USING FRUIT ENZYMES FOR EXTRACTION AND CHARACTERISATION OF COLLAGEN FROM BLACK TILAPIA (*Oreochromis niloticus)* SKIN

by

AN NABIL BINTI ZARINI

Thesis submitted in fulfillment of the requirements for the degree of

Master of Science in Food Technology

February 2016

CHAPTER 1

INTRODUCTION

1.1 Introduction

Collagen is a type of fibrous protein that serves as structural protein in vertebrates. It serves as building blocks and provides flexibility for skin tissue, bone, cartilage and tendons. In most organisms, collagen relates to the muscle toughness. Collagens find it most valuable application in biomedical and pharmaceutical area as it serves as biological scaffolds. Collagens are widely used in industries as edible and photographic gelatin, sausage casings, leather industries, cosmetics and pharmacy. It also serves as supports for enzymes or biologically active compounds due to its special characteristics such as biodegradability and weak antigency properties (Lee *et al.*, 2001).

Bovine and porcine bones and hides have been used as the main source for collagen due to their abundance and availability. However, over the years, demand for alternative collagen source has increased. Religious barrier, health concerns, increased price and high demand over supplies of pigs and cows hides have been identified as the main motivations for new alternative collagen sources. Due to the religious restriction, sources from pigs were restricted for the Muslims and Jews, whilst the Sikhs and Hindus were forbidden to consume collagen that was cow derived. Increased concerns on transmissible Bovine Spongiform Encephalopathy (BSE) or mad cow disease, swine flu and foot-and-mouth disease endemic (FMD) that infects cows and pigs has somehow results in declining and limited usage of collagen-derived products from both sources. Over the years, there is a trend in increasing price of pig and cow hides in consequence of increased raw collagen demand especially in China for gelatin and sausage casings

manufacturing (A'Court, 2012). However, the demand could not be met since there is reduction in cattle hides supplies from Europe and America (Ryan, 2013). Therefore, there is a need for new collagen source that can benefit and fulfill the needs of all human kinds regardless of their religion and ethnic.

Tilapia is one of the most used fish in filleting industry. Based on the data by FAO FishStat (2015), global production of tilapia increased from 233 802 tonnes in 1990 to 3 197 330 tonnes in 2012. In Malaysia, tilapia was listed as one of the most consumed fish species in 2013 (Aruna, 2014). Increased tilapia production and market value over the years indicates stability and continuous demand from customers. Tilapia was sold in various forms such as live fish, whole fish or in fillet form. The industrial interest on the edible meat as fillets requires removal of viscera, bones, skin, scale and fins leaving filleting by-products of about 75-80 % (FAO, 2012a). Waste such as viscera, skin, scales and bones are discarded due to its non-economical value. This, if not treated in good ways may lead to many other problems such as environmental problems due to bacterial contamination. Typically, the fish by-products are converted to compost and animal feed or landfilled (Chen & Jaczynski, 2007; Shahidi, 2006).

1.2 Rational of the study

Taking advantage of the facts that fish waste contains high amounts of protein, the wastes (such as fish skins) from the tilapia fish fillet industry could serve as an alternative and cheaper collagen source. Utilization of the fish wastes may help to reduce the waste disposal and also pollution problems. Thus, this study attempted to utilize the fish skins obtained from the fish filleting industry for the extraction of collagen. Extraction of collagen from animal sources is usually done using both organic

(acetic, chloroacetic, citric and lactic) or inorganic acids (hydrochloric) (Skierka & Sadowska, 2007). However, due to difficulties to fully degrade or hydrolyze raw material composed of keratin, low collagen yield was obtained. To overcome this, utilization of peptidase particularly porcine pepsin has been used widely to increase the collagen yield significantly (Khan *et al.*, 2009; Morimura *et al*., 2002; Tan *et al.*, 2013). Pepsin has shown to increase collagen yield by cleaving the intramolecular cross-linking which is located at telopeptide region without disrupting the collagen triple helix structures (Hickman *et al*., 2000). Usage of pepsin derived from porcine source cause doubts on the permissibility especially for Muslims. Thus, this creates interest to study using the use of plant based enzymes such as peptidases as an extraction aid to increase the extraction yields. Very limited studies have reported the use of pepsin from aquatic animal sources such as from yellow fin tuna, tongol tuna, skipjack tuna and albacore tuna (Ahmad *et al.*, 2010; Nalinanon *et al.*, 2008). To date, there is no report on collagen extraction with addition of bromelain and papain. Thus, in general, this study aims to extract collagen from fish skins using plant enzymes; such as bromelain and papain to obtain higher collagen yields.

