



**Faculty of Resource Science and Technology**

**EXTRACTION AND CHARACTERISATION OF COLLAGEN FROM THE SKIN  
OF BLUESPOTTED STINGRAY (*Dasyatidae kuhlii*)**

Mohamad Fais bin Che Nuddin (36940)

**Bachelor of Science with Honours  
(Resources Chemistry)  
2015**

**Extraction and Characterisation of Collagen from The Skin of  
Bluespotted Stingray (*Dasyatidae kuhlii*)**

**Mohamad Fais bin Che Nuddin (36940)**

A final project report submitted to fulfill the requirement for the  
degree of Bachelor of Science with Honours  
(Resource Chemistry)

**Supervisor: Mr. Razip Asaruddin**

Resource Chemistry  
Department of Chemistry  
Faculty of Resource Science and Technology  
Universiti Malaysia Sarawak  
2015

UNIVERSITI MALAYSIA SARAWAK

Grade: \_\_\_\_\_

Please tick (✓)

Final Year Project Report

Masters

PhD

DECLARATION OF ORIGINAL WORK

This declaration is made on the .....day of.....2015.

**Student's Declaration:**

I **MOHAMAD FAIS BIN CHE NUDDIN, 36940, FACULTY OF RESOURCE SCIENCE AND TECHNOLOGY** hereby declare that the work entitled **EXTRACTION AND CHARACTERISATION OF COLLAGEN FROM THE SKIN OF BLUESPOTTED STINGRAY (*Dasyatidae kuhlii*)** is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

\_\_\_\_\_  
30 June 2015

\_\_\_\_\_  
Mohamad Fais bin Che Nuddin (36940)

**Supervisor's Declaration:**

I **MR RAZIP ASARUDDIN** hereby certifies that the work entitled **EXTRACTION AND CHARACTERISATION OF COLLAGEN FROM THE SKIN OF BLUESPOTTED STINGRAY (*Dasyatidae kuhlii*)** was prepared by the above named student, and was submitted to the "FACULTY" as a \*partial/full fulfillment for the conferment of **Bachelor of Science with Honours (Resources Chemistry)**, and the aforementioned work, to the best of my knowledge, is the said student's work.

Received for examination by: \_\_\_\_\_  
(Mr. Razip Asaruddin)

Date: \_\_\_\_\_

I declare that Project/Thesis is classified as (Please tick (√)):

- CONFIDENTIAL** (Contains confidential information under the Official Secret Act 1972)\*  
 **RESTRICTED** (Contains restricted information as specified by the organisation where research was done)\*  
 **OPEN ACCESS**

### Validation of Project/Thesis

I therefore duly affirmed with free consent and willingness declare that this said Project/Thesis shall be placed officially in the Centre for Academic Information Services with the abiding interest and rights as follows:

- This Project/Thesis is the sole legal property of Universiti Malaysia Sarawak (UNIMAS).
- The Centre for Academic Information Services has the lawful right to make copies for the purpose of academic and research only and not for other purpose.
- The Centre for Academic Information Services has the lawful right to digitalise the content for the Local Content Database.
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis for academic exchange between Higher Learning Institute.
- No dispute or any claim shall arise from the student itself neither third party on this Project/Thesis once it becomes the sole property of UNIMAS.
- This Project/Thesis or any material, data and information related to it shall not be distributed, published or disclosed to any party by the student except with UNIMAS permission.

Student signature \_\_\_\_\_  
(30 JUNE 2015)

Supervisor signature: \_\_\_\_\_  
(30 JUNE 2015)

Current Address:

**NO 41A, TAMAN LKNP, PERINGKAT 1, 27000 JERANTUT, PAHANG.**

Notes: \* If the Project/Thesis is **CONFIDENTIAL** or **RESTRICTED**, please attach together as annexure a letter from the organisation with the period and reasons of confidentiality and restriction.

[The instrument is duly prepared by The Centre for Academic Information Services]

## **ACKNOWLEDGEMENT**

Alhamdulillah, thanks to ALLAH S.W.T. I am blessed for this opportunity to express my acknowledgments to a number of people for the support, excellent guidance and encouragements during my entire Final Year Project (FYP). In this first paragraph of my acknowledgements, I would give my sincere thanks and credits my supervisor, Mr. Razip Asaruddin, from Faculty of Resource Science and Technology (FRST), UNIMAS for providing excellent guidance and desires in helping me to complete this thesis.

I would love to thank my parents, Che Nuddin bin Che Ismail and Siti Eshah binti Mat Daud for their continual family support, encouragements and their love throughout three years of my study in UNIMAS.

