COMPARISON OF FUCOSYLATED CHONDROITIN SULFATE STRUCTURE FROM THREE HOLOTHURIAN SPECIES IN SABAH, MALAYSIA

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ABSTRACT

Fucosylated chondroitin sulfate (FuCS) is a unique compound from commercially important holothurians that possess pharmaceutical bioprospect through its many physicochemical activities but was not explored in non-targeted species of *Stichopus horrens*, *Holothuria atra* and *Holothuria arenicola*. This study has examined the levels and structure of FuCS in these three holothurian species of Sabah using combination of spectrometry, spectroscopy and chemometrics techniques. Through extraction and purification, the FuCS levels for *H. arenicola* was 66.24±0.307 µg/ml, *H. atra* was 73.16±0.293 µg/ml and *S. horrens* was 104.47±0.338 µg/ml. High performance liquid chromatography analysis confirmed each of the extracts had approximately equimolar of D-glucuronic acid (GlcUA) and D-N-acetylgalactosamine (GalNAc), resembling chondroitin sulfate analogs. *Stichopus horrens* had significant higher fucose ratio at 0.78 per mol GalNAc followed by *H. atra* at 0.45 per mol GalNAc and *H. arenicola* at 0.44 per mol GalNAc. Liquid chromatography quadrupole time-of-flight mass spectrometry (LCQTOF/MS) analysis had deduced the mode of mass breakage of the FuCS components and determined the extracts purity of >96.1%. Gas chromatography mass spectrometry (GCMS) analysis suggested sulfation was on the fourth and sixth carbon of acetylgalactosamine. Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) analysis showed additional evidence of sulfate and sulfation positions on the compound. The second derivatives IR spectra of each holothurian species had distinct absorptions difference in the region 860-800 cm^{-1}, indicating the sulfation position from each species was different. Principal component analysis (PCA) models had facilitated the IR discrimination and found the greatest influential variables were from carbonyl region, seconded by sulfation region and to a lesser extend of ring vibration region. Both PCA models and hierarchical clustering analysis (HCA) clustered the samples precisely to three clusters. The HCA analysis showed low degree of heterogeneity among the sample branches while samples from *S. horrens* forming an isolated branch at distance height of 285.234 using classical PCA scores and 0.057 using robust PCA scores. In conclusion, these three previously unexplored holothurian species had been proven to possess analogous FuCS chemical structure from commercially important species counterparts.

ABSTRAK

PERBANDINGAN STRUKTUR KONDROITIN SULFAT FUCOSYLATED DARIPADA TIGA SPECIES TIMUN LAUT DI SABAH, MALAYSIA

Kondroitin sulfat fucosylated (FuCS) merupakan sebatian unik daripada timun laut bermutu tinggi malah mempunyai nilai bioprospek farmaseutikal melalui pelbagai aktiviti fizikokimia tetapi belum dikaji dalam spesies kurang mutu seperti Stichopus horrens, Holothuria atra and Holothuria arenicola. Kajian ini meneliti tahap dan struktur FuCS dalam ketiga-tiga spesies timun laut daripada Sabah menggunakan kombinasi teknik spektrometri , spektroskopi dan chemometrik. FuCS telah diekstrak daripada Holothuria arenicola, Holothuria atra dan Stichopus horrens dengan memperolehi kepekatan 66.24±0.307, 73.16±0.293 dan 104.47±0.338 µg/ml, masing-masing. Analisis kromatografi cecair berprestasi tinggi menunjukkan kesemua ektrak timun laut mempunyai asid D-glucuronic dan D-N-acetylgalactosamine dengan nisbah yang equimolar, menyerupai struktur kondroitin sulfate. Stichopus horrens mempunyai nisbah fucose yang lebih tinggi pada nisbah 0.78/mol GalNAc diikuti H. atra pada nisbah 0.45/mol GalNAc dan H. arenicola pada nisbah 0.44/mol GalNAc. Analisis kromatografi cecair spektrometri jisim (LCQTOF/MS) berjaya menyimpulkan mod pecahan jisim komponen FuCS dan menghitungkan ketulenan ekstrak adalah lebih daripada 96.1%. Analisis kromatografi gas spektrometri jisim (GCMS) mencadangkan bukti ikatan ester sulfat pada karbon keempat dan keenam acetylgalactosamine. Analisis pelemahan jumlah pantulan - jelmaan Fourier spektroskopi inframerah (ATR-FTIR) menunjukkan corak sulfat dan kewujudan sulfat pada sebatian. Terbitan kedua IR spektrum setiap species mempunyai perbezaan penyerapan inframerah sekitar 860-800cm^{-1}, menunjukkan corak sulfat setiap species timun laut adalah berbeza. Model analisis principal component (PCA) telah memudahkan pembezaan IR dan mendapati pembolehubah paling berpengaruh merangkumi rantau karbonil, sulfat dan getaran cincin. Kedua-dua model PCA dan analisis pengelompokan hierarki (HCA) mengumpulkan sampel tepat kepada tiga kumpulan. Analisis HCA menunjukkan tahap rendah kepelbagaian di kalangan cawangan sampel manakala sampel dari S. horrens membentuk cawangan tersendiri pada jarak paksi-y 285.234 menggunakan skor PCA klasik dan 0.057 menggunakan skor PCA teguh. Kesimpulannya, ketiga-tiga spesies timun laut ini telah terbuki mempunyai ciri-ciri FuCS yang sama dengan spesies timun laut yang bernilai komersial tinggi.