1.3 Objectives

The specific objectives of this study are:

(i) To obtain collagen from tilapia (*Oreochromis niloticus*) skins using bromelain and papain enzymes as aids in the extraction process.

(ii) To evaluate the physicochemical characteristics and thermal stability of three types of collagen namely the acid solubilized collagen (ASC), bromelain solubilized collagen (BSC) and papain solubilized collagen (PSC) in comparison with Type I calf skin collagen.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to collagen

Collagen is a type of fibrous protein present in the body and provides structural function (Kadler *et al.*, 2007). Collagen synthesis takes place at both intracellular and extracellular, in which during intracellular stage, collagen precursor polypeptide were converted into procollagen which is later converted into collagen in the extracellular stage. Type I collagen, the most characterised collagen type were normally present in higher animals as the main organic components of skin, bone tendon and loose connective tissue (Eastoe, 1995). Collagen is categorised as sceleroprotein which is marked by low solubility in water and when heated (for example, above 60 °C), the collagen helical structure will denature irreversibly to single α chain with some β- and ɣ-chains forming gelatin. Amongst various types of identified collagen, Type I collagen has been widely used ranging as biomaterials in medicine and pharmaceutical industry to sausage casings in food industry (Kołodziejska *et al.*, 1999). Preference on collagen as biomaterials in various connective tissue applications is due to its excellent biocompatibality, low antigency, high biodegradability and good mechanical, hemortatic and cell binding properties (Lee *et al.*, 2001).

2.1.1 Structure of collagen

Madras Model or rope like ‘coiled-coil structure’ is the well accepted collagen structure that were introduced by Ramachandran and Kartha (1955), with the aid of X-ray diffraction technique on stretched collagen fiber. This structure re-defined the initial

collagen structure suggested by Astbury (1938) and Pauling and Corey (1951) that stated collagen were build of mixture of *cis* and *trans* peptide units. In contrast to earlier concept of collagen structure, Ramachandran and Kartha (1955) marked that; (i) collagen were build of *trans* peptide unit, as like other natural protein, (ii) glycine as every third amino acid and accommodated by proline and hydroxyproline, and (iii) collagen triple helix were stabilised by one direct interchain hydrogen bond and water mediated hydrogen bond instead of two hydrogen interchain hydrogen bond per tripeptide (Bhattacharjee & Bansal, 2005).

Arrangement of collagen fibril in collagen fiber is shown in Figure 2.1. Basic distinguished polypeptide configuration of collagen were build with at least one or more regions of repeating $(\text{-Gly-X-Y-})_n$ amino acid tandem where proline at either X or Y position while 4-hydroxyproline were only bound to Y. Three individual helix strands of collagen polypeptides (α-chains) will later coiled around each other in triple helix form called tropocollagen that are 300 nm long and 1.5 nm in diameter (Fratzl, 2007). The non helix part of tropocollagen (N- and C-terminal end) is called telopeptide (de Wolf, 2002). Collagen triple helical structure were primarily build by three left-handed polyproline-II-type helix coiled around each other, forming right-handed triple helical structure bonded by hydrogen bonding between adjacent –CO and –NH group (Benjakul *et al*., 2012). To allow maximal stability, glycine will align to centre of triple helix. Bulkier side chain (proline and 4-hydroxyproline) were relocated facing outer triple helix region and later helps stabilises polyproline-II-like collagen helices (Krane, 2008). High amount of proline and hydroxyproline further stabilise the structure since they allows close association of both monomers and chains thus facilitate H-bonding and formation of intramolecular cross-linking through presence of pyrollidine rings in

proline (Ikoma *et al.*, 2003; Jongjareonrak *et al.*, 2005; Li *et al.*, 2013) Meanwhile, hydroxyl group of hydroxyproline stabilises the helix by interchain hydrogen bonding via bridging water molecule as well as direct hydrogen bonding to a carbonyl group.

