ISOLATION AND PHYSICOCHEMICAL PROPERTIES EVALUATION OF PEPSIN-SOLUBILISED COLLAGEN AND ITS HYDROLYSATE FROM THE INTEGUMENT OF SEA CUCUMBER (*Stichopus vastus*)

by

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DEDICATED
TO
MY BELOVED FAMILY

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LIST OF SYMBOLS

Å	Angstrom
α	Alpha
β	Beta
λ	Gamma
Δ	Delta
%	Percentage
°C	Degree Celsius
C	Concentration
cm^{-1}	Per centimetre
g	Gram/Gravitational force
g^{-1}	Per gram
µg	Microgram
µl	Microliter
h	Hour
HBr	Hydrobromic acid
HCl	Hydrochloric acid
HCOOH	Formic acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HNO_3	Nitric acid
kDa	Kilodalton
K_2CO_3	Potassium carbonate

KOH	Potassium hydroxide
L	Liter
L^{-3}	Per cubic liter
M	Molar
mL	Milliliter
mM	Millimolar
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
NaOH	Sodium hydroxide
$(NH4)_2SO_4$	Ammonium sulphate
nm	Nanometre
p	Probability
pI	Isoelectric point
R^2	Coefficient of determination
s	Second
s^{-1}	Per second
t	Time
*T*d	Denaturation temperature
*T*m	Melting temperature
UV	Ultra Violet
V	Volt/Volume
v/v/v	Volume by volume by volume
w/v	Weight by volume
ΔH	Denaturation enthalpy
ζ	Zeta

LIST OF ABBREVIATIONS

A	Absorbance
AABA	Alpha amino butyric acid
ABTS	2,2'-Azino-bis (3-methylbenzothiazoline-6-sulfonic acid)
AccQ	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate Q
ACE	Angiotensin converting enzyme
AFM	Atomic force microscopy
APS	Ammonium persulphate
ANOVA	Analysis of variance
AOAC	Association of Analytical Communities
ATR	Attenuated total reflectance
BBD	Box-Behnken Design
BSA	Bovine serum albumin
CH	Collagen hydrolysates
DH	Degree of hydrolysis
DF	Dilution factor
DM	Dry matter
DSC	Differential scanning calorimetry
EAI	Emulsion activity index
EC	Enzyme Commission
ESI	Emulsion stability index
EDTA	Ethylenediaminetetraacetate
EFTEM	Energy filtered transmission electron microscopy

FE	Foam expansion
FESEM	Field emission scanning electron microscopy
FS	Foam stability
FTIR	Fourier transform infrared
Hip-His-Leu	Hippuryl-L-histidyl-L-leucine
HPLC	High performance liquid chromatography
MAR	Moisture absorption rate
min	Minute
MRR	Moisture retention rate
PAGE	Polyacrylamide gel electrophoresis
PSC	Pepsin-solubilised collagen
RSc	Radical scavenging
RSM	Response surface methodology
RH	Relative Humidity
S/E ratio	Substrate/Enzyme ratio
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid
TEMED	N,N,N′,N′-Tetramethylethylenediamine
Tris	Hydroxymethyl aminomethane
VPSEM	Variable pressure scanning electron microscopy

PENGASINGAN DAN PENILAIAN SIFAT-SIFAT FIZIKOKIMIA KOLAGEN TERLARUT PEPSIN DAN HIDROLISAT YANG TERHASIL DARIPADA LAPISAN ATAS TIMUN LAUT (*Stichopus vastus*)

ABSTRAK

Timun laut (*Stichopus vastus*) dianggap sebagai sumber yang kurang digunakan lantaran hanya perut dan ususnya yang dimakan secara mentah sebagai salad di beberapa negara Asia. Semua bahagian lain termasuk lapisan atas (yang tinggi kandungan kolagen) dibuang dan oleh itu, hasil sampingan yang bernilai dan berpotensi dalam aplikasi makanan, kosmetik dan farmaseutikal terbuang begitu sahaja. Sehubungan itu, kajian ini telah dirangka untuk mengasingkan kolagen terlarut pepsin (PSC) daripada lapisan atas *S. vastus* dan menghasilkan hidrolisatnya melalui hidrolisis tripsin untuk digunakan dalam aplikasi-aplikasi yang berpotensi. Analisis SDS-PAGE menunjukkan bahawa kolagen yang terhasil adalah daripada jenis I dan terdiri daripada tiga rantaian α_1 dengan hasil sebanyak 21.3%. Transformasi fourier spektroskopi inframerah (FTIR) mendedahkan bahawa struktur heliks ganda tiga masih kekal dalam kolagen tersebut. Komposisi asid amino menunjukkan bahawa glisin adalah asid amino dominan dalam PSC tersebut. Peta peptida PSC yang telah dicerna oleh protease V8 adalah berbeza daripada kolagen jenis I dari kulit anak lembu. Suhu denaturasi (Td) dan suhu peralihan haba maksimum (Tm) masing-masing adalah pada 21.23 °C dan 37.3 °C. PSC didapati mempunyai keupayaan pembentukan gel yang baik pada pH 6.5, kepekatan 300 mM NaCl, mempunyai pH isoelektrik pada 4.67, penyerapan dan keupayaan pengekalan kelembapan yang baik pada kelembapan yang tinggi. Keterlarutannya sangat

dipengaruhi oleh pH dan kepekatan NaCl. PSC juga mempunyai kandungan protein yang sangat tinggi dan ciri-ciri struktur ultra.

Jalur β dan α_1 PSC telah berjaya dihidrolisiskan oleh tripsin seperti yang ditunjukkan oleh analisis SDS-PAGE hidrolisat kolagen dengan menunjukkan karakter paten hidrolisis. Hidrolisat tersebut mempunyai jisim molekul antara 5 hingga 25 kDa dan kaya dengan residu glisin, alanin, glutamat, prolin dan hidroksiprolin. Hidrolisat tersebut menunjukkan keterlarutan, mengemulsi dan sifat pembuihan yang baik pada pH 7.0. Pengoptimuman keadaan hidrolisis (nisbah substrat kepada enzim (S/E), suhu tindak balas dan masa hidrolisis) telah dijalankan oleh RSM berdasarkan penyekatan enzim penukaran angiotensin I (ACE) dan aktiviti pemerangkapan radikal (RSc) daripada hidrolisat. Keadaan yang optimum diramalkan pada 55 °C, 1 jam dan nisbah S/E sebanyak 15. Dalam keadaan yang optimum, penyekatan ACE dan aktiviti RSc telah dianggarkan sebanyak 67.8% dan 77.9%, masing-masing. Urutan peptida juga mengenal pasti beberapa peptida bioaktif terbaru di dalam hidrolisat tersebut. Kesimpulannya, kolagen dan hidrolisat kolagen yang terhasil daripada timun laut (*S. vastus*) mempunyai potensi untuk digunakan sebagai bahan berfungsi dalam produk-produk makanan, kosmetik dan farmaseutikal.

