that all composites displayed an open and interconnected pore structure and the spicules appeared rod like structure. The chemotactic properties of collagen have many advantages in tissue engineering scaffolds (Postlethwaite et al., 1978). The sponge collagenous poriferan has provided a natural environment for cellular attachment and aggregation due to their connective tissue; it should be more complex organisms and it analogous to collagen type XIII (Green et al., 2003).

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