UNIVERSITI PUTRA MALAYSIA

PRODUCTION AND CHARACTERIZATION OF COLLAGEN AND GELATIN FROM EDIBLE JELLYFISH (Acromitus hardenbergi Stiasny, 1934) FOR FABRICATION OF BIOFUNCTIONAL SCAFFOLD

NICHOLAS KHONG MUN HOE

IB 2014 12

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By

NICHOLAS KHONG MUN HOE

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

December 2014

DEDICATIONS

To those whom I love dearly, and

To those who love me selflessly.

To those that finds competition in me, nonetheless.

To you, especially,

who is reading this,

May this little piece illustrates you some marvels of knowledge,

May it brings you joy of explorations,

May it brings you hope and inspirations.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

PRODUCTION AND CHARACTERIZATION OF COLLAGEN AND GELATIN FROM EDIBLE JELLYFISH (*Acromitus hardenbergi* Stiasny, 1934) FOR FABRICATION OF BIOFUNCTIONAL SCAFFOLD

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December 2014

Chair: Fatimah Md. Yusoff, PhD
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Jellyfish have always been an important fishery commodity and a multi-million business widely practised in China, Japan, Malaysia, Indonesia, Thailand, Vietnam and the Philippines. This study aimed to explore food and nutritional properties of indigenous jellyfishes; which contributes to the development of simple and efficient extraction procedures of jellyfish collagen and gelatin that would be practical in the production of biomedical-significant matrices for potential tissue engineering applications. It was found that edible jellyfishes were food of low calorific value (ranged from 1.0 to 4.9 kcal/g dry weight) and high protein (ranged from 20.0 to 53.9 g/ 100g dry weight) and minerals (total ash ranged from 15.9 to 57.2 g/ 100g dry weight) components. Collagen was estimated to comprise approximately half the total protein content of edible jellyfishes, hence the major protein component of edible jellyfishes. Among the jellyfishes, *A. hardenbergi* exhibited significantly ($p < 0.05$) better protein quality (higher total amino acids and collagen content) and richer in major minerals compared to other two species. Subsequently, two novel procedures were devised for efficient collagen and gelatin extraction from *A. hardenbergi*. It was found that this improved process significantly increased the production yield ($p < 0.05$) by approximately seven times and two times compared to the conventional acid-assisted and pepsin-assisted extraction, respectively. Proximate composition and amino acids profile of collagen extracted using the improved process was found to be similar ($p > 0.05$) to those extracted using the pepsin-assisted extraction. However, the collagen produced by the improved process retained high molecular weight distributions and polypeptide profiles similar to the conventional acid-assisted extraction. They possessed better appearance, instrumental colour and were found to be non-toxic *in vitro* and free of heavy metal contaminations. Findings from comparisons between the novel and the conventional procedures illustrated that different treatments of collagen extraction process imparts significant impacts to the quality, quantity and cost of the collagen produced. The study also managed to extract gelatin successfully from jellyfish tissues with satisfactory yield, gelling (Bloom) and sensory properties. Jellyfish gelatin has high protein content (>80%) with low moisture (<2.5%), ash (<2.0%) and negligible fat contents. All gelatins had very mild to undetectable fishy odour and were all whitish in appearance. Jellyfish collagen and gelatin were then used to fabricate porous scaffolds suitable for tissue engineering application by freeze-drying the mixture with different proportions of chitosan solutions. The addition of jellyfish gelatin into

the scaffold formulation, to partially substitute collagen, was able to reduce the production cost while improving functional characteristics of the scaffolds. The combinations of different jellyfish collagen and gelatin concentrations were observed to have significant effects on the cross-section morphology and the molecular integrity of the cross-linked scaffolds. Proportional combination of jellyfish collagen into the ternary scaffolds was found to significantly ($p < 0.05$) increase mean pore size and porosity. All ternary scaffolds exhibited mean pore size in the range of 200-300 µm and porosity above 90%. These results suggested that the chitosan: jellyfish-derived collagen and gelatin tripolymer matrix is a potential candidate for soft tissues equivalent with enhanced biostability and good biocompatibility. Overall, the findings of this study supported that jellyfish is potentially practical as a sustainable source of high quality collagen and gelatin. Jellyfish-derived biomaterials were also found to exhibits good biophysicofunctionality in the fabrication of scaffolds for tissue engineering. Scaffolds, fabricated in the current study is but a beginning for more forms of functional matrices such as hydrogel, sol, liquid, concentrate and powder.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN DAN PENCIRIAN KOLAGEN DAN GELATIN DARIPADA UBUR-UBUR (*Acromitus hardenbergi* Stiasny, 1934) UNTUK FABRIKASI PERANCAH BIOFUNGSIAN

