ULTRAVIOLET ASSISTED AUTOLYSIS OF SEA CUCUMBER *(STICHOPUS HORRENS)* BODY WALL FOR THE ENHANCEMENT OF SULFATED POLYSACCHARIDES RELEASE

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by

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LIST OF SYMBOLS

%	Percentage
a*	Redness-greenness
b*	Yellowness-blueness
cm	Centimetre
g	Gram
kg	Kilogram
L	Liter
L*	Lightness
mg	miligram
nm	nanometer
J/m^2	Joule per meter square
mm	milimeter
U/g	Unit enzyme per gram
W/m^2	Watt per meter square

LIST OF ABBREVIATIONS

Ac	Actin
ACE	Angiotensin-converting enzyme
AGEs	Advance glycation endproducts
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
CAT	Catalase
DNA	Deoxyribonucleic acid
DSC	Differential scanning calorimetry
DTG	Derivative thermogravimetry
ECM	Extracellular matrix
ER	Endoplasmic reticulum
FTIR	Fourier transform infrared spectroscopy
FuCS	Fucosylated chondroitin sulfate
GAG	Glycosaminoglycan
GMP	Gelatinolytic metalloproteases
GPx	Glutathione peroxidase
HPLC	High performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MAPKs	Mitogen-activated protein kinase
MCT	Mutable collagenous tissue
MHCs	Myosin heavy chain

MMP	Matrix metalloprotease
MYP	Major yolk protein
Pm	Paramyosin
ROS	Reactive oxygen species
RSM	Response surface methodology
S/N	Signal/noise
SEM	Scanning electron microscope
SHA	*Stichopus horrens* autolysate
SHA-M	*Stichopus horrens* with addition metal ion
SHBW	*Stichopus horrens* body wall
SHE	*Stichopus horrens* extract
SHH	*Stichopus horrens* hydrolysate
SOD	Superoxidase dismutase
TCA	Trichloroacetic acid
TEM	Transmission electron microscope
TGA	Thermogravimetry analysis
UV	Ultraviolet
WB	Without buffer
WHC	Water holding capacity

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AUTOLISIS DINDING BADAN GAMAT *(STICHOPUS HORRENS)* DENGAN BANTUAN SINAR ULTRAUNGU UNTUK PENGELUARAN POLISAKARIDA SULFAT

ABSTRAK

Gamat *(Stichopus horrens)* mengandungi sebatian bioaktif penting yang dipanggil polisakarida sulfat (PS), yang boleh diekstrak melalui proses autolisis menggunakan enzim endogennya. Walau bagaimanapun, proses ini lambat dan tidak cekap. Matlamat kajian ini adalah untuk menilai mekanisme autolisis bantuan UV dalam meningkatkan pembebasan PS dari dinding badan gamat (DBG). Dalam fasa I, kesan dos UV dan pH pada aktiviti protease endogen, biokimia, dan perubahan struktur DBG semasa autolisis telah dinilai. DBG beku telah dihomogenkan dengan dan tanpa penimbal (pH 6, 7, 8) dan terdedah kepada dos UV rendah (10,800 J/m^2) dan tinggi (324,000 J/m^2) sebelum autolisis pada 50 °C selama 4 dan 8 jam. Aktiviti protease endogen dan perubahan biokimia masing-masing dinilai daripada pecahan larut manakala pecahan tidak larut DBG dinilai untuk perubahan struktur protein sekunder dan kestabilan terma menggunakan Fourier-transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), dan Thermogravimetry Analysis (TGA). Peningkatan dos UV sebanyak 30x, meningkatkan aktiviti proteolitik sebanyak 4.5 kali ganda selepas autolisis 4 jam, mengakibatkan kandungan protein larut, hidroksiprolin dan glikosaminoglikan (GAG) meningkat sebanyak 5, 4 dan 3 kali ganda, masing-masing. Walaupun sampel tanpa penimbal menunjukkan aktiviti protease endogen yang serupa dengan sampel kawalan, jumlah protein larut, hidroksiprolin, dan GAG masing-masing lebih tinggi sebanyak 4, 0.1, dan 2.5 kali

ganda. Analisis FTIR mendedahkan pengurangan kandungan α-heliks yang lebih tinggi dalam sampel tanpa penimbal (13%) berbanding dengan penimbal (6%) yang disertai dengan pertambahan kandungan gegelung rawak (8%) dan kandungan helaian β (13%) dalam sampel dengan dan tanpa penimbal, masing-masing. Derivatif kedua spektrum IR bagi jalur amida I dan kawasan karbohidrat seterusnya mengesahkan nisbah GAG/kolagen yang lebih tinggi untuk sampel tanpa penimbal. Penyinaran UV yang tinggi mengurangkan kestabilan terma pecahan tidak larut DBG. Autolisis dibantu UV pada dos UV yang tinggi dinilai selanjutnya pada pH berbeza dengan memanjangkan masa. Aktiviti enzim endogen dan pembebasan PS tertinggi pada pH 7. Autolisis sehingga 8 jam disertai dengan penurunan aktiviti proteolitik dan GAG pada pH 7 tetapi hidroksiprolin larut meningkat pada pH 8. Dalam fasa II, autolisis dibantu UV kemudiannya dioptimumkan menggunakan metodologi permukaan tindak balas (PTB), dengan tiga parameter autolisis termasuk dos UV (324,000, 486,000, dan 648,000 J/m^2), suhu (40, 50, dan 60 ℃), dan masa (3, 4, 5 jam), untuk menghasilkan autolisat dengan maksimum kandungan PS dan aktivitas antioksidan. Dos UV menunjukkan pengaruh paling kuat terhadap aktiviti enzim endogen, pembentukan oligopeptida larut TCA, pembebasan hidroksiprolin dan GAG. Degradasi kolagen menunjukkan kesan sinergistik antara dos UV dan suhu. Aktiviti antioksidan sangat dipengaruhi oleh masa dan suhu tetapi berkurangan pada dos UV yang tinggi. Keadaan optimum pada dos UV 486,000 J/m^2, suhu 60 °C dan 4 jam autolisis dibantu UV menghasilkan 40.91 U/mg aktiviti proteolitik, 40.92 mg/g oligopeptida larut TCA, 0.68 mg/g hidroksiprolin, 37.33 mg/g kandungan GAG, dan 12.15 μmol TE/g DPPH. Peningkatan aktiviti protease endogen dan pembebasan GAG masing-masing sebanyak 85.46% dan 50.67% dengan penambahan ion logam divalen (Mn^{2+}). Keselamatan makanan, kandungan nutrisi, dan antioksidan autolisat (SHA) dan

autolisat yang dipertingkatkan dengan ion logam (Mn^{2+}) (SHA-M) kemudiannya dibandingkan dengan hidrolisis enzimatik papain (SHH) dan ekstrak air panas (SHE). Kadar pengeluaran PS dalam susunan menurun ialah 59.13%, 51.95% dan 32.36% untuk SHA-M, SHA dan SHE, masing-masing. Pembentukan 5-hydroxymethylfurfural (HMF) adalah tertinggi dalam SHH (5.13 mg/kg) diikuti oleh SHE, SHA, dan SHA-M pada 0.21, 0.12, dan 0.01 mg/kg konsisten dengan jumlah kandungan gula dan warnanya (a* dan b*). Autolisis dibantu UV dengan ketara mengurangkan kandungan merkuri, sementara meningkatkan kandungan fosforus berbanding mentah. Aktiviti antioksidan mengikut urutan SHH, SHA, SHA-M, dan SHE pada 23.82, 10.17, 9.36, dan 6.49 μmol TE/g sampel untuk ujian ABTS, manakala 30.67, 16.41, 13.07, dan 1.59 μmol TE/g sampel untuk ujian DPPH. Kesimpulannya, autolisis dibantu UV adalah kaedah alternatif yang berpotensi untuk pemprosesan gamat untuk menghasilkan makanan berfungsi yang kaya dengan protein dan polisakarida sulfat.

