Chapter 1

INTRODUCTION

Thailand is one of the largest surimi producers in the Southeast Asia. Twelve surimi factories are located in Thailand, with a total production of about 60,000 metric tons per year (Morrissey and Tan, 2000). Most of the fish used for surimi production includes threadfin bream (*Nemipterus* spp.), bigeye snapper (*Priacanthus* spp.), croaker (*Pennahia and Johnius* spp.) and lizardfish (*Saurida* spp.) (Benjakul *et al*., 2003). During surimi processing, a large amount of wastes including waste water and solid waste is generated (Morrissey *et al*., 2000). The solid wastes constitute 50-70% of original raw materials, depending on the processing used. These wastes are a mixture of heads, viscera, skin and bone (Morrissey *et al.*, 2000). Although the nutritional values of these wastes are fairly high, these useful resources have been mainly used as fish meal or fertilizer with low value. (Nagai and Suzuki, 2000a). Also, improper disposal of these wastes may cause pollutions and emit an offensive odor. Hence optimal utilization of surimi processing wastes, especially the production of value-added products, is the promising means to increase a revenue for producer and decrease the cost for disposal or management of these wastes.

Collagen and gelatin have a wide range of applications in leather and film industries, pharmaceutical, cosmetic and biomedical materials and food (Bailey and Light, 1989; Cavallaro *et al*., 1994; Hassan and Sherief, 1994; Hood, 1987; Nimmni, 1988; Slade and Levine, 1987; Stainsby, 1987). Generally, pig and cow skins and bones are the main sources of collagen and gelatin isolation. The outbreak of mad

cow disease has resulted in anxiety among users of cattle gelatin. Additionally, the collagen and gelatin obtained from pig skin or bones can not be used due to the religious constraint (Sadowska *et al*., 2003). As a consequence, more increasing interest and attempt have been paid to the alternative collagen and gelatin sources, especially fish skin and bone from seafood processing wastes. About 30% of these wastes consist of skin and bone, which are very rich in collagen (Shahidi, 1994; Montero and Borderias, 1989; Gomez-Guillen *et al*., 2002). However, fish collagens have lower thermal stability than mammalian collagens because fish collagens contain a lower imino acid content than mammalian collagens (Foegeding *et al*., 1996). So far, skin and bone collagens as well as gelatin from several fish species have been isolated and characterized (Yata *et al*., 2001; Sadowska *et al*., 2003; Nagai *et al*., 2002a; Kimura *et al*., 1987; Nagai and Suzuki, 2000a; Nagai and Suzuki, 2000b; Kimura *et al*., 1991; Gudmundsson and Hafsteinsson, 1997; Montero and Gomez-Guillen, 2000; Gomez-Guillen and Montero, 2001; Jamilah and Harvinder, 2002; Gomez-Guillen *et al*., 2002). However, no information regarding the collagen and gelatin from tropical fish particularly from surimi processing wastes, has been reported. Among all species used, bigeye snapper is one species commonly used for surimi production due to the high gel-forming ability (Benjakul *et al*., 2001), leading to a high amount of skin and bone generated. Therefore, extraction of collagen and gelatin would be a means to fully use of those wastes and the products with higher value could be produced and possibly commercialized for both local and international markets.

Literature Review

Collagen

Collagen is the major fraction of connective tissue and it contributes significantly to toughness in mammalian muscle. Also the partially denatured product of collagen, gelatin, is a useful ingredient in many food products because it serves as the functional ingredient in temperature-dependent gel-type desserts. Collagen is abundant in tendons, skin, bone, the vascular system of animals, and the connective tissue sheaths surrounding muscle. Collagen comprises one-third or more of the total protein of mammals. 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 tropocollagen, a long cylindrical protein about 2800 ^{O}A long and 14-15 ^{O}A in diameter (Foegeding *et al.*, 1996). It consists of three polypeptide chains (called α chains) wound around each other in a suprahelical fashion. Each α chain coils in a left-handed helix with three residues per turn, and the three chains are twisted right-handed to form the triple helix (Figure 1) (Wong, 1989). Each chain has a molecular mass of about 100,000 D, yielding a total molecular mass of about 300,000 D for collagen. The three chains are held together by hydrogen bonding (Foegeding *et al.*, 1996). Another unique feature about collagen is that each collagen molecule contains about 33% glycine, 12% proline, 11% hydroxyproline and alanine and 1% hydroxyproline and is devoid of tryptophan (Xiong, 1997; Pearson and Young, 1989). The amino acid sequence indicates that most polypeptide chain consists of glycine-led triplets with the following distribution:

Triplet	Proportion
Gly-X-X	0.44
Gly-X-I	0.20
Gly-I-X	0.27
Gly-I-I	0.09

Where I = imino acid residue (proline or hydroxyproline) and X = other amino acid residues. The segment of the polypeptide chain consisting of repeating triplets with imino acid residues are the nonpolar regions, and the segments containing Gly-X-X triplets are mostly polar (Wong, 1989).