Oleh

NICHOLAS KHONG MUN HOE

Disember 2014

Pengerusi: Fatimah Md. Yusoff, PhD
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Ubur-ubur merupakan komoditi perikanan yang penting dan pemakanannya diamalkan secara meluas di China, Jepun, Malaysia, Indonesia, Thailand, Vietnam dan Filipina. Kajian ini bertujuan untuk menerokai ciri-ciri makanan dan nutrisi ubur-ubur tempatan dan membangun prosedur cekap pengekstrakan kolagen dan gelatin yang akan menyumbang kepada pembuatan matriks bioperubatan untuk aplikasi kejuruteraan tisu. Hasil pengajian menunjukkan bahawa ubur-ubur ialah makanan berkalori rendah (antara 1.0 – 4.9 kcal/g berat kering) yang kaya dengan protein (antara 20.0 - 53.9 g/100g berat kering) dan mineral (jumlah abu antara 15.9 – 57.2 g/100 g berat kering). Kira-kira separuh daripada jumlah kandungan protein ubur-ubur dianggarkan terdiri daripada kolagen. Antara ubur-ubur yang dikaji, *A. hardenbergi* menunjukkan jumlah asid amino, kandungan kolagen dan mineral yang lebih tinggi berbanding spesies yang lain, dengan ketara ($p < 0.05$). Selepas itu, dua prosedur baru telah berjaya dicipta untuk penghasilan kolagen dan gelatin yang eficien daripada *A. hardenbergi*. Proses pengekstrakan kolagen baru mampu meningkatkan hasil pengeluaran dengan ketara ($p < 0.05$), kira-kira tujuh kali dan dua kali ganda berbanding dengan kaedah pengekstrakan konvensional yang menggunakan asid dan enzim (pepsin), masing-masing. Komposisi proksimat dan asid amino kolagen yang diekstrak menggunakan proses baru adalah seakan kolagen yang diekstrak dengan bantuan pepsin ($p > 0.05$). Pada masa yang sama, kolagen yang diekstrak dengan kaedah baru mengekalkan profil pengagihan berat molekul polipeptida seakan kolagen yang diekstrak dengan kaedah asid konvensional ($p > 0.05$). Kolagen tersebut mempunyai penampilan dan warna yang lebih baik dan didapati bebas daripada pencemaran logam berat serta toksik *in vitro*. Perbandingan antara kaedah baru dan konvensional menunjukkan bahawa rawatan yang berbeza semasa proses pengekstrakan kolagen memberi kesan yang ketara kepada kualiti, kuantiti dan kos kolagen yang dihasilkan. Kajian ini juga berjaya merumuskan kaedah penghasilan gelatin daripada tisu ubur-ubur yang mempunyai ciri gel (Bloom) dan sensori yang memuaskan. Gelatin ubur-ubur mempunyai kandungan protein yang tinggi (> 80%), air (<2.5%), abu (<2.0%) dan kandungan lemak (>0.1%) yang rendah. Gelatin yang terhasil mempunyai bau hanyir ikan yang sangat ringan dan mempunyai penampilan putih salji. Kolagen dan gelatin ubur-ubur kemudiannya digunakan untuk mereka perancah berliang yang sesuai untuk aplikasi kejuruteraan tisu dengan kaedah pengeringan beku campuran kolagen-gelatin dengan perkadaran yang berbeza dengan kitosan. Penambahan gelatin ubur-ubur ke dalam formulasi