ULTRAVIOLET ASSISTED AUTOLYSIS OF SEA CUCUMBER *(STICHOPUS HORRENS)* BODY WALL FOR THE ENHANCEMENT OF SULFATED POLYSACCHARIDES RELEASE

ABSTRACT

Sea cucumbers *(Stichopus horrens)* contain important bioactive compounds called sulfated polysaccharides (SP), which can be extracted by autolysis process utilizing its endogenous enzyme. However, this process is slow and not efficient. The aim of this study was to evaluate the mechanism of UV assisted autolysis in enhancing the release of SP from sea cucumber body wall. In phase I, the effects of UV dosage and pH on endogenous proteases activity, biochemical, and structural changes of SHBW during autolysis were evaluated. Frozen SHBW was homogenized with and without buffer (pH 6, 7, 8) and exposed to low (10,800 J/m^2) and high (324,000 J/m^2) UV dosage prior to autolysis at 50 °C for 4 and 8 hours. The endogenous protease activity and respective biochemical changes were evaluated from the soluble fraction while the insoluble fraction of SHBW was evaluated for secondary protein structural changes and thermal stability using Fourier-transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), and Thermogravimetry Analysis (TGA). Increase in UV dosage by 30x, increased proteolytic activity by 4.5-fold after 4 hours autolysis, resulting in increased soluble protein, hydroxyproline, and glycosaminoglycan (GAG) content by 5, 4, and 3 folds, respectively. Although sample without buffer showed similar endogenous protease activity with control sample, the total soluble protein, hydroxyproline, and GAG were higher by 4, 0.1, and 2.5 folds, respectively. FTIR analysis revealed higher reduction of α-helixes content in sample

without buffer (13%) compared to with buffer (6%) which accompanied with increment of random coil content (8%) and β-sheet content (13%) in sample with and without buffer, respectively. The second derivative of IR spectra of amide band I and carbohydrate regions further confirmed higher GAG/collagen ratio for sample without buffer. High UV irradiation reduced the thermostability of insoluble fraction of SHBW. The UV assisted autolysis at high UV dosage was further evaluated at different pH and prolong time revealed highest endogenous enzyme activity and release polysaccharides at pH 7 and autolysis up to 8 hours was accompanied by a drop-in proteolytic activity and GAG at pH 7 but increased in soluble hydroxyproline at pH 8. In phase II, UV assisted autolysis was optimized using response surface methodology (RSM), with three autolysis parameters including UV dosage (324,000, 486,000, and 648,000 J/m^2), temperature (40, 50, and 60 °C), and time (3, 4, 5 hours), for production autolysates with maximum SP and antioxidant activities. UV dosage exhibit strongest influence on endogenous enzyme activity, formation of TCA-soluble oligopeptide, release of hydroxyproline and GAG. Collagen degradation showed synergistic effects between UV dosage and temperature. The antioxidant activity was greatly influenced by time and temperature but reduced at high UV dosage. The optimum condition at 486,000 J/m^2 UV dosage, 60 °C and 4 hours UV assisted autolysis resulted in 40.91 U/mg proteolytic activity, 40.92 mg/g TCA soluble oligopeptide, 0.68 mg/g hydroxyproline, 37.33 mg/g GAG content, and 12.15 μmol TE/g DPPH. Further increment of endogenous proteases activity and release of GAG by 85.46% and 50.67%, respectively with the addition of divalent metal ions (Mn^{2+}). The safety, nutritional, and antioxidant activities of the autolysates (SHA) and autolysates enhanced with metal ion (Mn^{2+}) (SHA-M) were then compared with papain enzymatic hydrolysate (SHH) and hot water extract (SHE). The dissolution rate of SP in

descending order was 59.13%, 51.95%, and 32.36% for SHA-M, SHA, and SHE, respectively. The formation of 5-hydroxymethylfurfural (HMF) was highest in SHH (5.13 mg/kg) followed by SHE, SHA, and SHA-M at 0.21, 0.12, and 0.01 mg/kg consistent with their total sugar content and colour (a^* and b^*). The UV assisted autolysis significantly reduce the mercury content, while increased the phosphorus content compared to raw. Antioxidant activities following the order of SHH, SHA, SHA-M, and SHE at 23.82, 10.17, 9.36, and 6.49 μmol TE/g sample for ABTS assay, while 30.67, 16.41, 13.07, and 1.59 μmol TE/g sample for DPPH assay. In conclusion, UV assisted autolysis is a potential alternative method of *S. horrens* processing for the production of functional food rich in protein and SP.

CHAPTER 1

INTRODUCTION

1.1 Research background

Stichopus horrens is one of the dominant sea cucumber species found in shallow tropical waters which is known as an important fishery commodity (Purcell et al., 2012). They have been exploited for a long time as a main ingredient in nutraceutical products due to their high nutritional value and many valuable bioactive compounds (Bordbar et al., 2011; Kamarudin et al., 2017). The main component of this animal is body wall, which is rich in endogenous enzymes and mainly composed of extracellular matrix (ECM) making up the dermis tissue and connected to inner circular muscle layer for movement (Zhong et al., 2016). The organized and complex structural of ECM is comprised of collagen fibrils which interlinked together by proteoglycan interfibrillar bridge and surrounded with microfibrillar network (Thurmond & Trotter, 1996). The interfibrillar proteoglycan bridges are proteoglycans consisting of core proteins with covalently attached sulfated polysaccharides (Scott, 1992).

Sulfated polysaccharides from sea cucumber body wall including fucosylated chondroitin sulfate (FuCS) and fucoidan have recently received significant research focus due to its various potential benefits for human health (Xu et al., 2022). Sulfated polysaccharides exhibit wide spectrum of bioactivities such as antioxidant (Mou et al., 2018), anti-inflammation (Cui et al., 2016), anti-coagulant (Li et al., 2017), and immunomodulator (Jiang et al., 2021). In addition, sulfated polysaccharides isolated from sea cucumber especially FuCS are unique due to the existence of fucosyl branches unit that exhibit additional therapeutic properties such as anti-coagulant and

anti-thrombotic activities, which mammalian polysaccharides does not have (Pomin, 2014).

Sea cucumbers are very sensitive towards external stimuli. The body walls of sea cucumbers undergo massive tissue autolysis, also known as 'melting' or 'local degeneration', in response to changes in external environmental factors such as nutrient deficiencies, salinity, temperature, sunlight exposure, mechanical stimulation, and UV irradiation (Hill, 2001; Zhu et al., 2008). Autolysis is a complex sequential biochemical reaction resulting massive ECM breakdown and proteolysis as the action of endogenous enzyme. Autolysis has created major problem in the processing and storage of sea cucumber in relation to its quality and safety. On the other hand, proteolysis and breakdown of ECM structure during controlled autolysis condition can be employed as promising method to release functional bioactive compounds from sea cucumber body wall such as sulfated polysaccharides considering its simplicity and low cost (Liu et al., 2018a; Song et al., 2018).

