# EXTRACTION OF FISH GELATIN FROM SURIMI PROCESSING BY-PRODUCTS AIDED BY ENZYMES USING RESPONSE SURFACE METHODOLOGY

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**by**

**KEE HUEY YING**

**Thesis submitted in fulfillment of the requirements for the degree of Master of Science**

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## TABLE OF CONTENTS

**APPENDICES**

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF PLATES

## LIST OF SYMBOLS/ABBREVIATION

| Symbol/ Abbreviation | Caption |
|---|---|
| α | alpha |
| β | beta |
| δ | delta |
| γ | gamma |
| *a** | greenness/redness |
| ANOVA | one-way analysis of variance |
| *b** | blueness/yellowness |
| $Ca(OH)_2$ | calcium hydroxide |
| CCRD | central composite rotatable design |
| CBG | commercial bovine gelatin |
| CFG | commercial fish gelatin |
| CPG | commercial porcine gelatin |
| EFG | extracted fish gelatin |
| FMOC | 9- fluorenylmethyl chloroformate |
| FTIR | Fourier transform infrared spectroscopy |
| G′ | dynamic storage/elastic modulus (pronounced “G-prime”) |
| G″ | dynamic loss/viscous modulus (pronounced “G-double prime”) |
| HPLC | high performance liquid chromatography |
| IEP | isoelectric point |
| *L** | Lightness |
| OPA | o-phthalaldehyde |
| RFG | ribbon fish gelatin |
| SCG | sin croaker gelatin |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate polyacrilamide gel electrophoresis |
| SSG | slender shad gelatin |
| TBG | threadfin bream gelatin |

**PENGEKSTRAKAN GELATIN IKAN DARIPADA HASIL SAMPINGAN PEMPROSESAN SURIMI DENGAN ENZIM MENGGUNAKAN METODOLOGI PERMUKAAN RESPON**

## ABSTRAK

Gelatin telah diekstrak daripada hasilan sampingan pemprosesan surimi daripada timah yang terdiri daripada kulit, tulang dan sisik, dengan bantuan enzim bromelin dan papain, masing-masing. Proses ini telah dioptimakan menggunakan pendekatan kaedah respon permukaan (RSM) dengan rekabentuk komposit pertengahan berputar (CCRD) yang mempunyai 4-faktor dan 5-aras. Kesan kepekatan enzim ($X_1$), tempoh pra-rawatan asid ($X_2$), suhu pengekstrakan ($X_3$) dan tempoh pengekstrakan ($X_4$) ke atas respon hasil gelatin dan kekuatan gel telah dikaji. Kondisi optima diperolehi untuk pengekstrakan gelatin dengan bantuan bromelin (EFGB) ialah: kepekatan enzim, 0.03%; tempoh pra-rawatan asid, 1.5 jam; suhu pengekstrakan, 41 °C dan tempoh pengekstrakan, 5 jam manakala pengekstrakan gelatin dengan bantuan papain (EFGP) ialah: kepekatan enzim 0.03%; tempoh pra-rawatan asid 4 jam; suhu pengekstrakan 42 °C dan tempoh pengekstrakan 5.1 jam. Nilai ramalan untuk respon hasil gelatin dan kekuatan gel bagi EFGB ialah 19.2% dan 73.6 g manakala nilai eksperimen sebenar yang diperolehi ialah 18.3% dan 62.9 g. Nilai ramalan untuk respon hasil gelatin dan kekuatan gel bagi EFGP ialah 18.7% dan 37.0 g manakala nilai eksperimen sebenar yang diperolehi ialah 18.5% dan 34.6 g. Kedua-dua hasil gelatin EFGB dan EFGP telah meningkat sebanyak 50% berbanding dengan hasil gelatin yang diekstrak tanpa bantuan enzim (EFG). Namun kekuatan gel bagi EFGB dan EFGP telah mengurang berbanding dengan kekuatan gel bagi EFG yang sebanyak 76.0 g. Sifat fizikokimia dan reologi gelatin yang diekstrak

telah dicirikan dan dibandingkan dengan sifat fizikokimia dan sifat reologi gelatin komersil daripada ikan, babi dan lembu. Kedua-dua gelatin ikan yang diekstrak berwarna kuning keperangan. Kandungan lembapan, abu dan protein bagi EFGB ialah 5.8%, 2.4%, 88.4% dan 6.3%, 2.1%, 86.3% bagi EFGP. Kelekitan, kejernihan dan titik isoelektrik yang diukur ialah: 1.9 mPa.s, 73.5% transmitans, pH 8.0 and 1.6 mPa.s, 79.4% transmitans, pH 8.0 bagi EFGB dan EFGP. Kandungan asid imino (prolina dan hidroksiprolina) bagi EFGB dan EFGP ialah 14.5% dan 13.3% daripada jumlah residu asid amino. Suhu mengel dan melebur gel bagi EFGB ialah pada 9.1 ºC dan 17.4 ºC manakala itu bagi EFGP ialah pada 8.6 ºC dan 15.8 ºC, masing-masing.