It is also my pleasure to express my gratitude to the laboratory assistants of Natural Product Chemistry 19 Laboratory at FRST, UNIMAS; Mdm. Leida Anak Anthony for her cooperation throughout the project progress. Lastly, I would love to express my sincere appreciations to all my fellow chemistry friends, especially my laboratory partners whom always help me in guidance and moral support.

## DECLARATION

I hereby declare that this Final Year Project 2015 dissertation is based on my original work except for quotations and citations, which have been duly declared that it has not been or concurrently submitted for any degree at UNIMAS or other institutions of higher education.

---

Mohamad Fais bin Che Nuddin

Resource Chemistry Programme

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak (UNIMAS)

## TABLE OF CONTENTS

<b>ACKNOWLEDGEMENT.....</b>	<b>i</b>
<b>DECLARATION.....</b>	<b>ii</b>
<b>TABLE OF CONTENTS.....</b>	<b>iii</b>
<b>LIST OF ABBREVIATIONS.....</b>	<b>v</b>
<b>LIST OF FIGURES AND TABLES.....</b>	<b>vi</b>
<b>ABSTRACT/ABSTRAK.....</b>	<b>1</b>
<b>1. INTRODUCTION.....</b>	<b>2</b>
<b>2. LITERATURE REVIEW.....</b>	<b>5</b>
2.1 Bluespotted stingray ( <i>Dasyatidae kuhlii</i> ).....	5
2.2 Collagen.....	6
2.3 Properties of collagen.....	9
2.3.1 Amino acid composition.....	9
2.3.2 Thermal stability.....	10
2.3.3 Molecular weight.....	11
2.4 Molecular structure of collagen.....	12
2.5 Application of collagen.....	13
2.5.1 Osteoarthritis and joint disorders.....	13
2.5.2 Drug carriers.....	15
2.6 Extraction of collagen from the skin.....	16
2.6.1 Extraction of acid soluble collagen (ASC).....	16
2.7 Characterisation of collagen.....	17
2.8 Biological evaluation.....	18
2.8.1 Cytotoxicity test.....	18
2.8.2 Antioxidant test.....	19
<b>3. METHODOLOGY.....</b>	<b>20</b>
3.1 General procedure.....	20
3.2 Chemicals.....	21
3.3 Sample collection.....	22
3.4 Fish skin preparation.....	22
3.5 Extraction of collagen.....	23
3.5.1 Extraction of acid soluble collagen (ASC).....	23
3.6 Characterisation of collagen.....	25
3.6.1 Fourier Transform Infra Red (FT-IR) Spectroscopy.....	25
3.6.2 Scanning Electron Microscope (SEM).....	25
3.7 Biological evaluation.....	26

3.7.1	Brine Shrimp Lethality Assay.....	26
3.7.1.1	Sample preparation.....	26
3.7.1.2	LC50 Determination.....	26
3.7.2	Antioxidant activity test.....	28
3.7.2.1	Sample preparation.....	28
3.7.2.2	Statistical analysis.....	28
3.7.3	Antibacterial and Antifungal.....	29
3.7.3.1	Sample preparation.....	29
3.7.3.2	Zone Inhibition Determination.....	29
<b>4.</b>	<b>RESULTS AND DISCUSSIONS.....</b>	<b>30</b>
4.1	Extraction of Collagen from Bluespotted Stingray Skin.....	30
4.2	Characterisation of Collagen.....	31
4.2.1	Fourier Transform Infra Red (FT-IR) Spectroscopy.....	31
4.2.2	Scanning Electron Microscope (SEM).....	32
4.3	Biological evaluation.....	33
4.3.1	Brine Shrimp Lethality Assay.....	33
4.3.2	Antioxidant activity test.....	35
4.3.3	Antibacterial and antifungal assays.....	37
4.3.3.1	Antibacterial assay.....	37
4.3.3.2	Antifungal assay.....	38
<b>4.</b>	<b>CONCLUSION.....</b>	<b>39</b>
<b>5.</b>	<b>REFERENCES LIST .....</b>	<b>40</b>



## LIST OF ABBREVIATIONS

<i>D. kuhlii</i>	<i>Dasyatis kuhlii</i>
<i>A. salina</i>	<i>Artemia salina</i>
ASC	acid soluble collagen
PSC	pepsin soluble collagen
BSE	bovine spongiform encephalopathy
TSE	transmissible spongiform encephalopathy
FMD	foot-and-mouth disease
SDSPAGE	<i>SDS-polyacrylamide gel electrophoresis</i>
SEM	Scanning Electron Microscope
FTIR	Fourier transform infrared spectroscopy
DMSO	dimethyl sulfoxide
DPPH	1, 1-Diphenyl-2-picrylhydrazyl
EtOH	Ethanol
MeOH	Methanol
LC <sub>50</sub>	Lethal concentration for 50% of brine shrimp population
BSLT	Brine Shrimp Lethality Test