ISOLATION AND PHYSICOCHEMICAL PROPERTIES EVALUATION OF PEPSIN-SOLUBILISED COLLAGEN AND ITS HYDROLYSATE FROM THE INTEGUMENT OF SEA CUCUMBER (*Stichopus vastus*)

ABSTRACT

Sea cucumber (*Stichopus vastus*) is considered as an underutilised source, since only its stomach and intestines are eaten raw as salad in few Asian countries. All other parts including the integument (high content of collagen) are discarded and hence, a valuable by-product having potential food, cosmetic and pharmaceutical applications is wasted. In view of this, the present study was designed to isolate pepsin-solubilised collagen (PSC) from the integument of *S. vastus* and to prepare its hydrolysate through trypsin hydrolysis for their potential applications. SDS-PAGE analysis showed that the isolated PSC was of type I and consisted of three α_1 chains with a yield of 21.3%. Fourier transform infrared (FTIR) spectroscopy revealed that the structure of triple helix was well preserved in the PSC. Amino acid composition showed that glycine was dominant in the PSC. Peptide map of the PSC digested by V8 protease was different from that of calf skin type I collagen. Denaturation temperature (T_d) and maximum thermal transition temperature (T_m) of the PSC were at 21.23°C and 37.3 °C respectively. The PSC was found to have decent gel forming ability at pH 6.5 and 300 mM NaCl, isoelectric pH of 4.67, and high moisture absorption and retention capacity at higher humidity. Its solubility was greatly affected by pH and NaCl concentration. The PSC also has very high protein content and ultra-structural properties.

β and α_1 bands of the PSC were successfully destroyed by trypsin hydrolysis as reflected by SDS-PAGE analysis of collagen hydrolysate with a characteristic hydrolysis pattern. The hydrolysate had a molecular mass ranging from 5 to 25 kDa and was rich in glycine, alanine, glutamate, proline and hydroxyproline residues. The hydrolysate showed favourable solubility, emulsifying and foaming properties at pH 7.0. Optimisation of hydrolysis conditions (Substrate to enzyme (S/E) ratio, reaction temperature and hydrolysis time) was carried out by RSM based on angiotensin I converting enzyme (ACE) inhibitory and radical scavenging (RSc) activity of the hydrolysate. The optimal conditions were predicted to be at 55°C, 1 h and S/E ratio of 15. Under deduced optimal conditions, the ACE inhibitory and RSc activity were estimated to be as high as 67.8% and 77.9%, respectively. Peptide sequencing also identified some novel bioactive peptides in the hydrolysate. It is inferred that collagen and its hydrolysate obtained from the sea cucumber (*S. vastus*) might have the potential to be used as functional ingredients in food, cosmetics and pharmaceutical products.

CHAPTER ONE

INTRODUCTION

1.1 Research background

Collagen is an insoluble fibrous protein, most abundant (about 30%) in animal tissues having diverse applications in food, pharmaceutical, cosmetic and biomedical fields (Zhang et al., 2014; Mori et al., 2013; Zhu et al., 2012; Singh et al., 2011; Karim and Bhat, 2009; Kittiphattanabawon et al., 2005; Neklyudov, 2003; Lee et al., 2001). It is a large molecule consisting of three main polypeptide chains designated as α-chains and composed of a repeating amino acid sequence of Gly-X-Y, where most of the X is proline and Y is hydroxyproline, respectively. These polypeptide chains are intertwined to form right-handed triple helical structure (Ricard-Blum et al., 2005).

Primarily, collagen is the fundamental structural element for skin, bone, cartilage, tendon, ligament and other organs in vertebrates, whereas in invertebrates, it contributes to the structural integrity of integumental body walls and cuticles (Toral-Ganda et al., 2008; Conand and Sloan, 1989). Because of its special characteristics, such as ultrastructural features, gel forming ability, moisture absorption and retention capacity, especial amino acid compositional pattern, bioactivities, biodegradability and weak antigenecity, collagen has become a high value biomaterial for diffrent applications. Consequently it emerges as an increased global demand. Uzel and Buechler (2009) established a relationship between biochemical parameters and functional properties of collagen. Since collagen is

bound by intermolecular cross-linking and difficult to solubilise, it is often extracted from the animal tissues in acidic condition by pepsin digestion which cleaves the telopeptide region responsible for intermolecular cross-linking without damaging the structure of collagen triple helix (Hickman et al., 2000).

Bovine and porcine skin and bones have been the major sources of industrial collagen (Zhang et al., 2006). However, bovine collagen is a great cause of concern because of bovine spongiform encephalopathy (BSE), foot-and-mouth disease (FMD) and transmissible spongiform encephalopathy (TSE) while porcine collagen is forbidden for religious restrictions in Judaism and Islam. Therefore, researchers have focused their attention towards exploration of new sources of collagen and have identified marine organisms as prospective resources due to their vast availability, no religious disputation, free from disease transmission and the potential of high collagen yield (Liu et al., 2007). Subsequently, collagen with diverse characteristics has been extracted from a number of marine origin including squids, cuttlefish, sea-bass and oysters (Mizuta et al., 2002; Nagai et al., 2001; Nagai and Suzuki, 2000; Koladziejaka et al., 1999).

Textural properties of the sea cucumber integument largely depend on the physicochemical properties of collagen as it is a major protein on the body wall (Saito et al., 2002). These body wall proteins can be solubilised followed by salt disaggregation along with pepsin digestion treatments (Liu et al., 2010; Cui et al., 2007; Saito et al., 2002). Researchers have successfully extracted and characterised pepsin-solubilised collagen (PSC) from different species of sea cucumber such as *Parastichopus californicus, Stichopus japonicus* and *Cucumaria frondosa* (Liu et al.,

2010; Cui et al., 2007; Saito et al., 2002; Trotter et al., 1995). PSC isolated from these species of sea cucumbers have been reported to be $(\alpha_1)_3$ homotrimers except in the case of sea cucumber (*S. japonicus*) which is reported to contain $(\alpha_1)_3$ homotrimers (Cui et al., 2007) and $(\alpha_1)_2\,\alpha_2$ heterotrimers (Saito et al., 2002).

