CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Nowadays seafood have become an important income generator for Thailand. By the year 2000, Thailand earned more than 71.395 million US Dollars from frozen surimi (Department of export promoter, 2001). Surimi is one of seafood products, which has been exported to several countries. During processing, a large amount of wastes is generated. Fish solid wastes constitute 50–70% of the original raw material, depending on the method of meat extraction from the carcass (Morrissey and Park, 2000). This waste is an excellent raw material for the preparation of high-protein foods, besides helping to eliminate harmful environmental aspects (Shahidi, 1994). About 30% of such waste consist of skin and bone with high collagen content (Gomez-Guillen *et al*., 2002). Nagai and Suzuki (2000a) reported that the collagen content in fish waste skin of Japanese sea-bass, chub mackerel, and bullhead shark were 51.4%, 49.8%, and 50.1%, respectively, on the basis of lyophilized dry weight. Thermal denaturation of these collagens generally produces gelatin, which is a marketable value added product (Foegeding *et al*., 1996).

Collagen and gelatin have been widely used in food industries as ingredients to improve the elasticity, consistency and stability of foods. It can be used for encapsulation and edible film formation, making it of interest to the pharmaceutical, biomaterial-based packaging, and photographic industries, *etc*. (Stainsby, 1987). Recently, its use is expanding to new application such as health foods. Collagen and gelatin of land animal origin such as bovine and porcine has been mainly used (Yoshimura *et al*., 2000). The outbreak of mad cow disease has resulted in anxiety among users of cattle gelatin. Additionally, the collagen obtained from pig bones can not be used as a component of some foods for religious reason (Sadowska *et al*., 2003). Therefore, alternative sources, such as fish processing waste has been paid increasing attention. However, fish gelatins have considerably lower melting and gelling points than land animal (bovine and porcine) (Leuenberger, 1991). Moreover, the research on fish gelatins are few and limited in scope (Gudmundsson, 2002). Therefore, knowledge about the characteristics and functional properties including gelation and film forming ability as well as the improvement of those

properties will be beneficial for the further utilization of fish skin wastes from surimi processing, especially to produce the prime quality collagen and gelatin products.

1.2 Literature review

Collagen

Collagen is abundant in tendons, skin, bone, the vascular system of animals, and the connective tissue sheath surrounding muscle, contributing to toughness of muscle. About 10% of mammalian muscle protein is collagen but the amount in fish is generally much less. Some of the collagen is soluble in neutral salt solution, some is soluble in acid, and some is insoluble (Foegeding *et al.*, 1996).

The collagen monomer is a long cylindrical protein about 2,800 Å long and 14-15 Å in diameter (Foegeding *et al.*, 1996). It consists of three polypeptide units (called α-chains). Each α-chain coils is a left-handed helix with three residues per turn, and the three chains are twisted right-handed to form the triple helix held together by hydrogen bonding (Figure 1). Each α-chain contains ~1,000 amino acid residues and varies in amino acid compositions (Wong, 1989) and has a molecular mass of about 100,000 D, yielding a total molecular mass of about 300,000 D for collagen. (Foegeding *et al.*, 1996).

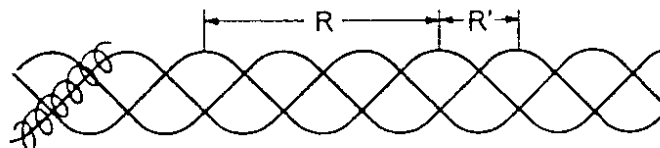

Figure 1. Schematic representation of the conformation of tropocollagen
Source: Burghagen (1999).

Various types of collagen are observed among different organs and connective tissue layers of muscular tissue (Table 1) (Burghagen, 1999).

Table 1. Types of collagen

Type	Peptide chains[a]	Molecular composition	Occurrence
I	α^1, α^2	$[\alpha^1(I)]_2\alpha^2(I)$	Skin, tendons,bones, muscle (epimysium)
II	α^1	$[\alpha^1(II)]_3$	cartilage
III	α^1	$[\alpha^1(III)]_3$	Fetal skin, cardio vascular system, synovial membranes, inner organs, muscle
IV	α^1, α^2	$[\alpha^1(IV)]_3$(?)[b]	Basal membranes, capsule of lens, glomeruli, lung, muscle (endomysium)
V	αA, αB, αC	$[\alpha B]_2\alpha A$ or $[\alpha B]_3$+ (αA) or $[\alpha C]_3$(?)	Placental membrane, cardiovascular system, lung, muscle (endomysium), secondary component of many tissues

[a] Since the α chains of various types of collagen differ, they are called α^1(I), α^1(II), αA etc.

[b] (?) Not completely elucidated.

Source: Burghagen (1999).

Polypeptides of collagen are mostly helical but differ from the typical α-helix due to the abundance of hydroxyproline and proline, which interfere with α-helical structure (Foegeding *et al.*, 1996). Collagen molecules link end to end and adjacently form collagen fibers as shown in Figure 2.

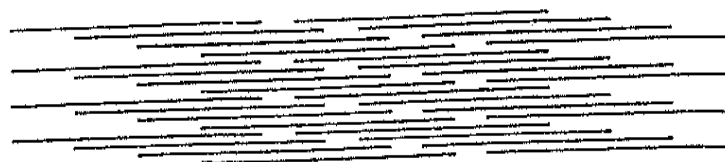

Figure 2. Overlap structure of the collagen

Source: Foegeding et al. (1996).