However, endogenous proteases in sea cucumber body wall exist as zymogen or inactive form that require long sequential biochemical reaction for activation (Qi et al., 2016; Yang et al., 2015). Autolysis was also shown to localize event as inhomogeneous distribution of endogenous proteases in sea cucumber body wall which denser in epidermis and outer dermis layer compared to inner dermis layer (Liu et al., 2016). Besides, the distribution of sulfated polysaccharides as targeted bioactive compound is buried within compact collagen structure (Scott, 1992) resulting in minimum accessibility of endogenous proteases to release sulfated polysaccharides. These cause the release of sulfated polysaccharides during autolysis involved long incubation time up to 72 hours (Song et al., 2018). The formation of aldehydes, ketones, amines, organic acid, and other substance from the degradation of nutritional

components due to the microbial reproduction and metabolism during uncontrolled long incubation period can lead to nutritional degradation, reduce product quality, and pose safety risk. In addition, long autolysis duration can activate endogenous glycosidases that involves in the degradation of sulfated polysaccharides (Dong et al., 2020).

Ultraviolet (UV) technology is a non-thermal and environmentally friendly technology that has been applied to assist autolysis of aquatic animals such as shrimp and sea cucumber (Cao et al., 2014; Zhu et al., 2008). Yang et al. (2015) reported low UV dosage (1008 J/m^2) induced autolysis of sea cucumber after 6 hours UV irradiation indicated by increasing amount of TCA-soluble oligopeptide. The formation of reactive oxygen species (ROS) as the key for endogenous enzyme activation increased by increasing UV dosage from 4,500 to 18,000 J/m^2 in homogenate sea cucumber body wall (Qi et al., 2016). In addition, UV irradiation at high UV dosage (220,000 J/m^2) resulted in the structural degradation of compact collagen structure [type I structure derived from rat tail] (Rabotyagova et al., 2008). These suggest the potential of high UV irradiation to enhance the release of sulfated polysaccharides during autolysis by accelerating endogenous enzyme activation and increase enzyme accessibility as the degradation of compact collagen structure. Nevertheless, the application of high UV dosage in autolysis-based processes of sea cucumber has not been extensively investigated.

Proteolysis and breakdown of ECM structure during autolysis resulted in the release of valuable bioactive compounds and various nutritious components such as protein, polysaccharides, and essential minerals. Release of protein and polysaccharides subjected to the high temperature during controlled autolysis possibly induce the formation of advance glycation end products (AGEs) that become safety

consent. AGEs are heterogeneous, complex group of compounds as the products of the Maillard reaction in thermally processed foods which known as non-enzymatic browning reaction between amine group of protein and free carbonyl group of reducing sugar (Inan-Eroglu et al., 2020). In recent decades there has been increasing evidence that high levels of AGEs in tissue and blood circulation in the body promote chronic degenerative diseases such as as diabetes mellitus, cardiovascular diseases, neuro-degenerative, and Alzheimer's disease (Luevano-Contreras & Chapman-Novakofski, 2010). However, besides the application of high UV dosage in autolysis-based processes of sea cucumber, the nutritional value, safety, and bioactivity of the resulted autolysis product have also not been fully studied. Therefore, this study aims to understand the mechanism of UV-assisted autolysis in enhancing the release of sulfated polysaccharides (SP) from sea cucumber body wall, and also evaluate the nutritional value, safety, and bioactivity of the resulted autolysis products.

1.2 Research objectives

The objectives of this study were:

1. To evaluate the effects of UV dosage and pH on endogenous proteases activity in relation to degree of proteolysis, release of sulfated polysaccharides, and structural changes of *S. horrens* body wall during autolysis.
2. To optimize UV assisted autolysis of *S. horrens* body wall for maximum endogenous proteases activity, release of sulfated polysaccharides, and antioxidant activity.
3. To compare safety, nutritional quality, and antioxidant activities of *S. horrens* autolysate with *S. horrens* papain enzymatic-assisted hydrolysate, and *S. horrens* hot water extract.

CHAPTER 2

LITERATURE REVIEW

2.1 Sea cucumber *(Stichopus horrens)*

Sea cucumber is group of echinoderms which is characterized as having soft body tissue, bilateral symmetry body, worm-like organism, and lying on one side with an elongated body axis between the mouth and anus (Purcell et al., 2012). Sea cucumber have been utilized for long time as source of food and nutraceutical ingredient particularly in some part of Asia due to its medicinal and health benefits attributed to the presence of various valuable bioactive compounds (Bordbar et al., 2011; Xu et al., 2018). More than a thousand species of sea cucumber distributed over the world has been identified. Among them, most valuable and important sea cucumber belong to 2 families and 7 genera of the *Aspidochirotids* including *Stichopus, Actinopyga, Bohadschia, Holothuria (Holothuridae), Isostichopus, Parastichopus* and Thelenota *(Stichopodidae)* and one family and genus of the *Dendrochirotids: Cucumaria (Cucumariidae)* (Bordbar et al., 2011).

The most abundant and top species of sea cucumber in Malaysia is belong to 5 genera of order *Aspirochorotida* namely, *Stichopus, Holothuria, Thelenota, Bohadschia* and *Actinopyga* (Baine & Choo, 1999). Further study about the distribution of sea cucumber in Malaysia also reported that around 18% from 50 species of sea cucumber found in Malaysia is from genus *Stichopus* with another reported genus includes *Holothuria, Actinopya, Bohadschia, Molpadia* and *Pearsonuthuria* (Kamarudin et al., 2009). In Malaysia, all species of sea cucumber from the family of *Stichopodidae* are refer to as *gamat* including the genus *Stichopus* and the genus *Thelenota* while other sea cucumber are refer to as *timun laut*

(Kamarudin et al., 2017). Recently, Woo et al. (2015), reported that 5 species belonging to family *Stichopus* sp. found in Malaysia including *Stichopus horrens, Stichopus fusiformiossa, Stichopus chloronotus, Stichopus vastus,* and *Stichopus hermanni*.

Stichopus horrens is one of important and valuable sea cucumber species which have been long time used as traditional medicine in Malaysia. They are distributed in many region in Malaysia such as Pulau Pangkor, Pulau Langkawi, Pulau Payar, Port Dickson, Pulau Tinggi, Pulau Tioman, Pulau Perhentian, Pulau Redang groups and Tunku Abdul Rahman Park (Ibrahim, 2009). The morphological identification of *Stichopus horrens* species has been done by Kamarudin et al., (2017). Their body form is rectangular in cross section with numerous papillae (Figure 2.1). The colouration of this species varies from tan to red, often variegated with patches and the maximum length of this animal is about 30 cm. The taxonomy of *Stichopus horrens* as follows.

