EXTRACTION OF COLLAGEN FROM QUAIL (*Coturnix japonica*) FEET: CHARACTERIZATION AND DEVELOPMENT OF ELECTROSPUN NANOFIBERS

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by

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ABSTRAK

Kilang pemprosesan burung (ayam, itik, dan puyuh) menghasilkan sisa buangan dalam jumlah yang besar. Pembuangan sisa ini menimbulkan masalah kepada persekitaran, kesihatan, dan pembaziran sumber biologi berharga seperti protein dan lipid. Penggunaan sisa ini dalam penghasilan produk ditambah nilai menawarkan penyelesaian yang mudah untuk dilaksanakan disamping memberi manfaat kepada ekonomi. Hal ini membuka ruang kajian dalam penggunaan sisa pemprosesan sebagai sumber kolagen. Dalam kajian ini, kaki burung puyuh daripada sisa pemprosesan burung digunakan sebagai sumber baru bagi kolagen disamping penggunaan lain seperti gentian nano yang di elektrospun. Untuk tujuan ini, kolagen yang larut dalam asid dan pepsin, dan kolagen yang larut dalam asid laktik dan pepsin asid laktik diekstrak daripada kaki burung puyuh dan dicirikan. Sifat fizikal-kimia, terma dan morfologi bagi kolagen yang diekstrak turut dikaji. Corak elektrophorosis menunjukkan semua sampel adalah merupakan kolagen type-I dengan tiga rantai yang berbeza. Kajian asid amino menunjukkan kandungan asid imino di dalam sampel kolagen burung puyuh adalah lebih tinggi daripada kolagen kulit anak lembu, kulit khinzir, dan kaki ayam. Spektra FTIR menunjukkan kolagen yang diekstrak mempunyai struktur tiga-heliks. Kajian potensi zeta menunjukkan takat isoelektrik kolagen-kolagen adalah di antara pH 5.24-5.61. Kajian pengimbas perbezaan calorimetri (DSC) dan termogravimetrik menunjukkan kolagen yang diekstrak mempunyai kestabilan terma yang lebih tinggi berbanding kolagen kulit anak lembu dan kulit khinzir. Keputusan juga menunjukkan kaitan di an-

tara kestabilan terma kolagen dengan kandungan asid imino. Gentian nano kolagen dihasilkan menggunakan teknik elektrospun dan dicirikan untuk mengetahui sifat fizikalnya. Kesan parameter teknik elektrospun terhadap gentian nano kolagen juga turut dibincangkan. Reka bentuk Box-Behnken dan kaedah tindakbalas permukaan (RSM) digunakan untuk mengkaji kesan pembolehubah tidak bersandar seperti voltan, jarak hujung ke pengumpul dan kadar aliran terhadap diameter gentian nano kolagen. Hasil kajian menunjukkan keadaan elektrospun yang stabil untuk penghasilan gentian nano kolagen dicapai pada 20 (W/V)% kepekatan kolagen. Julat yang tepat untuk parameter elektrospun adalah seperti berikut; jarak hujung ke pengumpul (10-15 cm), kadar aliran (1-5 μl/min) dan voltan (20-30 kV). Keadaan yang terbaik untuk penghasilan gentian nano kolagen yang berdiameter minimum (lebih rendah daripada 200 nm) adalah seperti berikut; 20 kV voltan, 1 μl/min kadar aliran dan berjarak 10 cm. Hasil kajian menunjukkan kolagen yang diekstrak mempunyai ketahanan terma yang tinggi menjadikan ia sesuai untuk digunakan sebagai pilihan lain bagi menggantikan kolagen berasaskan mamalia seperti kulit anak lembu dan kulit khinzir di dalam makanan berfungsi, farmaseutikal dan kosmetik. Keputusan daripada kajian teknik elektrospun bukan hanya untuk mengenalpasti keadaan teknik elektrospun yang terbaik untuk mendapatkan gentian nano kolagen, tetapi juga membuka ruang dalam penghasilan gentian kolagen untuk penggunaan dalam industri makanan dan bioperubatan.

EXTRACTION OF COLLAGEN FROM QUAIL (*Coturnix japonica*) FEET: CHARACTERIZATION AND DEVELOPMENT OF ELECTROSPUN NANOFIBERS

ABSTRACT

Poultry processing plants produce a huge amount of wastes. The discharge of these amounts of wastes has raised serious concerns regarding their environmental impacts, diseases, and loss of valuable biological resources like proteins and lipids. Utilization of these by-products for producing value-added products may offer a feasible and economically viable solution. This line of thought led to the research on the by-products as sources of collagen. In this study, quails' feet from poultry processing by-products was utilized as a new source of collagen along with other applications such as electrospun nanofibers. In this way, acid and pepsin soluble collagens and also lactic acid and lactic acid pepsin soluble collagens were extracted from quails' feet and characterized. The physicochemical, thermal and morphological properties of the extracted collagens are investigated. The electrophoretic patterns indicated that all samples were type-I collagen with three different chains. Amino acid analysis demonstrated that the imino acid contents of the samples are higher than those of calf-skin, pig-skin, and chicken feet collagens. The FTIR spectra suggested that the extracted collagens were in triple-helical structure. The zeta-potential analysis is shown the isoelectric points of the collagens are in the pH range of 5.24-5.61. Differential scanning calorimetry and thermogravimetric analyses indicated that the extracted collagens had higher thermal stability in comparison to calf-skin and pig-skin collagens. The result showed that the correlation between the thermal stability of collagen and the imino acid content. Collagen nanofibers produced by electrospinning and characterized to quantify their

