Chapter 1

INTRODUCTION AND REVIEW OF LITERATURE

Contents

1.1 General Introduction

The utilization of waste from fish processing industry for the production of value added products has attracted substantial attention. In fish processing industry, large amount of waste is generated. These wastes are a mixture of heads, viscera, skin and bone (Morrissey *et al.*, 2005). About 30% of such waste consists of skin and bone with high content of collagen (Gomez-Guillen *et al.*, 2002). Fish skin, which is a major byproduct of the fish-processing industry, could provide a valuable source of collagen (Badii and Howell, 2006). The solid waste from surimi processing, which may range from 50–70% of the original raw material (Morrissey *et al.*,2005), could also be the initial material for obtaining collagen from under-utilized fish resources.

Collagen is the most abundant protein of animal origin, comprising approximately 30% of total animal protein (Birk and Bruckner, 2005). Being a major constituent of the connective tissues, collagen plays an important part in increasing mechanical strength, integrity and rheological properties of the muscles and fillets. Collagen extracted from fish skin, a polymer that is a by-product of food manufacture, has various industrial applications in cosmetic technology and medicine. Collagens of fish skins studied in recent

years were mainly from marine species, such as black drum (*Pogonia cromis*) (Ogawa *et al.,* 2003), brown stripe red snapper (*Lutjanus vitta*) (Nagai *and Susuki,* 2000a), and ocellate puffer fish (*Takifugu rubripes*) (Nagai, Araki & Suzuki, 2002a). Isolation and characterization of collagen from fresh water fish, however, was rarely reported, except for the Nile perch (*Lates niloticus*) (Muyonga *et al.,* 2004a), grass carp (*Ctenopharyngodon idella*) (Zhang *et al.,* 2007) and channel catfish (*Ictalurus punctaus*) (Liu, Li, & Guo, 2007).

Collagen has a wide range of applications in leather and film industries, pharmaceutical, cosmetic and biomedical materials and food (Slade and Levine, 1987; Stainsby, 1987; Bailey and Light, 1989; Hassan and Sherief, 1994). Generally, pig and cow skins and bones are the main sources of collagen isolation. Fish offal, such as skins, scales, as well as bones is the tissues that are mainly structured by collagen. So far, skin and bone collagen from several fish species have been isolated and characterised (Kimura *et al.,* 1991; Nagai *et al.,* 2002; Nagai and Suzuki, 2000b).

Collagen could be extracted and further enzymatically hydrolysed by a process employing commercially available proteolytic enzymes to liberate physiologically active peptides. By selection of suitable enzymes and controlling the conditions, the properties of the end product can be selected. Specifically, some collagen derived peptides may exhibit interesting antioxidant activity, potent anti hypertensive activity, anti microbial activity against different strains of bacteria, protective effect on cartilage, or capacity to stimulate bone formation. Collagen hydrolysates from fish disposals may also exhibit other interesting activities (e.g., satiety, calciotropic, or opioid). The bioactive properties of collagen derived peptides, and also their resistance to protein digestion, make them potential ingredients of health promoting foods (Bailey and Light, 1989; Hassan and Sherief, 1994; Slade and Levine, 1987; Stainsby, 1987).

1.2 Significance of the study

In fish processing plants, there is huge amount of skin that is left as the waste. When this skin is taken and processed into fish collagen, it will save large amount of money that is used for extraction of collagen from other animals. Disposal of waste can cause pollution to environment. The waste not only causes pollution but also emits offensive odour (Takeshi and Nobutaka, 2000).

Fish collagen can be used as an alternative to replace mammalian collagen, especially collagen extracted from bovine, when we consider the outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and the foot-and-mouth disease (FMD) issues. BSE and TSE are progressive neurological disorders affecting cattles caused by proteinacious infectious particles called prions. FMD is viral disease causing fever and blisters inside foot and mouth of cattles (Shen *et al.*, 2007).

As a consequence, the alternative sources of collagen, especially from aquatic animals including fresh water and marine fish have received increasing attention. The use of fish collagen may contribute to the recycling of an unutilized resource, with consequent highly value added production.

The study aims in producing collagen that has been extracted from fish skin to replace other animal collagen so as to overcome the problem of other animal collagen issues. Also the study utilized the abandoned fish waste produced by fish processing industry since bone, skin, fin and scales of fish can be a useful source of collagen.

The study develops wound healing hydrogel and anti arthritic formulations from the purified fish collagen which are high value products of pharmaceutical importance. The products from the study can be effectively utilized in the wound care management and diseases associated with bone and joint degeneration.

1.3 Objectives of the study

The present study aims to

1. To isolate and characterize collagen from five different species of fishes.
2. To evaluate tissue regenerative potential of collagen.
3. To develop fish collagen hydrolysate - optimization of the process parameters and characterization of collagen peptide.
4. To evaluate the anti arthritic activity of fish collagen peptide in Complete Freunds Adjuvant (CFA) induced rat model systems.
5. To evaluate the stimulating effect of collagen hydrolysate on collagen synthesis in osteoblast cell lines.

1.4 Review of literature

Fish solid waste constitutes 50-70% of the original raw material, depending upon the method of meat extraction from the carcass (Morrisserry and Park, 2000). About 30% of such waste consists of skin and bone with high collagen content (Gomez-Guillen *et al.*, 2002). High value products can be developed with this fish waste, besides helping to minimise harmful environmental pollution.

As far as fish collagen is concerned, the huge number of species having very different intrinsic characteristics has aroused the interest of the scientific community in optimising the extracting conditions as well as characterising the yields, and physio-chemical and functional properties of the resulting collagens.

Fish collagen has lower denaturation temperatures compared to vertebrates' collagen. The denaturation temperature of mammalian collagen was higher than 30°C while most fish collagens denature at temperatures below 30°C (Ogawa *et al.*, 2003). Marine collagen had a lower denaturation temperature by about 10°C than that of the porcine skin collagen (Nagai *et al.*, 2008). This indicates that fish

collagen is generally less stable than mammalian counterparts (Ogawa *et al.*, 2003).

1.4.1 The Collagen Molecule

1.4.1.1 Distribution and molecular structure

Collagen is one of the most abundant biological macromolecules of extracellular matrix where it provides the major structural and mechanical support to tissues. The presence of collagen in all connective tissue makes it one of the most studied biomolecules of the extracellular matrix. This fibrous protein species is the major component of skin and bone and represents approximately 25% of the total dry weight of mammals (Alberts *et al.*, 2002).

Collagen molecules are comprised of three chains (two α and one β chains) that assemble together due to their molecular structure. Every α chain is composed of more than a thousand amino acids based on the sequence -Gly-X-Y-. X and Y positions are mostly filled by proline and 4-hydroxyproline (Whitford, 2005).

There are approximately twenty-five different chain conformations, each produced by their unique gene. The combination of these chains, in sets of three, assembles to form the twenty-nine different types of collagen currently known. Although many types of collagen have been described, only a few types are used to produce collagen based biomaterials (Brodsky and Persikov, 2005). Type I collagen is currently the gold standard in the field of tissue-engineering. The fibroblast is responsible for the majority of the collagen production in connective tissue. Collagen pro-α chain is synthesized from a unique mRNA within the rough endoplasmic reticulum and is then transferred to the Golgi apparatus of the cell. During this transfer, some proline and lysine residues are hydroxylated by the lysyloxydase enzyme. Specific lysine residues are glycosylated and then pro-α chain self-assemble into procollagen prior to their encapsulation in excretory vesicles. Following their

passage through the plasma membrane, the propeptides are cleaved outside the cell to allow for the auto-polymerisation by telopeptides. This step marks the initiation of tropocollagen self-assembly into 10 to 300 nm sized fibril and the agglomeration of fibril into 0.5 to 3 μm collagen fibers. Fibril-forming collagens are the most commonly used in the production of collagen-based biomaterials (Van der Rest and Garrone, 1991; Prockop and Kivirikko, 1995)

It is a unique protein, able to form insoluble fibers with a high tensile strength and contains right-handed triple super helical rod consisting of three polypeptide chains (Gelse *et al.*, 2003).

1.4.1.2 Collagen types

There are at least 27 different types of collagen, named type I–XXVII (Birk and Bruckner, 2005). The collagen variants vary in their macromolecular structure (Baily, 1998). Type I collagen is commonly found in connective tissues, including tendons, bones and skins (Muyonga *et al.*, 2004a). Type I collagen is predominant in higher order animals and especially in the skin, tendon and bone where extreme forces are transmitted. It is a compound of three chains, two of which are identical, termed α1, and one α2 chain with different amino acid composition. Type II collagen is essentially unique to hyaline cartilage. Type III is found in limited quantities (~ 10%) in association with type I. Thus, type III can be a minor contaminant of type I collagen prepared from skin (Piez, 1985). In addition, blood vessels predominantly contain type III. Collagen types I, II, and III have large sections of homologous sequences, independent of the species (Timpl, 1984). Type III collagen is mainly found in embryonic tissue, scar tissue, arteries and intra organ connections (Baily and Light, 1989). It is composed of identical α1 chains and contains intra and possibly intermolecular disulphide bonds.