Figure 1 Triple-helical structure of collagen.

Source: Wong (1989)

The α chains contain ~ 1,000 amino acid residues and vary in amino acid composition. These variations in the α chains constitute at least four major types of collagen (Table 1). More than one collagen type is usually present in a particular tissue (Wong, 1989). Different α chains, designated α1, α2, or α3, within the same type of collagen differ in their amino acid composition. The distribution of α1, α2, and α3 chains in collagen molecules varies depending on the specific genetic variants (Xiong, 1997).

Table 1 Collagen and their distribution.

Type	Triple helix	Distribution
I	two identical α1(I) chains + one α2 chain	skin, tendon, bone
II	three α1(II) chains	Intervertebral disc, cartilage
III	three α1(III) chains	Cardiovascular vessel, uterus
IV	three α1(IV) chains	basement membrane, kidney glomeruli, lens capsule

Source: Wong (1989)

Forgeding *et al.* (1996) reported that type IV collagen like type III collagen, contains oxidizable cysteine residues and is rich in hydroxyproline and hyroxylysine contents. Type V collagen, like type IV collagen, is rich in hydroxyproline and hydroxylysine contents but contains no cysteine.

Based on its macromolecular structures, collagen can be divided into three major groups : (a) striated fibrous collagen, which includes types I, II and III collagen, (b) nonfibrous collagen, which contains type IV or basement membrane collagen, and (c) microfibrillar collagen, which encompasses types VI and VII (the matrix microfibrils), type V, IX, and X (the pericellular collagen), and types VIII and XI, which are yet unclassified (Xiong, 1997).

Polypeptides of collagen are mostly helical with the exception of the few residues at each end. However, the helices differ from the typical α-helix due to the abundance of hydroxyproline and proline, which interfere with α-helical structure. Collagen molecules link end to end and adjacently to form collagen fibers, as shown in Figure 2. There is a periodicity in the cross-striations of collagen at about 640-700 ^{O}A intervals (about one-fourth of its length). Collagen fibers are sometimes arranged

in parallel fashion to give great strength, as in tendons, or they may be highly branched and disordered as in skin (Foegeding *et al.*, 1996).

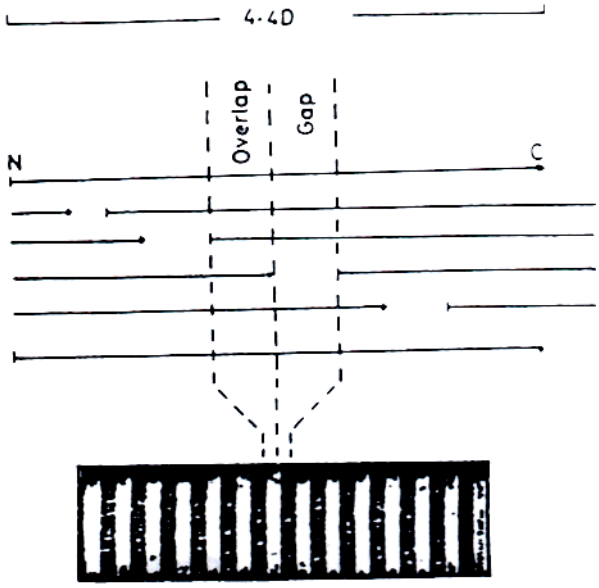

Figure 2 Illustration of the overlap structure of the collagen fiber responsible for the banding pattern of a negatively stained collagen fiber.

Source: Foegeding *et al.* (1996)

The amino acid composition of collagen is nutritionally unbalanced and is unusual in several other respects. Collagen is almost devoid of tryptophan; it is rich in glycine, hydroxyproline, and proline; and it is one of the few proteins that contains hydroxylysine. Glycine represents nearly 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 or so amino acid residues from the N-terminus and the first 10 or so from the C-terminus, with

these end portions being termed “telopeptides”. Collagen is the only protein that is rich in hydroxyproline (up to 10% in mammalian collagen); however, fish collagens contain less of this amino acid than do mammalian collagens. The presence of proline stabilizes the helix structure by preventing rotation of the N-C bond. Hydroxyproline also stabilizes the collagen molecule (Foegeding *et al.*, 1996). Because hydroxyproline is present in negligible amounts in other proteins, it is ofter used as a measure of the amount of collagen in food sample (Foegeding *et al.*, 1996).