Fish species are frequently used as a source of high collagen content (Zhuang et al., 2012; Liu et al., 2010; Shahidi et al., 1995) for the production of bioactive protein hydrolysates. A large number of protein hydrolysates (small peptides) are produced from proteins by controlled enzymatic hydrolysis and the resultant hydrolysates may enrich the physicochemical properties of proteins. The commercial proteases frequently used for the preparation of such hydrolysates are trypsin, protamex, flavourzyme, et-chymotrypsin, kojizyme, alcalase, neutrase, pepsin and papain (Hosomi et al., 2012; Lin et al., 2012; Ngo et al., 2011; Aspmo et al., 2005; Nilsang et al., 2005). Generally different enzymes have diverse active sites on the protein to catalyze the reactions. It is important to select the suitable enzyme to prepare the small hydrolysates based on the amino acid composition of parent protein.

It has been reported that small peptides display bioactive characteristics i.e., antioxidative, antimicrobial and antihypertensive activities (Song et al., 2012; Li et al., 2012; Chen et al., 2012) and the human body can easily absorb them. A number of fish and food proteins e.g., lobster, Northern shrimp, capelin, sardine, Pacific whiting, cod, tuna, jellyfish, yellow stripe trevally, wheat protein, soy protein, yellow fin sole frame, porcine myofibrillar protein, mackerel, herring and milk casein were used as protein source to prepare hydrolysates through enzymatic hydrolysis

(Himaya et al., 2012; Ovissipour et al., 2012; Mazorra-Manzano et al., 2012; Taheri et al., 2011; Zhuang et al., 2009; Klompong et al., 2007; Zhu et al., 2006; Moure et al., 2006; Jun et al., 2004; Saiga et al., 2003; Wu et al., 2003; Sathivel et al., 2003; Gildberg and Stenberg, 2001; Suetsuna et al., 2000; Amarowicz et al., 1997; Vieira et al., 1995). It is required to optimize the hydrolysis conditions for generating such bioactive peptides from the original raw materials. Collagen contains a high content of hydrophobic amino acid and this property of collagen promotes advanced affinity to oil and improved emulsifying capacity (Lin and Li, 2006; Rajapakse et al., 2005). Consequently, collagen is likely to supply natural ACE inhibitory and antioxidant peptides with superior effects as compared to other proteins. Peptides obtained from sea cucumber collagen following such hydrolysis have not been explored extensively, except few reports (Liu et al., 2011a; Wang et al., 2010; Zhao et al., 2007).

Processing conditions have immense consequences on the physicochemical characteristics of functional peptides. It is required to utilise a reliable tool to envisage the best possible conditions for producing ACE inhibitory and radical scavenging peptides through enzymatic hydrolysis of protein. Box-Behnken Design (BBD) is one of the response surface approaches engaged in optimising the biotechnological processes. Aslan (2008) has been reported that response surface methodology (RSM) is an inexpensive means with the minimum number of experiments and a short period of time for utmost information.

The sea cucumber (*S. vastus*) is rich in collagen and has never been explored as a latent resource of bioactive peptides. It is considered as an underutilised source

since only its stomach and intestines are used as edible raw (Pakoa et al., 2009) with salad in few Asian countries and the remaining parts, especially integument rich in collagen is discarded. Thus a valuable by-product having potential food and pharmaceutical applications is wasted. However, there are no reports found on the isolation of collagen from *S. vastus* which has wide distribution in Malaysia. In view of this, the present study was designed to isolate pepsin-solubilised collagen (PSC) from the integument of *S. vastus* and to prepare its hydrolysate through trypsin hydrolysis for their potential applications. Eventually, RSM was employed to investigate the optimum hydrolysis conditions (Substrate to enzyme ratio, temperature and time) based on biological activities and to explore bioactive peptides identification by mass spectrometry under optimised hydrolysis conditions.

1.2 Problem statements

At present, land-based mammalians especially bovine and porcine skin and bones are used as the major sources of industrial collagen and gelatin (Zhang et al., 2006). These raw materials are costly throughout the world. They have impact on the manufacturing of necessary leather products (e.g., shoes, bags, belts) and show-pieces. There is no report observed on the isolation of collagen from underutilised sea cucumber (*S. vastus*).

Collagen isolated from land-based mammalian origin has constraint related to safety and religious issues. It is well known that the usage of bovine collagen is a great cause of health concern because of bovine spongiform encephalopathy (BSE),

transmissible spongiform encephalopathy (TSE) and foot-and-mouth disease (FMD). On the contrary, porcine collagen is strictly forbidden for religious barriers.

Sea cucumber (*S. vastus*) belongs to stichopodidae family in the class of Holothuroidea. Most of these Holothuroidea species are classified as non-edible invertebrates (Saito et al., 2002) as they contained neurotoxins (Kim and Himaya, 2012; Stonik et al., 1999). They are usually thrown away after captured which subsequently pollute the local environment. Sea cucumber (*S. vastus*) is available in Malaysia particularly in the sea shore regions and is generally considered as underutilised source.

1.3 Scope of study

Collagen extraction consists of mainly three phases: pre-treatment, removal of non-collagenous protein and solubilisation as well as digestion of collagen. After extraction, dialysis is carried out successively against diluted disodium hydrogen phosphate and acetic acid to inactivate pepsin and to remove last minutes of contaminants, respectively for the isolation of PSC.

The properties and inert morphology of the isolated PSC were examined under high-resolution microscopy that includes cantilever based atomic force microscopy (AFM), variable pressure scanning electron microscopy (VPSEM) and energy filtered transmission electron microscopy (EFTEM) to determine topographical, structural build-up and similarity features and fiber type identification. Physicochemical properties of the isolated PSC were investigated in terms of SDS-

PAGE, FTIR, peptide mapping, amino acid composition, solubility, gel forming ability, viscosity, zeta potential, denaturation temperature, thermal transition temperature, moisture absorption and retention capacities.

Collagen hydrolysates can be prepared by controlled trypsin hydrolysis of isolated collagen in phosphate buffer. The physicochemical properties of resulting hydrolysates such as degree of hydrolysis, SDS-PAGE, amino acid composition, solubility, emulsion and foam properties were determined by stablished conventional methods with modifications. Bioassays were performed to evaluate their radical scavenging and ACE inhibitory activities. The parameters (S/E ratio, temperature and time) which affect the collagen hydrolysis were optimised using Box-Behnken design in order to determine the interactive effect of important factors toward the maximum activities of collagen hydrolysates. Peptide sequencing was conducted to identify bioactive peptides under optimised hydrolysis conditions.