Figure 1.1 *Schematization of a collagen α chain triple helix segment. (b) Assembled tropocollagen molecules. (c) Collagen fibril ranging from 10 to 300 nm in diameter. (d) Aggregated collagen fibrils forming a collagen fiber with a diameter ranging from0.5 to 3 μm (Alberts et al., 2002).*

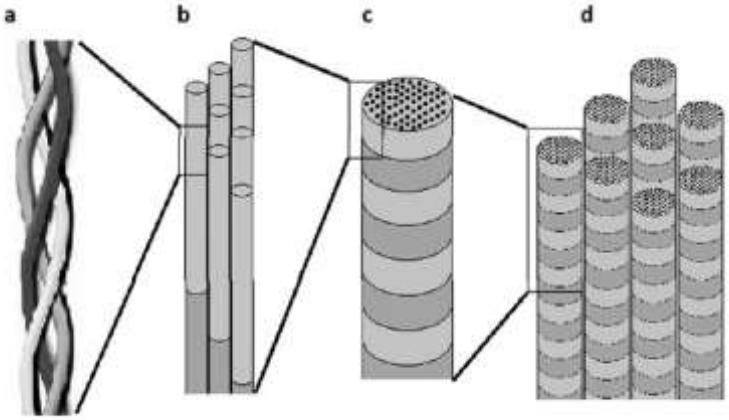
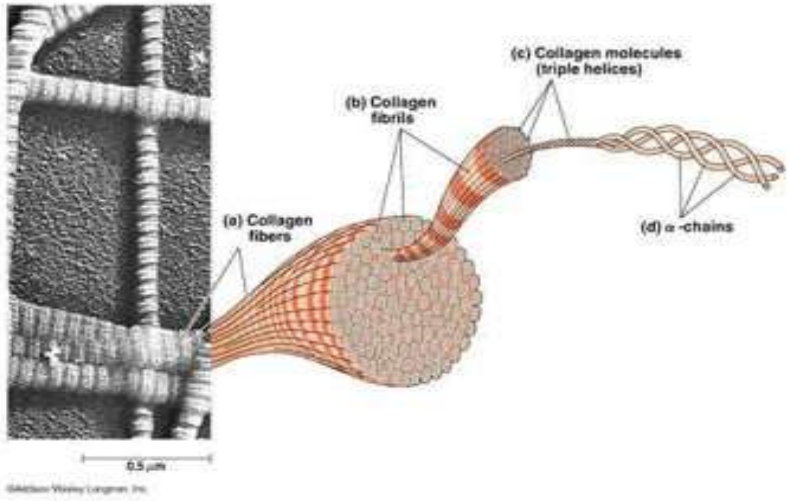


Figure 1.2 The ultra structure of collagen type I *Transmission electron microscopy and diagrammatic cross section of (a) collagen fibres. (b) Collagen fibres consist of collagen fibrils. (c) Collagen molecules make up the collagen fibril. (d) Collagen molecules are, in turn, triple helices of 3 α chains.*



Type IV collagen is a highly specialized form found only as a loose fibrillar network in the basement membrane. Type IV collagen is high in hydroxyproline and hydroxylysine. In addition to the usual 4-hydroxyproline, it also contains 3 hydroxyproline. Type V collagen contains α1 and α2 chains in the ratio of 1:2 as well as α3 chains. The α3 chains contain more cysteine than α1 and α2 chains (Kuhn, 1987).

Table 1.1 Different types of collagen

Type	Molecular formula	Polymerized form	Tissue distribution
I	[α1(I)]2α2(I)	fibril	bone, skin, tendons, ligaments, and cornea.
II	[α1(II)]3	fibril	cartilage, intervertebrate disc, notochord, vitreous humor in the eye.
III	[α1(III)]3	fibril	skin,blood vessels
V	[α1(V)]2α2(V) and α1(V)α2(V)α3(V)	fibril (assemble with type I)	idem as type I
XI	α1(XI)α2(XI)α3(XI)	fibril (assemble with type II)	idem as type II
IX	α1(IX)α2(IX)α3(IX)	lateral association with type II fibril	cartilage
XII	[α1(XII)]3	lateral association with type I fibril	tendons, ligaments
IV	[α1(IV)]2α2(IV)	Sheet-like Network	basal lamina
VII	[α1(VII)]3	anchoring fibrils	beneath stratified squamous epithelia

(RémiParenteau-Bareil *et al.,* 2010)

Collagen can be extracted from various sources considering that it is one of the most abundant proteins on earth. It can be extracted from almost every living animal, even including alligators (Wood *et al.,* 2008). Nonetheless, common sources of collagen for tissue engineering applications include bovine skin and tendons, porcine skin and rat tail among others. Marine life forms are also a considerable source of collagen.These collagens are widely used in the industry, but less for research and clinical usage. All these collagen sources are worth investigating considering that collagen properties differ from one animal to another (Lin and Liu, 2006].

1.4.1.3 Fish Collagen

Fish collagen is a complex structural protein that is mainly concentrated in skin, cartilage, airbladder and scales. Collagen is a unique protein compared to other fish muscle proteins and this uniqueness of fish lies in the amino acid content and they are rich in non-polar amino acids (above 80%) such as Gly, Ala, Val and Pro.

In fish, collagen is a major fraction of skin, scales and airbladder (Foegeding *et al.,* 1996). Collagen is the fibrous protein that contributes to the unique physiological functions of connective tissues in skin, tendons, bones, cartilage and others (Wong, 1989). Collagen contents vary considerably with fish species, age and season (Nagai *et al.,* 2002a). Collagen obtained from different species and habitats might be different in terms of molecular compositions and properties (Foegeding *et al.,* 1996).

Most fish collagens have been found to consist of two α - chain variants, which are normally designated as α1 and α2 (Nagai *et al.,* 2001; Gomez-Guillen *et al.,* 2002). The different collagen variants also vary in the nature of the constituent α chain. Different α chain types vary slightly in amino acid composition and as a result have small differences in hydrophobicity (Nagai and Suzuki, 2002). These chain variants, though having approximately the same molecular weight (95,000 Da), can be separated by SDS - PAGE due

to their different affinity for SDS. The α2 have a higher affinity for SDS and consequently exhibit a higher mobility than α1 (Hayashi and Nagai, 1980)

1.4.2 Isolation and purification of collagen from fishes

The major impediment to dissolution of collagen type I from tissue is the presence of covalent cross links between molecules. Collagen is insoluble in organic solvents. Water soluble collagen represents only a small fraction of total collagen and the amount depends on the age of the animal and type of tissue extracted. In some tissues, notably skin from young animals, cross linking is sufficiently low to extract a few percent under appropriate conditions. Furthermore, collagen molecules present within fibrillar aggregates can be dissociated and brought into aqueous solution. However, the nature of the cross links prevalent in different tissues determines the particular solvent to be used and the corresponding yields.

1.4.2.1 Acid soluble collagen

Dilute acidic solvents, e.g. 0.5 M acetic acid, citrate buffer are efficient to extract collagen from the tissues. The intermolecular cross links of the aldimine type are dissociated by the dilute acids and the repelling charges on the triple-helices lead to swelling of fibrillar structures (Trelstad and Birk, 1984)

Dilute acids will not disassociate less labile cross links such as keto-imine bonds. Therefore collagen from tissues containing higher percentages of keto-imine bonds, i.e. bone, cartilage, or tissues from older animals has a lower solubility in dilute acid solvents. In order to acid extract collagen, generally, tissue is ground in the cold, washed with neutral saline to remove soluble proteins and polysaccharides, and the collagen is extracted with a low ionic strength, acidic solution (Bazin and Delaumay, 1976). It is possible to solubilize ~ 2% of tissue collagen with dilute salt or acid

solutions. These collagen molecules can be reconstituted into large fibrils with similar properties as native fibrils by adjusting the pH or temperature of the solution (Piez, 1984). The remaining 98% is referred to as insoluble collagen although this dominant collagen material is not absolutely insoluble and can be further disintegrated without major damage to the triple-helical structures. The two most common approaches are the use of strong alkali or enzymes to cleave additional cross links and suspend or dissolve at first acid-insoluble structures.