Collagen contains high contents of glycine (33%), proline (12%) and the occurrence of 4-hydroxyproline (12%) and 5-hydroxylysine (1%) (Burghagen, 1999). Glycine generally represents mainly one-third of the total residues, and it is distributed uniformly at every third position throughout most of the collagen molecule. The repetitive occurrence of glycine is absent in the first 14 amino acid residues from the N-terminus and the first 10 from the C-terminus, with these end portions being termed "telopeptides". Collagen is the only protein that is rich in hydroxyproline; however, fish collagens contain less of this amino acid than do mammalian collagens (Foegeding *et al.*, 1996). Collagen is almost devoid of tryptophan.

The presence of proline stabilizes the helix structure by preventing rotation of the N-C bond. Hydroxyproline also stabilizes the collagen molecule. Collagen that contains small concentrations of both imino acids denature at lower temperatures than do those with large concentrations (Foegeding *et al.*, 1996). The imino acid content of fish collagens therefore affects their thermal stability and correlates with the water temperature of their normal habitat (Foegeding *et al.*, 1996).

Distribution of collagen in marine animals

Collagen contents in the muscles depend on the species as well as on the state of maturation and feeding of the fish (Regenstein and Regenstein, 1991). In starving fish, the sarcoplasmic and myofibrillar proteins undergo gradual degradation, while the connective tissues are not utilized. As a consequence, collagen is deposited in the myocommata and in the skin. Generally, the contents of collagen in fish muscles range from about 1 to 12% of the crude protein. Collagen at levels of 0.2 to 2.2% was found in fresh meat, and 1.7 to 4.6% of collagen was reported in the fish skin (Sikorski *et al.*, 1990).

Marine animal muscle and skin collagens differ from bovine meat and hide collagens in having significantly higher contents of seven essential amino acids and a considerably lower concentration of hydroxyproline residues (Table 2). The collagens of edible marine invertebrates are characteristic for a high content of carbohydrates (Kimura, 1972). Some fish and invertebrate collagens also contain glucose, galactose, mannose, and fucose (Sikorski *et al.*, 1990). Furthermore, small amount of arabinose, xylose, and ribose residues was also found as constituents in collagens (Kimura, 1972). The carbohydrates

are mainly linked O-glycosidically to hydroxylysine residues as glucosyl-galactosyl-hydroxylysine units. These hydroxylysine-linked carbohydrates may have an impact on the structure of the fibrils in the invertebrate collagens (Sikorski *et al.*, 1990).

Table 2. Amino acid compositions of collagen from fish and invertebrates

Amino acids	**Residues per 1000 residues**			
	Cod	Squid	Spiny lobster	Bovine L. dorsi
Alanine	106.0	88.8	43	107
Arginine	59.1	59.0	54	45
Aspartic acid	42.3	57.7	47	34
Cystine	-	1.8	0	-
Glutamic acid	82.2	86.4	102	83
Glycine	313.6	308	324	336
Histidine	16.3	7.4	7	5
Hydroxyproline	40.7	89.3	90	109
Hydroxylysine	8.2	16.1	24	8
Isoleucine	18.6	20.9	20	12
Leucine	32.3	33.9	46	25
Lysine	36.9	15.3	15	23
Methionine	20.4	7.7	12	5
Phenylalanine	14.5	11.5	8	14
Proline	87.6	96.0	108	113
Serine	62.9	46.9	49	36
Threonine	25.8	26.2	24	17
Tyrosine	6.0	4.5	4	3
Valine	26.1	21.1	20	25

Source: Sikorski *et al.* (1990).

An attempt has also been made to group commercially important fishes on the basis of their collagen content. The percent of total collagen on total protein of these 20 fish species was used to categorize fish into 3 groups by their collagen contents as shown in Figure 3 (Hassan and Mathew, 1996).

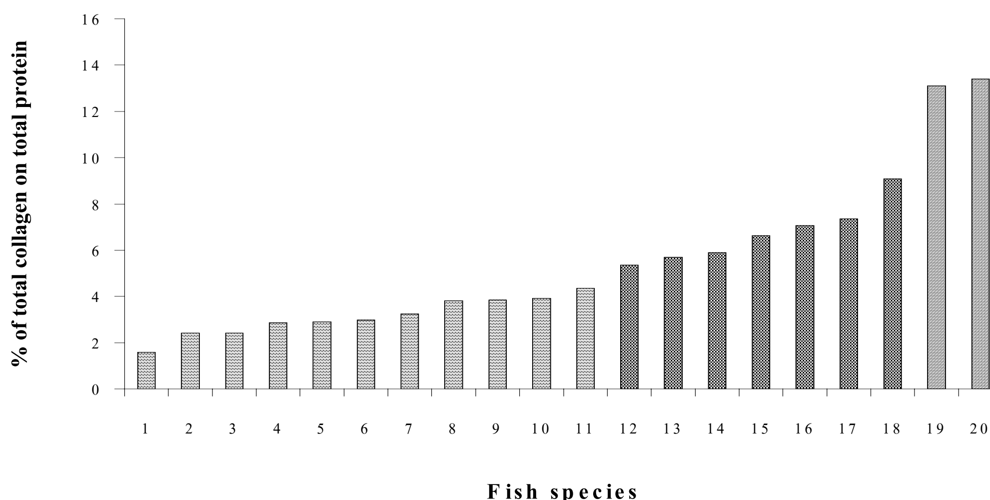

Figure 3. Classification of fish based on their collagen content

1. *P. argenteus* 2. *S. longiceps* 3. *R. Kanagurta* 4. *C. semifasciatus* 5. *Caranx* spp. 6. *T. savala* 7. *Anchoviella* 8. *O. mossambicud* 9. *C. carpto* 10. *L. rohita* 11. *M. cyprinoides*. 12. *Barbus* spp. 13. *N. japonicus* 14. *N. cephalus* 15. *K. koval* 16. *C. catla* 17. *E. affinis* 18. *S. sihama* 19. *Himantura* 20. *S. sorrakowa*.

Source: Hassan and Mathew (1996).