physical properties. Also, the effects of electrospinning parameters of the nanofibers are discussed. The Box-Behnken design and response surface methodology were employed to investigate the effect of independent variables such as voltage, tip to collector distance and flow rate on the diameters of collagen nanofibers. The findings indicate that the most stable electrospinning conditions for producing collagen nanofibers were achieved at 20 (W/V)% of collagen concentration. Also, the proper range of electrospinning parameters were obtained as follows: tip to collector distance (10-15 cm), flow rate (1-5 μl/min) and voltage (20-30 kV). The most favorable conditions for producing collagen nanofiber with a minimum diameter (lower than 200 nm) were obtained as follows: 20 kV of Voltage, 1 μl/min of flow rate and 10 cm of distance. The experimental results suggested that the extracted collagens with high thermal stability could be considered as an alternative to mammalian-derived collagen such as calf-skin and pig-skin collagens in functional foods, pharmaceuticals, and cosmetics. The results from the electrospinning experiments not only provide the proper electrospinning conditions to get collagen nanofibers but also open the possibility to produce collagen fibers for application in food and biomedical industries.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Collagen is one of the abundant protein in vertebrates, building up nearly 25-35% of total body protein. It is the main part of connective tissues and could be seen in cartilage, tendons, bone, ligaments, corneas, and skins presenting various structural and functional aspects (Lullo et al., 2002).

In vertebrates, there are around 29 different types of collagen molecules from the collagen superfamily (Sinthusamran et al., 2013) Collagen is classified according to its structure into fibril-forming collagens, anchoring fibrils, network-forming collagens, transmembrane collagens, fibril-associated. The fibril-forming collagen is one of the most dominant classes, consisted of nearly 90% of collagen population (Gelse et al., 2003). Collagen has been largely utilized in biotechnological industries as the raw material for biomedicine, foods, and cosmetics due to its low immunogenicity and high compatibility. Commonly collagen can be extracted by organic acids such as acetic acid, lactic acid, and citric acid or hydrochloric acid (Skierka and Sadowska, 2007). However, due to the intermolecular cross-linking in the fibrotic collagen molecules (Hickman et al., 2000), it is difficult to completely solubilize the collagen using acid in the extraction process. Therefore, enzymatic digestion is used to solubilize the collagen (Skierka and Sadowska, 2007). Some studies have demonstrated that pepsin digestion is an effective approach to solubilize the collagen and it could be attributed to the significant increase of the collagen yield (Khan et al., 2009; Skierka

and Sadowska, 2007). The enzyme cleaves the intermolecular cross-linking, which is located at the telopeptide region of collagen without damaging the triple helix (Hickman et al., 2000). The major sources of commercial collagen consist of cows and pigs. Due to outbreaks of transmissible spongiform encephalopathy (TSE), bovine spongiform encephalopathy (BSE), and foot-and-mouth disease (FMD), the application of collagen from these sources has become a concern. Moreover, the extracted collagens from calf-skin and bones are forbidden for use by Sikhs and Hindus, while collagen extracted from porcine skin and bones cannot be utilized by Jews and Muslims (Singh et al., 2011). Collagen has also been extracted from by-products of freshwater fishes, marine fishes and several aquatic animals such as squid, jellyfish, starfish, octopus and others (Bilgen et al., 1999; Kimura et al., 1991; Miura and Kimura, 1985; Nagai et al., 2004; Nagai and Suzuki, 2002; Swatschek et al., 2002). However, low thermal stability of marine-based collagens, limiting their broad application (Nagai and Suzuki, 2000; Nagai et al., 2000).

Collagen obtained from various sources depends on their thermal resistance, which could be correlated with the living habitat and body temperature of the origin species (Matmaroh et al., 2011). In comparison to the different sources, the literature information on the collagen extracted from poultry by-product is still limited (Cheng et al., 2009; Dhakal et al., 2018; Hashim et al., 2014; Huda et al., 2013; Lin and Liu, 2006a,0; Liu et al., 2001). The poultry industry plays a significant role in the Malaysian economy in the provision of a cheap source of the protein to its multi-ethnic population. The poultry industry accounts for 57.5% of the total value added in the livestock sector in 2012, higher than other livestock individually and collectively. The percentage share of poultry to the total livestock is highly likely to increase in the future (Abdurofi et al., 2017).