1.4.2.2 Enzyme treated collagen

Collagen material can be solubilized by treating connective tissue with an aqueous solution comprising of alkali hydroxide and alkali sulfate, e.g. approximately 10% sodium hydroxide and 10% sodium sulfate for ~ 48 h (Cioca, 1981; Roreger, 1995). Thus, fat associated with the insoluble collagen is saponified, non-helical telopeptide regions are truncated and the collagen fibers disintegrated. The size and molecular weight of the resulting collagen material depend on the time of treatment and alkali concentration (Roreger, 1995). The presence of alkali sulfate controls the swelling of the collagen structures and protects the native triple-helical characteristics. It has to be noted that similar to gelatin, the isoelectric point of the resulting material is shifted to lower pH as asparagine and glutamine are converted into aspartic and glutamic acid. Much higher yields compared with acidic extraction can be achieved by taking advantage of the fact that the collagen triple-helix is relatively resistant to proteases, i.e. pepsin or chymotrypsin below ~20°C (Piez, 1984).

The efficacy of enzymatic treatment arises from selective cleavage in the terminal non-helical regions breaking peptide bonds near cross links and releasing molecules which dissolve. Some cross links presumably remain, attaching small peptide remnants to the solubilized molecules (Miller *et al,* 1984). Thus, the telopeptide ends of the polymer chains are dissected but under appropriate conditions

the helices remain essentially intact. The resulting material, so-called atelocollagen, benefits from the removal of the antigenic determinant located on the non-helical protein sections and provokes milder immune response (Knapp *et al.,* 1977). Pepsin at a 1:10 weight ratio of enzyme to dry weight tissue in dilute organic acid (0.5 M acetic acid) provides a propitious medium in which collagen can be swollen and solubilized (Piez, 1985).

Soluble collagen is purified mainly by precipitation after pH, salt concentration or temperature adjustment (Li, 1995). The high viscosity of even dilute solutions interferes with purification methods such as chromatography, electrophoresis and differential sedimentation. Collagen solutions contain varying proportions of monomer and higher molecular weight covalently linked aggregates, depending on the source and method of preparation. Truly monomeric solutions are difficult if not impossible to obtain (Piez, 1985). Pepsin solubilized collagen usually contains higher proportions of monomer than salt- or acid extracted material (Piez, 1984). Soluble collagen can be stored frozen or lyophilized. In the course of drying, denaturation or non-specific cross linking can occur and the degree of association upon reconstitution can change (Lee, 1983).

1.4.2.3 Insoluble collagen

Instead of disintegration and transfer into soluble material, extensively cross linked collagen can be dispersed as opalescent, fine fibrillar suspensions by the use of mild denaturation agents and mechanical fragmentation usually at an acidic pH. Fibrillar collagen is more resistant to proteolysis than most other non-collageneous tissue constituents, which are removed during processing by selective proteolysis and washing (Li, 1995). In additional steps collagen material can be subjected to chemical modifications such as succinylation (Singh *et al.,* 1995) acetylation (Srivastava *et al.,* 1990), methylation (Wang *et al.,* 1978) or attachment to other polymers (Panduranga and Rao, 1995).

Due to their high biocompatibility, collagens extracted from land-based animal skins have been widely used in the pharmaceutical, food, healthcare, and cosmetic industries (Ogawa *et al.*, 2004). Commonly isolated from by-products of land-based animals, such as cows, pigs and poultry, collagen has been widely used in food, pharmaceutical, and cosmetic industries because of its excellent biocompatibility and biodegradability, and weak antigenicity (Liu *et al.*, 2009). However, the outbreaks of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), foot-and-mouth disease (FMD) and avian influenza have raised anxiety among some consumers of collagen and collagen-derived products from these land-based animals.

Therefore, the global demand for collagen from alternative sources such as aquatic animals has been increasing over the years. With the rapid development of the fish processing industry in China, large quantities of by-products are generated, accounting for 50–70% of the original raw material (Kittiphattanabawon *et al.*, 2005). Collagens from fish skin or swim bladders (a waste product in fish processing), may be good substitutes, because of their safety and solubility in neutral salt solutions and dilute acids. Also, the development of fish swim bladder-based collagens would add significant value to the fish processing industry (Trevitt and Singh, 2003). Consequently, optimal use of these by-products is a promising way to protect the environment, to produce value-added products, to increase the revenue to the fish processors, and to create new job/business opportunities.

1.4.3 Characteristics of Fish Collagen

The physical and chemical properties of collagen differ depending on the tissues such as skin, swim bladder and the myocommata in muscle. Fish collagen is heat sensitive due to labile cross links as compared to mammals. Different fish species contain varying amounts of collagen in the body tissue that reflect the swimming behaviour and it influences the textural characteristics of

fish muscle (Montero and Borderias, 1989). Collagen is unique in its ability to form insoluble fibres that have high tensile strength (Gelse *et al.*, 2003).

In addition to differences in molecular species, fish collagens have been shown to vary widely in their amino acid composition. In particular, the levels of imino acids (proline and hydroxyproline) vary significantly among fish species (Balian and Bowes, 1977; Poppe, 1997; Gudmundsson and Hafsteinsson, 1997). The amount of imino acids, especially hydroxyproline, depends on the environmental temperature in which the fish lives and it affects the thermal stability of the collagens (Rigby, 1968; Balian and Bowes, 1977). Collagens derived from fish species living in cold environments have lower contents of hydroxyproline and they exhibit lower thermal stability than those from fish living in warm environments. This is because hydroxyproline is involved in inter-chain hydrogen bonding, which stabilizes the triple helical structure of collagen (Darby and Creighton, 1993). In the absence of proline hydroxylation, the essential triple helical conformation of collagen is thermally unstable at well below physiological temperatures (Berg and Prockop, 1973).

1.4.3.1 Amino Acid Composition of collagen

For amino acid analysis, the strict condition for sample preservation is important and indispensable before collagen extraction. This means that the hydroxyproline content in relation to collagen stability strongly depends on these sampling procedures (Swatschek *et al.*, 2002). Several works showed that amino acid composition of fish collagens was almost similar to that of mammalian collagens (Nagai *et al.*, 2000a; Bae *et al.*, 2008). Furthermore, the degree of hydroxylation of proline was calculated to be 40-48%, which was also similar level to that of the mammalian (about 45%). A linear relationship between the stability of collagen and the hydroxyproline content has been reported. The difference in hydroxyproline amount might relate to the species, environment and

the fish body temperature (Zhang and Webster, 2009). It is very interesting that the degree of hydroxylation of proline of fishes in cold sea, for example chum salmon, was reported to be low (35-37%) (Matsui *et al.*, 1991) compared to that of fishes in relatively warm sea, from similar environments. Difference in collagen denaturation temperatures is also associated to the proline and hydroxyproline content. This is because proline and hydroxyproline can stabilize the triple helix due to the non-covalent bonding of their pyrrolidine ring. Greater the value of proline and hydroxyproline, greater is the thermal stability of the collagen (Lin and Liu, 2006).

Collagen contains two uncommon derivative amino acids not directly inserted during translation. These amino acids are found at specific locations relative to glycine and are modified post-translationally by different enzymes, both of which require vitamin C as a cofactor. One is hydroxyproline derived from proline and the other is hydroxylysine derived from lysine. Depending on the type of collagen, varying numbers of hydroxylysine are glycosylated (mostly having disaccharides attached). Glycine is the most abundant amino acid and accounted for more that 30% of all amino acids (Nagai *et al.*, 2000b).

1.4.3.2 Viscosity of collagen

High viscosity is one of the physico-chemical characteristics of collagen. Fish collagen may have a range of viscosity about 12 to 19 dL/g (Ogawa *et al.*, 2004). As collagen is made up by structured systems it is characterized by a high degree of viscosity due to greater electrostatic repulsion among the collagen molecular chains in solution even at low concentrations. The study showed that the relative viscosity of collagen decreased continuously on heating up to 30°C. Rate of decrease was retarded in the temperature range of 35-50°C. This is due to the breaking of hydrogen bonds during the high temperature which stabilize the collagen structure. As collagen a of protein, it can be denatured at above 40°C. This collagen would be denatured to a mixture of random-coil single, double and triple

strands (Kittiphattanabawon *et al.*, 2005). The triple helix structure of collagen stabilized by hydrogen bonds was converted into the random coil arrangement by the process of thermal depolymerization which accompanied by variations in physical properties like viscosity, sedimentation, diffusion, light scattering and optical activity (Ahmad and Benjakul, 2010).

Viscosity measurement is commonly used to determine the thermal stability of collagen. This tool is used to measure the transitions in polymers and to learn about the loss of viscosity with heating which is attributed to denaturation of collagen (Zhang *et al.*, 2007). Thermal denaturation measurement of collagen provided useful signs to the thermal stability of collagen in relation to environment and amino acid content (Li *et al.*, 2008). The temperature of denaturation of collagen solution from grass carp was 28.4°C (Zhang *et al.*, 2007) and 32°C (Li *et al.*, 2008), chub mackerel was 25 to 28°C (Kittiphattanabawon *et al.*, 2005) and nile perch was 36-36.5°C (Muyonga *et al.*, 2004b). Higher denaturation temperature for collagen of Nile perch may be attributed to the higher amino acid content than that of cold-water fish collagens (Muyonga *et al.*, 2004b). The denaturation temperature is proportional to the content of hydroxypoline. Hydroxypoline is believed to play an important role in the stabilization of the triple-stranded collagen helix due to its hydrogen bonding ability through its hydroxyl group (Li *et al.*, 2008).