Invertebrate marine animals collagen

Muscles of abalone (*Haliotis discus*) and top shell (*Turbo cornutus*), which are the gastropodian animals, consist of homotrimetric collagen molecules $(\alpha 1)_3$. On the other hand, the byssus threads of sea mussel (*Mytilus edulis*) and the adduction muscles of pearl oyster (*Pinctada fucata*) have been shown to possess $(\alpha 1)_2\alpha 2$ heterotrimers, which have similar compositional features to vertebrate Type I collagen (Yoshinaka and Mizuta, 1999).

Mantle muscle of common squid (*Todarodes pacificus*) has been reported to be composed of two genetically distinct types of collagen from pepsin-solubilized collagen preparations. The major collagen, named Type SQ-I collagen, is insoluble in 0.5 M acetic acid containing 0.45 M NaCl, and has a similar amino acid composition to those of vertebrate Type I collagen, and of major collagens in the cranial cartilage and skin of common squid (*Todarodes pacificus*), and in the skin and arm muscle of octopus (*Octopus vulgaris*). The minor collagen, called Type SQ-II, is very soluble in 0.5 M acetic acid

containing 0.45 M NaCl. Type SQ-II collagen has some compositional characteristics similar to vertebrate type V collagen, showing a low level of alanine and high level of hydroxylysine. Reducing treatment with 2-mercaptoethanol causes little change to SDS-PAGE pattern of type SQ-II collagen, indicating the absence of disulfide bonds in this molecular species (Yoshinaka and Mizuta, 1999).

Pepsin-solubilized collagen of edible jellyfish exumbrella was composed of $\alpha_1 \alpha_2 \alpha_3$-heterotrimers. This collagen was relatively stable at 26.0°C for 60 min (Nagai *et al.*, 1999). Yoshinaka and Mizuta (1999) demonstrated that at least two genetically distinct types of collagen are present in the muscle of kuruma prawn. The major type of collagen, referred to as Type AR-I, consists of two kinds of α components, named α1 (AR-I) and α2 (AR-I) components. Type AR-I collagen has three molecular forms of which subunit compositions are $[\alpha 1 \text{ (AR-I)}]_3$, $[\alpha 1 \text{ (AR-I)}]_2 \alpha 2 \text{ (AR-I)}$, $\alpha 1 \text{ (AR-I)} [\alpha 2 \text{ (AR-I)}]_2$. These molecular forms were referred to as type AR-Ia, AR-Ib and AR-Ic collagens, respectively, as shown in Figure 4. The minor type of collagen, Type AR-II collagen, found in kuruma prawn contained disulfide bonds, which occupied about 10% of the total pepsin-solubilized collagen (Yoshinaka and Mizuta, 1999).

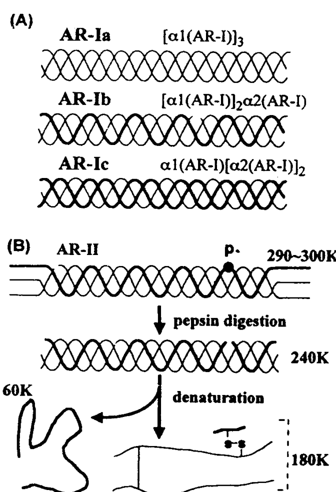

Figure 4. Structural models of molecular species of subunit composition of Type AR-I collagens

Source: Yoshinaka and Mizuta (1999).

For purple sea urchin (*Anthocidaris crassispina*), pepsin-solubilized collagen consisted of two α-chains and had a chain composition of $(\alpha1)_2\alpha2$ heterotrimer (Nagai and Suzuki, 2000b). The primary structure of the purple sea urchin collagen is different from that of a mammalian such as porcine. Sea urchin collagen has a denaturation temperature of 28°C, which is about 9°C lower than that of the porcine skin collagen. Saito *et al.* (2002) reported that about 70% of the total body wall protein of edible sea cucumber (*Stichopus japonicus*) was constituted by highly insoluble collagen fibers. The disaggregation with β-mercaptoethanol, 0.1 M NaOH treatment, and limited pepsin digestion of these collagen fibers resulted in complete solubilization. The solubilized collagen consisted of 2 distinct subunits, $\alpha1$ and $\alpha2$, which formed $(\alpha1)_2\alpha2$ heterotrimers and was rich in glutamic acid when compared with other fibrillar collagens.

Fish collagen

Muscle collagen

Fish muscle generally contains only one-tenth of the collagen found in red meat (Eskin, 1990). Total collagen content in teleost varied in the range of 1.52-9.08% of total protein whereas the elasmobranches was 13.11 - 13.39% of total protein (Hassan and Mathew, 1996). Eckhoff *et al.* (1998) determined the contents of total collagen, acid-soluble, pepsin-soluble and insoluble collagen in white muscle of farmed Atlantic salmon (*Salmo salar* L.) and found that the total collagen was 0.66% of flesh weight with a relative distribution of 6% acid-soluble, 93% pepsin-soluble and 1% insoluble collagen.

The solubility of the muscle collagen varies significantly among the various species of fish. The difference in solubility of collagen may reflect the difference in the degree and properties of intra- and inter-molecular cross-linking of collagen. The collagen was classified into acid soluble and insoluble fraction from each fish species as shown in Table 3.