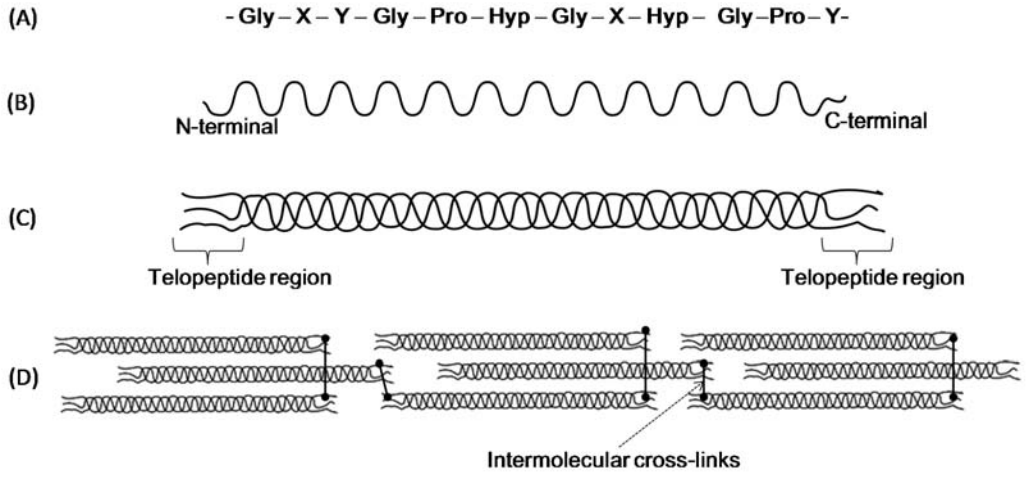

Figure 2.1 Arrangement of collagen fibril in collagen fiber. (A) Amino acid sequence of a collagen polypeptide. (B) Collagen polypeptide (C) Tropocollagen (combination of collagen polypeptides) (D) Collagen fibril.
(Source: Benjakul *et al.*, 2012)

2.1.2 Types of collagen

Collagens are products of different genetic loci and therefore non allelic (Bornstein & Sage, 1980). Up to date, at least 28 types of collagens has been reported and it is designated by the roman numbers (Brinckmann, 2005; Shoulders & Raines, 2009). According to Shoulders & Raines, (2009), those 28 collagen types were composed of at least 46 distinct polypeptide chains. Based on their chemistry composition, distribution and functions, collagen could be categorised into six-group namely fibrillar-forming collagen, network-forming collagen, anchoring fibrils-forming collagen, fibril-associated collagens with interrupted triple helices (FACITs),

membrane-associated collagens with interrupted triple helices (MACITs) and multiple triple helix domains and interruptions (MULTIPLEXINs) (Table 2.1). Most collagen types were build by specific encoded α-chains and assembled into supramolecular form either by itself or by aid of other extracellular matrices components (Koide & Nagata, 2005).

Table 2.1 Types of vertebrate collagens, their classes, compositions and distributions.

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

(Source: Shoulders & Raines, 2009)

2.1.3 Sources of collagen

Collagen could be extracted from various animals. Usually, industrial and animal by-products like hides and bones are used since they provide good protein sources at low cost. Two main sources of collagen used in the industry were mammals (bovine and porcine) and marine animals. Animal parts such as skin, hides and bones have been widely used as collagen sources. Other than marine and mammalian collagens, there is also increased interest in extracting collagen from other sources like star fishes, avian and reptiles. A summary on reported extraction and characterisation of collagen from various sources is given in Table 2.2.

Traditionally, the hides and bones of porcine and bovine have been used as main sources for collagen and gelatin due to its abundance and low costs. According to a report by Transparent Market Research, in 2011, 348.9 kilo tons of gelatin with market value worth $ 1.77 billion were produced. The production is expected to increase up to 450.7 kilo tons in 2018. Major sources used for gelatin production are pig skin (42.1 %), followed by bovine hides (28.7 %) and bones (27.4 %) (Anon, 2013). However, religious limitations, diseases related concerns like BSE and swine flu (Gomez-Guillen *et al.,* 2011) and increased demand over supply (Ryan, 2013) have changed the direction of collagen sources to other sources particularly marine based and other species as alternatives.

Table 2.2 List of literatures reporting on collagen extraction and characterisation**.**

Animal type	Source of collagen / Species	References
Land	Chicken	Cliche *et al.*, 2003
	Bovine	Wolf *et al.*, 2009
	Silky fowl	Cheng *et al.*, 2009
Amphibian	Bullfrog	Li *et al.*, 2004
Aquatic	Brownbanded bamboo shark (*Chiloscyllium punctatum*)	Kittiphattanabawon *et al.*, 2010
	Ornate treadfin bream (*Namipterus hexodon*)	Nalinanon *et al.* 2011
	Pig	Lin *et al.*, 2011
	Striped catfish (*Pangasianodon hypophtalamus*)	Singh *et al.*, 2011
	Catfish (*Clarias sp.*)	Kiew & Mat Don, 2012
	Cobia (*Rachycentron canadum*)	Zeng *et al.*, 2012
	Red sea cucumber (*Stichopus japonicas*)	Park *et al.*, 2012
	Sailfish (*Istiophorus platypterus*)	Tamilmozhi *et al.*, 2013
	Sea cucumber (*Stichopus vastus*)	Abedin *et al.*, 2013
	Lizard fish (*Saurida spp*.) , horse mackerel (*Trachurus japonicus*) , grey mullet (*Mugil cephalis*), flying fish (*Cypselurus melanurus*) and yellowback seabream (*Dentex tumifrons*)	Thuy *et al.*, 2014
	Amur sturgeon (*Acipenser schrenckii*)	Wang *et al.*, 2014
	Chilean mussels (*Mytilus Chilensis*)	Vallejos *et al.,* 2014
	Grass carp (*Ctenopharyngodon idellus*)	Wang *et al.*, 2014
	Whale shark (*Rhincodon typus*)	Jeevithan *et al*., 2015
	Seabass (*Lates calcarifer*)	Chuaychan *et al.*, 2015
	Haddock (*Melanogrammus aeglefinus*)	Dang *et al.*, 2015
	Atlantic salmon (*Salmo salar* L.)	Moreno *et al*., 2016