perancah, sebagai pengganti kolagen dalam nisbah terpilih, dapat mengurangkan kos pengeluaran sambil meningkatkan ciri-ciri fungsian perancah. Kombinasi nisbah kolagen dan gelatin ubur-ubur yang berbeza didapati mempunyai kesan yang ketara (p > 0.05) ke atas keratan rentas morfologi dan integriti molekul perancah. Turut diperhatikan adalah peningkatan saiz liang dan keliangan.perancah yang ketara (p < 0.05) hasil penambahan kolagen ubur-ubur. Semua perancah mempamerkan saiz purata liang dalam julat 200-300 μm dan keliangan melebihi 90%. Keputusan ini mencadangkan bahawa kolagen dan gelatin ubur-ubur mampu menambahbaik perancah perubatan kitosan dan merupakan calon yang berpotensi untuk penggantian tisu manusia seperti kulit, tulang dan hati. Secara keseluruhannya, hasil kajian ini menyokong usul potensi ubur-ubur sebagai sumber lestari kolagen dan gelatin yang berkualiti tinggi dan praktikal. Biopolimer yang diperolehi daripada ubur-ubur juga mempamerkan ciri-ciri fizikal dan fungsian yang baik dalam pembuatan perancah untuk aplikasi kejuruteraan tisu. Kolagen dan gelatin terhasil daripada kajian ini mampu berfungsi baik dalam pelbagai bentuk termasuk bentuk serbuk, matriks, hidrogel, sol mahupun cecair.

ACKNOWLEDGEMENTS

"We can only appreciate the miracle of a sunrise if we have waited in the darkness." Many thanks to whoever and whatever that have showed me kindness, patience and guidance throughout this roller-coaster ride. No words can express, no act of gratitude can relay and no gift can represent what your love and support have meant to me. Please accept this note as an indicator of my heartfelt appreciation for everything you are.

First and foremost, to my family: my mother Mdm. Chan Ooi Yee; my grandmothers Mdm. Chan King Chen and Mdm. Wong Swee Lan; my sisters Elaine and Evon; brother Eric, and uncle Foong Kin Tuck, many thanks for the unconditional love and understanding. For those you do not understand, you trust me nevertheless. This, I will be forever grateful.

My sincerest gratitude to my supervisors, Prof. Dr. Fatimah Md. Yusoff, Prof. Dr. Jamilah Bakar, Prof. Dr. Mahiran Basri and Prof. Dr. Maznah Ismail, for plenty of what I am today is born from your kindness and tolerance. Words cannot express all that I wish to say to you. The difference you make is nothing short of legendary.

To my mentor, Chan Kim Wei, my beloved brother concurrently, friend like you are a rare gift. Many thanks for all you have done for me. Your love and support will always be remembered, fondly in good times, and as encouragement in bad. Dearest Lai Wee Ting, thanks for everything and for being there. You are a gem to my life. I cannot imagine the world without you. I only know it would be a much different, much less comforting place.

To my sponsor, Khazanah Foundation (Yayasan Khazanah) and all dedicated people within. For your trust and expectations, maitaining steps ahead has gradually become my apparel. Thank you for all the memorable moments, the timely trainings and the generous encouragements you have provided me. Special thanks to my scholarship managers: Pn. Sariza Ibrahim, Pn. Salawati Awang and Pn. Raja Nordianna Raja Harun. My live has never been the same, ever, with all of you.

To truly appreciate life, we seek companionship. To those that share my enthusiasms, Siti Balqis Abdul Razak, Foo Su Chern, Yau Sook Kun, Dora Lai, Nur Leena Wong and Che Azurahanim. My IFTSA Developing Solutions for Developing Countries (DSDC) team, Prof. Dr. Lai Oi Ming, Lim Sue Shan, Lau Kah Yan, Hee Yen Yi and Sim Biow Ing; and my housemates, Khoo Li Teng and Liew Sien Yei. I appreciate the fights and quarrels but I especially treasure the kind words you spoke and the memorable challenges we cruised along. Thank you for accompany.

To those in the lab, Mr. Perumal Kuppan, Dr. Sanjoy, Dr. Helena, Farhan, Hasina, Suhayati, Adibah, KB, Tahhir, Kak Ita, Kak Farah, Nur Arifa, Aini, Shu Chee, Rozhin, Roazita, Umi Hartina, Sharmila, Fizah, Nana, Nia, Sarega, DJ, Jhi Biau, Hadiza, Umar, Aisyah, Nisah, Kak Siti, Kak Yat and Kak Norsha. I just wanted to let you know—the things you do for me do not go unnoticed. You're a necessary piece to this puzzle. I appreciate your dedication and service, and I know others, among us, do too.