Kingdom	: Animalia
Phylum	: Echinodermata
Class	: Holothuroidea
Family	: Stichopodidae
Genus	: Stichopus
Species	: *Stichopus horrens* (Selenka, 1867)

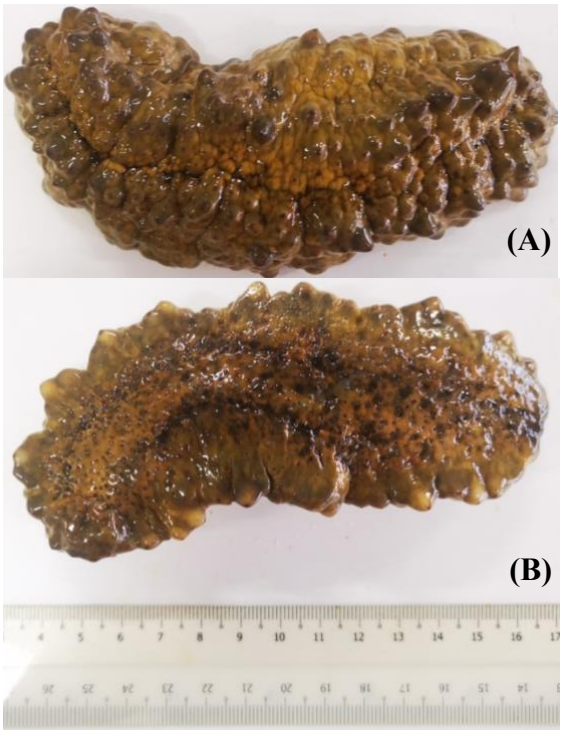

Figure 2.1 *Stichopus horrens*; dorsal (A) and ventral (B) views.

2.1.1 Utilization of *Stichopus horrens* as food and traditional medicine

In Malaysia, *Stichopus horrens* are traditionally processed by boiling in hot water to produce body fluid extract *(air gamat)* and oil extract *(minyak gamat)* (Kamarudin et al., 2017). The raw material of *air gamat* is only pure from *Stichopus horrens* which is used for oral consumption. Meanwhile, *minyak gamat* is produced from dried *Stichopus horrens* body wall with addition of coconut oil or other oil and herbs such as lemongrass which the resulted product only for external use. *Stichopus horrens* also often processed as food and consumed orally especially for patient who have undergone surgery for the purpose of healing. In Pulau Langkawi, on the west coast of Peninsular Malaysia, *Stichopus horrens* is one of the most threatened species due to its high demand in the pharmaceutical and cosmetic processing industries (Baine & Choo, 1999). A variety of sea cucumber-based products are available for purchase in Langkawi, including lotions, water, toothpaste, oils, tablets, and cosmetics.

In addition, *Stichopus horrens* are consumed raw including their body wall and viscera by the local people in Samoa (Eriksson et al., 2007). They placed the viscera of *Stichopus horrens* along with body wall strips in a bottle filled with seawater and sold at local markets and along the roadside.

2.1.2 Nutritional composition and bioactive compounds in *Stichopus horrens*

The nutritional composition and bioactive compounds from *Stichopus horrens* have been widely investigated. *Stichopus horrens* body wall contain high protein, ash, and low lipid in the range of 34.97 – 48.19%, 31.69 – 55.01%, and 1.46 – 5.55%, respectively as summarised in Table 2.1. In addition, *Stichopus horrens* body wall also was reported as good source for many essential minerals such as Mg, Fe, Zn, and Ca (Barzkar et al., 2017).

Table 2.1 Proximate composition of *Stichopus horrens*

Moisture (% wb)	**Ash (% db)**	**Protein (% db)**	**Fat (% db)**	**References**
93.10	39.13	41.01	3.04	(Forghani et al., 2012)
92.8	46.25	48.19	5.55	(Barzkar et al., 2017)
95.14	31.69	36.62	4.11	(Gianto et al., 2018)
94.51	55.01	34.97	1.46	(Fawzya et al., 2020)

Many bioactive compounds have been successfully extracted and characterized from *Stichopus horrens* including peptides (Forghani et al., 2016), triterpene diglycoside (Xuan et al., 2017), triterpene tetraglycosides (Vien et al., 2017), saponins (Torreno et al., 2021), fucosylated chondroitin sulfates (FuCS) (Myron et al., 2017; Ustyuzhanina et al., 2018), and fucan sulfates (FS) (Shang et al., 2018; Ustyuzhanina et al., 2018). Among these, sulfated polysaccharides (FuCS and FS) has become a

significant research focus recently (Xu et al., 2022). Myron et al. (2017), reported that *Stichopus horrens* contain relatively high sulfated polysaccharide (64 mg/g) which higher compared to other tropical sea cucumber species such as *S. vastus* (34.97 mg/g) and *S. hermanni* (32.23 mg/g) (Masre et al., 2012), but lower compared non-tropical species such as *S. japonicus* (110 – 190 mg/g) and *C. frondosa* (74.1 mg/g) (Li et al., 2021; Masre et al., 2012; Zhu et al., 2018).

2.2 Structure and composition of sea cucumber body wall

The major edible part of the sea cucumber animal is their body wall consisting of leathery mutable collagenous tissue (MCT) which contribute to unique texture characteristics of sea cucumbers. Since they make up the majority of sea cucumber body wall, structural protein is the most abundant component that accounted for 40 – 60% of sea cucumber body wall dry matter, while carbohydrate and lipid were recorded at 23% and 2-8%, respectively (Xu et al., 2018).

As shown in Figure 2.2, the structure of sea cucumber body wall is divided into four layers: cuticle, epidermis, dermis, and an inner circular muscle layer, with dermis tissue accounting for the majority part (Zhong et al., 2016). The epidermis layer which is covered by an acellular cuticle mainly consist of widely spaced epidermal cells and some granule containing cells. In the middle part, the dermis layer determine the sea cucumber body wall's thickness mainly consist of collagen fibers, microfibrils, ground substances, and scattered cells (Hill, 2001; Thurmond & Trotter, 1996). The collagen fibers are surrounded and separated by a microfibril network that maintains tissue and provides long-lasting restorative power which is the main building block of the MCT (Thurmond & Trotter, 1996; Trotter et al., 1995). The MCT of sea cucumber body wall consists of parallel aggregates of discontinuous, spindle-shaped collagen

fibrils where the proteoglycan and other glycosaminoglycan containing molecules are attached whose functions include serving as binding sites for molecules responsible for interfibrillar cohesion (Wilkie et al., 2021). The outer circular and inner longitudinal muscle tissues lay between the dermis and coelarium to support the body wall of sea cucumber and movement (Zhao et al., 2016). In addition, the structural protein in sea cucumber body wall can be classified into 3 categories including extracellular matrix (ECM) protein, muscle protein, and proteases (Wang et al., 2020).