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LIST OF SYMBOLS & ABBREVIATIONS

%	-	Percentage
(S/N)	-	Signal to noise ratio
(v/v)	-	Volume by Volume
°C	-	degree Celsius
®	-	Registered trademark
µm	-	micrometer
^{60}Co	-	Cobalt 60
Å	-	Angstrom
ACN	-	Acetonitrile
ANOVA	-	Analysis of Variance
aPTT	-	activated partial thromboplastin time
AT III	-	AntiThrombin type 3
ATR-FTIR	-	Attenuated total reflectance-Fourier transform infrared
AUFS	-	Absorbance Unit Full Scale
B.C.	-	Before Christ
BSTFA	-	N,O-Bis-trifluoroacetamide
Ca^{2+}	-	Calcium ion
CD4	-	Clusters of Differentiation 4
CITES	-	Convention on International Trade in Endangered species
cm^{-1}	-	Unit for wavenumber; # of waves per centimeter
CNS	-	Central Nervous System
CPC	-	Cetylpyridinium chloride
cPCA	-	Classical Principal Component Analysis
CS D	-	Chondroitin sulfate type D
CS E	-	Chondroitin sulfate type E or Chondroitin 4,6-di-sulfate
CS	-	Chondroitin Sulfate
CXCR4/ CCR5	-	Chemokine Receptor Type 4 or 5
DHG	-	Depolymerized holothurian glycosaminoglycan
DMMB	-	1,9-dimethylmethylene Blue
DS	-	Dermatan sulfate or Chondroitin sulfate type B
EC_{50}	-	Effective drug concentration at 50% maximal

ECM	-	Extra cellular matrix
EDTA	-	Ethylenediaminetetraacetic acid
FPLC	-	Fast Protein Liquid Chromatography
Fuc	-	Fucose
Fuc2S4S	-	Fucose, sulfated at carbon-2 and 4
Fuc3S	-	Fucose, sulfated at carbon-3
FuCS	-	Fucosylated chondroitin Sulfate
g	-	Relative centrifugal force to earth's gravity
GAGs	-	Glycosaminoglycans
Gal	-	Galactose
GalNAc	-	N-acetylgalactosamine
GCMS	-	Gas Chromatography Mass Spectrometry
GlcA	-	Glucuronic acid
GLUT4	-	Glucose Transporter 4
GPI	-	Glycosylphosphatidylinositol
GuHCl	-	Guanidine Hydrochloride
H_2SO_4	-	Sulfuric acid
HA	-	Hyaluronic acid
HC II	-	Heparin Cofactor type 2
HCA	-	Hierarchical Clustering Analysis
HCl	-	Hydrochloric acid
HDL	-	High density lipoprotein
Hep	-	Heparin
HG	-	Holothurian Glycosaminoglycan
HIV	-	Human Immunodeficiency Virus
HS	-	Heparan sulfate
HUVEC	-	Human umbilical vein endothelial cell
IdoaA	-	Iduronic acid
IU/mg	-	International Unit per milligram
kDa	-	kiloDalton or atomic mass unit
KS	-	Keratan sulfate
L-/D-	-	conformation L or D
LDL	-	Low density lipoprotein