Over the years, fisheries sector showed increased and stable numbers of production. In 2011, Asia Pacific region dominates world fisheries sector by supplying 55 % of total capture fisheries and 91 % of aquaculture (FAO, 2014a). Increased production of fish-based products has contributed to generation of large fish waste. In 2009, Malaysia's production of surimi and fish paste and other similar preparations were estimated around 900 tonnes with market value of RM 12.2 million (MOA, 2015). In 2010, a sum of US$ 403 million of inedible fish waste has been traded all over the world and used as fertilisers, fish oil and incorporated in animal feed (FAO, 2012b).

Throughout the decades, global fish production has increased steadily and was expected to continue in order to meet the demand. Increasing number of aquaculture that involves variety of species and techniques were adopted widely all over the world. Continuing growth of aquaculture in Asia has somehow contributed to higher farmed fish production, with expenditure of 7 % per annum during first decade of twenty-first century. In 2011, up to 76 million ton of farmed fish produced compared to 52 million ton of wild fish caught in Asia (FAO, 2014a). According to Food and Agricultural Organization of the United Nations, (2014) almost 20 % of world animal protein intakes were of fish particularly from captured fisheries and aquaculture. Out of 74 % of global fish production were used for direct human consumption while the remainder were turned into non-food consumption such as animal feeds and oil (Arvanitoyannis & Kassaveti, 2008).

In the year 2012, the fisheries sector had contributed market value of RM 11, 440.31 million to the nation's economy, showing an increase of 13.61 % compared to 2011. The food fish sector which comprises of marine capture fisheries, inland fisheries and aquaculture (excluding seaweed) produced 1,780,168 tonnes worth RM

10, 597.60 million, signifying an increase of 6.86 % and 12.98 % respectively in terms of quantity and value compared to 2011. For the non-food fish sector, seaweeds, ornamental fish and aquatic plants contributed RM 842.71 million, improving by 22.08 % compared to 2011. Fish production from the fisheries sector contributed 1.1 % or RM 7.822 billion to the nation Gross Domestic Products (GDP) in 2012 (Department of Fisheries, 2012). Between 2000 to 2011, Malaysia's aquaculture production marked increment of 11 % per annum in which 190 thousand tonnes produced by inland farm and 337 thousand tonnes produced by marine farm earning export fish trade value of $USD 911 million (FAO, 2014a).

In terms of consumption, from 2009 to 2011, Malaysia has been reported amongst top 20 country with highest consumption of fish, seafood and aquatic products with nearly 100 kcal/cap/day that contributes to 3.8 % of Malaysian dietary energy supply (Food and Agricultural Organization of the United Nations, 2014). It was reported that Malaysian consumes major species includes mackerel, shrimp, squid, tilapia and catfish (Aruna, 2014).

Tracking back the history, tilapia was first introduced to Malaysia from Thailand in 1979 for aquaculture purposes. Aquaculture of tilapia not only target on improving local fisheries stock enhancement but also provide live bait for tuna industry, contribute to aquaculture and also as biological control for aquatic weed and mosquitoes (FAO, 2015). Two strains of tilapia were cultivated in Malaysia namely Red Tilapia (*Oreochromis mossambicus*) and Black Tilapia (*Oreochromis niloticus*). Red tilapia were farmed either in brackish or freshwater cage while black tilapia only farmed in freshwater cage mainly in ex-mining pools and freshwater ponds (Department of Fisheries Malaysia, 2013). In Malaysia, black tilapia were cultivated mainly in Perak (2721.39 tonnes with retail value of RM 21.07 million)

and Sabah (1244.61 tonnes with retail value of RM 14.93 million) (Department of Fisheries, 2012). Figure 2.2 showed production and market value of Black Tilapia from 2005 to 2012.