## LIST OF FIGURES AND TABLES

<b>Figure</b>	<b>Caption</b>	<b>Page</b>
Figure 1	Bluespotted stingray	5
Figure 2	Thermal transition curve of grass carp skin	10
Figure 3	Thermal denaturation curve of collagen from grass carp skin	10
Figure 4	Molecular structure of fibrillar collagen	12
Figure 5	Brine shrimp larvae <i>Artemia Salina</i> sp.	18
Figure 6	Denuded skins	22
Figure 7	Storage of the skins	22
Figure 8	Flow chart of the extraction process	24
Figure 9	Flow chart of lethality test	27
Figure 10	The yield of ASC from Bluespotted stingray skin (7.3 %)	30
Figure 11	FT-IR spectra of ASC from bluespotted stingray skin (A) and FT-IR spectra of ASC from walleye pollock skin fish (B) (Mingyan <i>et al.</i> , 2007)	31
Figure 12	SEM images of ASC from Bluespotted stingray skin	32
Figure 13	SEM image of collagen sponges from grass carp skin	32
Figure 14	Cytotoxicity of ASC from bluespotted stingray skin after 48 hours	34
Figure 15	The changing color of DPPH	35
<b>Table</b>	<b>Caption</b>	<b>Page</b>
Table 1	The various collagen types as they belong to the major collagen families	7
Table 2	Amino acid composition of ASC	9
Table 3	Collagen drug delivery applications	15
Table 4	Cytotoxicity of ASC from bluespotted stingray skin after 48 hours	33
Table 5	Antioxidant scavenging percentage of ASC from bluespotted stingray skin	36
Table 6	Zone of inhibition of ASC from bluespotted stingray skin	37
Table 7	Antifungal activity of ASC from bluespotted stingray skin	38

# **Extraction and Characterisation of Collagen from The Skin of Bluespotted Stingray (*Dasyatidae khulii*)**

by

Mohamad Fais bin Che Nuddin

## **ABSTRACT**

Collagens were known for its function to replace the human collagen for sustainability of human health. The demand for the quality collagens in so many industry especially pharmaceuticals and cosmetics are extremely high. Acid soluble collagen (ASC) from the skin of bluespotted stingray, *Dasyatidae khulii* were extracted and characterised. The method developed by Nagai and Suzuki (2000a) with slightly modification were used for the extraction process. The yield of ASC was 7.3% based on the wet weight of the skin. The characterisations of the collagens were determined using Fourier Transform Infra Red and Scanning Electron Microscope. Biological evaluation was conducted using Brine Shrimp lethality test for cytotoxicity. The antioxidant activity, antibacterial and antifungal were also conducted. The results suggest that bluespotted stingray skin has potential as an alternative source of collagen for use in various fields.

Keywords: Collagens, acid soluble collagen, cytotoxicity, antibacterial and antifungal.

## **ABSTRAK**

*Kollagen sudah dikenali dengan fungsinya untuk menggantikan kollagen manusia demi mengekalkan kesihatan tubuh manusia. Permintaan kollagen yang berkualiti dalam pelbagai industri terutamanya farmasitikal dan kosmetik adalah tinggi. Asid larut kollagen dari kulit ikan pari bertompok biru telah diekstrak dan dipencirikan. Langkah yang dihasilkan oleh Nagai dan Suzuki (2000a) dengan sedikit pengubahsuaian telah digunakan untuk tujuan proses ekstraksi. Penghasilan asid larut kollagen adalah 7.3% berdasarkan berat basah kulit. Pencirian kollagen telah dikenalpasti dengan menggunakan Infra Merah Transformasi Fourier dan Mikroskop Pembias Elektron. Penilaian biologi telah dijalankan menggunakan penilaian kematian udang laut, untuk tahap toksik. Penilaian aktiviti antioksidan, antibakteria dan antikulat juga dijalankan. Data yang didapati mencadangkan kulit ikan pari bertompok biru mempunyai potensi sebagai sumber alternatif kollagen untuk kegunaan dalam pelbagai bidang.*

*Kata kunci: Kollagen, asid larut kollagen, tahap toksik, antibakteria dan antikulat.*