**EXTRACTION OF FISH GELATIN FROM SURIMI PROCESSING BY-PRODUCTS AIDED BY ENZYMES USING RESPONSE SURFACE METHODOLOGY**

**ABSTRACT**

Gelatin was extracted from surimi processing by-product of ribbon fish (*Trichiurus lepturus*) specie that consisted of skins, bones and scales, aided by enzymes, bromelain and papain. The extraction process was optimized using response surface methodology (RSM) approach with a 4-factor and 5-level central composite rotatable design (CCRD). The effects of enzyme concentration ($X_1$), acid pretreatment time ($X_2$), extraction temperature ($X_3$) and extraction time ($X_4$) on responses, gelatin yield ($Y_1$) and gel strength ($Y_2$) were investigated. Optimum conditions obtained for gelatin extraction aided by bromelain (EFGB) were: enzyme concentration, 0.03%; acid pretreatment time, 1.5 hr; extraction temperature, 41 ºC and extraction time, 5 hr whilst that for extraction aided by papain (EFGP) were: enzyme concentration, 0.03%; acid pretreatment time, 4 hr; extraction temperature, 42 ºC and extraction time, 5.1 hr. The predicted values for response gelatin yield and gel strength of EFGB were 19.2% and 73.6 g while the actual experimental values obtained were 18.3% and 62.9 g. The predicted values for response gelatin yield and gel strength of EFGP were 18.7% and 37.0 g while the actual experimental values obtained were 18.5% and 34.6 g. The gelatin yield of both EFGB and EFGP were increased by about 50% compared to the gelatin yield for gelatin extracted without enzyme (EFG). However the gel strength for both EFGB and EFGP were reduced compared to that of EFG, which was 76.0 g. Physicochemical and rheological properties of the extracted gelatin were characterized and compared to those

of commercial fish, porcine and bovine gelatin. Both extracted fish gelatin appeared to be brownish yellow in color. The moisture, ash and protein contents for EFGB and EFG were: 5.8%, 2.4%, 88.4% and 6.3%, 2.1%, 86.3%, respectively. The viscosity, clarity and isoelectric point measured for EFGB and EFGP were: 1.9 mPa.s, 73.5% transmittance, pH 8.0 and 1.6 mPa.s, 79.4% transmittance, pH 8.0, respectively. The imino acid content (proline and hydroxyproline) of EFGB and EFGP were 14.5% and 13.3% of the total amino acid residues. The gelling and melting point of EFGB were found to be 9.1 ºC and 17.4 ºC while that of EFGP were 8.6 ºC and 15.8 ºC, respectively.

# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Gelatin is a type of translucent, colorless or yellowish, flavorless consumable material that is available in film or powder form commercially. When added into water, gelatin tends to swell and eventually dissolves in water when heated, producing a highly viscous liquid. When this solution is cooled down to a certain temperature, it will set to a clear semi-solid colloid gel and that temperature is called the setting point or gelling point of the gelatin. Upon heating up, the gel again, starts to soften at its melting temperature and turns back into a gelatinous liquid. The thermo-reversible gelling-melting process is one of the important properties that define gelatin.

Gelatin is the most common of the hydrocolloids. It has wide applications in many fields, especially the food industry, since the early days. Despite the efforts from both manufacturers and scientists to find alternatives for hydrocolloids, gelatin remains irreplaceable in many applications due to its unique melting point. With a melting point below the human body temperature, gelatin gives a melt-in-the-mouth feel delivering an excellent sense of confection when consumed (Choi & Regenstein, 2000). Gelatin has also been applied in other areas, such as nutraceutical, pharmaceutical and cosmetic industries, photography, manufacturing of adhesives and in recent years, development of edible coatings.

Gelatin is not a naturally occurring protein (Jones, 1987), but it is a substance derived from collagen, a type of fibrous protein from animal, through an irreversible thermal hydrolysis process. Collagen composes of mainly glycine (Gly), alanine (Ala), proline (Pro), hydroxyproline (Hyp) and 14 others amino acids. Collagen is found in animal skins, connective tissues and bones. A gelatinous solution can simply be obtained by boiling animal skin in water and letting it to cool. In the manufacturing of gelatin, raw materials are pretreated with acid and other chemicals to increase the efficiency of gelatin extraction.

According to the Gelatin Manufacturers of Europe report, a total of 326,000 tons of animal-based gelatin is produced annually using pork skin (46 %), bovine hide (29.4 %) and bone (23.1 %) as the major raw material (GME, 2008). Some of the world largest gelatin manufacturers are Capsugel (US), Gelita Group (Germany), Nitta Corporation (Japan), Norland Products (US), Suheung Capsule (Korea) and Rousselot Group (France) (Global Industry Analysts, 2012). The global demand for gelatin which is expected to increase consistently and will reach 357,800 tons by 2015 (Jose, 2011) adds impetus for research on optimization of gelatin extraction.

The conventional gelatin extracted from the above raw materials is however rejected by Muslim, Jewish and Hindu consumers due to religious restrictions. Porcine gelatin is strictly forbidden for both Muslim and Jewish consumers because it does not have Halal and kosher status. Bovine gelatin is banned for Hindu consumers while the bovine gelatin obtained from cattle that has not been religiously treated is also considered to be not Halal for Muslim consumers (Choi & Regenstein, 2000). The global Halal food trade is estimated at US$ 547billion a year and is expected to have a consistent growth of 12 % to 15 % for the next ten years (DagangAsia Net, 2008).