1.4.3.3 Solubility of collagen

In general, fish collagen would be more soluble in the acidic pH ranges while at neutral pH it will show sharp decrease in solubility. On the other hand, solubility slightly decreased at extremely acidic pH. According to Ahmad *et al.* (2010) collagen was soluble in the pH range from 1 to 4 with the highest solubility at pH 2 and the lowest solubility at pH 6 to 7. When pH values are above and below isoelectric point (pI), a protein has a net negative or positive charge, respectively. Therefore, more water interacts with

the charged proteins (Kittiphattanabawon *et al.,* 2005). At pH near the pI, a collagen molecule is unstable and tends to coagulate. This is because of the increase in hydrophobic interaction among the collagen molecules. The higher solubility at lower pH would increase from the greater repulsive force between collagen molecules. In alkaline condition, the slight increase in solubility was observed (Ahmad *et al.,* 2010).

The solubility of collagen in 0.5M acetic acid can be maintained in the absence of NaCl. Increasing the NaCl concentration will reduce the solubility of collagen (Kittiphattanabawon *et al.,* 2005). The slight decrease in solubility was determined in the presence of 1 to 2% NaCl. The sharp decrease was observed as the salt concentration rise up to 6%. At 6% of NaCl, the solubility of 36.91% was determined. The lower solubility of collagen was mainly due to the salting out effect (Ahmad *et al.,* 2010). Higher concentration of NaCl might result in decreasing protein solubility by increasing hydrophobic interaction and aggregation. As a result, the proteins start to precipitate (Kittiphattanabawon *et al.,* 2005).

Fish skin collagens have been reported to develop minimal amounts of mature cross-links. By measuring hydrothermal isometric tensions that fish collagen cross-links do not mature to thermally stable bonds. As a result of its low content of stable cross-links, fish collagen can easily be solubilised (Muyonga *et al.,* 2004b).

1.4.4 Characteristics of fish collagen to be used as biomaterial

Biomaterials made of collagen offer several advantages: they are biocompatible and non-toxic and have well-documented structural, physical, chemical, biological and immunological properties (Chvapil, 1979; Ramshaw *et al.,* 1995). It has to be stressed that collagen properties like mechanical strength, fluid absorption volume or haemostatic activity differ depending on the

animal source and anatomical location of the raw material. For local antibiotic delivery, the goal should be able to maintain the highest possible, but not toxic, local drug concentration without achieving systemic effects. This can be achieved by physical and possibly also chemical incorporation of the drug into a collagen matrix in the course of the manufacturing process to assure drug immobilization. Drugs may be complexed to collagen through direct binding of the drug to free amino or carboxylic groups of the collagen molecule (Chvapil, 1979). Drug release occurs by diffusion from a collagen matrix implanted or injected as such or polymerized after intra-tissue injection (Stemberger *et al.*, 1997). For example, a tetracycline solution injected subcutaneously reached a maximum serum concentration after 3 h which slowly decreased within the next 20 h. When the same amount of tetracycline solution was soaked into a collagen sponge and inserted into a natural body cavity, the drug release was detected over a period of 14 days resulting in a relatively constant serum concentration of the drug (Chvapil, 1979).

1.4.4.1 Biocompatibility

The primary reason for using collagen as biomaterial is its excellent biocompatibility, low antigenicity (Pati *et al.*, 2012), high level of direct cell adhesion, and high degree of biodegradability (Lee *et al.*,2001). An immune response against collagen mainly targets epitopes in the telopeptide region at each end of the tropocollagen molecule (Steffen *et al.*, 1968). The application of fish collagen as a scaffold for tissue engineering has been attempted (Nagai *et al.*, 2008 ; Sugiura *et al.*, 2009). Atelocollagen is a processed natural biomaterial produced from bovine type I collagen. It inherits useful biomaterial characteristics from collagen, including a low rate of inflammatory responses, high level of biocompatibility, and high degree of biodegradability (Miyata *et al.*, 1992; Hanai *et al.*, 2006). The components of collagen that are attributed to its immunogenicity, namely, telopeptides, are eliminated during atelocollagen production. Therefore, atelocollagen exhibits little

immunogenicity (Sano *et al.*, 2003). The ability to obtain a substantial amount of collagen from fish waste (scales, skin, and bone) would result in the development of an alternative to bovine collagen for use in food, cosmetics, and biomedical materials. Elastic salmon collagen (SC) vascular grafts have been prepared by incubating a mixture of acidic SC solution and fibrillogenesis-inducing buffer containing a cross-linking agent, water-soluble carbodiimide. Upon subcutaneous placement in rat tissues, the SC grafts induced little inflammatory reactions (Nagai *et al.*, 2008). Tests of pellet implantation into the para vertebral muscle in rabbits have demonstrated that tilapia collagen rarely induces inflammatory responses at one or four weeks after implantation, a finding that is statistically similar to that of porcine collagen and high-density polyethylene as a negative control (Sugiura *et al.*, 2009).

1.4.4.2 Biodegradability

Biodegradability is a valuable aspect for most collagen-based biomaterials. Collagen biocompatibility and possible degradation by human collagenases are responsible for the widespread use of this material in many biomedical applications. Collagenases such as matrix metalloproteinase (MMP) are responsible for most collagen degradation *in vivo*. On the other hand, the rate of the degradation process often needs to be regulated using diverse methods such as crosslinking techniques (Weadock *et al.*, 1996). *In vitro* degradation studies (using collagenase solution) have demonstrated a higher level of stability among cross linked scaffolds derived from tropical fresh water fish scale collagen, with only a 50% reduction in mass after 30 days, whereas the uncross linked scaffold has been shown to degrade completely within four days (Pati *et al.*,2012). Upon placement in subcutaneous tissues in rats, grafts gradually biodegrade. One month after implantation, fibroblasts and macrophages begin to penetrate the surface of the graft, without signs of necrosis (Nagai *et al.*, 2008).

1.4.5 Collagen-Based Biomaterials

1.4.5.1 Types of collagen-based biomaterials

Collagen-based biomaterials can originate from two fundamental techniques. The first one is a decellularized collagen matrix preserving the original tissue shape and ECM structure, while the other relies on extraction, purification and polymerization of collagen and its diverse components to form a functional scaffold. Physical methods include snap freezing that disrupt cells by forming ice crystals, high pressure that burst cells and agitation, that induce cell lysis and used most often in combination with chemical methods to facilitate penetration of active molecules in the tissue. Chemical methods of decellularization include a variety of reagents that can be used to remove the cellular content of ECM. These substances range from acid to alkaline treatments, as well as chelating agents such as EDTA, ionic or non-ionic detergents and solutions of extreme osmolarity. Enzymatic treatments such as trypsin, which specifically cleaves proteins and nucleases that remove DNA and RNA, are also commonly used to produce acellular scaffold. However, none of these methods can produce an ECM completely free of cellular debris and a combination of techniques is often required to obtain a material free of any cell remnant.

A plethora of biomolecules can also be added to collagen solution to produce collagen-based biomaterials. These biomolecules, typically glycosaminoglycans, elastin and chitosan are added to the compound to potentially enhance the properties of collagen (Zhong and Young, 2009; Caissie *et al.*, 2006). The other type of collagen-based biomaterial is made by processing a collagen solution with other biomolecules like GAG (Chen *et al.*, 2005)

1.4.6 wound healing

Wound healing is a dynamic process and the performance requirements of a dressing can change as healing progresses. However, it is widely accepted that a warm, moist environment encourages rapid healing and most modern wound care products are designed to provide these conditions (Barnett and Irving, 1991). Fluid balance in burn injury is very important since heavy loss of water from the body by exudation and evaporation may lead to a fall in body temperature and increase in the metabolite. Besides this, dressing should have certain other properties like ease of application and removal, and proper adherence so that there will not be any area of non-adherence left to create fluid-filled pockets for the proliferation of bacteria (Quinn *et al.*,1985)

Wounds that exhibit impaired healing, including delayed acute wounds and chronic wounds, generally have failed to progress through the normal stages of healing. Such wounds frequently enter a state of pathologic inflammation due to a postponed, incomplete, or uncoordinated healing process. Most chronic wounds are ulcers that are associated with ischemia, diabetes mellitus, venous stasis disease, or pressure (Mathieu *et al.*, 2006; Menke *et al.*, 2007).

1.4.6.1 Biochemical processes in wound healing

The wound-healing process consists of four highly integrated and overlapping phases: hemostasis, inflammation, proliferation, and tissue remodeling or resolution (Gosain and DiPietro, 2004). These phases and their biophysiological functions must occur in the proper sequence, at a specific time, and continue for a specific duration at an optimal intensity (Mathieu *et al.*, 2006). There are many factors that can affect wound healing which interfere with one or more phases in this process, thus causing improper or impaired tissue repair.