# CHAPTER 1

## INTRODUCTION

Collagen is the most abundant protein of animal origin, comprising approximately 30% of total animal protein (Zhang, Liu, Li, Shi, Miao & Wu, 2007). It is widely distributed in the skins, cartilages, bones, ligaments, tendons, cornea, blood vessels, teeth and other organs of vertebrates (Senaratne, Park & Kin, 2006). A recent research by Liu, Oliveira and Su (2010) had listed at least twenty-six variants of collagen named types I-XXVI from different sources of animal and human including skin, cornea and blood vessels which each type has a distinctive amino acid sequence and molecular structure thus bring different roles in the tissue. Previous research was found that collagens from many marine species always had lower contents of hydroxyproline and lower denaturation temperatures compare to mammals collagen (Nagai *et al.*, 2002). The collagens from marine species contained two or three distinct  $\alpha$  chains compare to only two different  $\alpha$  chains in mammals collagens (Muyonga, Cole & Duodu, 2004a, 2004b; Nagai *et al.*, 2002; Kimura, Ohno, Miyauchi & Uchida, 1987).

Collagens of fish skins studied in recent years were mainly from marine species, such as brownstripe red snapper (*Lutjanus vitta*) (Jongjareonrak, Benjakul, Visessanguan, Nagai & Tanaka, 2005), ocellate puffer fish (*Takifugu rubripes*) (Nagai, Araki & Suzuki, 2002a) and black drum (*Pogonia cromis*) (Ogawa *et al.*, 2003). Isolation and characterisation of collagen from freshwater fish, however, was rarely reported, except for the grass carp (*Ctenopharyngodon idella*) (Zhang *et al.*, 2007), Nile perch (*Lates niloticus*) (Muyonga *et al.*, 2004).

Since early 20s collagen has been introduced into many industrial fields including leather industry, edible and photographic gelatine (Bailey *et al.*, 1998). Collagen also has been utilised to produce edible casings for meat processing industries while heat-denatured collagens, gelatin is a necessary component in food manufacturing (Senaratne *et al.*, 2006). Recently, soluble collagen has been introduced as a component in cosmetics and biomedical materials, as supports of enzymes activity due to the special characteristics including biodegradability and weak antigenicity and as food (Kolodziejaska, Sikorsi & Niecikowska, 1999; Zhang Li & Shi, 2006). While in pharmaceutical applications, collagen can be used for production of wound dressings, carrier for drug delivery and vitreous implants (Kittiphattanabawon, Benjakul, Visessanguan, Nagai & Tanaka, 2005).

At the very early of the research on collagen, it was first extracted from skins and bones of land animals like cattle and pigs. However, users of collagen and collagen-derived products claimed to experience various types of diseases which are outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and the foot-and-mouth disease (FMD) thus becoming a concern (Jongjareonrak *et al.*, 2005). In addition, extraction of collagens from land animals are prohibited for Sikhs and Hindus, whilst cannot be consumed by Muslims and Jews.

Although the physical and chemical properties of fish collagen are different from those of mammalian's collagen (Bailey & Light, 1989), collagens from fish offal are unlikely to be associated with infections such as BSE, TSE and FMD. Therefore, the global demand for collagen from alternative sources such as aquatic animals including freshwater and marine fish and molluscs have been increasing over the years due to their availability, the lack of dietary restriction or risk of disease transmission and the possibility of high collagen yields (Shen, Kurihara, & Takahashi, 2007; Chang-Feng *et al.*, 2013).

The type of fish water used in the collagen extraction is bluespotted stingray, *D. kuhlii* which is a species of stingray in the family of Dasyatidae, generally found on bottom near rocks or coral reefs. This species usually cover themselves in sand with eyes and tail visible. Growing to 70 cm across, this species has an angular disc and very short, angular and broad snouts. The body is dark green in colour with blue spots all over the body. Bluespotted stingrays are generally found in almost the entire continental waters of Asia including East China Sea, Java Sea, Philippine Sea and Arabian Sea.

The main objective of the project is to extract and isolate the collagen from the skin of bluespotted stingray, *D. kuhlii*, followed by characterisation of FTIR spectrometry, Scanning Electron Microscopy Analysis, cytotoxicity test, antioxidant activity, antibacterial and antifungal tests.

## CHAPTER 2

### LITERATURE REVIEW

#### 2.1 Bluespotted Stingray (*Dasyatidae kuhlii*)

Bluespotted stingray, *D. kuhlii* is a species of stingray in the family of Dasyatidae, generally found on bottom near rocks or coral reefs. This species usually cover themselves in sand with eyes and tail visible. Growing to 70 cm across, this species has an angular disc and very short, angular and broad snouts. The body is dark green in colour with blue spots all over the body.