1.4 Significance of study

These python-like echinodermata invertebrates that are indigenous to the coastal sanctuary of Terengganu are considered as the second largest marine invertebrates of its order (Nestor et al., 2013; Sulardiono et al., 2012). Mucopoly-saccharides and connective tissues are its main basic tissue component. Although grouped in the much exploited sea cucumber species called 'gamat', this invertebrate is less known and not commercial exploited as gamat (*Stichopus horrens*) or 'Trepang' (*C. frondosa*). However, this invertebrate has a much better weight volume of biomass as compared to the other sea cucumbers exploited. Thus, this

tangible weight per volume biomass is hypothetical of potential for sustainable exploitation to isolate collagen in pharmaceutical and food usage, and thus a research objective worth pursuing to better maximise this wasted biomass especially from the thickness of its integumental body wall. Since the emergence of bovine spongiform encephalopathy (BSE), the search for safer sources of collagen has intensified (Anonymous, 1998). Malaysia is situated within the most biodiverse hotspot in the geographical map. In tandem to the sea cucumber fisheries industry agenda, Malaysia is not just an importer but also an exporter. However, little innovation and sustainable exploitation of this wasted integumental biomass as tangible products of worthiness in food and pharmaceutical is still found wanting.

The sea cucumber (*S. vastus*) species are indigenous marine invertebrate echinodermata of Terengganu's exotic island, and have been aquacultured and the sea ranched. As such the sustainable exploitation of aquaculture invertebrates for tangible biocompounds product of worthiness for food and pharmaceutical purposes is now a possible green approach technology. Hence with this idea, collagen is hypothesised to be isolated from this species biomass especially from the integument. Moreover, the use of *S. vastus* as the raw material to isolate collagen will maximise the use of underutilised marine biomass resource and thus the preparation of high value exploitable natural based product that would be helpful for the betterness and sustainable management of local eco-environment signatures and the local aquaculture industry economic development.

Despite a broad range of application, the use of mammalian collagen as an ingredient in food and pharmaceutical products is limited due to its high price and

possibility to transmit viral diseases. The underutilised *S. vastus* would be a potential source of collagen due to their vast availability, no religious barriers and free from disease transmission. There is no report found on the isolation of collagen from the underutilised *S. vastus* species. Thus the integumental biomass of *S. vastus* utilisation as a starting raw material to extract tangible product may not only resolve the wasted biomass disposal problem but also able to generate a high worth product collagen. This study would be served as a benchmark for the production of commercially, economically and technologically viable substrate from which to extract structurally optimised collagen from the underutilised biomass marine sources.

1.5 Objectives of the research

The main objective of this study was to investigate the underutilised local marine derived source biomass (integument) which might be the potential alternative biosource of tangible isolation of collagen for various sustainable and exploitable applications in commercial products. This study consisted of the following specific objectives:

1. To isolate pepsin-solubilised collagen (PSC) from the integument of sea cucumber (*S. vastus*).
2. To investigate its morphological and physicochemical properties.
3. To produce collagen hydrolysates from the isolated PSC by trypsin hydrolysis and to investigate their physicochemical properties.
4. To optimise the hydrolysis conditions based on biological activities and to identify bioactive peptides under optimised hydrolysis conditions.

CHAPTER TWO

LITERATURE REVIEW

Relevant literature review is discussed in this chapter. The aim of Section 2.1 is to present the recent information on sea cucmber echinodermata. The morphology, physicochemical properties, sources, extraction methods and functional properties of collagen are reviewed in Section 2.2 to 2.6. Subsequently, the review on collagen hydrolysates in Section 2.7 is discussed. In addition, the applications and prospects of collagen and its hydrolysates are reviewed in Section 2.8 to 2.9, respectively.

2.1 Sea cucumbers

2.1.1 General biology

Sea cucumbers are soft-bodied marine invertebrates belonging to the phylum echinodermata. The echinodermata phylum have five principal classes namely Ophiuroidea (1,900 species), Crinoidea (650 species), Echinoidea (1,000 species), Asteroidea (1,500 species) and Holothuroidea (1,200 species) (Britannica online, 2000). Most of the Holothuroidea species are non-edible and unexplored. Holothuroidea species are wormlike with a bunch of tentacles in the region of the mouth at one edge. They are categorized by some unique characters generally not shown in other animal phylum. Sea cucumber (*S. vastus*) is a member of the family stichopodidae in the class of Holothuroidea (Table 2.1).

Table 2.1 Taxonomy of sea cucumber (*S. vastus*).

Taxonomic ranks	Taxonomic hierarchy
Kingdom	Animalia
Phylum	Echinodermata
Class	Holothuroidea
Order	Aspidochirotida
Family	Stichopodidae
Genus	*Stichopus*
Species	*S. vastus*
Generic name	Sea cucumber

Source: Sluiter, 1887.

The sea cucumbers are slow-moving invertebrates and are genomic to local geochemical signatures. Various fishery management is now taking initiative to aquaculture these invertebrates for a sustainable fisheries. Literatures have document but optimised that the collagen in echinodermata are mutable. There are morula cells, plasma cells and splenocytes present in its thick dermis.

2.1.2 Anatomy

The appearance of sea cuucmber (*S. vastus*) looks like a cucumber usually obtained from plant origin. It is a sea fish invetebrate (Figure 2.1). It has a characteristic green and yellow harlequin pattern and a firm body with a quadrangular section (Masre et al., 2012). Major portion of this species is body wall known as integument. Integument is divided into two parts, dorsal and ventral integument. Their dorsal integument cosists of calcium carbonated build-up skinny meshwork which is called 'ossicle'.