In adult humans, optimal wound healing involves the following events: (1) rapid hemostasis; (2) appropriate inflammation; (3) mesenchymal cell differentiation, proliferation, and migration to the wound site; (4) suitable angiogenesis; (5) prompt re-epithelialization (re-growth of epithelial tissue over the wound surface); and (6) proper synthesis, cross-linking, and alignment of collagen to provide strength to the healing tissue (Gosain and DiPietro, 2004; Mathieu *et al.*, 2006).

The first phase of hemostasis begins immediately after wounding, with vascular constriction and fibrin clot formation. The clot and surrounding wound tissue release pro-inflammatory cytokines and growth factors such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF). Once bleeding is controlled, inflammatory cells migrate into the wound (chemotaxis) and promote the inflammatory phase, which is characterized by the sequential infiltration of neutrophils, macrophages, and lymphocytes (Gosain and DiPietro, 2004; Broughton *et al.*, 2006; Campos *et al.*, 2008). A critical function of neutrophils is the clearance of invading microbes and cellular debris in the wound area, although these cells also produce substances such as proteases and reactive oxygen species (ROS), which cause some additional bystander damage. Macrophages play multiple roles in wound healing. In the early wound, macrophages release cytokines that promote the inflammatory response by recruiting and activating additional leukocytes. Macrophages are also responsible for inducing and clearing apoptotic cells (including neutrophils), thus paving the way for the resolution of inflammation. As macrophages clear these apoptotic cells, they undergo a phenotypic transition to a reparative state that stimulates keratinocytes, fibroblasts, and angiogenesis to promote tissue regeneration (Meszaros *et al.*, 2000; Mosser and Edwards, 2008). In this way, macrophages promote the transition to the proliferative phase of healing.

Table1.2 Normal wound healing process

Phase	Cellular and Bio-physiologic Events
Hemostasis	1.Vascular constriction
	2.Platelet aggregation, degranulation and fibril formation
Inflammation	1.Neutrophil infiltration
	2.Monocyte infiltration and differentiation to macrophage
	3.Lymphocyte infiltration
Proliferation	1.Re-epithelialization
	2.Angiogenesis
	3.Collagen synthesis
	4.Extra cellular matrix formation
Remodeling	1.Collagen remodelling
	2.Vascular maturation and regression

(Guo and Di Pietro, 2010)

Inflammation is a normal part of the wound-healing process, and is important to the removal of contaminating micro-organisms. In the absence of effective decontamination, however, inflammation may be prolonged, since microbial clearance is incomplete. Both bacteria and endotoxins can lead to the prolonged elevation of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α and elongate the inflammatory phase. If this continues, the wound may enter a chronic state and fail to heal. This prolonged inflammation also leads to an increased level of matrix metalloproteases (MMPs), a family of proteases that can degrade the extra cellular matrix. In tandem with the increased protease content, a decreased level of the naturally occurring protease inhibitors occurs. This shift in protease balance can cause growth factors that appear in chronic wounds to be rapidly degraded (Edwards and Harding, 2004; Menke *et al.*, 2007).

1.4.6.2 Moist wound healing theory

The most significant advancement in wound care came with Winter's (Winter, 1962) study in 60's, which showed that occluded wounds healed much faster than dry wounds and moist wound healing environment optimized the healing rates. He demonstrated that when wounds on pigs are kept moist, epithelialisation is twice as rapid as on wounds allowed to dry by exposure to air. Later Hinman and Maibach (2000) confirmed Winter's work on human beings in 1963. An open wound, which is directly exposed to air, will dehydrate and a scab or escar is formed. This forms a mechanical barrier to migrating epidermal cells and is then forced to move in a deeper level of tissue, which prolongs the healing process. Moist healing prevents the formation of scab as the dressing absorbs wound exudate secreted from the ulcer (Winter and Scales, 1963).

1.4.6.3 Collagen hydrogel

The term "hydrogel" refers to a broad class of polymeric materials that are swollen extensively in water but that do not dissolve in water. They have been used in a wide variety of biomedical applications and may be synthesized from monomers or monomers mixed with polymers. Hydrogels are attractive as biomaterials; they are highly permeable to water, ions, and small molecules (Peppas and Khare, 1993). Hydrogels comprised of naturally derived macromolecules and have potential advantages of biocompatibility, cell-controlled degradability, and intrinsic cellular interaction.

Hydrogels have structural similarity to the macromolecular-based components in the body and are considered biocompatible (Jhon and Andrade, 1973). Gels are formed when the network is covalently crosslinked (Hoffman, 2001). Biocompatible hydrogels are currently used in cartilage wound healing, bone regeneration, wound dress, and as carriers for drug delivery. Hydrogels are often favorable for promoting cell migration, angiogenesis, high water

content, and rapid nutrient diffusion (Bryant and Anseth, 2001). Some of the examples of hydrogel forming polymers of natural origin are collagen (Wallace and Rosenblatt, 2003), gelatin (Kim *et al.*, 2004) and chitosan (Francis and Mathew, 2000)

1.4.6.4 Collagen as wound healing biopolymer

Wound dressings are generally classified as

1. Passive products,
2. Interactive products and
3. Bioactive products, based on its nature of action.

Traditional dressings like gauze and tulle dressings that account for the largest market segment are passive products. Interactive products comprise of polymeric films and forms, which are mostly transparent, permeable to water vapour and oxygen but impermeable to bacteria. These films are recommended for low exuding wounds. (Schoof *et al*., 2001; Kuberka *et al*., 2002) Bioactive dressing is one which delivers substances active in wound healing; either by delivery of bioactive compounds or dressings constructed from material having endogenous activity. These materials include proteoglycans, collagen, non-collagenous proteins, alginates or chitosan. (Ruszczak, 2000; Hansen *et al*., 2001; Froget *et al*., 2003; Gomathi *et al*., 2003). In November 1999, Food and Drug Administration of the United States of America (FDA) reclassified the dressing categories as,

1. Non-resorbable gauze/sponge dressing for external use,
2. Hydrophilic wound dressing,
3. Occlusive wound dressing,
4. Hydrogel wound and burn dressing and
5. Interactive wound and burn dressings.

Both artificial and natural polymers have been used to reconstitute dermis. Collagen is a natural substrate for cellular attachment, growth and differentiation, and promotes cellular

proliferation and differentiation. Natural polymers such as fibrin (Keiser *et al.*, 1994; Siedler and Schuller, 2000), hyaluronic acid (King *et al.*, 1991; Murashita *et al.*, 1996), fibrinogen (Vacanti and Langer, 1998) and collagen (Ruszczak, 2000; Hansen *et al.*, 2001; Froget *et al.*, 2003; Gomathi *et al.*, 2003) have been recently tested in different matrix systems for local drug delivery and wound healing.

Collagen is unique in possessing different levels of structural order: primary, secondary, tertiary and quaternary (Ho *et al.*, 2001). In vivo, collagen molecules form fibers having a specific internal and structural orientation and strengthened together by two types of covalent crosslinking: intramolecular and intermolecular. Intermolecular cross-linking is essential to form macromolecular fibers and, consequently, for its mechanical stability and other physical properties.

Collagen is a natural substrate for cellular attachment, growth and differentiation in its native state. In addition to its desirable structural properties, collagen has functional properties. Certain sequences of the collagen fibrils are chemotactic and promote cellular proliferation and differentiation. Collagen provides considerable strength in its natural polymeric state. The source of collagen either purified from animal sources or as an integral component of a more complex extracellular matrix, and its treatment prior to use are important variables in the design of tissue-engineered devices. Biomaterials made of collagen offers several different advantages: they are biocompatible and nontoxic to tissues (including neural and brain tissue) and have well-documented structural, physical, chemical, biological and immunological properties. Additionally, mechanical and to some extent immunologic properties of collagen scaffolds can be influenced by modification of matrix properties (porosity, density) or by different chemical treatment affecting its degradation rate. Collagen contains a number of biological functional groups and has been clinically

used as a wound dressing. Its potential as artificial skin, bone grafts and pharmaceutics has been intensively investigated (Schoof *et al.*, 2001; Kuberka *et al.*, 2002).

Several methods of cross-linking and sterilization can be utilized to alter the rate of *in vivo* degradation or to change the mechanical properties of collagen (Friess and Lee, 1966). These methods include glutaraldehyde treatment, carbodiimide treatment, dye-mediated photooxidation, exposure to polyepoxy compounds and glycerol treatment.