LMWH	-	Low Molecular Weight Heparin
M	-	Mole
m/z	-	mass per charge
Man	-	Mannose
MeOH	-	Methanol
mg/g	-	milligram per gram
mg/kg	-	milligram per kilogram
mg/ml	-	milligram per milliliter
MS	-	Mass Spectrometry
MW	-	Molecular Weight
NaCl	-	Sodium Chloride
NIST	-	National Institute of Standards and Technology
nm	-	nanometer
NMR	-	Nuclear magnetic resonance
PAD	-	Pulsed Amperometry Detector
PF4	-	Platelet Factor 4
PI3K	-	Phosphoinositide 3-kinase
PKB	-	protein kinase B
pmol	-	Pico Mole
PMP	-	1-Phenyl-3-methyl-5-pyrazolone
ppm	-	Parts per Million
QTOF	-	Quadruple Time Of Flight
rPCA	-	Robust Principal Component Analysis
RP-HPLC	-	Reverse Phase-High Performance Liquid Chromatography
rpm	-	Revolution per Minute
RSG	-	Rosiglitazone drug
Ser	-	Serine, amino acid
T-20	-	Enfuvirtide drug
TFA	-	Trifluoroacetic acid
TMS	-	Trimethyl-silyl
™	-	Trademark
UFH	-	Unfractionated Heparin
ug/ml	-	microgram per milliliter

UV	-	Ultra Violet
Xyl	-	Xylose
µL	-	microliter

CHAPTER 1

INTRODUCTION

1.1 Overview

Among the twelve "mega-biodiversity" countries, Malaysia is accounted for its great biological diversity and species endemism. It is also stated in the Malaysia National Biodiversity Policy (1998) that East Malaysia is blessed with greater diversity and endemism of holothurian species compared to Peninsular Malaysia (MOSTI, 1998). Albeit sea cucumber is considered as small scale fisheries to compared with other fisheries sectors, such as marine finfish and shrimp, the sea cucumber fisheries in Sabah remain significant in terms of commodity contribution to sea cucumber fisheries in Malaysia (Choo, 2004). Sea cucumber harvesting is considered as artisanal fisheries in Malaysia, but plays an important part as a food source and traditional remedies such as for cuts and burns, impotence and rheumatism (Bordbar et al., 2011; Choo, 2004). Out of the estimated 80 species in Malaysia, only a handful are edible (Choo, 2008) such as *Holothuria scabra*, *Thelenota ananas*, *Thelenota anax* and *Stichopus hermanni*, while *Stichopus horrens* and *Stichopus hermanni* are choice species used for making traditional medicine (Choo, 2004). The body wall or internal organs are consumed depending on traditional medicinal beliefs or serves as a food source (Hartati and Yanti, 2006).

Sea cucumber possesses wide array of bioactive components such as saponins, proteoglycans, sulfated polysaccharides, sterols, phenolics, cerebrosides, lectins and fatty acids (Bordbar et al., 2011). These bioactives are gaining recognition in modern biomedical research to explain the properties exhibited in traditional medicine practices such as wound healing, rheumatism, body nourishment and asthma (Chen, 2003). The compound fucosylated chondroitin sulfate (FuCS) is a characteristic sulfated polysaccharide belonging to sea cucumber

species (Yamada et al., 2011). This compound is unique in various bioactivities having a research record for the last three decades from many research groups covering the structural characterization to physicochemical activities (Pomin, 2014a; Myron et al., 2014a). However, structural characterization of FuCS were mainly addressed in a few commercially important species such as *Thelenota ananas* (Wu et al, 2012), *Ludwigothuria grisea* (Mourão, 1996), *Apostichopus japonicus* (Kariya et al, 1997) and *Holothuria forskali* (Panagos et al., 2014).

1.2 A unique bioactive compound: fucosylated chondroitin sulfate (FuCS) in sea cucumber

Fucosylated chondroitin sulfate is an anionic mucopolysaccharide found in the body wall of holothurian (Pomin et al, 2014a). The origin of the compound is thought to be an integral part of holothurian stiffening-liquidation mechanism by being the complex glycosaminoglycan that interacts with core proteins to form proteoglycans found in the cellular environment (Mourão, 1991; Trotter et al., 1998; Koob et al., 1999). FuCS has demonstrated various biological activities such as antithrombotic and anticoagulant (Mourão et al., 1996; Kariya et al., 1997; Wu et al., 2012), angiogenesis and anti-angiogenesis (Tapon-Bretaudière et al., 2000), antitumor (Borsig et al., 2007; Zhang et al., 2009; Lu et al., 2010; Zhao et al., 2013a), wound healing (Masre et al., 2010; Patar et al., 2012a; Patar et al., 2012b), antihyperlipidemic (Tovar and Mourão, 1996; Liu et al., 2002; Liu et al., 2012), antidiabetic (Hu et al., 2013; Hu et al., 2014a) and anti-human immunodeficiency virus-HIV (Hoshino and Heiwamachi, 1990; Huang et al., 2013; Lian et al., 2013). Among the biomedical effects, anticoagulation-related physicochemical activity has been well documented in two species of holothurian namely: *Ludwigothurea grisea* (Mourão et al., 1996), and *Stichopus japonicus* (Kariya et al., 1997). Moreover, the sulfation patterns on the fucose side chain are responsible for the biomedicinal properties (Pomin, 2014a). Zhao et al. (2012) mentioned the elucidation of whole molecular sequence of FuCS remains a challenge and FuCS structures related to its physicochemical activity was only partially known.