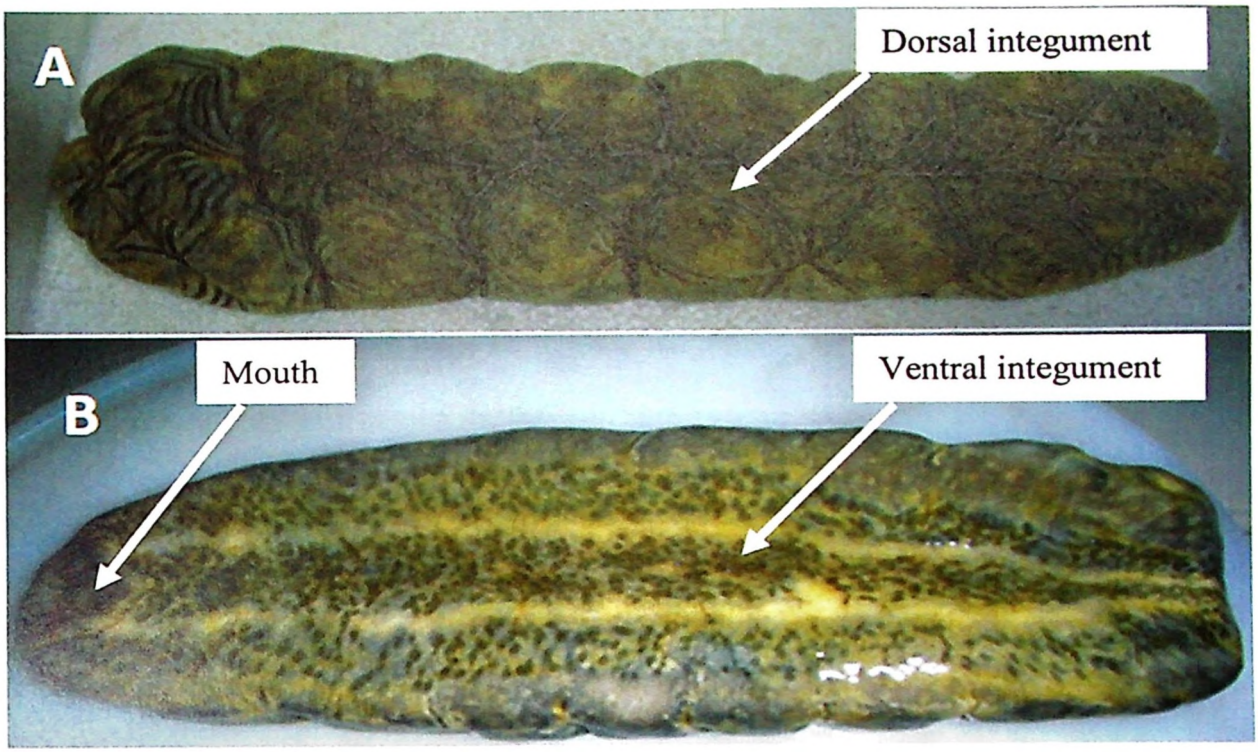

Figure 2.1: Taxonomy biodata of voucher specimen sea cucumber (*S. vastus*) echinodermata for the research. Dorsal integument (Figure A) and ventral integument (Figure B). Note: present of tube-like feet arranged in three parallel rows (Figure B). Gene bank accession: GQ 329871.

2.1.3 Traditional utilisation and economic value of sea cucumbers

The Chinese and Japanese consume sea cucumber as a delicacy, stewing them, stuffing them, and adding them to superior seafood soups. Although urban Malays do not consume gamat, those from the shore regions of Pulau Langkawi and Pulau Pangkor like gamat in kerabu (one kind of salad). The Malay term 'gamat' for sea cucumber, denotes to medicinal remedies obtained from some species of the Holothuroidea family (Lawrence et al., 1987). The golden sea cucumber (*S. horrens*) is largely used for these purposes. Gamat is generally the dried, powdered bodies of sea cucumbers prepared into a lotion or other topical medicinal ointment. It is now and then mixed into clay and used as a facial mask for beautification, or place in tea

and taken for stomach complications. Gamat is supposed to provide skin a soft and pleasant texture. The Malays boil and drink air gamat to treat cuts, inflammation and soress, peptic ulcers or to rejuvenate the body. Mothers who had freshly given birth also consume gamat as painkiller for quick healing. Consumers also believe that a solution of sea cucumbers may heal wounds, skin eruptions, and ulcers, and claim that it has a valuable influence on the immune system.

Trepanging is a form of the action of collection or harvesting of sea cucumbers, recognised in Indonesian, as 'trepang.' The collector or fisher of trepang is devised a trepanger. Trepanging is equivalent to clamming, crabbing, lobstering, musseling, shrimping and other customs of 'fishing' whose aim is acquirement of edible invertebrates rather than finfish.

Sea cucumbers have recently been over harvested in Malaysia to supply users of the traditional remedy, and as a consequence the species and its products are becoming rare. Efforts to replenish the fishery have not commonly been successful. Recently, a sea cucumber aquaculture operation was unlocked on the coasts of some Malaysian islands to boost the gamat supply. China, Japan and Brazil have also been opened sea cucumbers aquaculture plant as their demand increasing globally.

2.1.4 Medicinal biocompounds from Holothurian echinodermata

Marine origin invertebrates have long been purported as tangible biosource of biologically active substances and biocompounds which exhibit tangible food and pharmaceutical values worth exploited. It has also long been well-known that several

of these tangible Holothurian species possess inert toxic compounds. Cooper (1880) reported that some Holothurians released white filamentous structures, known as cuvierian tubules. Anaphylactic skin irritation tends to happen in having dermal contact with these tubules. Saponins (Holothurian toxins) are abundant in cuvierian tubules. On the contrary, in Holothurian species lacking these structures, saponins are allocated throughout the body wall. Dried sea cucumbers were applied as a home medication for skin diseases in some Asian countries including China and Japan. The pharmacological activity demonstrated is supposed to be due to saponins.

These bioactive glycolipids present in Asia-Pacific echinoderms have been examined extensively focusing on the advancement of exploitable novel medicinal biocompounds especially in wound healing (Masre et al., 2012; Yamada, 2002). Neuritogenic activity has been reported in gangliosides isolated from the echinoderms *S. japonicus*, *Holothuria leucospilota* (Yamada et al., 2001), *Asterias amurensis* (Higuchi et al., 1993). Marine organisms, including echinoderms draw attention because of their incredible and peculiar colors. The bodily coloration of these echinoderms is usually dependent on their inert chemical pigments present within the body wall. The compounds like carotenoids, porphyrins, melanins and naphthoquinones supply to the pigmentation of the body wall (integument). Carotenoids are well-known as antioxidants and extensively used in food as a supplement. De novo synthesis of carotenoids is absent in echinoderms, they usually achieve these pigments from algae or animals that catch them as food. Fox and Hopkins (1966) has been documented the occurrence of key carotenoids in echinoderms.

Many authors (Exposito et al., 2008; Garrone, 1999; Engel, 1997; Trotter et al., 1995) are consecrated especially to mutable collagens of marine invertebrate organisms including sponge, metazoans, molluscs and echinoderms. Type I and type V are the two major types of collagens reported to be found in these invertebrates (Liu et al., 2010; Sivakumar et al., 2000). The body wall (integument) of sea cucumbers is reported to be consisted of thick and mutable collagen fibres (Liu et al., 2010; Saito et al., 2002). Furthermore, type I collagen was successfully isolated from sea cucumbers, i.e. *P. californicus*, *S. japonicus* and *C. frondosa* (Liu et al., 2010; Cui et al., 2007; Trotter et al., 1995). Therefore, it is presumed that considerable amount of collagen could be isolated from the integument of sea cucumber (*S. vastus*) and they might have much needed enriched physicochemical and biochemical properties for a biotechnological sustainable exploitation.