On top of that, after the out burst of bovine spongiform encephalopathy (BSE, also called the mad cow disease) and foot-and-mouth disease, the consumer consensus was to avoid food products containing bovine or porcine as a source of ingredient which may carry a risk of infectious diseases (Wasswa, Tang & Gu, 2007). Awareness of health and purchasing power of the consumers have increased in recent days, consequently demand on the quality of food ingredients has set a higher bar. It is obvious that an alternative source of gelatin was required to solve the dilemma. Over the years, scientists found potential in gelatin extracted from fish that exhibited similar functional properties to mammalian gelatin whereas gelatin extracted from poultry was less desirable due to low collagen and high fat content in the skin (Schrieber & Gareis, 2007).

According to the Food and Agriculture Organization of the United Nations (FAO), the total global fish production was 38 million tons in 1960 and has increased to 143.6 million tons in 2006. According to an annual rate of growth of 3 %, global fish production is projected to reach 179 million tons by the year 2015 (FAO, 2009). By-products of the fisheries sector constitute about 35 % of the total harvest, but vary depending on the usage of the catch.

In a surimi manufacturing plan, fish meat is separated from other parts of the fish, with the fish heads removed prior to this, for further processing. The surimi processing by-product, comprised of fish skin, bone and scale, could reach 50-60 % of the total catch (Arvanitoyannis & Kassaveti, 2008). This by product, sometimes called fish waste, is conventionally turned into fishmeal for animal feed or discarded. However, fish waste discarded without proper waste treatment has a high environmental impact. As a rich source of naturally occurring collagen, fish waste can be utilized by making it

as the raw material for gelatin extraction, solving the problem of waste management and on the other hand, adding high commercial value to the by-product (Choi & Regenstein, 2000; Gudmundsson & Hafteinsson, 1997).

Studies were carried out to characterize and to improve the properties of gelatin extracted from different fish species, such as skin and bone of Tiger-toothed croaker and Pink perch (Jayappa et al., 2012), grey triggerfish skin (Kemel et al., 2011), unicorn leatherjacket skin ( Mehraj & Soottawat, 2011), walleye pollock skin (Yan et al., 2011), red tilapia skin, Walking catfish skin and Striped catfish skin (Jamilah et al., 2011), carp skin (Duan et al., 2011), hoki skin (Mohtar et al., 2010) and blue shark skin (Limpisophon et al., 2009). Functional properties of fish gelatin however, differ among various species due to the different amino acid composition. Fewer studies were carried out using surimi processing by-products (Norziah et al., 2009). Therefore, this study aims to develop value added products from the under-utilized surimi processing by products aided by plant based enzymes, bromelain and papain, to optimize the extraction conditions of gelatin. Thus producing an edible gelatin that would be appealing for people who emphasize on halal products.

## 1.2 Objectives

The objectives of this study are:

1) To extract fish gelatin from surimi processing by-product of fish specie, ribbon fish (*Trichiurus lepturus*) aided by proteolytic enzyme (bromelain and papain).

2) To optimize the extraction conditions for producing maximum gelatin yield with high gel strength using response surface methodology (RSM) approach.

3) To characterize the extracted fish gelatin for its physicochemical and thermal properties and to make comparisons to that of commercially prepared fish, bovine and porcine gelatin.

# CHAPTER 2
# LITERATURE REVIEW

## 2.1 Collagen the gelatin precursor

Collagen is a group of naturally occurring insoluble fibrous proteins that can be found in extracellular matrix, an entity surrounding and providing structural support to the animal cells, connective tissue, skin, bone and flesh of animals. Collagen is secreted by fibroblasts in connective tissue and epithelial cells and it is the most plentiful protein in the animal. The inelastic nature of collagen, one of its most distinctive mechanical properties, makes it the principle tensile force transmitter in animal bodies. Collagen fibre tends to shrink when it is heated to a certain temperature, known as the shrinkage temperature ($T_s$).

Out of 16 types of existing collagen, type I, II and III are the major fibrous collagen constituting 80 -90 percent of collagen in the body (Ferenc, 2010; Lodish et al., 2000). Regardless of its source, the fibrous collagen is a polypeptide macromolecule about 3000 Å in length and 14 Å in diameter with a molecular weight of about 350,000 Da (Grassmann, 1965) while the most abundant amino acid residues occur in collagen are glycine, alanine, proline and hydroxyproline (Szpak, 2011). The fundamental structure of collagen, known as tropocollagen, comprises three polypeptide α-chains arranged in triple helix structure which behaves as a firm rigid rod (Figure 2.1) (Linden & Lorient, 1999).

Figure 2.1 Tropocollagen triple helix. (Source: Linden & Lorient, 1999)

### 2.1.1 Structure

Tropocollagen, the principle structural molecule of collagen aggregates, is a left-handed triple helix composed by three polypeptide α-chains. Three of these tropocollagen polypeptide chains are held together by hydrogen bonds formed between the peptide amide group (NH) of a glycine residue and the peptide carbonyl (C=0) group in an adjacent polypeptide chain. The polypeptide chains are further twisted into a helix with a geometrically fixed-angle C-N peptidyl-proline or peptidyl-hydroxyproline bonds such that the three polypeptide chains can coil around a common axis and associate into a right-handed triple helix to form a quaternary collagen microfibril. The microfibrils align themselves side by side, forming intramolecular covalent aldol cross-links between lysine (or hydroxylysine) residues with adjacent microfibrils at each terminal site to generate a strong fibril (Figure 2.2). Neighboring fibrils are shifted by one quarter of its

length and form intermolecular covalent aldol cross-links to produce a highly ordered and very stable structure (Babian & Bowes, 1977; Lodish et al., 2000).