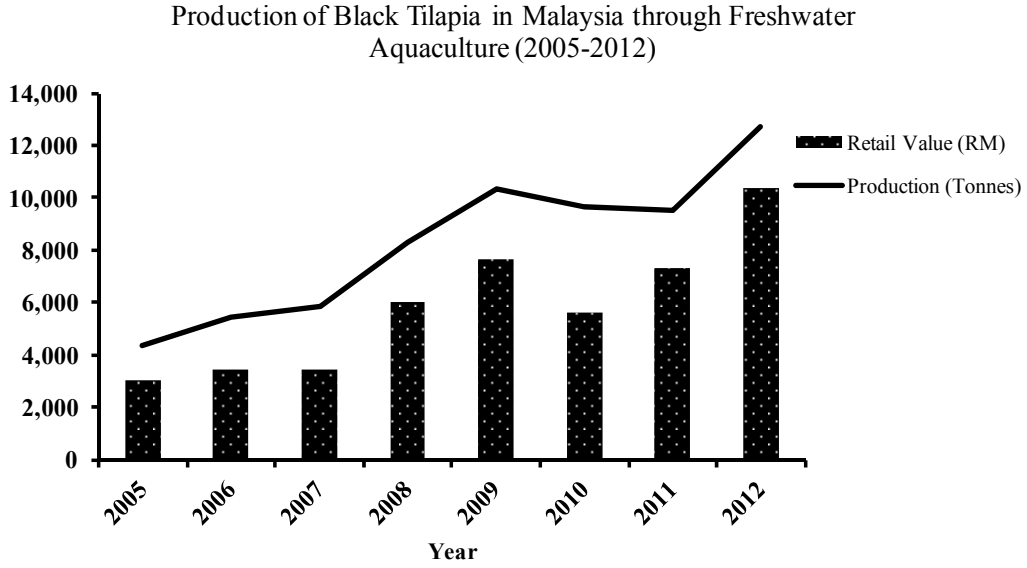

Figure 2.2 Production of Black tilapia in Malaysia (Source: Department of Fisheries Malaysia).

Other than being sold as live and in whole form for local market, tilapias were also sold as frozen fillets and loins. Up to 30 - 70 % of tilapia total weight were made into fillets, depending on their weight and fillet types (MyTilapia, 2015). Filleting add market values to fish products. During filleting process, machines cut along the upper and lower appendices on the spine, cutting the ribs, and vertebrae with a pair of symmetrical knives. Example of the trimmings standards and techniques available are; removal of the backbone, removal of visible fat, pin bones, and skin, and trimming technique produce different yields (Borderías & Sánchez-alonso, 2011). Different fish by-product was discarded throughout the filleting process. Bones and head were usually removed at the same stage while skin was removed later (Bechtel, 2003). Thus, it is possible to obtain fish by-products consisting of all heads, frames, viscera and skin. Borderías & Sánchez-alonso, (2011)

reported that tilapia (*Oreochromis* sp.) produce lowest fillet yield (33 %) compared to salmon (*Salmo salar*) (>50 %), channel catfish (*Ictalurus punctatis*) (> 38 %), and striped bass (*Morone saxatilis*) (> 40 %). Thus, approximately 67 % of total Tilapia fish waste that could be utilised for collagen production. Frozen fillets (Plate 2.1.(b)) that were produced in Malaysia were exported to North America, Europe, Asia and also domestically (FAO, 2013). In 2012, 112 tonnes of Tilapia from Malaysia were exported (FAO, 2014b). Therefore, there is a great potential to convert and utilise most of these by-products especially skin (Plate 2.1.(c)) as valuable products particularly collagen. Poor management of industrial by-products may cause pollution and emit offensive odours. By using the by-products as collagen source, multiple objectives could be met, particularly protects the environment, to produce value-added products to increase the revenue to the fish processors, and to create new job or business opportunities.

(a) (b) (c)

Plate 2.1 Production of tilapia fillet (a) Black Tilapia fish; (b) Tilapia fillets by-products; and (c) Tilapia fish skin waste

2.1.4 Collagen characterisation

The types and characteristics of a collagen vary according to their source of organism and parts. Based on an outline by ASTM, (2008), type I collagen shall contain characteristics of (i) two α_1-chains and one α_2-chain and (ii) high in glycine,

alanine, proline and hydroxyproline with low sulfur (low cysteine and methionine) and contains no tryptophan.