2.2 Collagen

Collagen is the major structural protein in animal origin, and comprises approximately 30% of total protein content (Shoulders and Raines, 2009; Muyonga et al., 2004). It is the primary constituent of the extracellular matrices which play an important role in determining the functional properties of connective tissues. It is the main constituent of the skin, bone, tendon and cartilage in animals (Shoulders and Raines, 2009; Kadler et al., 2007). Collagen is considered as a large protein family with a characteristic triple helical structure of three polypeptide chains (Kadler et al., 2007; Gelse et al., 2003). Gelse et al. (2003) reported that the members of collagen family varied considerably in their size, function and tissue distribution despite of a common triple helix domain. This contributes to the diversity of the physicochemical

properties of each connective tissue which is usually consisted of different types of collagen molecules (Asghar and Henrickson, 1982).

Nimni and Harkness (1988) have been reported that the native collagen molecule is a rod-shape with a length and width of about 3000 Å and 15 Å, respectively, and has a molecular weight of approximately 300 kDa. It consists of three polypeptide chains woven around each other as in a three stranded rope, with a principal feature of high content of glycine, and other amino acids that affect the formation of helix of polypeptides. Chen et al. (1995) investigated the structural and functional aspects of native collagen. The properties of the different types of collagen and their distribution *in vivo* as well as the knowledge of their functional properties have demanded the possibilities to isolate collagen from marine sources in a large scale for their commercial applications in various industries.

2.2.1 Types of collagen and their classification

Twenty eight genetically distinct types of collagen, numbered I-XXVIII (Shoulders and Raines, 2009) are catagorised to date. Each type of collagen is distinguished by a roman numeral based on the order in which they were discovered (Patino et al., 2002). Collagens are found in skins, cartilage, bones, ligaments, tendons, blood vessels, cornea, teeth and other organs of animal origin (Senaratne et al., 2006). Specifically, type I collagen is predominantly noticeable in every connective tissue including skin and bones. It holds a distinctive protein sequence and is capable to form insoluble fibres in conjunction with a superior tensile strength

(Gelse et al., 2003). It contributes to the structural integrity of integumental body walls and cuticles in invertebrates (Toral-Ganda et al., 2008; Conand et al., 1989).

On the basis of the structure and molecular arrangement, collagens can be listed into six major classes, namely, fibril-forming collagen, network-forming collagens, the fibril-associated collagens with interrupted triple helices (FACITs), membrane-associated collagens with interrupted triple helices (MACITs), multiple triple helix domains and interruptions (MULTIPLEXINs), and anchoring fibrils-forming collagen (Gelse et al., 2003). Table 2.2 represents the types of collagen in vertebrates and their respective class, distribution and subunit composition.

Among all forms of collagens, only type I, II and III (fibril-forming collagens) contribute for about 90% of total collagen found in animals (Gelse et al., 2003). The structural backbone of bone are mainly made up of type I and type V collagen while type II and type XI collagen are the principal components of the fibrillar matrix at articular cartilage (Gelse et al., 2003). Type IV, a class of network-forming collagen, is predominantly found in membrane basement. The microfibrils of type IV collagens have a elevated content of cross-links with disulphide bond which are involved in the network-forming of membrane basement and help in forming interlink with other collagen fibrils (Gelse et al., 2003). FACITs are comparatively small collagen, which according to their name, are interrupted by a short non-helical telopeptide domain (Kadler et al., 2007; Gelse et al., 2003). FACITs include type IX, XII and XIV collagen. The telopeptide domains of FACITs are also involved in network formation and aggregation of collagen fibrils (Gelse et

al., 2003). Hence, FACITs are thought to be a determinant of the diameter of collagen fibrils.

Table 2.2: Types of collagen in vertebrates and their respective class, subunit composition and distribution.

Type	Class	Composition	Distribution
I	Fibrillar	$\alpha1[I]_2\alpha2[I]$	Abundant and widespread: dermis, bone, tendon, ligament
II	Fibrillar	$\alpha1[II]_3$	Cartilage, vitreous
III	Fibrillar	$\alpha1[III]_3$	Skin, blood vessels, intestine
IV	Network	$\alpha1[IV]_2\alpha2[IV]$ $\alpha3[IV]\alpha4[IV]\alpha5[IV]$ $\alpha5[IV]_2\alpha6[IV]$	Basement membranes
V	Fibrillar	$\alpha1[V]_3$ $\alpha1[V]_2\alpha2[V]$ $\alpha1[V]\alpha2[V]\alpha3[V]$	Widespread: bone, dermis, cornea, placenta
VI	Network	$\alpha1[VI]\alpha2[VI]\alpha3[VI]$ $\alpha1[VI]\alpha2[VI]\alpha4[VI]$	Widespread: bone, cartilage, cornea, dermis
VII	Anchoring fibrils	$\alpha1[VII]_2\alpha2[VII]$	Dermis, bladder
VIII	Network	$\alpha1[VIII]_3$ $\alpha2[VIII]_3$ $\alpha1[VIII]_2\alpha2[VIII]$	Widespread: dermis, brain, heart, kidney
IX	FACIT	$\alpha1[IX]\alpha2[IX]\alpha3[IX]$	Cartilage, cornea, vitreous
X	Network	$\alpha1[X]_3$	Cartilage
XI	Fibrillar	$\alpha1[XI]\alpha2[XI]\alpha3[XI]$	Cartilage, intervertebral disc
XII	FACIT	$\alpha1[XII]_3$	Dermis, tendon
XIII	MACIT	-	Endothelial cells, dermis, eye, heart
XIV	FACIT	$\alpha1[XIV]_3$	Widespread: bone, dermis, cartilage
XV	MULTIPLEXIN	-	Capillaries, testis, kidney, heart
XVI	FACIT	-	Dermis, kidney
XVII	MACIT	$\alpha1[XVII]_3$	Hemidesmosomes in epithelia
XVIII	MULTIPLEXIN	-	Basement membrane, liver
XIX	FACIT	-	Basement membrane
XX	FACIT	-	Cornea (chick)
XXI	FACIT	-	Stomach, kidney
XXII	FACIT	-	Tissue junctions
XXIII	MACIT	-	Heart, retina
XXIV	Fibrillar	-	Bone, cornea
XXV	MACIT	-	Brain, heart, testis
XXVI	FACIT	-	Testis, ovary
XXVII	Fibrillar	-	Cartilage
XXVIII	-	-	Dermis, sciatic nerve

Source: Shoulders and Raines (2009)

MACITs including type XIII, XXIII and XXV collagens are the transmembrane protein (Kadler et al., 2007; Maertens et al., 2007) which have an elongated extracellular interrupted triple helical domain and a short intracellular N-terminal domain (Maertens et al., 2007). The anchoring fibrils-forming collagens, usually found beneath the epithelial tissue, are mainly composed of a single type namely type VII collagen (Kadler et al., 2007).