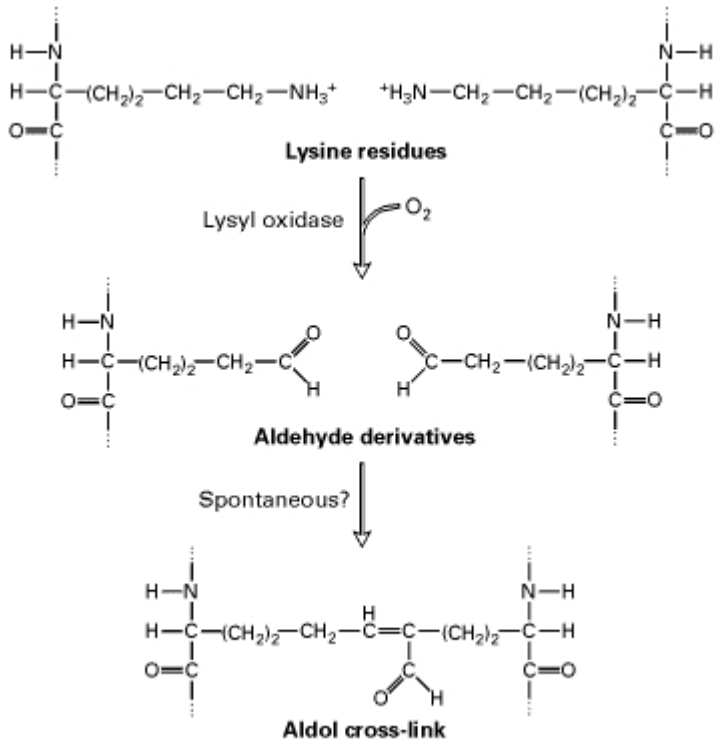


Figure 2.2 The side-by-side aldol cross-link of microfibrils between two lysine (or hydroxylysine) side chains. (Source: Lodish et al., 2000)

2.1.2 Amino acid composition

Collagen comprises 18 amino acids and has a distinctively high content of glycine and cyclic amino acids, proline and hydroxyproline compared to the other proteins (Table 2.1). Glycine is required at every third position in the collagen helix. Consisting of only an H atom, the side chain of glycine is the only one that can fit into the heavily packed centre of a triple helix α-chain forming hydrogen bonds with adjacent α-chain to stabilize the collagen helix. The non-proteinogenic hydroxyproline is generated by enzymatic hydroxylation of the proline amino acid following protein

synthesis (known as the post-translational modification). The proline and hydroxyproline side group rings which contribute to the formation of the helical structure are directed outward for the same reason.

Consequently, Glycine-Proline-X and Glycine-X-Hydroxyproline are the most common repeating motifs found in the amino acid sequence of collagen, where X is any amino acid other than glycine, proline and hydroxyproline. Alanine often incorporates into the X position in the Glycine-Proline-X triplets while glutamic acid and leucine prefers the X position in the Glycine-X-Hydroxyproline triplets (Gorres & Raines, 2010; Lodish et al., 2000; Szpak, 2011). The sulfur content of collagen is very low. Cysteine, the amino acid contributes to keratin structure by disulfide cross-link, is absent in collagen while methionine is the only amino acid containing sulfur group side chain (Johnston-Banks, 1990).

Table 2.1 The types of amino acid present in the collagen of mammal skin and fish skin and their composition.

| Amino Acid | Abundance in Mammal Skin (Residues/1000) | Abundance in Fish Skin (Residues/1000) |
|---|---|---|
| Aspartic acid | 447 | 47 |
| Hydroxyproline | 95 | 67 |
| Threonine | 19 | 26 |
| Serine | 36 | 46 |
| Glutamic acid | 74 | 76 |
| Proline | 126 | 108 |
| Glycine | 329 | 339 |
| Alanine | 109 | 114 |
| Valine | 22 | 21 |
| Methionine | 6 | 13 |
| Isoleucine | 11 | 11 |
| Leucine | 24 | 23 |
| Tyrosine | 3 | 3 |
| Phenylalanine | 13 | 14 |
| Hydroxylysine | 6 | 8 |
| Lysine | 29 | 26 |
| Histidine | 5 | 7 |
| Arginine | 49 | 52 |

(Source: Lodish et al., 2000)

### 2.1.3 Types of collagen

There are at least 16 types of collagen, each with their own characteristic polypeptide chains, but type I, II and III collagen constitute 80-90 % of the collagen in animal bodies (Table 2.2). The type I triple helix collagen molecule is composed of two α1(I)-chains with an identical amino acid sequence and a α2(I)-chain with an amino acid sequence slightly different from the α1-chain. Types II and III collagen triple helices are generated by three identical polypeptide chains, α1(II)-chains and α1(III)-chains, respectively. These three types of collagen are fibrous collagen that has a structure similar to thin long fibrils. Type IV collagen forms a sloppy two-dimensional reticulum

due to the lack of the glycine in every third position of the amino acid triplets sequence. Other types of collagen have different segments of interaction in their polypeptide chains and give rise to various three dimensional structures (Ashgar & Hendrickson, 1982; Lodish et al., 2000).