Different approaches to utilize animal-derived collagen for tissue substitution have been developed in the past 20 years: (a) the collagen gel, made of a mixture of fibroblasts and bovine collagen, (b) the collagen sponge based upon the production of a lyophilized collagen matrix in which fibroblasts are cultured and migrate, (c) the synthetic mesh composed of a nylon or a polyglactic acid mesh on which fibroblasts are cultured, (d) the collagen membrane used alone or with reconstructed epidermal sheet, and (e) the *in vitro* reconstructed skin-like products based on collagen matrix (Ruszczak, 2000).

The use of collagen sponges or pads either plain or containing antimicrobial drug has been reported in successful regeneration of dermal component and acceleration of wound healing. Especially, the use of drug containing collagen sponges was found beneficial in both partial-thickness and full-thickness burn wounds.

1.4.6.5 Immunology and biocompatibility of xenogenic collagen material

The presence of an immune response to collagen or any other biomaterial must be viewed in the context of its clinical performance. The immune response to xenotransplants includes both natural and induced humoral components, while a humoral response to allo transplants is generally seen only after sensitization.

The level of natural antibodies that react with organ xenotransplants increases proportionally with the phylogenic distance between the xenogenic species involved. In organ transplantation, the presence of such antibodies leads to hyper acute rejection, which occurs within minutes to hours after revascularization, and, consequently, to the loss of the transplanted tissue. This negative phenomenon can be avoided if an acellular and avascular tissue or a purified connective tissue matrix made from a natural biologic polymer such as collagen is used (Sachs, 1998; Timpl, 1984; De Lustro *et al*., 1990)

Collagen is a naturally occurring, highly conserved protein that is ubiquitous among mammalian species and accounts for approximately 30% of all body proteins. Since it is one of the first proteins synthesized during embryogenesis and then during organogenesis, its homology between species is very high.

Bovine and porcine type I collagen provide a readily available source of scaffold material for numerous applications and have been shown to be very compatible with human systems. The traditional and still widely used method of collagen extraction from tissues such as skin, tendons and ligaments is solubilization, and then reconstitution into injectable low-osmotic gels, fibrils and pads (Schoof *et al*., 2001).

Empiric observation based on the widespread use of xenogenic collagen and collagen-derived products for more than 50 years indicates that, in the case of these highly purified or native xenogenic collagens, no danger of acute or latent immunologic reaction occurs. Thus, appropriately purified xenogenic collagen has little or no significant immunogenicity and no discernible threat of inducing a systemic autoimmune disease.

Experimental and clinical studies have shown that both a sponge and a film consisting of xenogenic collagen, which was applied to the injured surface of the skin, did not cause any foreign-

body reaction, nor any immune rejection reaction or sensitization (Vacanti and Langer, 1998; Soo *et al.*, 1993). The xenogenous collagen did not promote any extensive inflammatory reactions or immunologic rejection. The take of collagen implants and tissue remodelling was complete and quick, allowing them to be even immediately combined with autologous thin split-skin grafting as well as with full-skin grafting.

The implanted collagen sheet enhanced the initial adhesion of keratinocytes allotransplants, supporting biological activity of the cells. Moreover, collagen membranes have been successfully used to speed-up the healing and re-epithelialization of split-skin donor sites showing a benefit over other currently used methods (Horch and Stark, 1998; Ruszczak and Schwartz, 1999). The implantation of collagen-based dermis substitutes protect against the contracture of wound borders and against typical scar formation.

1.4.7 Fish collagen hydrolysate

Fish processing waste, which otherwise cause serious environmental pollution, is a promising cost effective collagen source (Arnesen and Gilberg, 2007). Fish collagen from skins, bones, fins and scales could be extracted and hydrolyzed by chemical pre-treatment and subsequent heating at temperatures higher than 45ºC (Najafian and Babji, 2012).

Collagen contains bioactive peptides inactive within their sequence which can be released during gastrointestinal digestion or by controlled enzymatic hydrolysis. These collagen and gelatin derived peptides may exert a wide variety of physiological activities in the body, and could have potential applications in functional foods. Interest in nutraceuticals is growing rapidly worldwide, as they are a safe alternative to pharmaceutical drugs, which use is sometimes limited by toxicity or intolerance reactions.

Collagen and collagen hydrolysates could be attractive nutraceuticals for their interesting bioactive properties. The beneficial effect of collagen or gelatin hydrolysates on different diseases has been reported in animal or clinical studies, and actually several supplements including collagen-derived peptides have been patented and are currently commercialized in the USA, Japan and many European countries. Moreover, hydrolyzed collagen products have received GRAS status (Generally Recognized as Safe) by the US Food and Drug Administration (FDA). Although mammalian collagen are widely used in the field of nutraceuticals, the use of collagen from marine-discarded sources for preparing protein hydrolysates is nowadays increasing, as they are not associated with the risk of outbreaks of bovine spongiform encephalopathy.

The resistance of some collagen-derived peptides to protein digestion is one of the most interesting properties of collagen hydrolysates. Several studies focused on the effect of oral intake in both animal and human models have revealed the excellent absorption and metabolism of Hyp-containing peptides. Some of these collagen-derived peptides have revealed biological activity *in vivo* after absorption from the digestive tract (Moskowitz *et al.*, 2000).

Extensive researches have reported that the collagen peptides derived from variety of fish have various bioactivities (Fahmi *et al.*,2004; Mendis *et al.*,2005; Jung *et al.*,2006; Huo *et al.*,2009; Gómez *et al.*,2010; Ngo *et al.*,2010).

1.4.7.1 Collagen hydrolysates production

The main source of collagen peptides are bovine hide, bone, pig skin or fish bones and fish skin. Marine sources are an alternative to bovine or porcine and they are not associated with the prions related to risk of Bovine Spongiform Encephalopathy (BSE) (Karim and Bhat, 2009). Collagen hydrolysates are manufactured in controlled hydrolysis process to obtain soluble peptides. The raw

material is washed, homogenized and demineralized with diluted mineral acid or alkaline. The raw material is extracted in several stages with warm water. Further enzymatic degradation of gelatin results in a final product which is collagen hydrolysate (Moskowitz, 2000). Clemente (2000) has presented enzymatic hydrolysis as the most appropriate method for preparation of tailor-made peptides.

Enzymatic hydrolyzing process can produce small fragments of collagen peptides. Furthermore, some of its bioactivity increased obviously (Huo and Zhao, 2009) and its antigenicity decreased (Fujita and Yoshikawa, 1999) after hydroysis.

A number of commercial proteases have been used for the production of these hydrolysates, including trypsin, chymotrypsin, pepsin, alcalase, properase E, pronase, collagenase, bromelain and papain. Besides commercial proteases, enzymatic extracts from fish viscera have been used to obtain bioactive hydrolysates from skin and bones of different fish. Protease specificity affects size, amount, free amino acid composition and, peptides and their amino acid sequences, which in turn influences the biological activity of the hydrolysates (Nam *et al.*, 2008)

1.4.7.2 Optimization of hydrolysate production

The functional properties of peptides are highly influenced by their molecular structure and weight, which are greatly affected by processing conditions. Enzymatic hydrolysis has become a valuable tool for modifying the functionality of proteins (Korhonen *et al.*, 1998). Several factors, like pH, time, enzyme activity, and temperature influence enzyme function, offering possibilities to control the process. Depending on the specificity of the enzyme, conditions applied in hydrolysis process, and the extent of hydrolysis, wide variety of peptides will be generated. The resultant protein hydrolysate will possess particular properties according to the new peptides generated (Zhang and Webster, 2009).

Response surface methodology has been a quite effective method of statistical and mathematical analysis for experiment data since it was first proposed by Box and Wilson (1951). It can evaluate the influence of all the variables in the multiple factor experiment design. The mutual interaction among factors can also be estimated simultaneously (Myers *et al.*, 2008). Hong *et al.*,2013; Emna *et al.*,2013; Yong *et al.*,2009, in their studies successfully used RSM methods for optimizing enzyme hydrolysis conditions in the production of collagen hydrolysate.

1.4.7.3 Purification and identification of bioactive peptides

Enzymatically hydrolyzed fish peptides exhibit different physicochemical properties and biological activities depending on their molecular weight and amino acid sequence. Therefore the molecular weight of the bioactive peptide is one of the most important factors in producing bioactive peptides with the desired biological activities (Kim and Mendis, 2006; Kim and Wijesekara, 2010). An ultrafiltration membrane system can separate the peptides that have the desired molecular weights and functional properties from fish protein hydrolysates (Je *et al.*, 2005). Such a system can also control the molecular weight distribution of the appropriate peptide. Small peptides with different bioactivities are concentrated from the higher molecular weight fractions and remaining enzymes using a membrane with a low-molecular mass cut off such as 500, 1000 or 3000 Da. Nanofiltration, ion exchange membranes, and column chromatography can also be used (Pihlanto and Korhonen, 2003)

Often the most useful method for peptide separation is HPLC. Commercially available reversed-phase columns allow rapid separation and detection of their hydrophilic and hydrophobic characteristics (Shahidi and Zhong, 2008). Ferreira *et al.* (2007) recommended that peptides with different surface hydrophobicities can be separated by reversed-phase columns with a polystyrene divinyl benzene copolymer based packing. Choosing the right pore

size to achieve optimal separation of peptides is important, as the wrong pore size will result in poor resolution. In addition to pore size, the ligand on the gel also plays an important role in obtaining effective separation. Another essential factor for effective separation is determining the appropriate hydrophobicity of the gel. The appropriate pore size, hydrophobicity, particle size and column size should be combined to achieve high recovery and resolution in the isolation of peptides and proteins (Kuriyama *et al.*, 2005). HPLC is usually used in conjunction with other analyzing equipments including a UV detector or mass spectrometer.