In collagen characterisation, SDS-PAGE is commonly used as a tool to identify collagen types based on presence of α-chains. SDS-Page method were developed by Laemmli in (1970), whom discovered separation of Bacteriophage T4 protein components by electrophoresis. Initially, protein will be denatured into polypeptides using sodium dodecyl sulphate (SDS), leaving all protein components anionic and be in the same charge. Thus, during electrophoresis, the polyacrylamide gel separates the protein based on the polypeptides molecular mass (Neumann, 1996). SDS-Page gel matrix was built of two separate system namely (i) stacking gel (pH 6.8) that have lower acrylamide concentration and bigger pore size and (ii) resolving gel (pH 8.8) consists of higher acrylamide concentration and smaller pore size. Protein molecular mass were calculated in comparison to several protein marker with known protein molecular weight such as high range molecular weight marker, broad range molecular weight protein marker and low range molecular weight protein marker were available as reference (Gallagher, 2012).

As mentioned earlier in Section 2.1.2, each collagen type composed of different molecular composition. The most characterised collagen is Type I collagen, comprised of two α_1-chains and one α_2-chain giving total molecular mass of about 30,000Da (Foegeding *et al.*, 1996). However, presence of α_3-chain could not be precisely determined by electrophoresis since the mobility is highly similar to α_1 chain (Kimura & Ohno, 1987; Su *et al.*, 2009). For collagen separation, 4 % stacking gel and 7.5 % resolving gel were usually used as the gel matrix (Liu *et al.*, 2015; Nagai, 2010; Sinthusamran *et al.*, 2013).

Other than SDS-Page, fourier transform infra-red (FTIR) spectroscopy are useful to determine collagen purity and backbone pattern. The FTIR technique lies upon the absorption of infra-red (IR) radiation by vibrational transitions in covalent bonds of the biomolecules. The intensities of IR absorptions provide quantitative information about the sample contents, depending on the nature of the molecular bonds, structure, and environment (Belbachir *et al.*, 2009). Generally, formation of H-bonds will reduce stretching vibrations frequency since it lowers the restoring force while bending vibration frequency will increase as results of increased additional force (Barth, 2007). Typically, collagen would show five types of prominent peak namely the Amide I, Amide II, Amide III and Amide A and Amide B (Belbachir *et al*., 2009; Nazeer *et al*., 2014). Other than purity and backbone of the collagen, level of collagen triple helix could be accessed through the absorbance ratio between Amide III and 1450 cm^{-1} bands (Anand *et al.*, 2013; Zhang *et al.*, 2009).

Peptide mapping is an analysis that compares the primary structures of proteins; to characterize the families of protein from the same organism or similar proteins from different organism and cloned gene products (Gautambhai, Patelia, & Shah, 2015). It also could be viewed as a fingerprint of a protein. The main concept of this analysis is, each protein of similar primary structures will show undifferentiated peptide fragments after hydrolysis (Chen, 2014). Peptide mapping involve protein hydrolysis using chemical (i.e CnBr and acid cleaving), endopeptidase (trypsin, α-chymotrypsin and V8 protease from *S.aureus*) or Edman degradation method (key reagent used is phenyl isothiocyanate) (Buxbaum, 2007; Rehm, 2006). Conventionally, in-solution proteolysis of targeted protein were performed by mixing the denaturized protein (usually by heating) with endopeptidase (i.e trypsin) at weight ratio between protease and protein in the range of 1:100 to

1:20, and the mixtures were incubated in water bath at 37 °C until the peptides fragments are generated (Chen, 2014). Peptidase actions on the protein was terminated through heat shock, by boiling the mixture for several minutes (Sinthusamran *et al.*, 2013). After the proteolysis, protein and peptide fragments were separated using techniques like SDS-Page gel electrophoresis, high performance liquid chromatography and capillary electrophoresis (Dong, 2006). Separation using SDS-Page allows visual comparison of proteins, provide molecular mass information and can be combined with immunoblotting to verify the protein amino acid sequences through protein sequencing (Buxbaum, 2007; Chen, 2014). The obtained peptide maps (SDS-Page gel or chromatogram) will be compared to a reference standard or reference material that has been similarly treated, to confirm the primary structure of the protein (Gautambhai *et al.*, 2015).

Collagen contains the unusual composition of amino acid, hydroxyproline and hydroxylysine. Both hydroxyproline and hydroxylysine were rarely found in other type of protein, making them as one of the key indicator for collagen detection and quantification (Mc Anulty, 2005). Thus, hydroxyproline and hydroxylysine content could indirectly indicate collagen content in food (Foegeding *et al*., 1996). ASTM International (2008), outlined that collagen has notably high in glycine, alanine, proline and hydroxyproline, with low sulfur content and contains no tryptophan. Glycine governs 1/3 of total amino acid content since it present at every third residue of collagen Gly-X-Y pattern (Zayas, 1997). This trend was almost true for all collagen types regardless of the sources (Table 2.3). Piez & Gross, (1960) showed that imino acid content affects collagen shrinkage temperature rather than hydroxyproline content alone and pyrollidine rings (of both proline and hydroxyproline) protects collagen secondary structure more than hydrogen bonding

through OH groups of hydroxyproline. Rigby, (1968) suggested that the imino acid of collagen highly depend on the type of organism and their habitat.