Bluespotted stingrays are generally found in water of depth about 0-90 meters. At high tide, the stingray moves out into the shallow lagoons and reef flats. Bluespotted stingrays can be found almost the entire continental waters of Asia including East China Sea, Java Sea, Philippine Sea and Arabian Sea.

The skins from this species are thick and tough, which may be associated with the collagens. However, no information on composition and molecular properties of collagen from the skin of these species has been reported. Figure 1 below showed the bluespotted stingray species.



**Figure 1:** Bluespotted stingray

## 2.2 Collagen

Collagens are essential in our daily life since the component is needed in our daily life to sustain our health. According to the study of Bailey *et al.*, (1998) has stated that collagen is the most abundant protein of animal origin, comprising approximately 30% of total animal protein. The name collagen is used as a generic term for proteins that forms a characteristic of triple helix of three polypeptide chains. These supramolecular structures are form in the extracellular matrix with different size, function and tissue distribution. So far, 26 genetically distinct collagen types have been described (Gelse, Pöschl & Aigner, 2003).

Bama *et al.*, (2010) also stated that collagen is the major structural protein in the connective tissue of animal skin and bone in its purified form collagen is a biomaterial. Based on the title of this study that will only highlighted the collagen in fish, there is almost 40% of total collagen yield on the basis of lyophilised dry weight (Bama *et al.*, 2010). Table below show the types of collagens that have been described (Table 1).



**Table 1:** The various collagen types as they belong to the major collagen families

Type	Molecular composition	Genes (genomic localisation)	Tissue distribution
<b><i>Fibril-forming collagens</i></b>			
<b>I</b>	[ $\alpha 1(I)_2\alpha 2(I)$ ]	COL1A1 (17q21.31 – q22) COL1A2 (7q22.1)	bone, dermis, tendon, ligaments, cornea
<b>II</b>	[ $\alpha 1(II)$ ] <sub>3</sub>	COL2A1 (12q13.11 – q13.2)	cartilage, vitreous body, nucleus pulposus
<b>III</b>	[ $\alpha 1(III)$ ] <sub>3</sub>	COL3A1 (2q31)	skin, vessel wall, reticular fibres of most tissues (lungs, liver, spleen, etc.)
<b>IV</b>	$\alpha 1(V), \alpha 2(V), \alpha 3(V)$	COL5A1 (9q34.2– q34.3) COL5A2 (2q31) COL5A3 (19p13.2)	lung, cornea, bone, fetal membranes; together with type I collagen
<b>XI</b>	$\alpha 1(XI)\alpha 2(XI)\alpha 3(XI)$	COL11A1 (1p21) COL11A2 (6p21.3) COL11A3 = COL2A1	cartilage, vitreous body
<b><i>Basement membrane collagens</i></b>			
<b>IV</b>	[ $\alpha 1(IV)$ ] <sub>2</sub> $\alpha 2(IV)$ ; $\alpha 1$ – $\alpha 6$	COL4A1 (13q34) COL4A2 (13q34) COL4A3 (2q36– q37) COL4A4 (2q36– q37) COL4A5 (Xq22.3) COL4A6 (Xp22.3)	basement membranes
<b><i>Microfibrillar collagen</i></b>			
<b>VI</b>	$\alpha 1(VI), \alpha 2(VI), \alpha 3(VI)$	COL6A1 (21q22.3) COL6A2 (21q22.3) COL6A3 (2q37)	widespread: dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
<b><i>Anchoring fibrils</i></b>			
<b>VII</b>	[ $\alpha 1(VII)$ ] <sub>3</sub>	COL7A1 (3p21.3)	skin, dermal– epidermal junctions; oral mucosa, cervix

***Hexagonal network-forming collagens***

**VIII**     $[\alpha 1(\text{VIII})]_2\alpha 2(\text{VIII})$     COL8A1 (3q12– q13.1)  
COL8A2 (1p34.3– p32.3)    endothelial cells,  
Descemet's membrane

**X**     $[\alpha 3(\text{X})]_3$     COL10A1 (6q21– q22.3)    hypertrophic cartilage

***FACIT collagens***

**IX**     $\alpha 1(\text{IX})\alpha 2(\text{IX})\alpha 3(\text{IX})$     COL9A1 (6q13)  
COL9A2 (1p33– p32.2)    cartilage, vitreous  
humor, cornea