Table 3. Collagen content of white muscle of fishes

Common name	Scientific name	Acid soluble collagen (%)	Insoluble collagen (%)	Total collagen (%)
White Pomfret	*Pampus argenteus*	0.19	0.11	0.30
Sardine	*Sardinella longiceps*	0.36	0.09	0.45
Mackeral	*Rastrelliger kanagurta*	0.38	0.09	0.47
Sole	*Cynoglossus semifasciatus*	0.40	0.18	0.58
Vatta	*Caranx spp.*	0.50	0.08	0.58
Ribbon fish	*Trichurus savala*	0.12	0.51	0.63
White bait	*Anchoviella*	0.09	0.60	0.69
Tilapia	*Oreochromis mossabiscus*	0.47	0.22	0.69
Common carp	*Cyprinus carpio*	0.49	0.21	0.70
Rohu	*Labes rohita*	0.28	0.46	0.74
Palankanni	*Megalops cyprinoides*	0.38	0.41	0.79
Paral	*Farbus spp.*	0.66	0.31	0.97
Veloori	*Kowala koval*	0.66	0.62	1.22
Kilimeen	*Nempterus Japonicus*	0.09	1.01	1.10
Mullet	*Mugil cephalus*	0.94	0.25	1.19
Catla	*Catala catla*	0.71	0.55	1.26
Tuna	*Euthynnus affinis*	1.06	0.39	1.45
Whiting	*Sillage sihama*	1.00	1.08	1.08
Ray	*Himantura*	2.30	0.50	2.80
Shark	*Scoliodon sorrakowah*	2.13	0.86	2.99

Source: Hassan and Mathew (1996).

Skin collagen

Collagens in fish skin such as hake and trout were identified as Type I collagen (Ciarlo *et al.*, 1997; Montero *et al.*, 1990). Nagai *et al.* (2002) extracted the pepsin-solubilized collagen from the skin of ocellate puffer fish (*Takifuga rubripes*) and found that it comprised heterotrimer with a chain composition of $(\alpha_1)_2\alpha_2$. The

denaturation temperature was 28°C, about 9°C lower than that of porcine collagen as determined by the changes in of collagen solution viscosity in 0.1 M acetic acid. From peptide mapping study using lysyl endopeptidase, peptide fragments patterns were different from porcine collagen, suggesting the difference in amino acid sequence among collagens from different species (Figure 5).

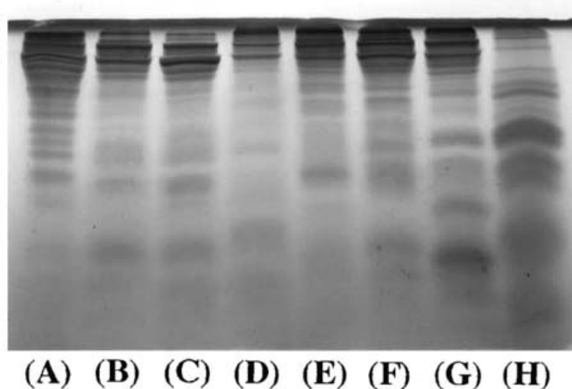

Figure 5. Comparison, by peptide mapping, of lysyl endopeptidase digests from several fish skin collagens. (A) Porcine collagen; (B) ocellate puffer fish acid-solubilized collagen; (C) ocellate puffer fish pepsin-solubilized collagen; (D) chub mackerel collagen; (E) Japanese sea bass collagen; (F) yellowtail collagen; (G) bullbead shark collagen; and (H) ayu collagen
Source: Nagai *et al.* (2002).

α-chains in fish or shellfish collagens can be varried with species. Kimura (1992) studied the distribution of α-3 chain in fish skin type I collagen and found that SDS-PAGE of collagen from the skin of frilled shark, eagle ray, South America lung fish, white sturgeon, bester, and longnose gar comprised at least two distinct αchain, $\alpha1(I)$ and $\alpha2(I)$. The peptide maps of type I collagen from these fish also had a characteristic peptide pattern after limited digestion with *Staphylococcus aureus* V8 protease and were quite similar to each other as indicated by being compatible with their close taxonomic positions. However, the subunit composition of skin collagen type I from South American lungfish, longnose gar, and eagle ray were different from white sturgeon, sturgeon, and bester, as determined by using CM-cellulose column chromatography. An elution pattern of South American lungfish, longnose gar, frilled shark, and eagle ray showed the heterotrimer molecules with $[\alpha1(I)_2\alpha2(I)]$, where $\alpha1(I)\alpha2(I)\alpha3(I)$ heterotrimer were found for

white sturgeon, sturgeon, and bester. The thermal denaturation temperature (T_D) of skin type I collagen from South American lungfish, longnose gar, and eagle ray were 33.8, 35, and 31°C, respectively. The high T_D values in these fish were found due to the presence of the high contents of imino acids (184, 198, and 183 residues/1000 residues, respectively), proline (114, 115, and 109 residues/1000 residues, respectively), and hydroxyproline (70, 83, and 74 residues/1000 residues, respectively). On the other hand, the T_D value of white sturgeon and frilled shark collagen were lower, 25.8 and 17.0°C, respectively. This was in agreement with the lower contents of imino acid (174 and 158 residues/1000 residues, respectively), proline (104 and 102 residues/1000 residues, respectively), and hydroxyproline (70 and 56 residues/1000 residues, respectively). Kimura *et al.* (1987) reported that the subunit α-3 chain was also detected in the skin collagen type I of eel, sardine, chum salmon, rainbow trout, carp, angler, Alaska pollack, cod, halfbeak, common mackerel, tilapia, red barracuda, nothern dab, and file fish, as determined by using CM-cellulose chromatography and SDS-PAGE of their elution profile. This collagen was $\alpha1\alpha2\alpha3$ heterotrimer. On the other hand, ayu, saury, and flying fish skin collagen consisted of two distinct α-chain ($\alpha1$, $\alpha2$) and the molecular structure existed as $(\alpha1)_2\alpha2$ heterotrimer.