Liquid chromatography followed by tandem mass spectrometry detection (LC–MS/MS) is commonly used to identify peptide sequences (Perkins *et al.*, 1999). Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometric analysis is also useful for generating peptide profiles of protein hydrolysates or semipurified fractions.

Collagen hydrolysates vary from each other in respect of peptides molecular weight, ranging from 2 to 6 kDa (Moskowitz, 2000; Zague, 2008). Its molecular weight is less than the average molecular weight of peptones. After purification, the product is concentrated and dried. The most common post-dried procedures are related to the control of molecular size and the elimination or reduction of bitterness in the resulting hydrolysates. The most efficient procedure to remove residual high molecular weight peptides and proteins or to reduce the antigen content of hypoallergenic formulas, is ultrafiltration (Clemente, 2000)

Several analyses may be done for the quality control of these products: the osmolarity, analysis of the hydrolysis degree, the molecular weight distribution, the total nitrogen, amino acid composition and the presence of toxic compounds (e.g. biogenic amines or pathogens). Protein hydrolysate qualitative analysis use different techniques based on spectrophotometric, chromatographic and electrophoretic methods (Emna *et al.*, 2013; Yong *et al.*, 2009)

1.4.7.4 Bioactive properties and applications of collagen hydrolysates

Collagen hydrolysates have been reported to have beneficial biological functions. Despite the fact that collagen hydrolysate has been generally regarded as having a low biological value, because it does not contain all of the essential amino acids, it's a reputable nutritional component often used to supplement other proteins because of its superb digestibility and high consumer tolerance (Zague, 2008). Its excellent properties are result of their amino acid composition and molecular structure. Peptide fractions from protein hydrolysates may vary in their effectiveness for a given biological activity. The average molecular weight of protein hydrolysates is one of the most important factors which determine their biological properties. Bioactive peptides usually contain 2–20 amino acid residues per molecule; the lower their molecular weights, the higher their chances of crossing the intestinal barrier and exerting a biological effect (Kim and Wijesekara, 2010)

According to the opinion of many researchers, beneficial effects of oral administration of collagen hydrolysates results by crossing the intestinal barrier, by dietary bioactive peptides, which reach the blood circulation and become available for metabolic processes (Zague, 2008). Collagen hydrolysates are used in medical applications, such as high-energy supplements, geriatric products and enteric, therapeutic or weight-control diets. Applications of protein hydrolysates are in treatment of patients with specific disorders of digestion, absorption and amino acid metabolism. Tests also included clinical cure of patients with malnutrition attached with trauma, burns, cancer and hepatic encephalopaties (Clemente, 2000). Collagen hydrolysates are good source of amino acids for people suffering from anorexia, anaemia and for vegetarians (because of absence of meat in their diet). Diet supplements containing collagen hydrolysates are considered as improvement

agents in tendon or joint regeneration in physically active athlets with activity related joint pain (Moskowitz, 2000 and Zague, 2008)

Orally consumed collagen hydrolysate has been shown to be absorbed intestinally and to accumulate in cartilage. Specifically, collagen hydrolysate ingestion stimulates a significant increase in the synthesis of extracellular matrix macromolecules by chondrocytes (Bello and Oesser, 2006). According to medical data clinical investigations suggest that ingestion of collagen hydrolysates reduces pain in patients suffering from osteoarthritis and osteoporosis. It is considered that about 15% of world populations suffer from joint pain-related diseases.

Increasing risk agents are senility (over 50% of elderly people suffer from rheumatism), gender (a high amount of patients are women, particularlly after menopause), body weight (huge body weight is a reason of joint overload and results in joint pain), constantly excessive sport activity, joint injury (e.g. dislocations), metabolic diseases (e.g. diabetes). Collagen hydrolysates are involved in cartilage matrix synthesis (Zague, 2008). For the last two decades scientists have studied a relationship between therapeutic trials in joint diseases and collagen, gelatin or collagen hydrolysates. In numerous studies researchers accepted dose of 10g of collagen hydrolysates daily as a safe and well tolerated by patients. Additionally clinical tests have proved that this level of daily ingested proteins can reduce the pain in comparison with placebo group patients (Moskowitz, 2000).

Several scientific reports have presented good bioavailability of hydrolyzed collagen, by animals and human beings after oral administration. Oesser *et al.* (1999) discovered that about 95% of orally applied collagen hydrolysate was absorbed within the first 12h. Zague, (2008) described the high safety of eating collagen hydrolysates in an animal model (1.66 g/kg of body weight per day). Studies related with preparations consisting gelatin derivated peptides showed good tolerance and little side effects including a

sensation of unpleasant taste, a feeling of heaviness in the stomach, and a bloated feeling or pyrosis after oral administration (Oesser *et al.*, 1999)

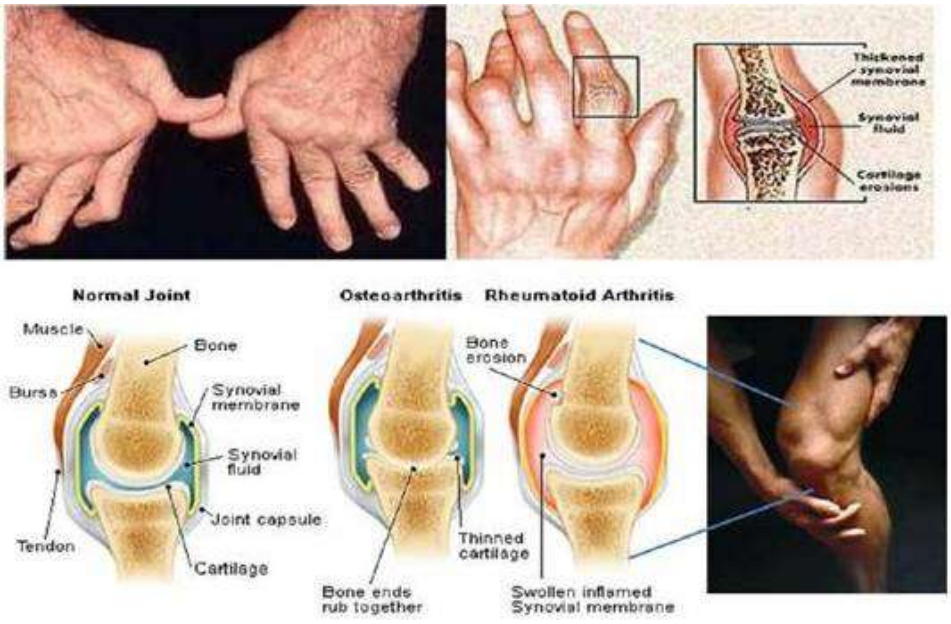
1.4.8 Role in bone and joint disease – Arthritis

Arthritis is a form of joint disorder that involves inflammation of one or more joints. There are different forms of arthritis. The most common form, osteoarthritis is a result of trauma to the joint, infection of the joint, or age (Felson *et al.*, 2000; Leyland *et al.*, 2012). Osteoarthritis is characterized by progressive destruction of joint cartilage and its associated structures (bone, synovial and fibrous joint capsules), remodeling of the periarticular bone, and inflammation of the synovial membrane (Blagojevic *et al.*, 2010). This disorder is basically produced by an imbalance between the synthesis and degradation of the articular cartilage. This imbalance leads to the classic pathologic changes of wearing away and destruction of cartilage. (Kuptniratsaikul *et al.*, 2002; Loeser *et al.*, 2012)

Rheumatoid arthritis (RA) is characterized by inflammation of the synovial membrane of diarthrodial joints. Early indications of RA are swelling and pain of the proximal inter-phalangeal and later, the larger joints become affected, especially those of the knee, elbow and ankle. (Mc Innes and O'Dell, 2010) Hyperplasia or thickening of the synovial membrane is promoted by cytokines and growth factors released from migrating cells. The synovial membrane becomes revascularised making it redder than normal. The cytokine enriched environment produced by pro-inflammatory cytokines (IL-1α, IL-6 and TNF-α) results in the aberrant growth of complex vessels known as pannus which invades the cartilage resulting in the degradation of the articular surfaces (Astusi *et al.*, 2005; McInnes and Schett, 2011).