**XII**     $[\alpha 1(\text{XII})]_3$     COL12A1 (6q12– q13)    perichondrium,  
ligaments, tendon

**XIV**     $[\alpha 1(\text{XIV})]_3$     COL9A1 (8q23)    dermis, tendon, vessel  
wall, placenta, lungs,  
liver

**XIX**     $[\alpha 1(\text{XIX})]_3$     COL19A1 (6q12– q14)    human  
rhabdomyosarcoma

**XX**     $[\alpha 1(\text{XX})]_3$           corneal epithelium,  
embryonic skin, sternal  
cartilage, tendon

**XXI**     $[\alpha 1(\text{XXI})]_3$     COL21A1 (6p12.3– 11.2)    blood vessel wall

***Transmembrane collagens***

**XIII**     $[\alpha 1(\text{XIII})]_3$     COL13A1 (10q22)    epidermis, hair follicle,  
endomysium, intestine,  
chondrocytes, lungs,  
liver

**XVII**     $[\alpha 1(\text{XVII})]_3$     COL17A1 (10q24.3)    dermal– epidermal  
junctions

***Multiplexins***

**XV**     $[\alpha 1(\text{XV})]_3$     COL15A1 (9q21– q22)    fibroblasts, smooth  
muscle cells, kidney,  
pancreas,

**XVI**     $[\alpha 1(\text{XVI})]_3$     COL16A1 (1p34)    fibroblasts, amnion,  
keratinocytes

**XVIII**     $[\alpha 1(\text{XVIII})]_3$     COL18A1 (21q22.3)    lungs, liver

## 2.3 Properties of Collagen

It was found that many properties of collagens can be known. The properties are amino acid composition, molecular weight, thermal stability and viscosity of collagen. Each property can be determined by using different types of specific procedures.

### 2.3.1 Amino Acid Composition

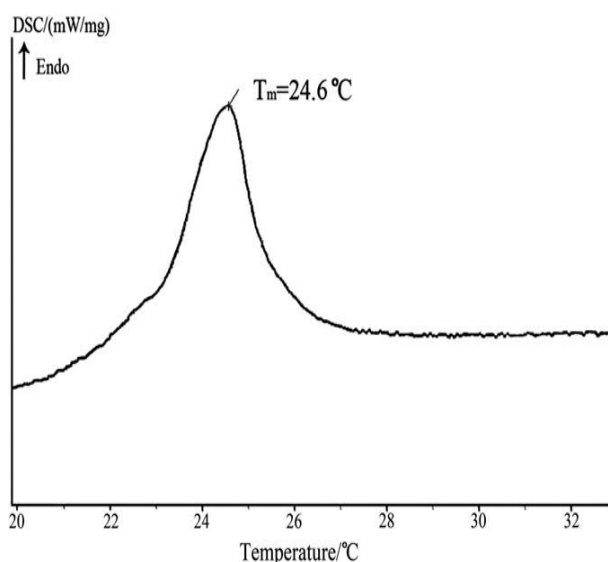
The amino acid composition can be determined by using amino acid analyser (Hitachi 835-50) as mentioned by Zhang, Duan, Tian and Konno (2009). The ASC were hydrolysed approximately in 6 M of HCl at 110°C for 24 hours in the absence of oxygen (Morimura *et al.*, 2002). There were so many amino acid analyses have been done from different species of marine species to study its amino acid composition. Table below shows the amino acid composition of the ASC from skins of silver carp, carp and cod from the research by Zhang *et al.*, (2009) (Table 2).