Collagens are most likely dissolved in the acidic pH ranges and the ionic strength affects the collagen solubility. Montero *et al.* (1999) reported that solubility of hake skin collagenous material in 0.5 M acetic acid was not greatly affected by pH between 1 to 5. However, it was found that the higher pH levels cause the solubility to decline. Moreover, at pH up to 6, it practically becomes insoluble. The apparent viscosity of the skin collagenous material of hake also showed the similar pattern to that of solubility in acetic acid. Beyond the pH 3, apparent viscosity declines progressively until at pH 5 where it was zero. The high solubility in 0.5 M acetic acid indicates a low degree of molecular cross-linking, or the predominance of weak bond in the skin collagenous material of hake (Montero *et al.*, 1999). An increase in ionic strength of 0.5 M acetic acid in the solubility test from 0 to 0.17 by the addition of NaCl caused the increase in solubility of skin collagenous material. However, upon ionic strength of 0.17, the solubility decreased, and at 0.51 the% solubility was around zero.

Bone collagen

Subunit structures of collagen from bone of various species were quite different as follows: Japanese sea bass; $(\alpha 1)_2\alpha 2$, horse mackerel; $(\alpha 1)_3$ and ayu; $\alpha 1\alpha 2\alpha 3$. These results suggest a wide distribution of molecular forms in fish bone collagen (Nagai and Suzuki 2000c). Nagai and Suzuki (2000c) extracted the acid-soluble collagen from various fish bones using EDTA solution to completely decalcify for 5 days, 10% butyl alcohol to remove fat and 0.5 M acetic acid to extract the acid-soluble collagen. The yields of the collagens were very high as follows: Japanese sea bass, 40.7%, horse mackerel, 43.5% and ayu, 53.6% on the basis of lyophilized dry weight.

Collagen Type I from fish waste including skin, bone and fins of various kind of fish were extracted and the yields were as follows: (1) skin collagen, 51.4% (Japanese sea-bass), 49.8% (chub mackerel) and 50.1% (bullhead shark), respectively; (2) bone collagen, 42.3% (skipjack tuna), 40.7% (Japanese sea-bass), 53.6% (ayu), 40.1% (yellow seabream) and 43.5% (horse mackerel), respectively; (3) fin collagen, 5.2% (Japanese sea-bass acid-soluble collagen) and 36.4% (Japanese sea-bass acid-insoluble collagen), on the basis of lyophilized dry weight (Nagai and Suzuki 2000a).

Factors affect collagen functional properties

Aging and living period

As cross-linking of collagen increases, it becomes less soluble in a variety of solvents, such as salt and acid solutions. With advancing age, the amount of insoluble collagen often increases many fold in mammalian muscle, whereas the amount of insoluble collagen of cod increases only slightly with age and the amount of soluble collagen actually increases (Foegeding *et al*., 1996). Starving fish produce more collagen and collagen with a greater degree of cross-linking is found, compared to well fed fish (Sikorski *et al*., 1990; Foegeding *et al*., 1996). Love *et al*. (1976) reported that starvation causes the myocommata and skin of cod to be thickened. Collagen prepared from the thick tissue appears to have identical properties to normal collagen in term of molecular shape, intramolecular cross-linking, amino and imino acid composition, and thermal denaturation

temperature (Love *et al.*, 1976). An exception is the intermolecular cross-linking which appears to be greater in starving myocommata collagen (Foegeding *et al.*, 1996).

Processing

During storage fish on ice, a progressive change in solubility of muscle collagen was found (Eckhoff *et al.*, 1998). For insoluble collagen, significantly lower values were detected at day 15 compared to day 0. A minor, but even increase in acid-soluble collagen was found from day 0, while no changes were seen in pepsin-soluble collagen during storage. These results show that some cleavage of intermolecular cross-links seems to occur during storage on ice. Montero *et al.* (1995) studied four methods for stabilizing the extracted collagen involving 1) freezing, 2) freeze-drying, 3) partial solubilization with 0.05 M acetic acid prior to freezing, and 4) partial solubilization with 0.05 M acetic acid prior to freeze-drying. Only freeze-drying caused reduction in solubility and emulsifying capacity. Viscosity was greatest when samples were pre-solubilized. Emulsifying capacity was not changed when samples were frozen and decreased when they were either freeze-dried or presolubilized. Optimum water-holding capacity occurred in samples, which had been previously solubilized.

pH and NaCl

pH and salt concentration are the important factor determining the functionality of collagen. Where pH was between 2 and 4, solubility and water binding capacity of trout (*Salmo irideus Gibb*) collagen was greatest but in the addition of NaCl, functionality was reduced (Montero *et al.*, 1991). Montero *et al.* (1999) also reported that solubility, apparent viscosity, and water binding capacity of collagenous material from hake and trout showed the maximum values at pH levels between 2 and 4, and at concentrations of less than 0.25 M NaCl.

Gelatin

Gelatin is obtained by thermal denaturation or physical and chemical degradation of collagen. The process involves the disruption of noncovalent bonds and it is

partially reversible in agreement with the gelling properties of gelatin (Bigi *et al.*, 1998). Collagen fibrils shrink to less than one-third of their original length at a critical temperature, known as the shrinkage temperature. This temperature varies, depending on species from which the collagen is derived (Burghagen, 1999). This shrinkage involves a disassembly of fibers and a collapse of the triple-helical arrangement of polypeptide subunits in the collagen molecule. Essentially, the same type of molecular changes occurs when collagen is heated in solution, but at a much lower temperature (Foegeding *et al.*, 1996). The midpoint of the collagen-to-gelatin transition is defined as the melting temperature (Figure 6). During the collagen-to-gelatin transition, many covalent bonds are broken along with some covalent inter and intramolecular bonds (Shiff-base and aldo condensation bonds). This results in conversion of the helical collagen structure to a more amorphous form, known as gelatin. These changes constitute denaturation of the collagen molecule but not to the point of a completely unstructured product (Foegeding *et al.*, 1996).