Among all types of collagen, the type I collagen (found in skin, joints and bones) and type III collagen (present in skin, muscle and blood vessels) are the main source of gelatin. Ossein gelatin obtained from de-mineralized bones consists solely of type I collagen while gelatin derived from animal skins composes of type I and type 3 collagens. Great interest has been drawn from conventional industrial sources of gelatin, such as pig skins, cattle hides and bones, to study and develop gelatin from fish sources (Nomura et al., 1996).

Table 2.2 The types of major collagen molecule in animal body.

| Type | Molecule Composition | Structural Features | Representative Tissues |
|---|---|---|---|
| Fibrillar Collagens | | | |
| I | $[\alpha1(I)]_2[\alpha2(I)]$ | 300-nm-long fibrils | Skin, tendon, bone, ligaments, dentin, interstitial tissues |
| II | $[\alpha1(II)]_3$ | 300-nm-long fibrils | Cartilage, vitreous humor |
| III | $[\alpha1(III)]_3$ | 300-nm-long fibrils; often with type I | Skin, muscle, blood vessels |
| V | $[\alpha1(V)]_3$ | 390-nm-long fibrils with globular N-terminal domain; often with type I | Similar to type I; also cell cultures, fetal tissues |
| Fibril-Associated Collagens | | | |
| VI | $[\alpha1(VI)][\ \alpha2(VI)]$ | Lateral association with type I; periodic globular domains | Most interstitial tissues |
| IX | $[\alpha1(IX)][\ \alpha2(IX)][\ \alpha3(IX)]$ | Lateral association with type II; N-terminal globular domain; bound glycosaminoglycan | Cartilage, vitreous humor |
| Sheet-Forming Collagens | | | |
| IV | $[\alpha1(IV)]_2[\alpha2(IV)]$ | Two-dimensional network | All basal laminaes |

(Source: Lodish et al., 2000)

2.1.3a Collagen from mammalian sources

The most common sources of collagen in the manufacture of gelatin are from bovine and porcine sources. Collagen rich tissues such as pig skins, cattle hides and bones are often used as the raw materials rather than pure collagen. Thus the manufacture of gelatin involves the removal of non-collagenous materials prior to the gelatin extraction process.

The amount of collagen in total skin for mature cattle is about 65-75 % of skin dry weight. Skin gelatin is prepared mainly from corium layer, the portion of the skin consisting mainly of collagen that supports the skin structurally. Mills & Bavetta (1966) found that the bone collagen (ossein) becomes crosslinked and insolubilized by a process called calcification as the animal grows. As a consequent, an additional step of demineralization has to be carried out to pre-treat the mature bone in order to extract ossein gelatin. Ossein gelatin obtained from de-mineralized bones consists solely of type I collagen while gelatin derived from animal skins composes of type I and type III collagens. Other sources of collagen include chicken, rats and kangaroos. Great interest has been drawn from conventional industrial sources of gelatin, such as pig skins, cattle hides and bones, to study and develop gelatin from fish sources (Nomura et al., 1996).

2.1.3b Collagen from marine sources

Fish collagen prepared from the swim bladder of sturgeon has been used for the clarification of alcoholic beverages centuries ago. Fish collagen of type I and III can be isolated from fish skin, scales and bones to produce gelatin. The amino acid composition for fish collagen varies from that of mammal collagen and thus, mechanical and rheological properties of fish collagen differ from mammal collagen. The number of interchain hydrogen bonding between the side chain of hydroxyproline and the backbone carbonyl group is accounted for the stability of the collagen structure. A lower concentration of proline and hydroxyproline is required in cold-water fish as the body temperature is lower than that of mammals and it is compensated for by other amino acids such as serine and threonine while warm-water fish tends to have similar amounts of proline and hydroxyproline compared to mammals. As a result, cold-water fish collagen is more thermally labile compared to warm-water fish and mammal collagens (Eastoe, 1957; Kimura & Ohno, 1987; Lodish et al., 2000).

## 2.2 Gelatin

Gelatin is a water soluble protein derived from the thermal hydrolysis of native collagen (Bailey & Paul, 1998). The word gelatin originated from the Latin '*gelata'*, which means frozen, congealed or stiff. Gelatin identification and purity standards had been recommended by the World Health Organization (WHO) Report No. 48 B, where gelatin is classified as foodstuff rather than food additives. Gelatin is defined by the

United States Pharmacopeia (USP, 1990) as a substance obtained by the partial hydrolysis of collagen derived from the skins, white connective tissues and bones of animals. According to the Malaysian Food Act 1983 and Food Regulation 1985 no. 153, edible gelatin is identified as a clean, wholesome product, containing not more than 16 % moisture and 3 % ash, obtained by processing the skin, bone or other collagenous material from animals for human consumption in general (Food Regulations 1985, 1985). A 5 % (w/v) edible gelatin solution will form a gel that is translucent, light to color and free from offensive taste and odor after maintaining at 18.5 ºC for 2 hours (Anon, 2007).