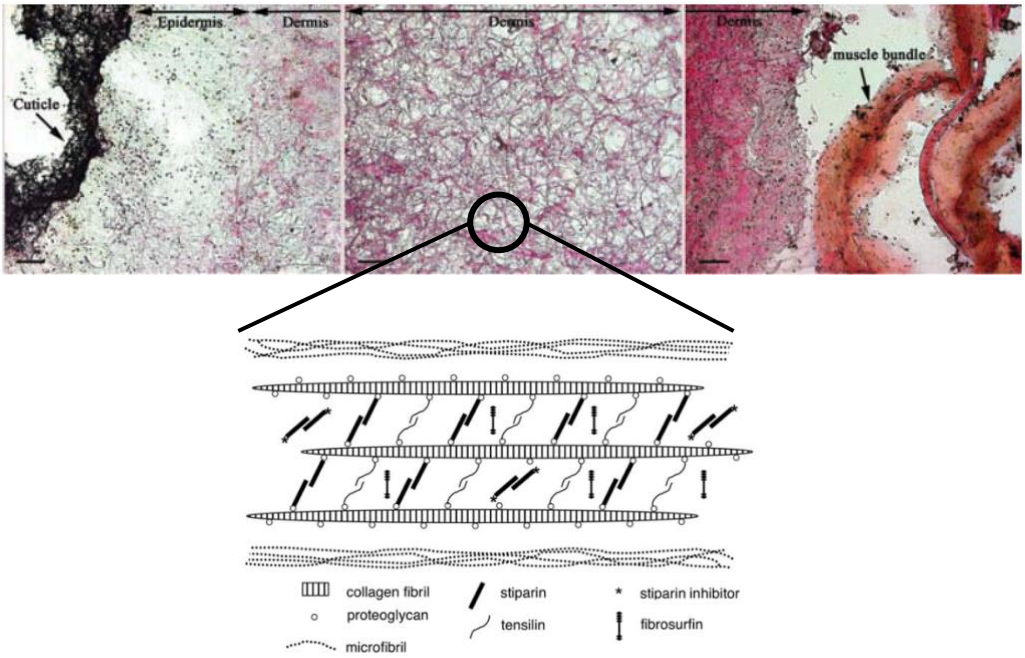

Figure 2.2 Light microscopy of the *Stichopus monotuberculatus* body wall (VG stain) (Zhong et al., 2016) and model of mutable collagenous tissue (MCT) structure (Wilkie et al., 2021).

2.2.1 Extracellular matrix (ECM) protein

Extracellular matrix is well organized large network of protein and other molecules that surround, support, and give structure to the tissue (Theocharis et al., 2016). The ECM in sea cucumber body wall consist of a large variety of matrix macromolecules including collagen, proteoglycan, and ECM glycoprotein (Wang et

al., 2020). Collagen in the form of collagen fibers is the largest constituent of sea cucumber body wall which accounted 70% of total protein (Are et al., 2002). Collagen fibers have a distinct structural hierarchy from micro to macro structure namely alpha polypeptide chains, collagen, collagen fibrils and collagen fibrils (Senadheera et al., 2020). Three alpha polypeptide chains intertwine together to form right-handed triple helices that form collagen. Collagen is connected from the head to the tail, one quarter of which is dislocated and arranged in parallel. Their molecule is spirally wound to form a stable collagen fibril through covalent cross-linking. Collagen fibrils are intertwined and arranged in parallel to form collagen fibers (Liu et al., 2019).

The second abundant part of sea cucumber body wall is proteoglycan. They consist of a core protein into which one or more glycosaminoglycan (GAG) chain of the same or different type are covalently attached (Trotter et al., 1995). At the molecular level, sea cucumber collagen is considered as bipolar collagen fibrils with surface associated proteoglycans. The proteoglycan interfibrillar bridge between adjacent collagen are primarily responsible for holding collagen fibrils together within collagen fibers (Puxkandl et al., 2002). Five basement membrane proteoglycans including heparan sulfate proteoglycan, agrins, aggrecan, and condroadherin were identified in *S. japonicus* body wall (Wang et al., 2020).

Glycoproteins are large group of biomolecules which composed of protein and carbohydrate chain. In sea cucumber body wall, glycoprotein functions as protein linkages between collagen fibrils and responsible for MCT stiffening (Mo et al., 2016) (Figure 2.2). For instance, tensilin is one of the protein effectors in the MCT found in sea cucumber body wall which have been well characterized as a fibril-aggregating and tissue stiffening protein (Mo et al., 2016). The collagen fibrils in sea cucumber body wall also held together to form collagen fibers by stiparin. Recently, Wang et

al., (2020) reported 213 predicted ECM glycoprotein found in sea cucumber body wall including fibrilin, laminins, fibronectin, tensilins, fibrinogens, and fibulins which fibrilin become the major constituents of microfibrils and most likely perform structural roles in the formation of microfibril scaffold. Proteomic analysis of *S. japonicus* body wall further revealed that new ECM glycoprotein such as laminin, fibronectin, fibrinogen, and fibulin which have not been previously reported in sea cucumber (Wang et al., 2020).

2.2.2 Muscle protein

Muscle protein are key organs and are closely related to the sea cucumber's movement. This protein belongs to non-collagenous protein structure which distributed in the inner part of sea cucumber body wall near to the coelom (Figure 2.2). The muscle protein in sea cucumber body wall has been successfully identified and characterized using SDS-PAGE which primarily consist of myosin heavy chains (MHCs) or major yolk protein (MYP), paramyosin (Pm), and actin (Ac) with the molecular weight of approximately 200, 98, and 42 kDa, respectively (Wu et al., 2013; Zhao et al., 2016). The proteomic analysis further provides information on the 8 different categories of myofibrillar protein found in sea cucumber *(S. japonicus)* body wall including myosins, actins, troponins, tropomyosins, actin-binding proteins, myosin-binding protein, titins, obsucurins, and twitchins (Wang et al., 2020).

2.2.3 Endogenous enzymes

Sea cucumber is one of unique marine animal which reported rich in various endogenous enzymes. Different type of endogenous proteases including matrix metalloprotease (MMP) and cathepsin have been identified in sea cucumber body wall

and gut. Each of the endogenous protease have different molecular weight and performed optimum activity against specific substrate in the certain range of pH and temperature as summarised in Table 2.2.

Table 2.2 Identification and characterization sea cucumber endogenous proteases

Enzyme	Source	Optimum condition		MW (kDa)	Reference
		Temperature (°C)	pH		
Cystein-like protease	Body wall	50	7	35.5	(Qi et al., 2007)
Cathepsin-L like enzyme	Body wall	50	5	63	(Zhu et al., 2008)
Gelatinolytic metalloproteinase (GMP)	Body wall	40 – 45	8-9	45	(Wu et al., 2013)
Collagenase	Body wall	40	8	45	(Zhong et al., 2016)
High alkaline protease	Gut	-	12–14	20.6; 39.1; 114.1	(Fu et al., 2005)
Cathepsin B	Gut	45	5.5	23; 26	(Sun et al., 2011)
Cathepsin L-like proteinase	Gut	50	5–5.5	30.9	(Zhou et al., 2014)
Serine protease	Gut	35–40	6–9	34	(Yan et al., 2014)

Matrix metalloproteinases (MMP) are a large family of calcium-dependent zinc containing endopeptidases that responsible for the tissue remodelling and degradation of the ECM (Visse & Nagase, 2003). Recent studies revealed the role of MMP as key enzyme in textural degradation of sea cucumber body wall during autolysis responsible to completely depolymerize collagen fibres into smaller collagen fibrils (Liu et al., 2019a). They are classified into 6 groups including collagenases, gelatinases, stromelysins, matrilysins, MT-MMPs, and other MMPs which the classification based on the common features such as substrate specificity, domain organization, and sequence similarity (Theocharis et al., 2016). Initially, MMP are secreted from cell to ECM as inactive zymogen or pro-enzyme form (Löffek et al., 2011). MMP pro-enzyme can be activated by either ECM proteinase, several chemical agents such as thiol-modifying agents (4-aminophenylmercuricacetate, $HgCl_2$, and N-

ethylmaleimide), oxidized glutathione, SDS, chaotropic agents, and reactive oxygen species (ROS), or external factor including heat treatment and low pH (Visse & Nagase, 2003).