After gelatin is produced and the temperature is lowered to below the critical value, there is a partial renaturation of the collagen molecule, involving what is called the "Collagen fold". Apparently, those parts of collagen that are rich in proline and hydroxyproline residues regain some of their structure, following which they can apparently interact (Foegeding *et al.*, 1996). When many molecules are involved, a three-dimensional structure is produced and responsible for the gel observed at low temperatures. This collagen fold is absolutely dependent on temperature. Above the melting temperature, the structure degrades and so does the gel. The strength of the gel formed is proportional to the square of the concentration of gelatin and directly proportional to molecular weight (Burghagen, 1999). Circular dichroism analysis reveals that gelling involves a refolding of denatured collagen chains into the typical triple helix conformation and, conversely unfolding upon reheating. The folding process seems to be directly related in the stabilization of the gels without disregarding its role in triggering the gelation process (Gomoz-Guillen *et al.*, 2002).

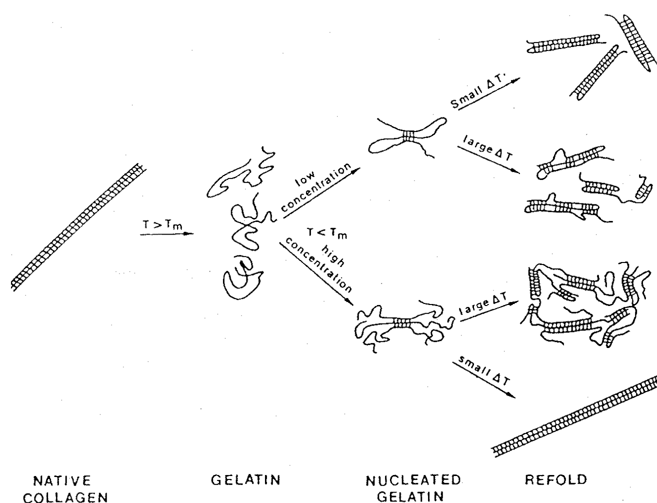

Figure 6. Collagen conversion into gelatin

Source: Wong (1989).

Functionality of gelatin

Gel formation, which is obtained by cooling gelatin aqueous solution after the collagen is heated, is accompanied by some characteristic changes which have been ascribed to a partial regain of collagen triple-helix structure. Gelatins from different fish species have different characteristics and properties. Gelatins from flat-fish species (sole and megrim) presented the best gelling ability and the gels were more thermostable than those from cold-adapted fish (cod and hake) (Gomoz-Guillen *et al.*, 2002). This different behaviour may be caused by the differences in the amino acid composition, the $\alpha 1/\alpha 2$ collagen-chain ratio, and the molecular weight distribution. Cod gelatin presented a lower alanine and imino acid content, and a decreased proline hydroxylation degree. Cod and hake gelatins had a low $\alpha 1/\alpha 2$ ratio (~1) and hake gelatin showed a highly significant decrease in β-components and other aggregates (Gomoz-Guillen *et al.*, 2002). Squid gelatin showed viscoelastic properties between those from flat-fish and cold-adapted fish species (Gomoz-Guillen *et al.*, 2002).

Fernandez-Diaz *et al.* (2003) reported that gelatin from the flounder (*Platichthys flesus*) skins frozen at -12°C had lower gel strength values when compared to that from fresh skins but showed the higher melting point value. SDS-PAGE revealed that gelatin from frozen skins showed some high molecular weight aggregates and clear bands corresponding to α, β and γ -components. Gelatin from frozen skin showed less α and

β-chains but more bands corresponding to lower molecular weight fragments. γ-components are less evident when both frozen at -12°C and -20°C, especially pronounced in the case of skin frozen at -12°C.

Fish gelatin has been known to possess the low gel strength, compared to gelatin from porcine or bovine collagen (Norland, 1990). Fennandez-Diaz *et al.* (2001) studied the gel properties of two different kinds of fish gelatins prepared from cod (*Gadus morhua*) and hake (*Merluccius merluccius*) and modified by the coenhancers, glycerol, salt and microbial transglutaminase. Gel strength was substantially increased by the addition of coenhancers although results varied, depending on the species. For gelatin from hake skin, the highest values were obtained with 10 mg/g of transglutaminase, whereas magnesium sulphate was more effective at both concentrations (0.1 and 0.5 M) in gelatin from cod skin. Although, in both gelatins, the addition of any ingredient increased the viscosity modulus, the elastic modulus was only increased by the addition of glycerol 15% (w/v) and $MgSO_4$ 0.5 M in hake gelatins. For cod gelatin, it was increased by all ingredients. Sarabia *et al.* (2000) also examined the effects of various salts on the viscoelastic properties of a class A gelatin from megrim (*Lepi dorhombus boscii*) skins in comparison with commercial tilapia skin gelatin. Although salts generally extended the setting time of gelatins, it was found that the melting temperature was increased considerably by the addition of $MgSO_4$, $(NH)_2SO_4$, or NaH_2PO_4. Of all the salt assayed, only $MgSO_4$ improved the rheological characteristics under suitable conditions of pH and ionic strength, which differed between megrim and tilapia gelatin.

Protein Films

Protein films are generally formed from solutions or dispersions of the protein as the solvent/carrier evaporates. The solvent/carrier is limited to water, ethanol, or ethanol-water mixtures (Krochta, 1997). Generally, globular proteins must be denatured by heat, acid, base, and/or solvent in order to form the more extended structures that are required for film formation. Once extended, protein chains can associate through hydrogen, ionic, hydrophobic, and covalent bonding. The chain-to-chain interaction that produces a cohesive film is affected by the degree of chain extension (protein structure) and the nature as well as sequence of amino acid residues. Uniform distribution of polar, hydrophobic,

and/or thiol groups along the polymer chain increases the likelihood of the respective interactions (Krochta, 1997).