Protein wastes or poultry by-products have not been utilized properly in Malaysia. A large amount of poultry by-products are only considered as waste and they were just being thrown away from the poultry farms, markets, food processing industries, and homes (Afiq et al., 2017).

A Large production scale of the poultry processing industry, leads to huge amounts of natural by-product in different forms, such as feathers, viscera, bones, blood, feet, and head are produced, which, if not correctly managed, may lead to environmental problems (Lasekan et al., 2013). Consequently, the use of poultry by-products to utilize a new alternative source of collagen, generate an economic return of the poultry industry and mitigate environmental effects has greatly expanded. In this regard, some studies have been done to find out the property of several poultry by-products to extract value-added materials, especially collagen (Cheng et al., 2009; Lin and Liu, 2006b; Liu et al., 2001). In Malaysia, quail production is a very important business. There are 12 million quails has been produced in 130 quail farms in Malaysia and produce 350 million eggs per year (Muhammad and Hassan, 2018). The purpose of this research is to use the quail processing by-product, quails' feet, to extract collagen.

As the first step to elucidate this idea in this thesis, the extraction and characterization of collagen from quails' feet are described to provide baseline data for next steps. In the next steps, the electrospinning technique was used to produce ultrafine fibers and fibrous mats from the collagen that might be used for biotechnological applications like drug release, tissue engineering, enzyme immobilization and wound dressing. Electrospinning is a novel fabrication technique that can be used to generate fibers from biopolymer solutions with diameters below than 1000 nm. Electrospinning is

associated with different types of parameters. These parameters are broadly classified into three groups: solution parameters (polymer concentration, viscosity, and surface tension), processing parameters (applied voltage, flow rate, and distance) and ambient parameters may influence the spinnability, fiber diameter and morphology of electrospun mats (Pezeshki et al., 2015). Solution concentration is one of the most important parameters because the stable Taylor cone is achieved, when the suitable concentration was used and also the elongation happen by whipping instability mechanism (Bhushani and Anandharamakrishnan, 2014). In the electrospinning technique, the diameter of nanofibers are important and controlled by several process parameters that may influence the spin ability and fiber morphology of electrospun mats. Undoubtedly, there is some degree of interactions between these parameters. Therefore, it is important to determine the impact of essential parameters on the fiber diameter. Response surface methodology (RSM) is one of the statistical optimization methods, used to study the interactive and individual effects of the process parameters on the fiber morphology.In this research, RSM was applied to identify an experimental correlation among electrospinning parameters. Also, it was employed to investigate the effects of parameters in an easy way and predict the consequences of the experiments with varied parameter combinations.

Furthermore, some experiments were applied to characterize collagen fiber mat achieved from electrospinning. Clearly, it is important to analyze the thermal characteristics of the obtained electrospun nanofibers to compare with pure extracted collagen. The thermal degradation is a sign of structural change which might be occurred after electrospinning. Thus, the final step of this thesis is devoted to enhance and characterize the fabricated nanofibers mat, which is supported by carefully designed experiment

to fabricate collagen nanofibers with diameters close to natural collagen fibers in the extracellular matrix (ECM).

1.2 Research Objectives

The general objective of this study is to evaluate the potential application of quails' feet as a new source of collagen.

This research study is designed to carry out the following specific objectives:

- To extract quails' feet collagen using acetic acid and lactic acid with/ without pepsin digestion and characterize the extracted collagens.

- To determine a suitable concentration of collagen to fabricate collagen nanofibers mat by electrospinning.

- To specify the appropriate range of electrospinning parameters.

- To obtain the optimum electrospinning parameters using Response Surface Methodology (RSM) technique.

- To fabricate collagen nanofibers and characterize the produced collagen nanofibers mat.

CHAPTER 2

LITERATURE REVIEW

2.1 Quail

Quail as a food source has been farmed since ancient times. The earliest observed of quail can be found in the Egyptian hieroglyphics. During the last decade, quail farming has achieved economic significance as a species to produce eggs and meat (Kayang et al., 2004).

In worldwide, Japanese quails can be found in many countries which influenced by its good performance in meat and egg production that can be described as small size, low feed requirements, short life cycle, good reproductive potential, good meat taste, better laying ability, rapid growth rate, shorter time of hatching and resistant to common poultry diseases as compared with different species of poultry. Thus, quail production is an important poultry business. Broiler quails (Coturnix coturnix japonica) are one type of poultry that presents in various environments, from tropical rainforest and Europe temperate to the Himalayan mountain area. In Malaysia, quail production is a very important business. There are 12 million quails has been produced in 130 quail farms in Malaysia and produce 350 million eggs per year (Muhammad and Hassan, 2018).