Cathepsins are distinct family of proteases from three different mechanistic classes: (1) cathepsins D and E (aspartic proteases), (2) cathepsins A and G (serine proteases), and (3) cathepsins B, C, F, H, L, K, O, S, V, X, and W (cysteine proteases) (Theocharis et al., 2016). They are synthesized as inactive precursors inside lysosome in endoplasmic reticulum then activated by proteolytic removal of the pro-domain (Fonović & Turk, 2014). Activated cathepsin can be released from the lysosome to the cytosol, translocated to the surface of the cell, or secreted into ECM under certain physiological condition such as cell apoptosis (Fonović & Turk, 2014). UV irradiation has been reported initiate cell apoptosis in sea cucumber body wall through the formation of ROS resulting in the released of cathepsin L to ECM (Liu et al., 2016; Yang et al., 2015).

Furthermore, proteomic study provide further information on endogenous protease in sea cucumber body wall including 33 serine peptidase, 65 metallo peptidase, 31 cystein peptidases, 4 threonine peptidases, and one aspartic peptidase, which aspartic peptidase, cysteine peptidase, metallo peptidase, serine peptidase presence relatively abundance (Wang et al., 2020).

2.3 Sulfated polysaccharides in sea cucumber body wall

Sulfated polysaccharides are the second most abundant component of sea cucumber body wall after collagen (Li et al., 2021). Two types of sulfated polysaccharides found in sea cucumber are fucosylated chondroitin sulfate (FuCS) and sulfat fucan or fucoidan (Li et al., 2021a). Much research has been done on the

structure elucidation, extraction method, bioactivity, and structure-bioactivity relation of sulfated polysaccharides isolated from sea cucumber body wall (Li et al., 2021; Xu et al., 2022).

2.3.1 Structure

2.3.1.1. Fucosylated chondroitin sulfate (FuCS)

Fucosylated chondroitin sulfate (FuCS) is a glycosaminoglycan (GAG) with unique structure which exclusively found in sea cucumber body wall (Bordbar et al., 2011). The major constituent monosaccharides of FuCS are glucuronic acid (D-GlcA), galactosamine (D-GalNAc), and fucose (L-Fuc*p*) (Pomin, 2014) as shown in Figure 2.3. Structurally, holothurian FuCS backbone is composed of a common polysaccharide found in mammalian chondroitin sulfates which composed of the repeating disaccharides of glucuronic acid (GlcA) and galactosamine (GalNAc), but the glucuronic acid residue (GlcA) has an unusual sulfated fucosyl (L-Fucp) branch O-linking to its carbon-3 (Myron et al., 2014). Due to the presence of these sulfated fucosyl branching units, FuCS exhibits additional therapeutic properties such as anti-coagulant and anti-thrombotic activity, which the mammalian polysaccharides does not have (Pomin, 2014).

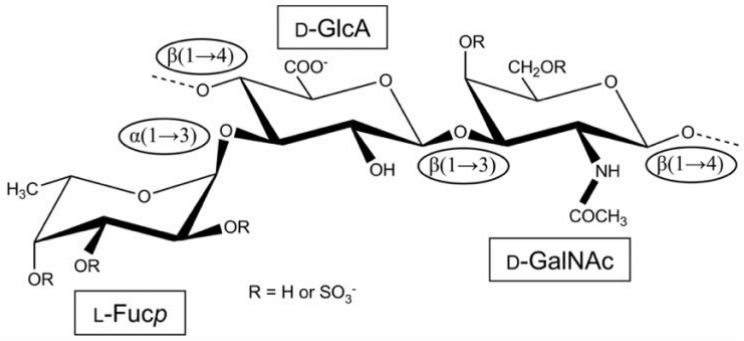

Figure 2.3 The basic structure of sea cucumber FuCS with possible sulfate ester substitution on R (Pomin, 2014).

The structure of FuCS from different species of sea cucumber vary in sulfation pattern and the presence of fucosyl branching. Gong et al. (2021) reported novel di-fucosyl branch namely Fuc2, 4Sα1→3Fuc4S from *Stichopus japonicus* FuCS which not presence in FuCS isolated from *Stichopus chloronotus* and *Stichopus horrens* (Ustyuzhanina, 2018). Mou et al., (2018), reported that FuCS from three sea cucumber species including *A. japonicus, S. chloronotus,* and *A. molpadioidea* have different sulfation pattern on their fucosyl branches which the O-4 sulfation pattern of fucosyl branch significantly influence FuCS antioxidant bioactive ability. In addition, the 2,4-O-disulfation pattern of fucosyl branches important for anticoagulant activity of the sulfated polysaccharides from different species including *Pearsonothuria graeffei*, *Stichopus tremulus*, *Holothuria vagabunda*, and *Isostichopus badionotus*. Therefore, the structure of fucosyl branches in sea cucumber FuCS have a considerable effect on their bioactivity.

2.3.1.2. Fucoidan

Sea cucumber sulfate fucan or fucoidan essentially consists of L-fucose and sulphate ester groups (Figure 2.4). Sulfated fucans in sea cucumbers are often linear polymers, consisting of repeated structural fragments (Shang et al., 2018; Ustyuzhanina et al., 2018; Yu et al., 2014). Structures of sea cucumber fucoidan were also found to be species-specific which differ on the sulfation pattern and backbone repeating unit. It is evident from comparative biological activity studies, that all the structural features of sulfated fucoidan, such as linear or branched carbohydrate moiety, sulfation pattern and several macromolecular characteristics, are important for the biological activity of these biopolymers (Luo et al., 2013). Unlike the structure of marine alga fucoidan which have complex structure including occurrence of non-

sulfated fucose branches, random substitution of sulfate groups, different types of glycosidic linkages and monosaccharide composition, as well as the presence of other possible heterogeneities such as O-acetylation and methylation (Berteau & Mulloy 2003), fucoidan isolated from sea cucumber are structurally less complex, as they only comprise only one kind of monosaccharide (Thinh et al. 2018; Ustyuzhanina et al. 2018; Yu et al. 2014; Zayed et al. 2020).

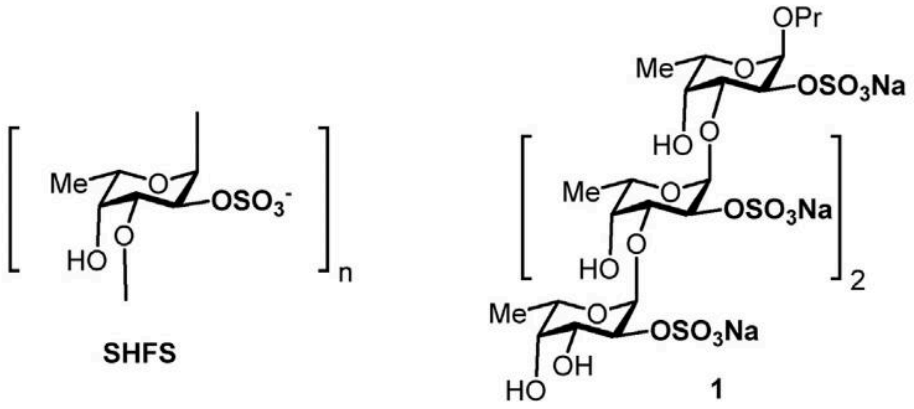

Figure 2.4 The structure of fucoidan purified from *Stichopus horrens* (Ustyuzhanina, et al., 2018).