2.2.2 Structure and properties

In general, type I collagen is consisted of two α_1(I) and one α_2(I) polypeptide chains and found mainly as a heterotrimer. Both the α_1(I) and α_2(I) chains share a noteworthy sequence homology, however α_2(I) chain is more hydrophobic and has less proline and hydroxyproline residues than α_1(I) chain. The α_2(I) chain was emerged in early vertebrates and is remained in all higher vertebrates through the evolution (Kelly et al., 1988; Brodsky et al., 1982). Besides heterotrimer, type I collagen of α_1(I) homotrimer has also been found in marine organisms (Liu et al., 2010; Cui et al., 2007; Trotter et al., 1995). The polypeptide chains of homotrimer

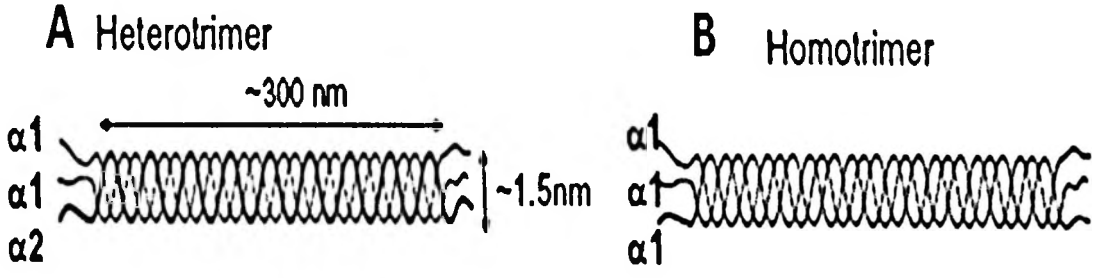

Figure 2.2: Schematic representation of type I collagen triple helix. (A) hetero-trimeric triple helix and (B) homotrimeric triple helix.

Source: Uitto (1979); Jimenez et al. (1977)

are less capable of mutual recognition and have weaker intermolecular attraction (Kuznetsova et al., 2003 and 2001). Homotrimer fibrils are morphologically analogous to heterotrimer fibrils (Figure 2.2) (McBride et al., 1997 and 1992) but they have lower tensile strength (Misof et al., 1997) and less precise packing of the helices (McBride et al., 1997).

The three α-chains of collagen remain twisted as a supercoiled structure in the region of central axis in a right-handed manner to construct the triple helix with one residue-stagger relative to the adjacent chain (Shoulders and Raines, 2009; Kadler et al., 2007). The supercoiled structure of the collagen triple helix is established by the formation of inter-chain hydrogen bonds amid the three α-chains. Each α-chain

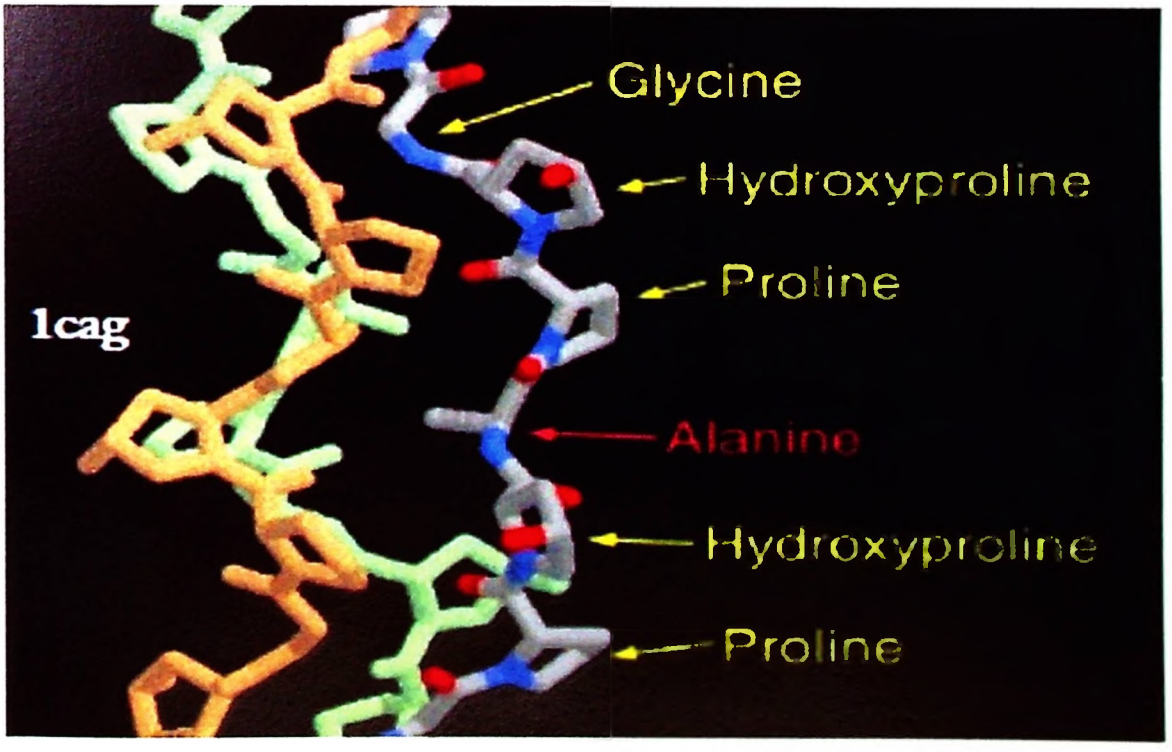

Figure 2.3: Structural organisation of collagen.

Source: Bella et al. (1994)

conserves a unique repeating amino acid sequence of Gly-X-Y, where glycine resides in every third position of the amino acid sequence while X and Y mostly represents proline and 4-hydroxyproline, respectively (Figure 2.3) (Kadler et al., 2007).