2.1.1 Species of quail

More than 70 domestic strains and 20 species of wild quail can be found around the world, including laboratory and commercial quails (Chang et al., 2005). Quail belongs to the order Galliformes and the family Phasianidae, as detailed in Table. 2.1

Table 2.1: Taxonomic classification of quail in the Animal Kingdom.

Phylum	Chordata
Subphylum	Vertebrata
Class	Aves
Order	Galliformes
Family	Phasianidae
Subfamily	Perdicinae (Old World quail)
	Odontophorinae (New World quail)
	Phasianinae (True Pheasants and Peafowls)

Ref. (Shanaway, 1994)

Shanaway (Shanaway, 1994) reported that the family of Phasianidae is very diverse which is hard to divide into natural groups, and it includes the Old World quails, pheasants, chickens, partridges and peafowls, which explains why they are all quite similar. However, three subfamilies are recognized: the Perdicinae (the Old World quail), the Odontophorinae (the New World quail) and the Phasianinae (the true pheasants and peafowls). The differences between pheasants, quails and chickens can be seen in their size and shape, as well as in their habitat and state of domestication.

2.1.2 The old world quail

The broadly scattered subfamily of the Old World quail is the Coturnix species, which is supposed to be popular species in the global and is the only truly migratory members of the order Galliformes. The Coturnix genus includes various species such as: Common Quail (*Coturnix coturnix*), Japanese Quail (*Coturnix japonica*), Stubble Quail (*Coturnix pectoralis*), Rain Quail (*Coturnix coromandelica*), King Quail or Button Quail or Chinese Quail (*Coturnix Chinensis*), Brown Quail (*Coturnix ypsilophora*), Harlequin Quail (*Coturnix delegorguei*) and Blue Quail (*Coturnix adansonii*)(Tavaniello, 2013). However, the most important species which produce egg and meat and widely distributed in the world is the Japanese quail (*Coturnix japonica*).

2.1.3 Japanese quail (*Coturnix Japonica*)

The Japanese quail (*Coturnix japonica*) is a little bird as shown in Fig. 2.1.

Figure 2.1: Japanese quail (adult female on the right; adult male on the left). The image is adopted from http://www.crescentquail.co.uk/quail-pedia.asp

However, males are almost as small as females. Adult males weight is in the range

of 100 to 140 g, but females are somewhat heavier, about 120 to 160 g. Shanaway claim that the weight of quail strains for meat production can weigh up to 300 g (Shanaway, 1994). Some of the characteristics of quail, are detailed in Table. 2.2.

Table 2.2: The characteristics of the Japanese quail.

Trait	Range
Body weight at one-day-old	6-8 g
Adult male	100-140 g
Adult female	120-160 g
Egg weight	9-10 g
Quail for meat production	300 g
Egg number/100 days	80-90
Age at sexual maturity	38-42 days of age
Life span	Max: 7 years in male
	Mean: 3-4 years

Ref. (Shanaway, 1994)

The plumage color of the Coturnix japonica is mainly brown (dark cinnamon). The eggshell color of the quail is white, light brown or speckled brown. The color, size and shape pattern on the eggs vary considerably among females as shown in Fig. 2.2.

Figure 2.2: Eggshell color of the quail.
The image is adopted from https://www.exportersindia.com/kaniraji-quail-farm/quail-egg-4367112.htm

The initial evidence of Japanese quail domesticated was in the 8th century in East Asia (Japan). The Japanese quail $(Coturnixjaponica)$, was successfully introduced from Japan to America, Europe, and the Near and Middle East since the 12th century. In fact, Japanese quail is grown to produce meat and egg and also it is used as a laboratory animal for animal and biomedical researches. More recently, consumption of quail meat and egg has been gaining popularity among users because of the global similarity in physical characteristics and behavior with other poultries such as chicken and duck (Rule et al., 2002; Tavaniello, 2013; Wood et al., 2004). For example, Hu et al. reported that a closer relationship between quail and chicken egg white proteome patterns. Indeed, Japanese quail is phylogenetically closely related to the chicken (Hu et al., 2016). Also, the hearing range and sensitivity of quail and chicken are remarkably similar (Niemiec et al., 1994).

According to the information obtained from poultry production in 2007, the largest producer of quail meat countries was China (with over 160,000 tons), followed by Spain (9,000 tons), France (8,000 tons), Italy and USA (3,000 tons each). Australia, Portugal, and Brazil are other countries that produce meat quail with over 1,000 tons each (da Cunha, 2009). With this massive production of quail, huge amounts of by-products in various forms, such as feet, blood, viscera, feathers, bones, and head are produced (Lasekan et al., 2013). In this case, if not properly employed these huge amounts of by-products, could be cause serious environmental problems. In fact, these large amounts of waste from poultry slaughterhouses into the environment, polluting land and surface waters as well as posing a serious human health risk. The discharge of biodegradable organic compounds may reduce the amount of dissolved oxygen in surface waters, which may lead to reduced levels of activity or even the death of aquatic life. Macronutrients (nitrogen, phosphorus) may cause eutrophication of water bodies. Therefore, animal by-products utilization has a big influence on public health and economic development. In spite of this, published information in this area is relatively scant. Therefore, it has become a basic need to promote animal by-products utilization through research in this field. This will not only help to solve pollution problems but will also meet the challenges of the carcass by-products industries through innovations and find better opportunities in the future.