The covalent cross-links involved in the β and γ components of collagen and the intermolecular cross-links between collagen molecules form spontaneously by the condensation of aldehyde groups. This may involve an aldol condensation-type reaction, or formation of a Schiff base when the aldehyde reacts with an amino group. When hydroxylysine reacts with hydroxylsine aldehyde, the reaction product undergoes an Amadori-type rearrangement to form a “keto” structure, hydroxylysino-5-keto-norleucine (Figure 3) (Foegeding *et al.*, 1996).

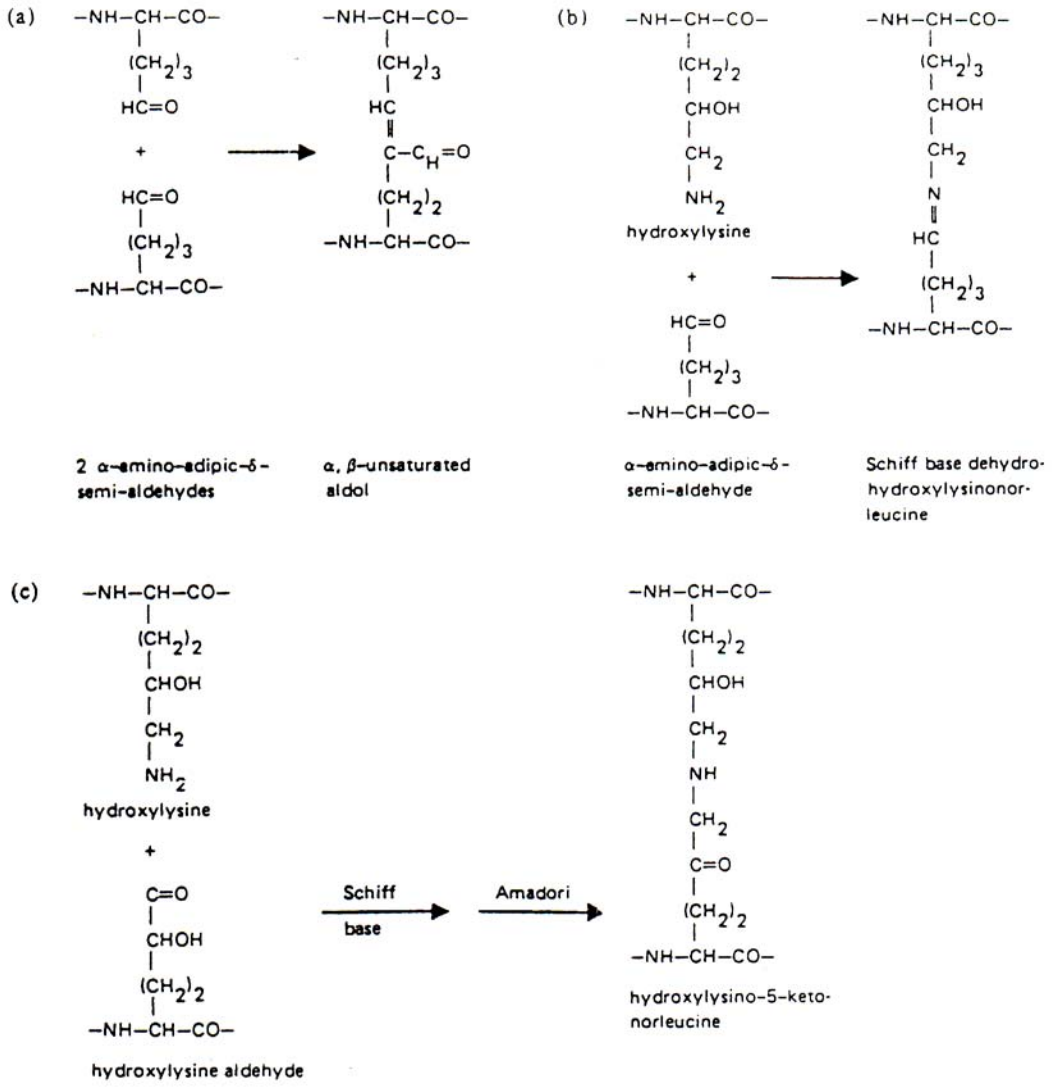

Figure 3 Cross-link formation in collagen by side chain groups.

(a) Aldol condensation followed by loss of water.