2.3.2 The distribution of sulfated polysaccharides in sea cucumber body wall

The major distribution of sulfated polysaccharides in sea cucumber are covalently attached to the either or both of two main core protein including proteoglycan interfibrillar bridge and collagen fibrils within the unique structural of mutable collagenous tissue (MCT) (Scott, 1992; Wang et al., 2018). The distribution of FuCS in sea cucumber body wall is dependence on the sea cucumber species. For instance, most FuCS distribution in *Cucumaria frodosa* (Trotter et al., 1995) and *Stichopus japonicus* (Wang et al., 2018) body wall covalently attached to the collagen fibrils. On the other hand, Pedrosa & Paulo, (1993), reported in the sea cucumber *Ludwigothurea grisea*, FuCS present in proteoglycans with highly heterogeneous core

proteins and there is no covalent association between FuCS and collagen fibrils in this organism.

Figure 2.5 shows the model distribution of sulfated polysaccharides including FuCS and fucoidan in sea cucumber body wall particularly in mutable collagenous tissue (MCT). As shown in Figure 2.5, the distribution of FuCS in sea cucumber body wall are covalently attached to the collagen fibrils via *O*-glycosidic linkage (orange dot) and attached to the proteoglycan interfibrillar bridge between adjacent collagen fibrils (orange bar) (Pedrosa & Paulo, 1993; Vieira et al., 1993; Wang et al., 2018). The green bar represents fucoidan in sea cucumber body wall which mainly bound with the proteoglycan core protein. Besides, sulfated polysaccharide in most of the aquatic animal also found inside cell (Xiong et al., 2020).

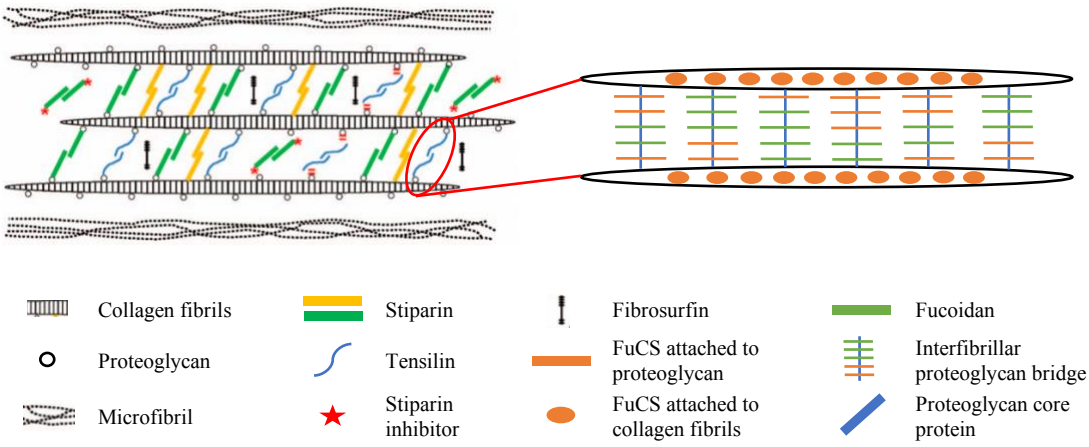

Figure 2.5 Model distribution of FuCS and fucoidan in sea cucumber body wall.

2.3.3 Bioactivity

2.3.3.1. Fucosylated chondroitin sulfate (FuCS)

Fucosylated chondroitin sulfate isolated from sea cucumber body wall has been widely reported exhibit wide spectrum of bioactivity. Mou et al., (2018) reported that FuCS isolated from edible sea cucumbers *A. japonicus*, *S. chloronotus* and *A.*

molpadioidea showed the antioxidant activity against DPPH and NO radical scavenging and inhibit lipid peroxidation which significantly influence by the sulfation patterns of the fucose branches in FuCS. The O-4 sulfation is particularly important for its antioxidant activities. The FuCS extracted from *S. japonicus* body wall using the mixture of pepsin and trypsin showed anti-inflammation activity (Cui et al., 2016b). Li et al., (2017), reported that FuCS isolated from *Isostichopus badionotus* body wall exerted anticoagulant activity only through the intrinsic pathway and had no or little effect on extrinsic and common coagulation pathways. The sulfation pattern of the fucose branch plays an important role in the anti-coagulant activity, but its impact on anticoagulant activity is less than molecular weight.

Furthermore, Gong et al. (2021), investigated the bioactivity of FuCS isolated from *S. japonicus* body wall which proven to have the potential antidiabetic activity by ameliorate the glucose uptake and glucose consumption of glucosamine-induced IR-Hep G2 cells and promote glycogen synthesis in the IR-Hep G2 cells. In addition, anti-hyperlipidemic bioactivity of FuCS isolated from *I. badionotus* have been reported by Li et al., (2017b) which showed that the outstretched linear conformation of FuCS show greater hypolypidemic activity.

2.3.3.2. Fucoidan

The sulfate fucan or fucoidan extracted from sea cucumber body wall also has been reported exhibited many bioactive potentials including anti-inflammatory, anti-coagulant, immunomodulator, and anti-thrombotic activity. Wang et al., (2016) reported that fucoidan isolated from *I. badionotus* alleviated inflammatory response and signaling as well as improved insulin resistance in the liver of obesity mice. The highly regular homogeneous fucoidan extracted from *H. fuscopunctata*, *T. ananas* and

S. horrens showed anti-coagulant activity which influenced by its structure particularly specific sulfation pattern and position of the glycosidic linkage (Shang et al., 2018). In addition, fucoidan isolated from sea cucumber body wall also reported have immunomodulator activity and anti-thrombotic activity (Chen et al., 2012; Jiang et al., 2021).

2.3.4 Extraction methods of sulfated polysaccharides

Sulfated polysaccharides in sea cucumber body wall exist as free and widely attach to two types of core proteins including proteoglycan interfibrillar bridge between adjacent collagen and collagen fibrils which buried within compact MCT structure. The challenge is to extract them effectively from this compact MCT without causing polysaccharides degradation and losing their functionality. Thus, the yield, extraction speed, purity, structure, and bioactivity of sulfated polysaccharide are highly influenced by the extraction method used (Li et al., 2021a). As shown in Table 2.3, the extraction method of sulfated polysaccharides from sea cucumber body wall includes hot water extraction, enzymatic-assisted extraction, combination of enzymatic-assisted and alkaline extraction, and autolysis. Most widely used method is enzymatic-assisted extraction and its combination with alkaline extraction due to higher yield, purity, and lower cost compared to other methods (Li et al., 2021c).

Table 2.3 Extraction method of sulfated polysaccharides from different sea cucumber species.