Studies in Malaysia have covered some promising results from the sulfated polysaccharide of three locally available species namely *Holothuria scabra, Stichopus hermanii* and *Holothuria leucospilota*. Extracts from *S. hermanii* have

shown potent bioactivity on wound healing (Masre et al., 2010; Patar et al., 2012b) and spinal astrocytes cell line growth (Patar et al., 2012a); An unknown sulfated polysaccharides compound from *H. leucospilota* exhibited antitumor metastasis (Zhang et al., 2009); and extracts from *H. scabra* showed hypolipidemic activity (Liu et al., 2002). The structural characteristics of their extract were not identified in aforementioned studies. Nevertheless, the authors suggested the active compound could be FuCS or FuCS analogs (Masre et al., 2010; Patar et al., 2012a; Liu et al., 2002; Zhang et al., 2009). The general aim of this study is to explore the structural characteristics and levels of FuCS in non-targeted holothurian species found in Sabah using separation techniques and infrared spectroscopy. Understanding the structural characteristics of FuCS will provide fundamental information for future pharmaceutical applications in regards to drug synthesis.

1.3 Research objectives

This study were focused on two main objectives to achieve the aim of providing fundamental structural characterization of FuCS from non-targeted holothurian species:-

I. To compare the FuCS fine structure extracted from three non-targeted local holothurian species.
II. To Determine the FuCS levels in three non-targeted local holothurian species.

The specific objectives of this study were:

a. To optimize FuCS extraction method.
b. To determine FuCS monosaccharide composition using 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization through reverse phase high performance liquid chromatography (RP-HPLC).
c. To elucidate the structure of FuCS using mass spectrometry techniques.
d. To elucidate the structure of FuCS using ATR-FTIR technique.
e. To compare amongst the FuCS IR spectra of three holothurian species using chemometric analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Sea cucumber status in Sabah

Sea cucumber or its interchangeable common name, holothurian belongs to the taxonomy class Holothuroidea. The class is further subdivided into three subclass namely Aspidochirotacea, Apodacea and Dendrochirotacea. The usual external morphology of the body shape is elongated longitudinally with different height of papillae. The stout, thick and firm body has a variable degree of tegument roughness dorsally and usually darker than the ventral (Figure 2.1). Microscopic spicules or "ossicles" are enclosed within the connective tissues in the dermis and used as identification key of species.

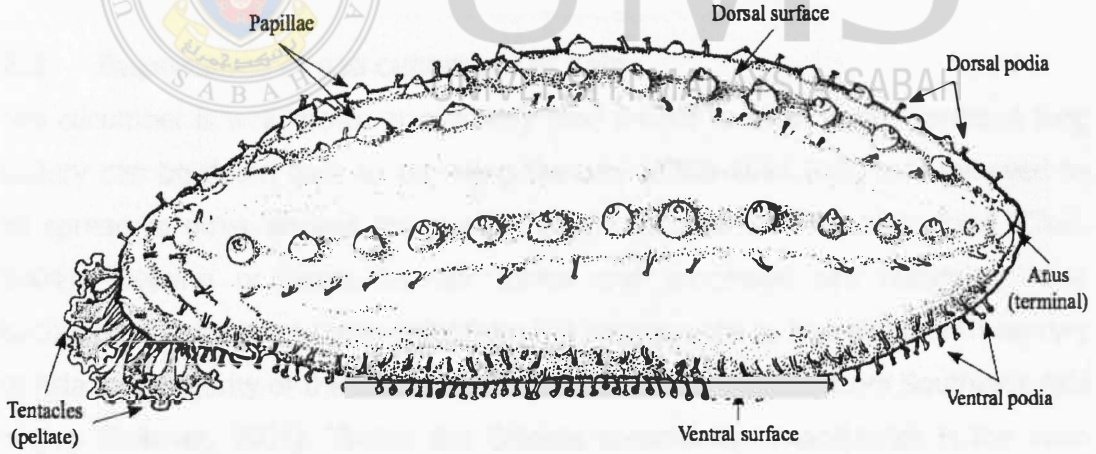

Figure 2.1: External morphology of holothurians (dorsal view).
Source: Purcell et al., 2012

A total of 62 species belonging to three Orders and five families were reported by Choo (2008) in Malaysian waters. Kamarudin et al., (2010) reviewed the species diversity and estimated there were more than 80 species in Malaysia. The Southeastern coast of Sabah around Semporna which located in the Wallace's