Two theories on collagen thermal stabilization by hydroxyproline have been proposed. Bella *et al.*, (1994) suggested, the "water bridge model" helps maintain collagen stability under thermal influence; where ordering of water around the collagen triple helices were done by extensive hydrogen bonding between water molecules and hydroxyproline. During thermal denaturation of collagen triple helices, increment of entropy happens due to the macromolecule and water. In contrasts to "water bridge model", Shoulders and Raines (2009) proposed that hydroxyproline stabilizes the helix via stereoelectronic forces. To conclude both theories, hydrogen bond was the most important basis for hydroxyproline to exerts it influence on collagen triple helix thermal stability (Hofman & Newberry, 2011).

Table 2.3 Comparison of amino acid content among different types of collagens.

Amino acid	Bovine collagen[(a)]	Porcine collagen[(a)]	Chicken skin collagen[(b)]	Spanish mackerel skin collagen[(a)]
Alanine (Ala)	120	114	130	135
Arginine (Arg)	51	51	85	51
Aspartic acid / aspargine (Asp)	46	44	91	50
Cysteine (Cys)	0	0	2	3
Glutamic acid / glutamine (Glu)	76	72	101	67
Glycine (Gly)	331	341	327	342
Histidine (His)	5	5	99	7
Isoleucine (Ile)	11	11	24	13
Leucine (Leu)	23	22	46	24
Lysine (Lys)	27	27	42	21
Hydroxylysine (Hyl)	8	7	-	5
Methionine (Met)	6	6	12	11
Phenylalanine (Phe)	3	13	21	15
Hydroxyproline (Hyp)	95	97	-	69
Proline (Pro)	122	123	121	109
Serine (Ser)	32	33	32	33
Therionine (Thr)	18	15	33	20
Tyrosine (Tyr)	4	1	8	3
Valine (Val)	22	22	47	24

Source: (a) Li *et al.*, 2013; (b) Liu *et al.*, 2001

2.1.5 Applications of collagen

Collagen has been used in food industries as water and fat binders. It is also used as emulsifier for acidic food products (Santana *et al.*, 2011).Usage of collagen as sausage casings, has replaced the traditional natural casings made of sheep, hog and beef intestines, which is cheaper and more sustainable (Harper *et al.*, 2012). About 80 % of current sausages casings were made of collagen obtained from corium layer of bovine hides, mixed with other cross linking agents, to improve their swelling ability (Amin & Ustunol, 2007). Besides that, collagen were found to improve water-binding properties that stabilise shrinkage and increase the cooking yield of meat (Schilling *et al.*, 2003).

As the age increased, human collagen production reduced by approximately 1.5 % per annum from the age 25-30 years onwards (Diggs, 2008). Utilisation of raw collagen as cosmetic material may not produce desirable effect since their large molecular volume (MV) or molecular weight (MW) of ≈300kDa will somehow prevent collagen molecules to reach the fibroblast cell in the dermal layer (Wu *et al.*, 2010). Thus, for cosmetics application, collagens were hydrolysed to reduce their MW, allowing absorption to dermal layers. Ingestion of collagen peptides has shown increased hydration level on skin by providing stimulatory effects of naturally occurred Type I collagen that present in skin (Zague *et al.*, 2011).

2.2 Extraction and recovery of collagen

Due to various types of tissue matrix and collagen types, there is no standard method that was used to extract collagen. Suitability of a collagen extraction method on specified matrices were affected by the solubility of tissue in the solvent used (Mocan *et al.*, 2011). Through time, number of covalent intermolecular interaction in

tissues increases, causing the tissues to be tougher and harder for collagen to be extracted (Duan *et al.,* 2009). Combinations of acid and enzyme extraction were widely used in both research and industrial collagen production due to its increased yield (Liu *et al.*, 2015; Sampath & Nazeer, 2013; Skierka & Sadowska, 2007). However, Mocan *et al.*, (2011), mentioned that combination of acid and enzyme extraction method have some drawbacks such as the collagen were prone to proteolysis/hydrolysis during isolation, and the collagen need to be stored in cold acid or dried to maintain their characteristics.