2.1.4 Poultry by-products

Poultry by-products comprise heads, feet, and inedible viscera (intestines, lungs, pancreas, spleen, and the reproductive organs). Zhu et al., showed that, about 22 to 30% of poultry products considered as by-products (Zhu et al., 2010). There are sev-

eral methods to manage poultry by-products. Commonly, animal by-products were used as agricultural compost and also used to make animal feed (Bolan et al., 2010). According to the Regulation (EC) No 1069/2009 of the European Parliament and of the Council of October 21, 2009, which came into force on March 04, 2011, the wastes are included in category 3 based on their risk level for transmitting pathogens and toxic materials. Accordingly those animal by-products should be reclassified as Category 3 material, so as to allow their use for certain feeding purposes.

Category 1 material shall include the following animal by-products: (a) entire bodies and all body parts, including hides and skins of the following animals: a-1) animals suspected of being infected by a TSE. a-2) animals slaughtered in the context of TSE eradication measures. a-3) animals other than farmed and wild animals, including in particular pet animals, zoo animals, and circus animals. a-4) animals used for experiments. a-5) wild animals when suspected of being infected with diseases communicable to humans or animals. (b) entire bodies or parts of dead animals containing specified risk material at the time of disposal. (c) animal by-products obtained from animals that have been submitted to illegal treatment. (d) animal by-products containing residues of other substances and environmental contaminants. (e) animal by-products collected during the treatment of wastewater. (f) catering waste from means of transport operating internationally. (g) mixtures of Category 1 material with either Category 2 material or Category 3 material or both.

Category 2 material (a) manure, non-mineralised guano and digestive tract content. (b) animal by-products collected during the treatment of wastewater. (c) animal by-products containing residues of authorized substances or contaminants exceeding the permitted levels. (d) products of animal origin that have been declared unfit for human

consumption due to the presence of foreign bodies in those products; (e) products of animal origin, other than Category 1 material, (f) animals and parts of animals that died other than by being slaughtered or killed for human consumption, including animals killed for disease control purposes; (g) mixtures of Category 2 material with Category 3 material; (h) animal by-products other than Category 1 material or Category 3 material. Category 3 material (a) carcases and parts of animals slaughtered or, in the case of game, bodies or parts of animals killed, and which are fit for human consumption. (b) carcases and the following parts originating either from animals that have been slaughtered in a slaughterhouse and were considered fit for slaughter for human consumption following an ante-mortem inspection or bodies and the following parts of animals from game killed for human consumption in accordance with Community legislation: (i) carcases or bodies and parts of animals which are rejected as unfit for human consumption in accordance with Community legislation, but which did not show any signs of disease communicable to humans or animals; (ii) heads of poultry; (iii) hides and skins, including trimmings and splitting thereof, horns and feet, including the phalanges and the carpus and metacarpus bones, tarsus and metatarsus bones, of animals, other than ruminants requiring TSE testing, and ruminants which have been tested with a negative result in accordance with Article 6(1) of Regulation (EC) No 999/2001; (iv) pig bristles; (v) feathers; (c) animal by-products from poultry and lagomorphs slaughtered on the farm. (d) the blood of animals which did not show any signs of disease communicable through blood to humans or animals. (e) animal by-products arising from the production of products intended for human consumption, including degreased bones, greaves, and centrifuge or separator sludge from milk processing.(f) products of animal origin, or foodstuffs containing products of animal origin. (g) pet food and feeding

stuff of animal origin, or feeding stuff containing animal by-products or derived products. (h) blood, placenta, wool, feathers, hair, horns, hoof cuts and raw milk originating from live animals that did not show any signs of disease. (i) aquatic animals, and parts of such animals, except sea mammals, which did not show any signs of disease communicable to humans or animals; (j) animal by-products from aquatic animals originating from establishments or plants manufacturing products for human consumption; (k) the following material originating from animals which did not show any signs of disease communicable through that material to humans or animals (l) aquatic and terrestrial invertebrates other than species pathogenic to humans or animals; (m) animals and parts thereof of the zoological orders of Rodentia and Lagomorpha, except Category 1 material. (n) hides and skins, hooves, feathers, wool, horns, hair and fur originating from dead animals that did not show any signs of disease. (o) adipose tissue from animals which did not show any signs of disease communicable through that material to humans or animals (Regulation, 2009). Then the category 3 material includes animal wastes such as; skin, bone, heads, and feet gathered from healthy animals are allowed to put to use for certain advantageous materials (Barrena et al., 2009; Lasekan et al., 2013).