(b) Schiff base formation. Lysine reacts in a manner analogous to hydroxylysine.

(c) Schiff base formation followed by Amadori rearrangement.

Source: Foegending *et al.* (1996)

Cross-links in collagen increase with an increase in animal age. Furthermore, as the animal age increase, the collagen cross-links are converted from a reducible form to amore stable, nonreducible form. The extent of cross-links, as well as the exact composition and structure of collagen, are also dictated by animal species, breed, sex, and nutritional status (Xiong, 1997).

Collagen in marine animals

Collagen is the major connective tissue of muscle and is the most ubiquitous protein in the animal kingdom. Fish have much less collagen content than higher animals. The shrink temperature at which connective tissue undergoes a reversible elastic shrinkage leading to an organoleptic texture-toughening is lower for fish collagen. Normal cooking of fish melts its collagen, eliminating the need to be concerned with cooking methods that tenderize the connective tissue, a concern that often exists with red meats (Regenstein and Regenstein, 1991).

The content of collagen in the muscles depends upon the species as well as on the state of maturation and feeding of the fish. The collagen content of cod constitutes about 2% of the fish by weight (Regenstein and Regenstein, 1991). Fish species such as shark have as much as 10% of their body weight as connective tissue (Regenstein and Regenstein, 1991). Generally, the contents of collagen in fish muscles range from about 1 to 12% of the crude protein, i.e., 0.2 to 2.2% of the wet weight of the meat, and 1.7 to 4.6% of collagen nitrogen in the fish skin. Raw fish meat rich in collagen is tougher than that containing less collagen. The muscle tissues of some edible marine invertebrates contain somewhat larger amounts of collagen. In the skinned mantle and

skinned arms of squid *Illex argentinus*, collagen constitute 2 to 11% and 2 to 16% of true proteins, respectively (Sikorski *et al.*, 1990).

Fish 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). A characteristic property of shrimp collagen is a high content of tryptophan residues. The collagens of edible marine invertebrates are characteristic for a high content of carbohydrates. Some fish and invertebrates collagens also contain, besides the sugars listed in Table 3, small amounts of arabinose, xylose, and ribose residues. The carbohydrates are mainly linked O-glycosidically to hydroxylysine residues as glucosylgalactosylhydroxylysine 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 composition of muscle collagens of marine fish and invertebrates.

Amino acid	Residues per 1000 residues			
	Cod	Spuid	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)

Table 3 Main neutral sugars in collagens of marine fish and invertebrates.

Collagen source	Total sugar (%)	Glucose (%)	Galactose (%)	Mannose (%)	Fucose (%)
Bigeye tuna, skin	0.40	0.17	0.19	0.01	0.02
Carp, skin	0.43	0.18	0.20	0.01	0.02
Octopus, body wall	2.89	1.50	1.00	0.05	0.18
Squid, body wall	3.96	2.16	1.44	0.10	0.26
Abalone, foot part	4.18	2.19	1.32	0.13	0.48
Spiny lobster, subcuticular tissue	10.19	5.76	3.84	0.17	0.22
Blue crab, subcuticular tissue	12.45	6.90	4.82	0.52	0.21

Source: Kimura (1972)

Collagen in invertebrates

Collagens with different types and contents have been found in invertebrates. The presence of homotrimetic collagen molecules $(\alpha 1)_3$ has been demonstrated in the muscle of abalone *Haliotis discus* and top shell *Turbo cornutus*. On the other hand, bivalves have been shown to possess $(\alpha 1)_2\alpha 2$ heterotrimers, which have similar compositional features to vertebrate type I collagen. The byssus threads of sea mussel *Mytilus edulis* and the adductor muscles of pearl oyster *Pinctada fucata* contained heterotrimer collagen $(\alpha 1)_2\alpha 2$ (Yoshinaka and Mizuta, 1999).

Collagen in the mantle muscle of common squid *Todarodes pacificus* consisted of two genetically distinct types of pepsin-solubilized collagen. 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. Type SQ-I collagen is the major collagen in the cranial cartilage and skin of

common squid *Todarodes pacificus*, and in the skin and arm muscle of octopus *Octopus vulgaris*. In addition, similar collagens have been isolated from the sucker of cuttlefish and octopus. 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. Both type SQ-I and SQ-II collagens are heterotrimers, of which the subunit compositions are represented as $[\alpha1(SQ\text{-}I]_2\alpha2$ (SQ-I) and $[\alpha1(SQ\text{-}II]_2\alpha2$(SQ-II), respectively (Yoshinaka and Mizuta, 1999).