I have had the privilege to be helped by too many good people. You know who you are. To those seen or unseen, recalled perhaps later or forgotten nonetheless, you have bent over backwards for me. I will try my best to repay all of your kindness by means of reflecting them upon others just like how I like them to be done on myself. This ride has never been smooth but at the end of it all, I am glad I had it with you.

Thank you.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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&	and
×g	gravitation force
°C	degree Celcius
µg	microgram
µL	microliter
µm	micrometer
AB	acid solubilized collagen from the bell of jellyfish
ANOVA	analysis of variance
AOA	acid solubilized collagen from the oral arms of jellyfish
ASC	acid soluble collagen
ca.	approximately; *circa*
cm	centimeter
e.g.	for example; *exempli gratia*
et al.	and others; *et alii*
etc	and other things; *et cetera*
g	gram
h	hour
HCl	hydrochloric acid
i.e.	that is; *id est*
IB	improved acid solubilized collagen from the bell of jellyfish
IOA	improved acid solubilized collagen from the oral arms of jellyfish
kDa	kilo Dalton
L	liter
M	mole
mg	milligram
min	minutes
mL	milliliter
mm	millimeter
MW	molecular weight
N	normality
NaOH	sodium hydroxide
PB	pepsin solubilized collagen from the bell of jellyfish
pI	isoelectric point
POA	pepsin solubilized collagen from the oral arms of jellyfish
PSC	pepsin soluble collagen
SD	standard deviation
SEM	scanning electron microscopy
v/v	volume (mL) per volume (mL)
v/w	volume (mL) per weight (g)
w/v	weight (g) per volume (mL)
w/w	weight (g) per weight (g)

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Jellyfishes, to the public, would always be associated to poisonous stinging nuisance that normally blooms in polluted waters, something which is not necessarily true. However, the fact that the blooms of jellyfishes are rapidly increasing is undeniable. Richardson et al. (2009) and Purcell et al. (2007) stressed that environmental changes (climate change and habitat modification) as well as anthropogenic developments (eutrophication, overfishing, translocation and reclamations) which took place rapidly nowadays, are among factors promoting jellyfish blooms to the disadvantage of other marine organisms. Damages inflicted by jellyfish blooms are increasingly experienced worldwide (Uye, 2008; Dong et al., 2010; Kim et al., 2012; Quinones et al., 2013). Among the negative impacts of jellyfish blooms includes diminished fishery production, malfunctionings of coastal energy plants; fatal consequences of envenomations and reduction of tourism and eco-activities. In Malaysia, Sim & Khairun (2008) reported the concerns of the public and the state government of Penang with the overflowing numbers of jellyfish now found in Penang waters that negatively affect the beach activities of the coastal areas. To address this issue which is steadily haunting the nation, the project intends to make use of these jellyfishes to produce *halal* and diseases-free value added biomaterial where none in Malaysia has been done so far.

Aside deadly venomous jellyfishes, some jellyfishes are edible and have long been served as delicacies in the world especially the Asian countries. Jellyfish fisheries is one of the oldest capture fisheries practised widely especially in Southeast Asia (Yusoff et al., 2010; Nishikawa et al., 2009). However, jellyfish fishery in Malaysia receives very little attention, regardless in scientific researches or socio-economical development, although Chinese all over the world are enjoying jellyfish as food and fine gourmet. Malaysia is one of the major exporters of jellyfish to Japan, Korea and China, which contributes to a multi-million dollars business in Southeast Asia. About three decades ago, trades of jellyfish commodities from Southeast Asia to Japan valued at about US$25.5 million (Omori and Nakano, 2001). There are generally four edible species of jellyfish commercially available in Malaysia which are better known i.e. obor merah (*Rhopilema esculentum*), obor pasir (*Rhopilema hispidum*) and lambuh air (*Acromitus hardenbergi*), and lambuh cendol (*Lobonema smithii*) (Figure 1). The river jellyfish, *Acromitus hardenbergi* could be found in abundance in the Perak estuary, Malaysia and is the only known source in the world where their occurrence is not restricted by any periods of time. Known only as the river type for a very long time, scientific descriptions and taxonomic confirmation of this species of jellyfish have only been published recently (Kitamura & Omori, 2010). The fishery of *Acromitus hardenbergi* in the Perak River, Malaysia has been carried out continually for no less than three generations. There is very little information and study on jellyfish in Malaysia even though its industry provides a very important source of revenue to the country. To the fishing community, jellyfish could be a good source of income given more efforts in studying the organism and potential benefits it could offer to the world.