Figure 2.3: Quail feet as a by-product of quail meat processing.

Hence, the utility of by-products from poultry industries as a source of valuable materials not only increases the economic value but also decreasing the environmental pollution difficulties. As poultry skin is composed of a high value of fat components and the concentration of collagen is low, it is thus preferable to use other material such as feet (Schrieber and Gareis, 2007). Fig. 2.3 shows quail feet as a by-product of quail meat processing.

2.2 Collagen Background

The extracellular matrix (ECM) in human and all multicellular animal bodies, provides storage of some essential biomolecules, structural support, and the connection between the cells of multicellular organisms. It consists of three major types of molecules: structural insoluble proteins (collagens, and elastin), specialized proteins (fibrillin, fibronectin, and laminin) and proteoglycans (decorin and aggrecan).

Collagen is a major fibrous protein, an essential component of the various connective tissues found in all multicellular animals, and exists in a variety of different forms (Bailey et al., 1998; Voet and Voet, 2015). In fact, it is the plentiful protein in vertebrates (nearly 25% to 35% of total protein mass). Collagen has fundamental functions and its related to the molecular architecture, and mechanical characteristics of tissue, for example; the tensile strength in the skin (Ricard-Blum and Ruggiero, 2005). Moreover, collagens interact with cells through various receptor families and regulate cell growth, differentiation, and migration. Collagen has the ability to maintain and to provide structural integrity in various connective tissues (Bigi et al., 2000).

2.2.1 Biochemistry of collagen

2.2.1(a) Molecular structure of collagen

In contrast to spherical globular proteins, collagen is composed of linear, fiber-like structures. Collagen is not a uniform substance but is rather a family of proteins. Like all other proteins, collagen exhibits primary, secondary, and tertiary structural elements. It also has a quaternary structure similar to other complex oligomeric proteins that are characterized by having multiple polypeptide chains or subunits.

Collagen units are constituted of three (α-chain) polypeptide chains with left-handed polyproline II (poly-Pro II) helical structure, wound around each other to form a right-handed triple helix (Shoulders and Raines, 2010). The left-handed polyproline II helical structure of each α-chain in the collagen molecule, there are 18 amino acids per turn. Three polypeptide chains are coiled around a central axis in the right-handed direction to form the triple helical structure with one residue-stagger about the next chain (Shoul-

ders and Raines, 2010). Moreover, the three α-chains in the triple helix are kept close by the interchain hydrogen bonds. Each α-chain is constructed from a unique repeating amino acid sequence of Gly-X-Y, where glycine employed in every third position of the amino acid sequence of the α-chain while proline and 4-hydroxyproline mostly occupy X and Y position, respectively (Brodsky and Shah, 1995).

Proline and hydroxyproline are responsible for the unique secondary structure of collagen. These amino acids limit the rotation of the polypeptide backbone and thus contribute to the stability of the triple helix. The hydroxyl group of hydroxyproline plays a crucial role in stabilizing the triple helix of collagen. Collagen polypeptides lacking hydroxyproline fold into triple-helical conformations at low temperatures, but these are not stable at body temperature.

Besides that, α-chain is made up of some 1300-1700 amino acids residues and with 285 nm long and the diameter of 1.4 nm. Additionally, most collagens demonstrate a unique 67 nm periodicity that obtains from large assemblies of quarter staggered molecules (4-5 in a cross-section) with a unique banding pattern when formed into microfibrils (Bailey, 2001; Prockop and Kivirikko, 1995).

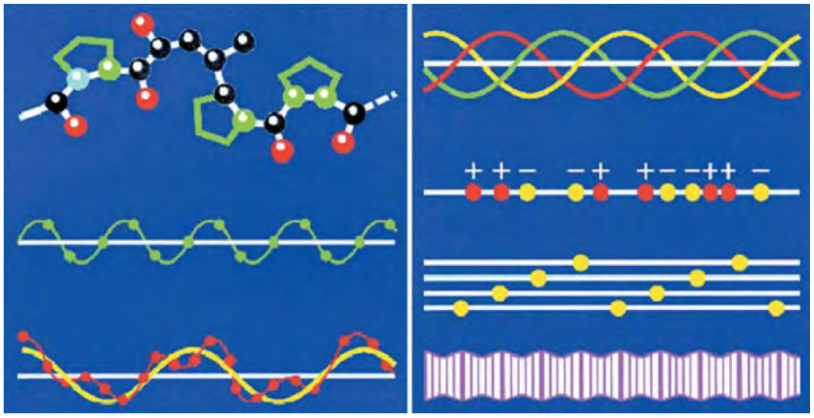

Figure 2.4: From the primary structure through the quaternary structure. The steps in collagen formation. The image is adopted from (Schrieber and Gareis, 2007).