Methods	Species	Conditions	Yield (%) *	References
Hot water extraction	*S. japonicus*	Distilled water ratio 1:20 (w/v); 80 ℃; 2 hours	9.1 (ddb)	(Cao et al., 2017)
	T. ananas, S. variegatus, S. choloronotus, H. nobilis, H. albiventer, H. scabra, H. tubulosa, H. impatiens, Y. bitentaculata and C. frondosa	Deionized water; 105 ℃; 1 hours	3.9 – 11.4 (db)	(Zhu et al., 2020)
Enzymatic extraction	*S. horrens, H. Arenicola, and H. atra*	Papain hydrolysis at 60 ℃ for 24 hours	5.7 – 6.6 (ddb)	(Myron et al., 2017)
	S. horrens	8.9% papain, 45 – 50 ℃; 24 hours	11.9 (db)	(Ustyuzhanina et al., 2018)
	S. horrens and S. chloronotus	8.9% papain, 45 – 50 ℃; 24 hours	5.0 – 11.9 (db)	(Ustyuzhanina, 2018)
	S. variegatus	10% papain, 60 ℃; 24 hours	6.8 (dft)	(Thinh et al., 2018)
	S. japonicus	0.4 % pepsin and 0.5% trypsin; 50 ℃; 5 hours	0.11 (ft)	(Zhang et al., 2010)
	C. frondosa	0.4% alcalase; 60 ℃; 24 hours	0.27 (ft)	(Kale et al., 2013)
Combination of enzymatic and alkaline extraction	*S. chloronotus*	KOH and disaste vera enzyme	-	(Mou et al., 2020)
	S. chloronotus, A. japonicus, and A. molpadiodea	1 M KOH; 60 ℃; 1 hours followed by 5% disaste vera; 50 ℃; 3 hours	6.4 – 8.1 (db)	(Mou et al., 2018)
	S. variegatus and H. fuscopunctata	0.1% papain; 50 ℃; 6 hours followed by 0.5 NaOH; 60℃; 2 hours	-	(Liu et al., 2020)
	S. hermanni	0.5% NaOH followed by 5% papain	7.5 (db)	(Li et al., 2017)
Autolysis	*S. japonicus*	Induce by cutting body wall; room temperature; 72 hours	4.5 (db)	(Song et al., 2018)

*Note: fresh or frozen tissue (ft); defatted fresh or frozen tissue (dft); dry biomass (db); defatted dry biomass (ddb).

2.3.4.1. Hot water extraction

Hot water extraction is a traditional and widely used method which play important role in the extraction of water-soluble sulfated polysaccharides. The principle of this method is accelerating the diffusion rate and improve the extraction efficiency of sulfated polysaccharide with the assistance of temperature. Compared with other methods, the hot water extraction method is simple, easier to control, lower in cost and using simpler equipment. However, because hot water extraction cannot completely degrade cell membranes, it can only extract extracellular polysaccharides. Hence, the production and efficiency are often low (Xiong et al., 2020). Only few studies use hot water method for sulfated polysaccharide extraction from sea cucumber body wall. For instance, the hot water extraction of *S. japonicus* with the water ratio 1:20 (w/v) at 80 ℃ for 2 hours produced crude polysaccharide with lower yield (9.1%) (ddb) (Cao et al., 2017) compared to enzymatic extraction method with the highest yield was 11.9% (db) as shown in Table 2.3. Zhu et al., (2020) extracted sulfated polysaccharides from 10 low edible sea cucumber species using combination of enzymatic extraction method with papain for 4 hours followed by hot water extraction at 105 ℃ for 1 hour also resulted in considerably low yield of sulfated polysaccharides between 3.90 – 11.44% dry weight.

2.3.4.2. Enzyme-assisted extraction

Enzyme-assisted extraction can hydrolyze selective glycopeptide linkage including core protein which polysaccharides are attached while maintaining glycosidic bonds. In addition, the enzyme-assisted extraction also can efficiently hydrolyze components of cell membranes which facilitate the release of polysaccharides distributed inside cells (Xiong et al., 2020). Therefore, enzyme-

assisted extraction significantly improves extraction rate and has the advantages to reduce solvent used and energy. Enzyme-assisted extraction is widely used to extract polysaccharides from sea cucumbers using different types of enzymes including papain, pepsin, trypsin, alcalase, and disaste vera as described in Table 2.3 (Li et al., 2021a). Among the proteases, papain is the most frequently adopted enzyme due to its specificity to hydrolyze core protein to which sulfated polysaccharide attached and disintegrate the cell membrane without polysaccharides degradation (Li et al., 2021a). As shown in Table 2.3, enzymatic extraction using papain resulted in the highest yield (11.9, db) of sulfated polysaccharides compared to other methods.

2.3.4.3. Combination of enzymatic and alkaline extraction

In order to enhance the extraction efficiency and obtain faster extraction time of sulfated polysaccharides, the enzymatic extraction is commonly combined with alkaline extraction (Li et al., 2021a). Dilute alkali solution facilitates the breakdown of glycopeptide bond between polysaccharide and core protein via β-elimination reaction (Xiong et al., 2020). The synergic effect of enzyme to hydrolyze the core protein which polysaccharide attached, disintegrate the cell membrane, and the ability of alkaline to cleave the glycopeptide bond between polysaccharides and its core protein resulted in the shorter extraction time and improve extraction rate (Li et al., 2021a). As shown in Table 2.3, the combination of enzymatic (papain or disaste vera) and alkaline extraction can be either enzymatic extraction followed by alkaline extraction or alkaline extraction followed by enzymatic extraction. The combination of enzymatic extraction and alkaline extraction method is usually conducted with low concentration of alkaline and lower amount of enzyme (8.9 – 10%) compared to only enzymatic extraction process (0.1 – 5%). In addition, the combination of enzymatic

and alkaline extraction method resulted in the shorter extraction time (4 – 8 hours) with slightly lower yield compared to only using enzymatic-assisted extraction method (24 hours) as shown in Table 2.3.

2.3.4.4. Autolysis

Autolysis of sea cucumber body wall is potential to release sulfated polysaccharides from the action of endogenous enzymes including cathepsin and matrix metalloprotease that play role in the disintegration of collagen fibers to collagen fibrils by cleave proteoglycan interfibrillar bridge (Liu et al., 2017, 2018a; Liu et al., 2019a). Song et al. (2018), reported that 65% sulfated polysaccharides release from sea cucumber body wall after 72 hours autolysis at room temperature. Autolysis is simple and low cost but need longest incubation time (72 hours) and produced polysaccharides with lowest yield (4.5%, db) compared to other methods as shown in Table 2.3 which makes this extraction method is less efficient. Hierarchical complex biochemical reaction of autolysis contributes to slow proteolytic reaction that usually involves endogenous enzyme exist as inactive form or zymogen which require specific stimulation or activation (Qi et al., 2016).

2.4 Autolysis of sea cucumber body wall

The body wall of sea cucumber easily undergo massive tissue autolysis due to its unique structural elements of mutable collagenous tissue (MCT) with reactive endogenous proteolytic enzymes that regulate its dynamic motion and regeneration including melting phenomena or ‘‘local degeneration” (Hill, 2001). Autolysis or self-digestion is defined as the physiological process of destruction of living organism in a series of complex biochemical reactions through the action of its endogenous enzyme