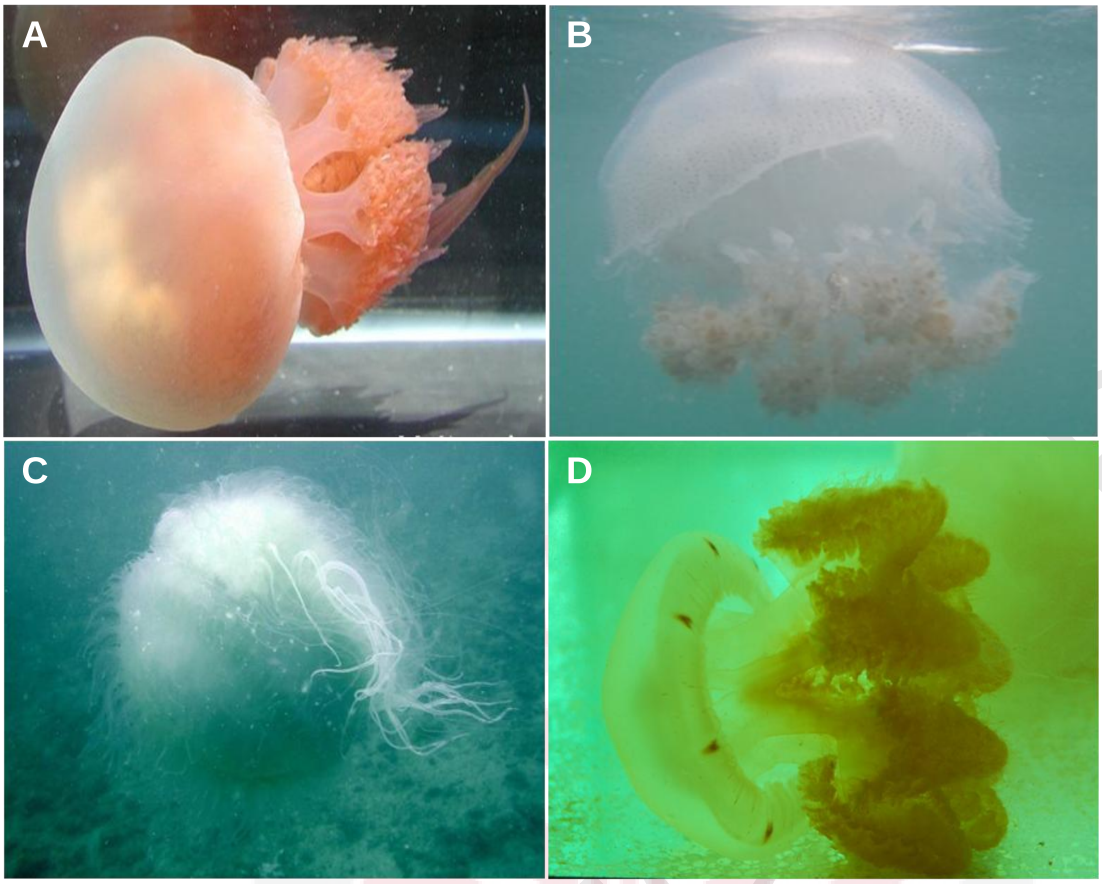

Figure 1. Edible jellyfishes fished in the waters of Malaysia. (A) *Rhopilema esculentum*; (B) *Rhopilema hispidum*; (C) *Lobonema smithii*; and (D) *Acromitus hardenbergi*.

Marine-derived biomolecules display arrays of functional activities, with compounds of lucrative economic importance having promising potentials of commercial and indutrial relevances. Southeast Asia especially, is a mega diversified region in aquatic and marine bioresources. The phylum Cnidaria includes over 11,000 extant species of unique and unexplored marine invertebrates. Spesies from tropical waters, specially, poses great potentials as 84% of the most potent Cnidarian biomolecules where previously isolated from Cnidarians collected from tropical waters (mostly from Southeast Asia and the Caribbean Sea) as compared to occupying temperate waters (e.g., European countries and Japan) (Rocha et al., 2011).