Because of the charge distribution, these basic structures are staggered by about one-quarter of their length (quaternary structure) as is shown in Fig. 2.4. Four to eight collagen molecules in cross-section constitute the basic unit to form so-called collagen fibrils. The entire structure is stabilized and reinforced by cross-links in the form of covalent bonds. Many of these collagen fibrils are then further cross-linked to form the basic structure for tissues such as skin and bone.

The outside peptide groups in α-chains are called the N-terminal (amine group-containing) end while the other end is referred to as the C-terminal end as it contains the carboxylic acid group. In these regions of each α-chains, the polypeptide chains do not form a triple helix because they consist of lysine and hydroxylysine residues and contributed to the intra- and intercross links of the collagen molecules (Gómez-Guillén et al., 2011). This non-triple helical domain of collagen is known as the non-collagenous domain or telopeptides. Another sign is that the non-collagenous regions are much shorter, typically on the order of 20-30 Gly-X-Y residues, compared to the $\sim$333 Gly-X-Y residues in collagen (Brodsky and Shah, 1995).

2.2.1(b) Amino acid composition of collagen

Much the same to other proteins, the building blocks of collagen are amino acids. Amino acids have a considerable role in the creation of the initial structure of collagens. Various types and several sequences of amino acids are responsible for structural, physicochemical and functional properties of collagens. Different types of collagen and collagens from different sources contain several amino acid compositions. However, in different sources of collagen, there is a common and unique feature of the amino acid composition. Collagen molecules are composed of the repetitive of three amino acids sequence Gly-X-Y where X and Y are mainly proline and hydroxyproline, respectively. This sequence is essential for the formation of a stable triple helix and is the main characteristic used to designate a protein as collagen. Glycine is necessary because it is small and allows the procollagen chains to come together in close proximity. Glycine appears as every three residues of collagen molecule except for the first $\sim$14 amino acids from N-terminal and the first $\sim$10 amino acids from C-terminal (Kittiphattanabawon et al., 2005).

Collagen mainly consists of considerably high amounts of proline and hydroxyproline, which are not found in other mammalian proteins (Karim and Bhat, 2009). Prolines are essential for many reasons. Proline residues help to stabilize the conformation of triple helix because they provide limited conformational possibilities to the unfolded state. Prolines also become hydroxylated, which stabilizes the triple helix by increasing hydrogen bonding. A unique feature of collagen is the existence of imino acids (proline+hydroxyproline), which are responsible for the secondary structure of this protein. Indeed, the structural stability of collagen depends on the imino acid content.

Table 2.3 shows the amino acid profile of the collagen from different sources (mammalian, poultry and marine). From this table can be observed that the mammalian collagens, for example, bovine and porcine collagen, contain high amounts of imino acid content compared to the collagen from other sources.

Table 2.3: Amino acid profiles of Collagens from Calf-skin, Pig-skin, Deep sea redfish, Grass Carp, Chicken feet and Bird feet collagens.

Amino acid	Calf-skin [a]	Pig-skin [a]	Deep sea redfish [b]	Grass carp [c]	Bird feet [d]
Hyp	95.1	97.2	64	65	136.6
Asp	45.7	43.8	50	42	57.1
Ser	33.2	32.6	61	39	6.2
Glu	75.9	72.4	78	61	46.5
Gly	330.6	340.7	335	334	382.1
His	5.3	5.2	4	5	9.1
Arg	51	47.5	50	57	23.8
Thr	18.4	15.4	23	24	16
Ala	119.7	114.4	105	135	127.8
Pro	121.5	122.8	101	121	42.9
Cys	0	0	-	4	1.7
Tyr	3.7	1.2	6	2	1.1
Val	21.5	22	25	31	14.6
Met	6.1	5.7	14	10	8
Lys	26.5	27.1	28	23	49.3
Ile	11.4	10.6	9	10	23.9
Leu	23.4	22.2	21	22	37.3
Phe	3.3	12.4	20	17	12.2
Hyl	7.7	6.8	-	-	-
Total	1000	1000	1000	1000	1000
Imino acid	216.6	220	165	186	179.5

[a] (Li et al., 2013)

[b] (Lingzhao et al., 2008)

[c] (Zhang et al., 2007)

[d] (Lin and Liu, 2006a)

According to the literature studies, the higher content of imino acid in mammalian collagen contributes to the high denaturation temperature compared to marine-based

collagen (Lingzhao et al., 2008; Nagai and Suzuki, 2000; Zhang et al., 2007). Some studies have reported a correlation between the thermal stability of collagen and the imino acid content (proline and hydroxyproline) (Bae et al., 2008; Cui et al., 2007; Kittiphattanabawon et al., 2005; Piez and Gross, 1960; Wong, 1989).