**Table 2:** Amino acid composition of ASC

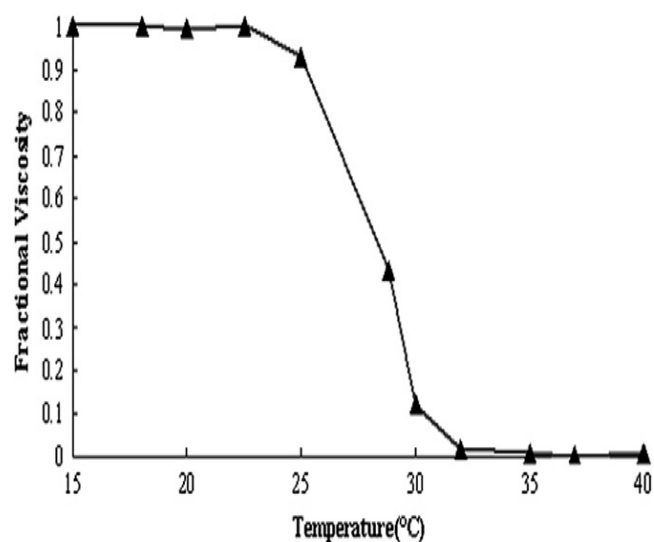
<b>Amino acid</b>	<b>Silver carp skin collagen</b>	<b>Carp skin collagen</b>	<b>Cod skin collagen</b>	<b>Calf skin collagen</b>
<b>Aspartic acid</b>	51	49	53	45
<b>Threonine</b>	20	24	23	18
<b>Glutamic acid</b>	77	76	80	75
<b>Glycine</b>	329	332	342	330
<b>Alanine</b>	112	118	107	119
<b>Valine</b>	20	19	19	21
<b>Methionine</b>	16	14	15	6
<b>Isoleucine</b>	14	10	12	11
<b>Leucine</b>	23	22	22	23
<b>Tyreonine</b>	5	3	4	3
<b>Phenylalanine</b>	14	13	12	3
<b>Hydrolysine</b>	8	7	7	-
<b>Lysine</b>	28	28	29	26
<b>Histidine</b>	5	5	8	5
<b>Arginine</b>	54	55	54	50
<b>Hydroxyproline</b>	78	76	51	94
<b>Proline</b>	114	114	103	121

### 2.3.2 Thermal Stability

The thermal stability of collagen was determined by using differential scanning calorimetry (DSC) (Netzsch DSC 200PC, Germany). According to Zhang *et al.*, (2007), the collagen is dissolved in 0.05 M of acetic acid with a concentration of 5 mg/ml. The graph was recorded from 20 - 40°C at heating rate of 2°C/min in nitrogen atmosphere. The data is interpreted as disintegration of the collagen triple helical structure into random coils. This includes a change in physical properties such as sedimentation, viscosity, optical activity and also diffusion (Zhang *et al.*, 2007). DSC was first to investigate the thermal stability of collagen, but with the well-developed analytical tools, viscosity and the denaturation temperature of the collagen can also be studied (Zhang *et al.*, 2007). Figure 2 and 3 below shows the thermal transition curve obtained from grass carp skin by DSC (Zhang *et al.*, 2007).



**Figure 2:** Thermal transition curve of grass carp skin



**Figure 3:** Thermal denaturation curve of collagen from grass carp skin

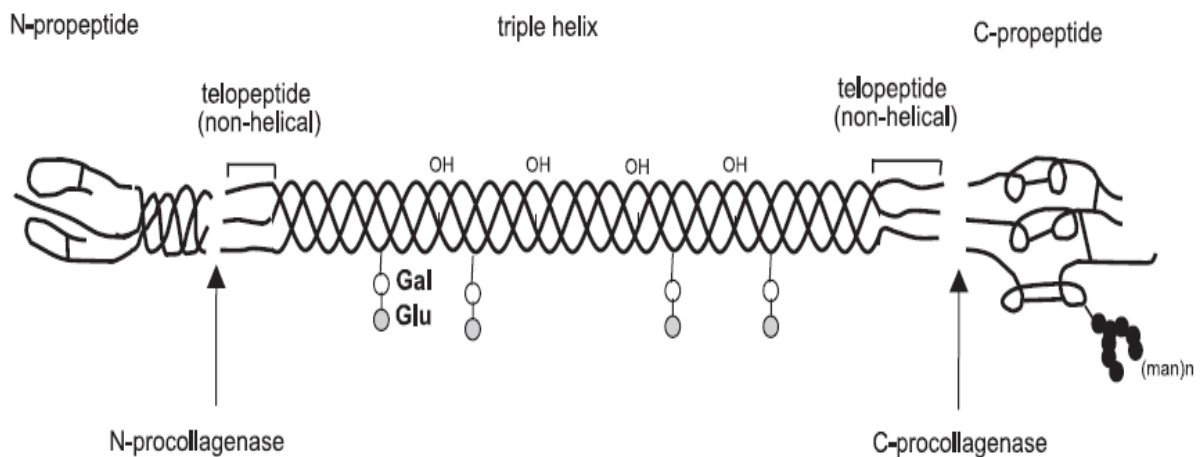
### 2.3.3 Molecular Weight

Molecular weight of collagen can be determined by using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This is a well known method to determine the molecular weight of every single protein in the collagen (Nagai *et al.*, 2001). SDS-PAGE works by separating the proteins from the denatured collagen according to its size. By this, the molecular weight of each protein contain in the collagen can be determined. The most well known method was proposed by Laemmli (1970) in his research on the “Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4”.