Gelatin is a digestible protein often incorporated into other food ingredients in the preparation of desserts (Stevens, 1992). It swells in water and dissolves upon heating to give a thick solution. The high viscosity gelatin solution sets to a thermo-reversible semi-solid gel on cooling. The low melting point of gelatin gel (< 35 ºC), a typical property for hydrocolloids, makes it melt in the mouth when consumed, releasing flavor and delivering a feel of smoothness.

In the process of producing gelatin, the collagenous raw material is subjected to an alkaline or acid pretreatment prior to thermal extraction. During the thermal extraction step, the triple helices collagen molecule undergoes a helix-to-coil deformation to produce the loosely coiled gelatin polypeptide chains (Ward & Courts, 1977). The resultant gelatin can be classified into two types, acid pretreated Type A gelatin with an isoelectric point of 7-9 and alkaline pretreated Type B gelatin with an isoelectric point of about 5.

Gelatin manufacturers use pig skin, cattle hides and their bones as the traditional raw materials for gelatin extraction. However, consumer concerns on religious and health issues largely restrict the usage of the gelatin derived from these raw materials. Polysaccharide-based gelatin alternatives, which form gel based on cation-induced junction zones, have less flexible molecular backbones, leading to the major drawback of their usage, that is polysaccharides cannot stimulate the melting feel in the mouth like gelatin (Glicksman, 1969; Morrison et al., 1999). In this case, gelatin extracted from fish seems to be able to satisfy most of the consumer requirements.

### 2.2.1 Structure

Unlike its native collagen, gelatin is a heterogeneous protein mixture of polypeptide chains obtained by the irreversible hydrolysis of the larger collagen molecule. The other factors such as source of collagen, pretreatment and extraction conditions also imply effects on the weight distribution of the polypeptide chains. Gelatin molecules thus, have a molecular weight distribution ranging from a few thousand up to several hundred thousand daltons, which corresponds to the α-chain oligomers. Oligomers consisting of three α-chain will exist mainly as intact triple helices while others are randomly bonded end-to-end or side-to-side as extended α-chain polymers (Johnston-Banks, 1990).

The primary structure of the gelatin peptide chain, with Gly-X-Y triplets as the typical repeating sequence is shown in Figure 2.3, resembles the native collagen though slight differences are induced by the source of raw material as well as pretreatment and extraction condition used. Glycine (21 %), proline (12 %) and hydroxyproline (12 %) are the major amino acids of the gelatin, whilst it contains no tryptophan and is deficient in isoleucine, threonine and methionine (Stevens, 1992; Veis, 1964).

During the pretreatment process of raw materials, the amide groups of asparagine and glutamine side chains are partially hydrolyzed by alkaline solutions. The number of free carboxyl groups in the side chains is thus increased among the amino acids in the gelatin, leading to the increase of net negative charges in the gelatin molecule that lowers its isoelectric point. Tyrosine and isoleucine in the gelatin are relatively lower in content compared to the native collagen due to the removal of some telopeptide during crosslink cleavage in the pretreatment step (Ames, 1944).

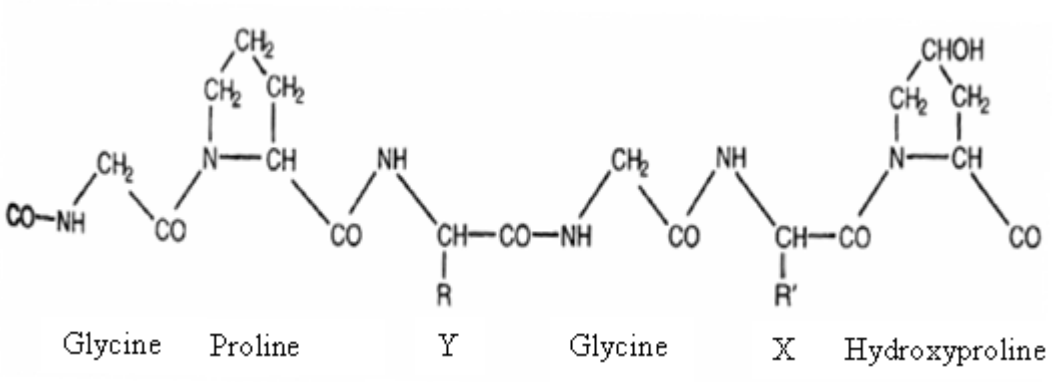


Figure 2.3 Amino acid sequence in gelatin. (Source: Veis, 1964)

## 2.3 Composition and properties of gelatin

Gelatin is usually available in granular or powdered form, containing 8-12 % moisture, 2-4 % mineral salts and the remainder being protein (85-90 %). It contains no fat, carbohydrate, preservatives nor additives. Gelatin comprises 21% glycine, 12%proline, 12% hydroxyproline, 10% glutamic acid, 9% alanine, 8% arginine, 6% aspartic acid, 4% lysine, 4% serine, 3% leucine, 2% valine, 2% phenylalanine, 2% threonine, 1% isoleucine, 1% hydroxylysine, < 1% methionine and histidine and < 0.5% tyrosine. However the values vary, especially the minor amino acids, according to the source of collagen and processing technique.