Edible jellyfishes are a promising ingredient for the development of nutraceutical and functional food. It is highly believed that jellyfish is effective to improve the conditions of arthritis, hypertension, backpain and ulcers (Hsieh et al., 2001). Basic chemical categorizations of different jellyfishes are essential for initial screening, bioprospecting and comparative bioefficacy prior to any developments of lead, products or value-added compounds. Proximate values of jellyfishes are relatively scarce and non-standardized worldwide. Proximate analysis allows justifiable comparisons of ingredients on the basis of specific nutrients, allowing one to judge how much better one is than another, in standardized terms. Informations on the proximate components of various jellyfishes, and their processed products, would aid in the development of regression equations to help predict nutritional performance of these food ingredients. In the more advance case, these proximate components could be utilised to predict performance-related factors e.g. bioavailability, digestibility and intake; that could be related to the development of novel products as well as the estimates of performance of these products. Many workers have presented on the potentials of jellyfishes as source of collagen (Barzansky et al., 1975; Kimura et al., 1983; Hsieh et al., 2001). However, similarly to proximate content, the comparison of total collagen content from different jellyfishes, especially commercially important ones, is yet to be performed.

Collagen exists in the skin, tendon, cartilage, blood vessels, bone and teeth of almost all vertebrates and recent researches have revealed that they are highly available in invertebrates as well. The degraded form of collagen is gelatin. The application of collagen stretches from making sausages in the food industry to delivering drugs in biomedical practices. It is most useful and convenient of collagen as compared to other biomaterials due to its well-defined structural, physical and biochemical properties as well as its biocompatibility and lack of allerginicity and toxicity. According to Foegeding et al. (2001), collagen exists in several polymorphic forms with collagen type I, III and V as the common ones while collagen type II and IV uncommon. Miura & Kimura (1985) found that the mesoglea of the jellyfish *Stomolophus nomurai* is composed of collagen chemically similar to vertebrate type V collagen. Meanwhile, Hsieh et al. (2005) isolated type II collagen from the jellyfish, *Stomolophus meleagris* which is capable of treating severe rheumatoid arthritis. Both experiments hinted that jellyfish could be a good source for rare and healthful collagen. Derivation of collagen from aquatic source is an exciting field with lots of enthusiasm from the world. However, the quality of collagen extracted is highly dependent upon its extraction process. Jamilah et al. (2010) have identified that many of the processing steps applied to mammalian collagen extraction are not directly applicable for the extraction of collagen from aquatic animals since the treatment may be harsh, or too strong for the matrix of aquatic animals. It would be highly desirable if a simplified extraction process which eliminates unnecessary extraction steps to increase yield and to reduce denaturation of the extracted collagen could be derived. Elimination of insignificant extraction steps could also save cost of chemicals and time. Therefore, it is necessary to comprehend the consequence s of extraction conditions to achieve higher recovery of good quality collagen. Extraction of collagen from jellyfish has always been low yielding while extraction of gelatin from jellyfish has never been reported successful previously.

1.2 Problem statements

Malaysia is one of the countries where the activities of jellyfish fisheries were most active worldwide. Yet, the amount of research performed on jellyfish as a source of sustainable resources is scarce. The exotic nature of this organism stirred numerous claims and rumours behind their near miraculous efficacy in the treating Acquired Immune Deficiency Syndrome (AIDS), infertility and other chronic illnesses. Meanwhile, local fishermen have been attributing the cure of joint problems and prevention of aging, fatigue to the consumption of jellyfish. Proper experimentations are necessary for the verification and solid discovery of pro-health claims and medical leads from these indigenous jellyfish which is easily available.

Fundamental understanding on the chemical constituents of jellyfish is obligatory as the basis of nutritional and biotechnological exploitations. Earlier, jellyfishes have been reported to contain substantial amount of collagen, antioxidants, bioactive peptides and terpenoids (Hsieh et al., 2001, Rocha et al., 2011). However, the production yields of those bioactive compounds are poorly reported. Moreover, none have performed comparisons between different jellyfishes, especially edible ones, on their proximate compositions, nutritional contents and total bioactive contents. Chemical variations between different edible jellyfishes, if any, would provide significant insights into their potential utilizations and developments, and thus should be categorised standardizingly among other seafoods. Nonetheless, identification of jellyfish with noteworthy amount of highly-demanded compounds such as collagen and antioxidants would be a practical approach to create value from this underutilized fishery resource.