Edible films are thin materials based on a biopolymer (Sobral *et al.*, 2001b). Biopolymer films derived from renewable sources (proteins, carbohydrates and lipids) have gained considerable research interest. Such films may be used as food coatings or stand-alone film wraps to retard unwanted mass transfer in food products (Kester and Fennema, 1989b; Miller and Krochta, 1997).

Many protein-based materials including wheat gluten, corn zein, soybean, ovalbumin, whey protein, casein and collagen have been used (Cuq *et al.*, 1995b). The mechanical and barrier properties of protein-based films are generally better than that of polysaccharide-based films. This is due to the fact that, contrary to polysaccharides which are homopolymers, protein has a specific structure (based on 20 different monomers) which confers a wider range of potential functional properties, especially high intermolecular binding potential (Cuq *et al.*, 1995). Proteins can form bonds at different positions, with different types and energies as a function of temperature, solvation conditions, pH and additive characteristics (plasticizers, bonding agents, etc.). Protein concentration, pH, temperature, time, ionic strength, and presence of additives affect the strength of protein-protein and protein-water interactions and thus modify functional properties (Cuq *et al.*, 1995). Cuq *et al.* (1995) found that pH and protein concentration had strong interactive effects on film forming solution (FFS) viscosity. During FFS was stored before casting, partial degradation of high molecular weight protein components led to decreased viscosity, allowing thin layer casting.

Plasticizers were found to affect mechanical properties of β-lactoglobulin (β-Lg) films (Sothornvit and Krochta, 2001). Propylene glycol-plasticized (β-Lg) films were the most brittle with mechanical properties independent of plasticizer content. Films with other plasticizers (glycerol, sorbitol, polyethylene glycol, PEG 200 and PEG 400 and sucrose) exhibited negative exponential dependence on plasticizer concentration for elastic modulus and tensile strength, while they exhibited linear dependence on plasticizer concentration of % elongation. Anker *et al.* (2002) reported that the water vapor barrier of whey protein films could be improved by adding a lipid. The laminated whey protein-lipid film decreased the water vapor permeability (WVP) 70 times compared with the WPI film. Lipid content did not affect WVP of the film, whereas an increased homogenization led to a slight reduction in WVP. Lipid functioned as an apparent

plasticizer by enhancing the fracture properties of the emulsion films. The maximum strain at break was 117% compared with 50% for the less-homogenized emulsion films and 20% for the pure WPI films.

Collagen and Gelatin films

Collagen film

Collagen can form the film with some pretreatments to induce the unfolding and subsequent cross-linking of molecules (Yamauchi *et al*., 2001). Collagen (type I from calf skin) was chemically modified by 4-butyrothiolactone to obtain the mercapto group-bearing collagen (collagen SH), which possessed 8-19% SH groups of a total amino acid residues. The triple helical strands of the collagen were not completely perturbed to exhibit the rotary dispersion. In the presence of the oxygen dissolved in water, the collagen SH was cross-linked by disulfide bonds. Collagen SS exhibited the tensile strength as high as 36 MPa and was insoluble in most organic solvents including water.

Ultraviolet irradiation (254 nm), which is the widely used for sterilization of collagen products, affected the collagen films by decreasing the denaturation temperature and causing the formation of wrinkles and micro cracks in the film surface of collagen during irradiation as determined by using differential scanning calorimetry (DSC) and scanning electron microscope (SEM), respectively (Sionkowska, 2000). Changes of denaturation temperature and surface of films under UV irradiation were pointed to the loss of water bonded to collagen. These were confirmed by IR spectra of the irradiated collagen films. Rhim *et al*. (1999a) reported that ultraviolet irradiated (253.7 nm, 51.8 J/m^2) treatment increased tensile strength of gluten, zein and albumin films suggesting the occurrence of UV radiation-induced cross-linking within film structures. For caseinate films, UV-curing did not affect tensile strength but substantially reduced total soluble matter (Rhim *et al*., 1999a).

Gelatin films

Gelatin films have been used in various fields such as an ideal delivery system for a wide range of medicine, food industry as a sausage casing component or

coating materials with antioxidants. Recent studies have shown that gelatin-based films with various additives have good potential for applications in a number of integrated optics devices such as holographic recording materials (Iwamoto *et al.*, 1999; Arvanitoyannis *et al.*, 1998a). To maximize the properties of film, many approaches have been implemented.

Plasticizers have been reported to affect the properties of protein based films. Sobral *et al.* (2001b) studied the effects of sorbitol content on mechanical, water vapor barrier and thermal properties of edible films based on bovine hide (BHG) and pig skin gelatins (PSG). An increased sorbitol content (from 15 to 65g sorbitol/100g gelatin) decreased the puncture force from 16.0 to 8.2 N and from 16.2 to 9.0 N, for the films of BHG and PSG, respectively. Cuq *et al.* (1997b) also observed a linear reduction of the puncture force of edible films based on myofibrillar proteins of Atlantic sardine from 5.1 to 2.6 N, with the addition of 0-40 g of glycerol/100 g of protein. On the other hand, the increase of sorbitol content from 15 g to 65 g sorbitol/100 g gelatin content increased the puncture deformation of both BHG and PSG films from 1.2 to 5.3% (Sobral *et al.*, 2001b). Gontard *et al.* (1993) also observed an increased puncture deformation of edible films of gluten from 6 to 20% by the increase of glycerol content from 16 to 33 g glycerol/100 g dry matter. The increase in the plasticizer concentration causes a reduction of the puncture force due to the decrease in the intermolecular interactions and causes an increase of the puncture deformation due to the increase in the mobility of the macromolecules (Sobral *et al.*, 1999). The increase in the plasticizer concentration increases the moisture content of the film because of its high hygroscopic character, which also contributes to the reduction of the forces between the adjacent macromolecules. The water vapor permeability of the PSG and BHG films increased linearly from 1.8 to 3.2×10^{-8} $g.mmh^{-1}cm^{-2}Pa^{-1}$, and from 1.7 to 3.8×10^{-8} $g.mmh^{-1}cm^{-2}Pa^{-1}$, respectively, with an increased sorbitol content from 15 to 65 g sorbitol/100 g gelatin. The increase in total plasticizer content (water, glycerol, sorbitol and sucrose) in the polymer matrix was found to result in a proportional increase in water vapor transmission rate (Arvanitoyannis *et al.*, 1998a). Cuq *et al.* (1997b) reported that the proteins network becomes less dense, and consequently more permeable with the plasticizer incorporation. The increase in free volume of the system also raises the solvent mobility, consequently increasing the water diffusion in the matrix of the film (Cuq *et al.*, 1997b).