Gelatin is not considered as a complete protein for the human diet due to the lack of certain essential amino acids which is tryptophan, while isoleucine, threonine and methionine are only present in small amounts. Edible gelatin should contain not more than 1 ppm arsenic, not more than 50 ppm heavy metals, not more than 200 ppm sulphur dioxide, not more than 100 ppm peroxides (as $H_2O_2$) and should be free of phenolic preservatives. It should also meet the bacteriological standard where 1 g of gelatin must be free of *Escherichia coli* and 10 g of gelatin must be free of salmonella and have a total viable aerobic count of less than $10^3$ microorganism per gram, determined by plate count (Glicksman, 1969; Ledward, 2000; Poppe, 1997).

The physicochemical and functional properties of gelatin depend much on the raw material, pretreatment method and extraction condition (Johnston-Banks, 1990). Some of the important characteristics of gelatin are its ability to give a viscous solution when dissolved in solution and to form a thermoreversible gel that retains its shape and elasticity when a gelatin solution is cooled to its gelling point (Glicksman, 1969; Stainsby, 1977a).

### 2.3.1 Solubility

Gelatin is less soluble in cold water but swells to form large visible swollen particles when immersed in water and dissolves completely in water as the temperature rises, usually in the range of 30-40 ºC. Gelatin can also dissolve in polyhydric alcohols such as sorbitol, mannitol and glycerol with the presence of water but it is insoluble in alcohol and non polar organic solvents such as carbon tetrachloride and petroleum ether. Gelatin forms a translucent, colorless to faint yellow solution when dissolved in water with its clarity dependent on the extraction and post-extraction conditions. Gelatin obtained from the first extraction generally gives the highest quality in clarity and color while the later extractions give yield to more turbid gelatin with a more intense brownish orange color. Appearance of the gelatin obtained in the later extraction though, can be improved by clarification and filtration process (Glicksman, 1969; Ledward, 2000; Ockerman & Hansen, 2000; Poppe, 1997).

### 2.3.2 Isoelectric point

The pH value of commercial gelatin, ranging from pH 4 to 7, varies according to the different acids and/or bases used in the pretreatment process (Jamilah & Harvinder, 2002; Johnson-Banks, 1990). Having the ability to donate a hydrogen proton from the acidic carboxylic acid group and receive hydrogen proton by a basic amine group, gelatin molecule is both zwitterionic and amphoteric. When dissolved in water, the gelatin is capable of acting either as acid (carboxyl group) or base (amino group) depending on pH of the solution. If the solution is acidic, gelatin will be positively charged and migrates as a cation. On the contrary, gelatin will be negatively charged and migrates as an anion in an alkaline solution. The pH at which the net charge of the gelatin molecule equals to zero is known as the isoelectric point (IEP). Acid pretreated type A gelatin has a wider range of isoelectric region from pH 7-9 whereas alkaline pretreated type B gelatin has an isoelectric region from pH 4.8-5.5 (Foegeding, Lanier & Hultin, 1996).

### 2.3.3 Gelation mechanism and gel strength

Gelatin forms thermoreversible gels by forming a microstructural network similar to those of carbohydrates. During the gelation process, the randomly coiled α-chains undergo coil helix reversion to form triple helices (Djabourov, 1989). The reversion can be interpreted as a three stage process as described in Figure 2.4. In the first stage, imino acid rich chain segments of single-chain gelatin molecules are rearranged into a collagen fold. The imino acid rich regions (Gly-Pro-Hyp) of the α-

chains act as junction zones that take up a helical conformation upon cooling similar to that proposed for native collagen.

In the second stage, a three dimensional gel is formed by interactions between the separate chains of the collagen fold and other non-helical regions of the gelatin chains. In the last stage, these triple helix structures are stabilized by lateral inter-chain hydrogen bonds in a way similar to those found in parent collagen. Water molecules trapped within the structure also help to stabilize the triple helix by forming hydrogen bonds like its parent collagen. As the gelatin gel network is held together by physical bonding formed among macromolecules, the system can undergo the transition from the gel to liquid state or vice versa by changing the external variables such as temperature (Haug & Draget, 2009; Borchard & Burg, 1990).

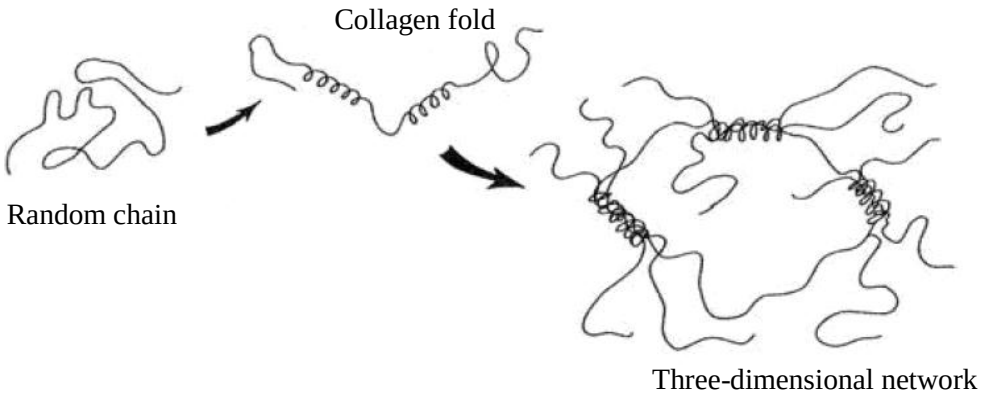


Figure 2.4 Model of gel formation.
(Source: Haug & Draget, 2009)

Gelatin gel melts upon heating as it loses its three dimensional network. The triple helix conformations start to disrupt and the chains are eventually able to move more freely (Figure 2.5). The melting temperature of gelatin is slightly lower than the human body temperature (27-34 ºC) thus enabling the gelatin to give the melt-in-mouth feel and to release flavor instantly when consumed. This is a desirable property for ready to eat food products such as marshmallow and dessert jellies (Haug & Draget, 2009; Choi & Regenstein, 2000; Poppe, 1997).