The 4-hydroxyproline residue is thought to contribute in the stability of the triple helix structure by forming intermolecular hydrogen bonds (Shoulders and Raines, 2009). The N- and C-terminal regions of the polypeptide chains of collagen are mainly made up of lysine and hydroxylysine residues, and their aldehyde derivatives. These residues resist forming triple helix structure in the terminal regions. These non-triple helical terminal domains of collagen are termed as telopeptides which take part in the formation of intra and inter cross-links of the collagen molecule (Gómez-Guillén et al., 2011).

The molecular arrangement of the collagen structure is maintained in a conserved fashion where the α-chains are twisted around the central axis in such a way that the side chains of gylcine residues are positioned toward the central of the triple helix whilst the side chain of other amino acid residues are positioned toward the outer surface of the triple helix (Gelse et al., 2003). Therefore, the collagen molecules achieve a close compact packing. Approximately four to eight molecules of collagen coupled by intermolecular covalent bond, are packed together to form collagen fibril (Gómez-Guillén et al., 2011). The cross-links of collagen molecules give a basic structure of collagen fibrils that contribute in the typical rigid properties of skin, tendon and bone (Gómez-Guillén et al., 2011).

The major structural protein is type I collagen usually observed in the extracellular matrix of animal origin which accounts almost three-fourth of dried skin. It assembles into fibrils which form the structural scaffold of skin, bone, and other connective tissues. It comprises mainly three α-chains which intertwine forming collagen triple helical structure (Ricard-Blum et al., 2005). Each chain within the helix rotates anticlockwise. The triple helix is about 300 nm in length with short nonhelical terminal peptides (telopeptides) and molecular weight of each chain is around 105 kDa (Papon et al., 2006). The aforementioned inter-chain hydrogen bonds stabilise the triple helices. Denaturation of collagen destroys the hydrogen bonds which cause separation of the rods and the chains totally or partially. Consequently, collagen loses its triple-helix conformation and finally the polymers exist in a coiled form.

The composition of collagen encompasses all 20 amino acids (Karim and Bhat, 2009; Schrieber and Gareis, 2007) as their building blocks. Although some variations in amino acid composition are evident among collagens derived from different sources, few features that are general and exclusively found in all collagens. Collagen possessed a high content of hydroxyproline and hydroxylysine, and the total imino acid (proline and hydroxyproline) contents which are not usually found in other mammalian proteins (Karim and Bhat, 2009; Balian and Bowes, 1977). This unique characteristic is in close agreement with the amino acid sequence of the α-chain in collagen molecule which is Glycine-X-Y, where most of the X and Y are proline and hydroxyproline, respectively.

Cysteine is not seen in extracted type I collagen as reported in a number of studies as acid is used during extraction process. Acid hydrolysis destroys cysteine completely. Cysteine is solely found in isolated collagen type III and IV (Asghar and Henrickson 1982). The amount of hydroxylysine, methionine, isoleucine, tyrosine, and histidine is very less in all types of the extracted collagen. The hydrophobic amino acids are dominant in the extracted collagens. Mammalians and fish collagens equally hold these relationships. These unusual characteristics are seen in this class of proteins.

2.3 Sources of collagen

Collagens are most abundant and ubiquitously distributed proteins found on earth. Collagens can largely be extracted from a variety of living animals. Therefore, collagen has been extensively used as a raw material in food, cosmetic and pharmaceutical industries (Zhang et al., 2006). Moreover, industrial scale of collagens is produced from the raw collagenous materials that are mostly wastes or byproducts of an industrial process which can be obtained at a very low cost. It is noteworthy that the byproducts including skin, bone, tendon and scale are highly rich in collagen. Currently, two major sources of collagen, mammals and marine organisms, are widely used for production of collagen in industries. Table 2.3 represents the list of literatures that reported on the extraction as well as characterisation of collagen from various sources.

Table 2.3: Literatures that report on the extraction and characterisation of collagen from various sources.

Source of collagen	Reference
Mammalian collagen	
Calf	Zhang et al. (2007b)
Pig	Zhao et al. (2005)
Marine collagen	
Barramundi (*Lates calcarifer*)	Jamilah et al. (2013)
Leather jacket (*Odonus niger*)	Muralidharan et al. (2013)
Pacific saury (Cololabis saira)	Mori et al. (2013)
Peru Squid (*Dosidicus gigas*)	Fu et al. (2013)
Sea cucumber (*Stichopus japonicus*)	Zhu et al. (2012)
Striped catfish (*Pangasianodon hypophthalmus*)	Singh et al. (2011)
Giant red sea cucumber (*Parastichopus californicus*)	Liu et al. (2010)
Brownbanded bamboo shark (*Chiloscyllium punctatum*)	Kittiphattanabawon et al. (2010)
Unicorn leatherjacket (*Aluterus monocerous*)	Ahmad and Benjakul (2010)
Squid (*Ommastrephes bartrami*)	Yan et al. (2009)
Nile tilapia (*Oreochromis niloticus*)	Zeng et al. (2009)
Deep-sea red fish (*Sebastes mentella*)	Wang et al. (2008a)
Grass carp (*Ctenopharyngodon idella*)	Zhang et al. (2007a)
Scallop (*Patinopecten yessoensis*)	Shen et al. (2007)
Channel catfish (*Ictalurus punctaus*)	Liu et al. (2007)
Brown backed toadfish (*Lagocephalus gloveri*)	Senaratne et al. (2006)
Bigeye snapper (*Priacanthus macracanthus*)	Jongjareonrak et al. (2005a)
Brownstripe red snapper (*Lutjanus vitta*)	Jongjareonrak et al. (2005b)
Nile perch (*Lates niloticus*)	Muyonga et al. (2004)
Sea cucumber (*Stichopus japonicus*)	Saito et al. (2002)
Puffer fish (*Takifugu rubripes*)	Nagai et al. (2002)
Paper nautilus (*Argonauta argo*, Linnaeus)	Nagai and Suzuki (2002)
Pearl oyster (*Pinctada fucata*)	Mizuta et al. (2002)
Cuttlefish (*Sepia lycidas*)	Nagai et al. (2001)
Purple sea urchin (*Anthocidaris crassispina*)	Nagai and Suzuki (2000)
Jellyfish (*Rhopilema asamushi*)	Nagai et al. (2000)
Sea cucumber (*Cucumaria frondosa*)	Trotter et al. (1995)

2.3.1 Mammalian collagen

Mammals especially bovine and porcine serve as the major sources of collagen for industrial demand. It is reported that the annual production of gelatine (partially hydrolyzed collagen) is about 326,000 tons globally, where the maximum (52.5%) gelatine yields from bovine followed by pig-skin (46%) and other source (1.5%) (Karim and Bhat, 2009). It is reasonable that the high availability of cow and