2.2.2 Types of collagen

Collagen is the major structural protein in creatures, with 29 individual types of collagen identified by NCBI (National Center for Biotechnology Information). Various types of collagen differ in amino acid composition, structure, assembly, type of additional non-helical domains, and functions. Moreover, collagens can be split up into various subfamilies: fibril-forming collagens, fibril-associated collagens with interrupted triple helices (FACIT), network-forming collagens, membrane collagens, multiplexing, and others with unique functions (Gelse et al., 2003). Collagens I-XXVIIII were evaluated according to the α-chains and molecular species. Table 2.4 shows the different collagen types defined by their structural diversity, variants of non-helical domains, assembly, and functions.

Table 2.4: Chain composition and distribution of the various collagen types.

Collagen Type	Molecular Composition	Tissue Distribution
Fibril-forming collagens		
I	$\alpha1(I)_2\alpha2(I)$	bone, dermis, tendon, ligaments, cornea
II	$[\alpha1(II)]_3$	cartilage, vitreous body, nucleus pulposus
III	$[\alpha1(III)]_3$	skin, vessel wall, reticular fibers of most tissues (lungs, liver, spleen, etc)
V	$\alpha1(V),\alpha2(V),\alpha3(V)$	lung, cornea, bone, fetal membranes;together with type I collagen
XI	$\alpha1(XI),\alpha2(XI),\alpha3(XI)$	cartilage, vitreous body
Basement membrane collagens		
IV	$\alpha1(IV)_2,\alpha2(IV);\alpha1-\alpha6$	basement membranes
Microfibrillar collagen		
VI	$\alpha1(VI),\alpha2(VI),\alpha3(VI)$	widespread, dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
Anchoring fibrils		
VII	$[\alpha1(VII)]_3$	skin, dermal-epidermal junctions; oral mucosa, cervix
Hexagonal network-forming collagens		
VIII	$[\alpha1(VIII)]_2\alpha2(VIII)$	endothelial cells, descemet's membrane
X	$[\alpha3(X)]_3$	hypertropic cartilage
FACIT collagens		
IX	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	cartilage , vitreous humor, cornea
XII	$[\alpha1(XII)]_3$	perichondrium, ligaments, tendon
XIV	$[\alpha1(XIV)]_3$	dermis, tendon, vessel wall, placenta, lungs, liver
XIX	$[\alpha1(XIX)]_3$	human rhabdomyosarcoma
XX	$[\alpha1(XX)]_3$	corneal epithelium, embryonic skin, sterna cartilage, tendon
XXI	$[\alpha1(XXI)]_3$	blood vessel wall
Transmembrane collagens		
XIII	$[\alpha1(XIII)]_3$	epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver
XVII	$[\alpha1(XVII)]_3$	dermal-epidermal junctions
Multiplexins		
XV	$[\alpha1(XV)]_3$	fibroblasts, smooth muscle cells, kidney, pancrease
XVI	$[\alpha1(XVI)]_3$	fibroblasts, amnion, keratinocytes
XVIII	$[\alpha1(XVIII)]_3$	lungs, liver

Ref. (Gelse et al., 2003).

2.2.2(a) The fibril-forming collagen (Types I, II, III, V, and XI)

Fibril-forming collagens are rod-like collagens with a large triple helical region (nearly 300 nm). This group is an abundant group of collagens and comprise of 90% of collagens superfamily (Friess, 1998). The fibril-forming collagens are the first collagens have been identified (Ricard-Blum and Ruggiero, 2005). The primary types of this group are types I and V as the structural backbone of the bone, types II and XI as a fibrillar matrix of articular cartilage and type III collagen is a homotrimer of three α1(III) chains and is widely distributed in collagen I containing tissues except bone.

These collagens can be found in and around many organs in the human and animal's body (Birk and Bruckner, 2005). This group of collagens is mostly accountable for the stability and integrity of the human and animal tissues (Friess, 1998).

2.2.2(b) The FACIT collagen (Type IX, XII, XIV, XIX, XX and XXI)

The FACITs do not form fibrils by themselves, but they are associated with the surface of collagen fibrils. Collagen IX is a heterotrimer of $\alpha 1(IX)\alpha 2(IX)\alpha 3(IX)$ chains, which is covalently linked to the surface of cartilage collagen fibrils mostly containing collagen type II. Moreover, collagen types XII and XIV are structurally close to type IX collagen and associated to type I collagen in skin, perichondrium, tendon, lung etc.(Olsen, 1997).

2.2.2(c) Microfibrillar collagen (Type VI)

Type VI collagen is a heterotrimer of $\alpha 1(VI), \alpha 2(VI), \alpha 3(VI)$ chains. Type VI collagen can be found in all connective tissues except bone (Gelse et al., 2003).

2.2.2(d) Hexagonal network-forming collagens (Type X and VIII)

Collagen types X and VIII are assembled to create hexagonal networks in different tissues (Sawada et al., 1990). Type X collagen is identified in hypertrophic cartilage in the ribs and vertebrae. Type VIII collagen is close to type X but with different functions. It is generated by endothelial cells and assembled in the form of hexagonal lattices and found in Descemet's membrane of cornea (Gelse et al., 2003).