Generally, collagen extraction involved three main steps namely sample preparation and pretreatment, collagen isolation, and collagen recovery. Each step is crucial and could directly affect the yield and properties of the extracted collagen. It is very important that the steps taken during collagen extraction to be done delicately without destroying the macromolecule, preventing its fibrils and fibers reconstitution by only imparting sufficient removal of numerous inter and intramolecular covalent cross-links (Skierka *et al.*, 2007). It is very important for the processes to be done at low temperature in order to reduce heat denaturation on the extracted collagen and to preserve their native structure (Lin & Liu, 2006).

Prior to treatment for the extraction of collagen, raw materials were cleaned and washed. The size of raw materials are reduced (for example, skins are cut into smaller sizes and bones are smashed into smaller chunks) and followed by appropriate pretreatment depending on the source or parts of animal used. Size reduction of the sources not only facilitates in cleaning and removing non collagen compound on the raw material, but also increases the surface area of the material during the extraction process.

After cleaning, the raw materials were subjected to pretreatment. At this stage, non-collagenous elements such as lipid and pigments are removed to increase the purity of collagen. In alkali pre-treatment step, pigments and non-collagenous compounds were removed mainly using sodium hydroxide (NaOH), hydrogen peroxide or sodium chloride (NaCl) (Moreno *et al.*, 2012; Zeng *et al.*, 2009; Zhang *et al.*, 2009). Consequently, removal of fat in raw materials were done using various solvents like butanol, chloroform or ethanol (Ahmad & Benjakul, 2011; Liu *et al.*, 2001; Nalinanon *et al.*, 2010). For bones, scales and any calcium containing raw material, decalcification is crucial. Usually, decalcification are done using ethylenediaminetetraacetic acid (EDTA) as the chelating agent or hydrochloric acid (HCl) that further causing porous decalcified raw material with increased surface area, making it ready for collagen extraction (Kittiphattanabawon *et al.*, 2005; Ogawa *et al.*, 2004; Wood *et al.*, 2008).

Cleaned and pretreated collagenous material will be subjected to extraction process that could be done by acid, enzyme, combination of acid and enzymes, salt or alkali as extraction medium. Utilisation of salt and alkali for collagen extraction was not as common as acid and pepsin. Salt extraction method was least favorable since it requires high salt concentration, longer extraction time and limited to young tissues only (Mocan *et al.*, 2011). Besides that, there is a risk of contamination of codistributed non-collagenous proteins from the salt extracted collagen (Eckhardt *et al.*, 2003).

Acid are the most common medium used to extract collagen and various types of acids were reported (Liu *et al*., 2001; Skierka & Sadowska, 2007). Acetic acid is usually used and the obtained collagen using acid extraction is termed as acid soluble collagen (ASC) (Liang *et al.*, 2014; Nalinanon *et al.*, 2011; Park *et al.*, 2012).

During acid hydrolysis, the positive charge of collagen polypeptide will become dominant thus increasing the repulsion among tropocollagen that further leads to increasing solubilisation of the polypeptide into the acid solution. Generally, in this condition, tropocollagen is still in the triple helix form with negligible changes (Benjakul *et al.*, 2012).

Effect of organic acid (like acetic, chloroacetic, citric and lactic acid) and inorganic acid (hydrochloric acid) on collagen extraction efficiency from Baltic Cod (*Gadus morhua*) skin revealed that HCl have the lowest collagen extractability while acetic and lactic acid have the highest collagen extractability (≈90 %) (Skierka & Sadowska, 2007). This phenomenon of weak organic acid i.e acetic acid, gave higher yield compared to HCl, aligned with findings by Caputo *et al.*,(2012), whom extracted collagen from archeological bone remains using both acids. Weak organic acid such as acetic and lactic acid helps in forming hydrotropic swelling in which undissociated molecules of organic acid will replace existing hydrogen bonds and later remove hyrdrophobic bondings and cause denaturation of dissolved collagen (Skierka & Sadowska, 2007). In terms of acetic acid concentration used, Caputo *et al.,* (2012) and Wang *et al.,* (2008) reported that 0.5 M acetic acid gave a better collagen extraction efficiency compared to 1 M acetic acid. Wang *et al.,* (2008) and Kiew and Mat Don, (2012) had optimized the extraction conditions for collagen from grass carp skin and cultured catfish, respectively. They postulated that at acetic acid concentration of (≤ 0.5 M), higher yields were obtained due to the presence of high amounts of positively charged amine groups of collagen but at higher acetic acid concentration (when pH becomes less than 2), lower yields were obtained due to denaturation of collagens. The low pH (≈pH3) of 0.5 M acetic acid could trigger repulsion of collagen amine group, resulting in porous and loose structures (Skierka