According to Laemmli (1970) SDS-PAGE was performed to determine the high molecular weight of proteins from ASC. ASC was dissolved in 5% SDS and the mixtures were incubated at 85°C for 1 hour in controlled temperature (water bath). The mixture then was centrifuged at 4,000 g for 5 minutes using a micro-centrifuge with condition of room temperature to remove undissolved debris. Solubilised samples were mixed at a ratio of 1:1 (v/v) with the sample buffer (0.5 M TrisHCl, pH 6.8, containing 4% SDS and 20% glycerol) containing 10% b-ME. The mixtures were kept in boiling water for 2 minutes. Samples (15 lg ASC) were then loaded onto polyacrylamide gels comprising a 7.5% running gel and a 4% stacking gel and were subjected to electrophoresis at a constant current of 15 mA/gel for 1 hour and 30 minutes. After electrophoresis, the gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and was destained with 30% (v/v) methanol and 10% (v/v) acetic acid. High molecular weight markers were used to estimate the molecular weight of proteins.

## 2.4 Molecular Structure of Collagen

Although the molecular structures are different among collagen types, all members of the collagen family have one same characteristic feature; a right-handed triple helix composed of three  $\alpha$ -chains (Gelse *et al.*, 2003). Each of the three  $\alpha$ -chains forms an extended left-handed helix with a pitch of 18 amino acids per turn (Hofmann & Fietzek, 1978). Each chain is staggered by one residue relative to one of each other, are supercoiled around a central axis in a right-handed manner to form a triple helix (Fraser, MacRae & Suzuki, 1979). Gesel *et al.*, (2003) stated that a structural in charge for assemble of the triple helix is a glycine residue which is the smallest amino acid. The assembly of glycine residue in a third position of the polypeptide chain gives a (Gly-X-Y)<sub>n</sub> repeat structure which characterised the “collagenous” domains of all collagens. Figure 4 below shows the molecular structure of fibrillar collagen with the various subdomains.



**Figure 4:** Molecular structure of fibrillar collagen

## **2.5 Application of Collagen**

Collagen has been applied in so many fields including pharmaceuticals, cosmetics even in foods production. The most recent application of collagen in medicinal field is used as a healing material for osteoarthritis and joint disorders also as drug carriers (Vigier *et al.*, 2010).

### **2.5.1 Osteoarthritis and Joint Disorders**

Osteoarthritis is one type of the most common form of arthritis, which have been affecting most of the people worldwide. This disorder is commonly affecting the joints in hands, hips, knees and spines. This disease can be happen due to the wears down of the protective cartilage on the ends of the bones. Cartilage is a firm, slippery tissue that permits nearly frictionless joint motion. A person with this disorder has a rough slick surface of the cartilage. The wears down of the cartilage caused the bone to be rubbing on each other which causes the pain. There is still no cure exists for this disorder. However, staying fit, maintaining healthy weight may slow down the progression of the disease, and reduce the pain from time to time.

The symptoms are developed slowly and worsen over time. Signs and symptoms of osteoarthritis include:

- Pain: The joint may hurt during or after movement.
- Stiffness: Joint stiffness may be the most noticeable when waking up in the morning or after a period of inactivity.
- Loss of flexibility: May not be able to move the joint through its full range of motion.
- Tenderness: The joint may feel tender when light pressure is applied.

- Bone spurs: Formation of extra bits of bone around the affected joint, which feel like hard lumps.
- Grating sensation: May hear or feel a grating sensation when the joint is used.

The factors are different from one patient to another patient. There are several factors that may increase the risk of osteoarthritis.

- i. Older age: The risk of osteoarthritis is increases with age.
- ii. Sex: Women are more likely to develop osteoarthritis but the explanation still remains unknown.
- iii. Obesity: Extra body weight contributes to osteoarthritis in many ways. Obesity added stress on weight-bearing joints especially in hips and knees.
- iv. Joint injuries: Injuries from both accident or during sport may increase the risk of osteoarthritis.
- v. Genetics: Osteoarthritis has to possibility to be inherited.
- vi. Bone deformities: Some people are born with malformed joints or defective cartilage which can be the factor of development of osteoarthritis.
- vii. Other diseases: Diabetic patients or other rheumatic diseases including gout and rheumatoid arthritis can increase the risk of osteoarthritis.

A recent research by Vigier *et al.*, (2010) stated that collagen can be beneficial for bone healing materials to replace the damaged cartilages. Type I collagen (skin) based scaffolds are excellent materials for tissue engineering due to their high biocompatibility and weak antigenicity (Vigier *et al.*, 2010).