Collagen and its degraded counterpart gelatin are valuable biofunctional ingredients in many industries especially of food, nutricosmetics, nutraceutical, cosmeceutical, pharmaceutical and medical relevances. Unfortunately, collagen and gelatin available in the market nowadays are either from porcine sources which possess significant limitations by religious dilemmas or bovine sources which equally suffer religious constraints as well as threat of deadly transmissible diseases such as bovine spongiform encephalopathy (BSE) and transmissible spongiform encephalopathy (TSE). Constant fluctuations of raw materials availability and market price led to numerous adulterations as well as fraudulent products that cause severe financial loss and health detriments to the unknowing public. Bioprospection of sustainable alternative collagen source would be vital to satisfy the increasing demand for collagen to the future. Hence, it would be most favourable if the highly demanded collagen could be extracted from the highly unwanted mass of jellyfish. The current study proposed to utilize jellyfish as a source for rare, *halal*/*kosher* and high quality collagen and gelatin.

Although collagen has been extracted from jellyfishes previously, their yield is pitifully low and impractical for industrial up scaling. In efforts to increase the yield of collagen extraction from jellyfishes, costly protease enzymes are commonly utilised. However, lack of standardizations in the use of enzymes produces collagens of variable quality due to under- or over- enzymatic cleavages of the protein.

Moreover, the most common protease used is pepsin which is from porcine gastric mucosa and thus, again brought forth religious limitations. Others utilise hazardous and potentially lethal chemicals and irradiation to increase the yield of collagen extraction. It would be much profitable and consumer friendly given a simpler and green procedure could be devised for rapid and efficient extraction of collagen from jellyfish. Successful extraction of gelatin from jellyfish has yet to be reported so far.

Jellyfish blooms are steadily increasing from year to year due to rapid anthropogenic activities. For now, jellyfish blooms are already very much a nuisance to the fishermen, breaking nets and greatly reduce fish catch. Given the intensity of human developments, it would be most unlikely that the volume of jellyfish blooms would be shrinking at all. The removal of jellyfish is a no longer a matter of choice and it maybe a compulsory routines in coastal areas especially those with ports, industrial facilities, and power plants. Thereupon, it would be beneficial to derive something useful out these nuisances which may present the food, cosmetics and pharmaceutical industries with interesting new additives. Studies delving onto pro-health utilizations would also manifest prosperous and expensive products (outcomes) which would eventually compensate for the costly management of jellyfishes from the environment and upgrade the lifestyle of downtown community of poor jellyfish fishermen.

1.3 Hypothesis

Based on the problem statements above, it is hypothesized that:

1.3.1 Jellyfishes possess significant nutritional properties which pose potentials as functional food and different jellyfishes would exhibit different proximate content and nutritional properties.

1.3.2 Collagen could be extracted more effectively and efficiently by slight modification of the existing procedures.

1.3.3 Different extraction processes influenced the quality of jellyfish collagen produced. Functional properties of collagens will be different amongst those extracted using physical assisted processes and chemical (enzyme) assisted processes.

1.3.4 Gelatin of good sensory properties and high Bloom strength could be extracted from jellyfish.

1.3.5 Jellyfish collagen and gelatin possess the superior quality required to be applied as biomedical devices.

1.4 Objectives

The study embarked on the general aim to develop value-added biomaterials from indigenous underutilized edible jellyfish for biomedical applications. Specifically, the objective of the study involved:

1.4.1 To evaluate the nutritional quality and nutraceutical potentials of indigenous commercially available edible jellyfishes;

1.4.2 To assess effective processes for the production of high quality collagen with high yield from jellyfish tissues;

1.4.3 To characterize the physiochemical and biophysical properties of jellyfish collagen as affected by different extraction processes;

1.4.4 To develop effective processes for the extraction of highly functional jellyfish gelatin from jellyfish tissues and their physiochemical characterizations.

1.4.5 To fabricate and characterize jellyfish-derived scaffolds suitable for biomedical applications.

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