The DSC traces obtained in the first scan of BHG and PSG films with 15-35 g sorbitol/100 g gelatin showed a well visible glass transition followed by a sol-gel

transition. However, with the increase of sorbitol concentration more than 35 g sorbitol/100 g gelatin, the glass transition become broader, typical characteristic of the system presenting a phase separation (Sobral *et al.*, 2001b). Souza *et al.* (1999) also observed this phase separation phenomenon in edible films of meat myofibrillar protein plasticized with glycerin.

Chain anisotropic distribution in gelatin films has been obtained by uniaxial streching at constant relative humidity, followed by air drying and successive cross-linking with a glutaraldehyde (Bigi *et al.*, 1998). The Young's modulus, E and the stress at break, σ_b, of the drawn gelatin films were found to increase linearly with the drawn ratio and reach values which are about five times those characteristic of undrawn samples. Furthermore, on stretching the alignment of the gelatin strands along the direction of deformation increases, while the thickness of the layers decreases significantly. The renaturation level, that is the fraction of gelatin in a collagen-like structure, has been calculated as the ratio between the melting enthalpy of gelatin samples and that of tendon collagen. The renaturation level increased from 34.5% to 74.1% with increased drawn ratio from 1.0 to 3.0.

Bigi *et al.* (2001) studied the influence of glutaraldehyde (GTA) concentration on the stability of GTA-cross-linked gelatin films. Air-dried gelatin films were submitted to treatment with GTA solutions at concentrations ranging from 0.05 to 2.5%. At the smallest GTA concentration, the cross-linking degree, determined by trinitrobenzensulfomic acid (TNBS) assay, was about 60% and increased up to values near 100% with GTA concentrations $\geq$1 wt%. Simultaneously, the deformability of the films decreased, whereas the stress at break, σ_b, and the Young's modulus, E, increased. A cross-linking degree of about 85% obtained by using 0.25% GTA, was enough to prevent gelatin release in buffer solution and to provoke a significant reduction of the swelling in physiological solution. Furthermore, cross-linking greatly increased the thermal stability of the films (Bigi *et al.*, 2001).

Film forming solution of gelatin treated with TGase contained high molecular weight polymer aggregates that did not enter the stacking gel with an increasing the incubation time (Lim *et al.*, 1999). This was accompanied by a decrease in band intensities for the unpolymerized protein fractions. In contrast, no polymerization products were observed for the control. These observations confirmed the formation of intermolecular cross-linked polymers of gelatin catalyzed by TGase.

Incorporation of antimicrobial and antioxidant in edible film

Active packaging is an innovative concept that can be defined as a type of packaging that changes the condition of the packaging to extend shelf-life or improve safety or sensory properties while maintaining the quality of the food (Vemeiren *et al.*, 1999). To control undesirable flavor from oxidation deterioration and micro-organisms on the surface of packed food products, that lower the product quality and spoilage, edible film and coating containing antioxidants and antimicrobials have been paid more attention. Inclusion of these compounds in film and coating concentrates them at the product surface, which is the place where protection is needed. Therefore, only very small amount of additive is required (Guilbert *et al.*, 1996). Miltz *et al.* (1988) reported that oatmeal cereals packed in high-level (0.32%) BHT-impregnated HDPE had an extended shelf-life (less oxidation) compared with those packed in low-level (0.022%) BHT-impregnated HDPE. After six weeks, the HDPE film was free of BHT and 19% of that originally present in the film remained in the cereal. Wessling *et al.* (2000) showed that incorporation of high levels of α-tocopherol into LDPE film could inhibit oxidation of a linoleic acid emulsion stored in contact with the film. The use of controlled release of antioxidants from polymeric films to reduce lipid oxidation in milk has also been reported (Van Aardt *et al.*, 2001). Cha *et al.* (2002) reported that films prepared from Na-alginate incorporated with various type of antimicrobial agent (lysozyme, isin, grape seed extract and EDTA) both alone and in combination exhibited larger inhibitory zones against *Micrococcus luteus, Listeria innocua, Salmonella enteritidis, Escherichia coli,* and *Staphylococcus aureus* on the plate diffusion agar, compared to κ-carrageenan-based films even within similar combinations and levels of antimicrobial agents. These result indicated that the releasing of antimicrobials from incorporated film prepared from Na-alginate was better than film prepared from κ-carrageenan.

1.3 Objectives of study

1. To characterize the collagen from bigeye snapper (*Pricanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*) skins.
2. To characterize the gelatin and to improve the properties of skin gelatin from both species.
3. To study some factors affecting the film properties of skin gelatin from both species.
4. To elucidate the role of antioxidant incorporation on the properties and antioxidative activity of skin gelatin film from both species.