Rheumatoid arthritis progresses in three stages. The first stage is the swelling of the synovial lining, causing pain, warmth, stiffness, redness and swelling around the joints. Second is the rapid division and growth of cell, or pannus, which causes the synovium to thicken. In the third stage, the inflamed cell releases enzyme that may digest the bone and cartilage, often causing the joints to lose its shape and alignments, more pain and loss of movements (Scott *et al.*, 1998; Mc Innes and Schett, 2011).

Figure 1.3 *showing deformities in small joints of rheumatoid arthritis patient (top). Schematic diagram showing the comparison among normal and the joints affected with osteoarthritis and rheumatoid arthritis (below)*



1.4.8.1 Autoantibodies and anti CCP assay

The rheumatoid factor (RF) and anti-cyclic citrullinated peptide (CCP) antibodies are considered clinically useful as disease markers (Mewar and Wilson, 2006). The rheumatoid factor is probably the most studied autoantibody in RA. It binds to the Fc region of IgG and contributes to the formation of immune complexes. Antibodies specific to the post-translational modification (citrulline) on proteins like, keratin, filaggrin, fibrinogen, vimentin,

fibronectin, α-enolase, CII etc, referred as anti-citrullinated protein antibodies (ACPAs) also monitored in the arthritic conditions. ACPAs are rarely found in healthy individuals but in RA patients they seem to be 70–90% and have high disease specificity. Proteins are citrullinated during apoptosis and inflammatory process in RA, they are found years before disease onset in the plasma and levels seem to be elevated in the synovial fluid (Schellekens *et al.*, 2000).

Citrullination is the post-translational conversion of positively charged peptidyl arginine to neutral peptidyl citrulline. The conversion is catalyzed by Peptidyl Arginine Deamiminase (PAD) enzyme in the presence of calcium ions. In humans, five PAD isotypes (PAD1,PAD2, PAD3, PAD4 and PAD6) are described with varied tissue expression and only PAD2 and PAD4 have been found to express in inflamed synovial tissue of RA and in other inflammatory arthritides (Foulquier *et al.*,2007).

An ELISA was developed to detect antibodies directed against filaggrin derived from human skin and has high specificity and sensitivity for the diagnosis of RA (Palosuo *et al.*, 1998). The target amino acid in filaggrin is citrulline, a post-translationally modified arginine residue (Schellekens *et al.*, 1998). Subsequently, an ELISA assay for the detection of antibodies to a cyclic peptide containing citrulline was made comercially available, which was easier to standardize, and also had high sensitivity and specificity for the diagnosis of RA. This became the assay for the detection of anti-cyclic citrullinated peptide (anti-CCP) antibodies. Anti-citrullinated protein antibodies are highly specific for RA (De Rycke *et al.*, 2004). The citrullination is catalyzed by peptidyl arginine deiminase; arginine residues on fibrin and fibrinogen may be favored sites for deimination within rheumatoid joints (Kinloch *et al.*, 2008). Intracellular citrullinated proteins colocalized with the deimidase in 59 percent of RA synovial samples versus 17 percent of control samples. However, citrullinated proteins may also be found in the synovium of other forms of arthritis, in nonsynovial tissue from

patients with RA (e.g. pulmonary rheumatoid nodules), in the lungs of patients with interstitial pneumonitis, in brain from patients with multiple sclerosis, and in normal brain (Bongartz *et al.*, 2007).

1.4.8.2 Cycloxygenases

Cyclooxygenase (COX, also called Prostaglandin H Synthase or PGHS) enzymes contain both cyclooxygenase and peroxidase activities. COX catalyzes the first step in the biosynthesis of prostaglandins (PGs), thromboxanes, and prostacyclins; the conversion of arachidonic acid to PGH_2. It is now well established that there are two distinct isoforms of COX.

Cyclooxygenase-1 (COX-1) is constitutively expressed in variety of cell types and is involved in normal cellular homeostasis. A variety of mitogenic stimuli such as phorbol esters, lipopolysaccharides, and cytokines lead to the induced expression of a second isoform of COX, cyclooxygenase-2 (COX-2). COX-2 is responsible for the biosynthesis of PGs under acute inflammatory conditions. This inducible COX-2 is the target enzyme for the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs. Pharmacological inhibition of COX can provide relief from the symptoms of inflammation and pain. Non-steroidal anti-inflammatory drugs, such as aspirin and ibuprofen, exert their effects through inhibition of COX. However these NSAIDS inhibit the activities of both COXs (COX I and COX ll). COX I is involved in the normal homeostasis mechanisms in the body and its inhibition can lead to the development of dyspepsia, erosions, gastric ulcers and renal dysfunction (Smith *et al.*, 2000).

1.4.8.3 Chondroprotectives

There are different classes of anti-arthritic drugs available like non-steroidal anti-inflammatory drugs (NSAIDS), Monoclonal antibodies, uricosuric agents, gold compounds, anti-cytokinine immunosuppressant like glucocorticoids, etc. Though the goal of

these drugs has been to relieve pain and to decrease joint inflammation, these drugs are known to produce various side effects including gastrointestinal disorders, organ damages, immunodeficiency and humoral disturbances. (Roth, 2005; Harirforoosh and Jamali, 2009) Selective COX-2 inhibitors make alternative approach to arthritic treatment with reduced GI side effects, but on long term treatment leads to serious cardiovascular and thrombotic side effects. Accordingly, reducing side effects should be considered while designing improved therapeutics for arthritis, besides enhancing medicinal effectiveness (Moore, 2007).

The extracellular framework and two-thirds of the dry mass of adult articular cartilage are polymeric collagen. Treatment with chondroprotectives, such as glucosamine sulfate, chondroitin sulfate, hyaluronic acid, collagen hydrolysate, or nutrients, such as antioxidants and omega-3 fatty acids are being increasingly recognized as an alternate approach to arthritic treatment (Jerosch, 2011; Henrotin *et al.,* 2012). Numerous clinical studies have demonstrated that the targeted administration of selected micronutrients leads to a more effective reduction of OA symptoms, with less adverse events. Their chondroprotective action can be explained by a dual mechanism: (1) as basic components of cartilage and synovial fluid, they stimulate the anabolic process of the cartilage metabolism; (2) their anti-inflammatory action can delay many inflammation-induced catabolic processes in the cartilage. These two mechanisms are able to slow the progression of cartilage destruction and may help to regenerate the joint structure, leading to reduced pain and increased mobility of the affected joint (Sawitzke *et al.,* 2010).

Treatment of arthritis includes analgesics and anti-inflammatory agents, lubricating, cushioning agents and nutritional supplements. Treatment for osteoporosis includes oestrogenic hormone replacement, bisphosphonates, calcitonin, selective oestrogen receptor agonists, fluorides, and parathormone derivatives.

Nonetheless, therapeutic responses are limited in many patients and it has adverse effects affecting health and organ systems of the body. Advances in treatment of osteoarthritis and osteoporosis include new and safer compounds (e.g., glucosamine, chondroitin sulphate, or methyl-sulfonyl-methane) capable of repairing damaged articular cartilage or at least decelerating its progressive degradation (Brief *et al.*, 2001).

Oral administration of collagen hydrolysate from shark skin has increased production of newly synthesized type I collagen and proteoglycan in the bone matrix of ovariectomized rats (Nomura *et al.*, 2005). Guillermin *et al.* (2010) administered a diet enriched with a collagen hydrolysate too variectomized mice during 12 weeks, and observed that osteoblast activity was increased at the end of the experiment, while differentiation and maturation of osteoclasts was lowered. These effects on osteoblasts and osteoclasts led to a significant stimulation of bone formation and mineralization.

Experimental studies have suggested that some collagen-derived peptides orally administered are absorbed intact in the intestine. Subsequently, these peptides would accumulate preferably in cartilage, where finally may stimulate cartilage metabolism (Oesser *et al.*, 1999). Some evidences exist on the ability of collagen hydrolysates to stimulate biosynthesis of type II collagen and proteoglycans in chondrocytes (Oesser *et al.*, 2003). Raabe *et al* (2010) have reported the marked effect of a fish collagen hydrolysate on chondrogenic differentiation of equine adipose tissue-derived stromal cells. These studies suggest that effectiveness of collagen hydrolysates on biosynthesis of macromolecules would be based on their unique amino acid composition, very similar to that of type II collagen. Oral administration of collagen hydrolysates would provide high levels of glycine and proline, two amino acids essentials for the stability and regeneration of cartilage (Walrand *et al.*, 2008). The therapeutic effect of collagen hydrolysates on osteoarthritis could also be mediated by the effect of specific

peptides on gene expression and function of chondrocytes. The effect of bioactive peptides on chondrocytes metabolism could be mediated by interaction with specific receptors on cell membranes (Nakatani *et al.*, 2009). To conclude, collagen hydrolysates are safer compounds that could provide, with less overall toxicity, a greater symptomatic relief than pharmaceutical drugs.