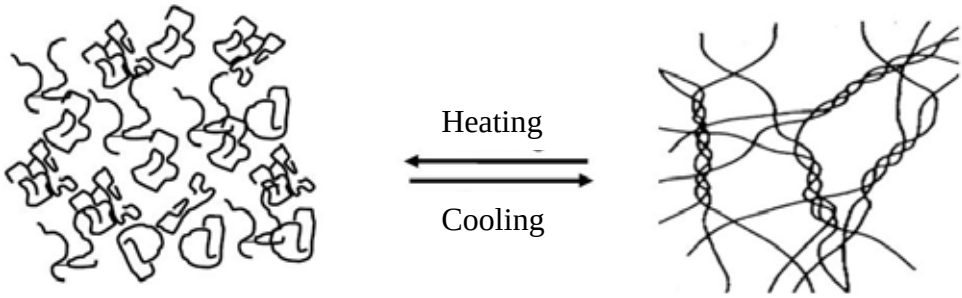


Figure 2.5 Gelling and melting mechanism of gelatin gel.
(Source: Haug & Draget, 2009)

The formation of gelatin gel is different from that of most polysaccharide hydrocolloids in that it is independent of the pH except in acidic medium and other reagents such as sucrose, salt, cation, etc. are not required. Gelatin can form gels at concentrations as low as 1 % (w/w) and as the concentration of gelatin increases, the gel strength of the gel also increases while it is disproportionate to increasing temperature. Besides concentration of the gelatin, thermal history, amount of electrolytes present and intrinsic strength, which is a function of both the structure and the molecular weight of the gelatin, also have an effect on the gelatin gel.

The standardized measurement for the gel strength, or Bloom strength, of a gel is carried out at the concentration of 6.67 % (w/w) and the gel is allowed to mature for 16-18 hours at 10 ºC before the measurement. As the gel reaches equilibrium along the maturation process, rigidity of the gel increases. Gel strength is defined as the weight in grams required to cause a depression of 4 mm deep into the surface of the gel using a probe of 12.7 mm in diameter (Linden & Lorient, 1999; Johnston-Banks, 1990). The commercial value of a gelatin is usually classified according to its gel strength, which lies usually within the range from less than 100 to more than 300 (Holzer, 1996). Gelatin with a gel strength about 250-260 is commonly used by the food industry to prepare desserts and confectionaries such as marshmallow, gummy candies and ice cream while the pharmaceutical industry uses it to make hard capsules.

### 2.3.4 Viscosity

One of the most important functional properties of gelatin is its ability to form a viscous solution when dissolved in water that naturally displays Newtonian rheological properties at a wide range of temperature and concentration. Viscosity of the gelatin solution depends primarily on the hydrodynamic interactions between the gelatin molecules; the contribution from the solvent and from the individual gelatin molecules; and interactions between individual gelatin molecules and the solvent. The gelatin molecular weight distribution is also important to the viscosity of the gelatin solution while gel strength of the gelatin, according to Glicksman (1969), is not directly related to its viscosity as gelatin of higher gel strength may exhibit lower viscosity than gelatin

with lower gel strength. Viscosity of the gelatin solution is proportionate to the gelatin concentration but decreases exponentially with rising temperature and reaches a minimum at the IEP of the gelatin (Poppe, 1997; Stainsby, 1977b). The viscosity of gelatin varies at different pH values due to changes in the molecular shape and charge distribution. Viscosity of commercial gelatins normally range from 2-7 mPa.s and may reach up to 13 mPa.s for specialized ones (Johnston-Banks, 1990). Low viscosity gelatin produces short and brittle texture gels with a slower setting rate, lower melting and gelling temperatures than high viscosity gelatin that produces tough and extensible gels.

The viscosity of gelatin is important in certain food systems, for example in the manufacturing of starch-moulded confectionary, the high working speeds generated by modern processing equipment require a low viscosity gelatin to prevent the formation of 'tails', together with a rapid distribution in the moulds (Ferenc, 2010). Gelatin with higher viscosity is preferred when it comes to the production of pastille, to give the 'tailing' effect and jelly, for chewier texture. The viscosity of the finished product is however, not affected as much by the viscosity of gelatin as the concentration of gelatin used and the total solids concentration (Badii & Howell, 2006; Glicksman, 1969).

### 2.3.5 Surface-active / protective-colloid properties

Like other proteins, gelatin is an amphiphilic molecule possessing surface-active properties that are able to lower the interfacial tension between two liquids, such as water and oil, or that between air and water. During the process of emulsification, where two or more immiscible liquids are mixed together, gelatin is used as a surfactant to