Octopus, *Callistoctopus arakawai* arm consisted of acid-solubilized collagen (ASC) and (PSC) at levels of 10.4 and 62.9%, respectively. The PSC had a chain composition of $\alpha1\alpha2\alpha3$ heterotrimer while the ASC showed only a single α-chain, α 1. The denaturation temperature of those collagens was lower than porcine collagen (Nagai *et al*., 2002b).

Nagai *et al.* (2000) found that rhizostomous jellyfish (*Rhopilema asamushi*) contained 35.2% collagen on the basis of the lyophilized dry weight. The primary structure was very similar to that of pepsin-solubilized collagen from edible jellyfish mesogloea, but it was different from those of the collagen from edible jellyfish exumbrella and the acid-soluble collagen from its mesogloea. This collagen contained a large amount of a fourth subunit, designated as $\alpha4$. This collagen may have the chain composition of an $\alpha1\alpha2\alpha3\alpha4$ heterotetramer. Mizuta *et al.* (1998) reported that collagen from muscle of antartic krill, *Euphausa superba*, referred to as $\alpha1$(Kr) component, constituted more than 80% of the total pepsin-solubilized collagen and showed typical compositional feature of crustacean major collagens, with low alanine and high hydroxylysine contents. The $\alpha1$(Kr) component was mainly distributed in

relatively thick connective tissues, epimysium and perimysium. These results suggest that the α1(Kr) component may functionally correspond to the major α component of collagen in decapods, α1(AR-I) (AR-I : Arthropod-type I), and comprise a major homotrimeric collagen molecule, [$\alpha1(AR\text{-}I)_3$].

Collagen in fish

Hassan and Mathew (1996) classified twenty species of commercially importanat fishes based on the percentage of total collagen on total proteins into three categories:

(1) The fishes having total collagen contents up to 5% of total proteins as low collagen fishes.

(2) The fishes having total collagen contents in the range of 5-10% of total proteins as medium collagen fishes.

(3) The fishes having total collagen contents above 10% of total proteins as high collagen fishes.

Muscle from different fish contained varying acid soluble collagen and in soluble collagen (Table 4). Total collagen content in the range of 0.30-2.99% of wet tissue with a corresponding range of 1.58-13.39% of the total proteins was shown in Table 4. This might contribute to the differences in textural properties among fish species.

Table 4 Collagen content in white muscle of fishes.

Common name	Acid soluble collagen (% of wet tissue)	Insoluble collagen (% of wet tissue)	Total collagen (% of wet tissue)	Total collagen (% of total protein)
White pomfret	0.19	0.11	0.30	1.58
Sardine	0.36	0.09	0.45	2.42
Mackerel	0.38	0.09	0.47	2.41
Sole	0.40	0.18	0.58	2.98
Vatta	0.50	0.08	0.58	2.89
Ribbon fish	0.12	0.51	0.63	2.87
White bait	0.09	0.60	0.69	3.82
Tilapia	0.47	0.22	0.69	3.85
Common carp	0.49	0.21	0.70	3.25
Rohu	0.28	0.46	0.74	3.92
Palankanni	0.38	0.41	0.79	4.36
Paral	0.66	0.31	0.97	5.36
Veloori	0.66	0.62	1.28	7.06
Kilimeen	0.09	1.01	1.10	5.69
Mullet	0.94	0.25	1.19	5.89
Catla	0.71	0.55	1.26	6.63
Tuna	1.06	0.39	1.45	7.35
Whiting	1.00	1.08	2.14	9.08
Ray	2.30	0.50	2.80	13.39
Shark	2.13	0.86	2.99	13.11

Source: Hassan and Mathew (1996)

The distribution of total collagen, acid-soluble collagen and crude protein in different tissues (white muscle, dark muscle, viscera, skin + scales, bones + fins) of 4 fish species including Japanese eel (*Anguilla japonica*); stone flounder (*Kareius bicoloratus*); red sea bream (*Pagrus major*); and chub mackerel (*Scomber japonicus*) was varied (Yoshinaka *et al*., 1990). Total collagen content of the whole body ranged from 3.26% for chub mackerel to 6.97% for Japanese eel. The acid-soluble collagen content in the total collagen of the whole body ranged from 13.1 (Japanese eel) to 56.6% (stone flounder). In all species, collagen solubility was relatively high in white muscle but was low in viscera. The skin + scale and bone + fin were the major source of total collagen (76.2-91.1%) (Yoshinaka *et al*., 1990).

Isolation of native acid-soluble collagen from fish muscle could be achieved by removing selectively non-collagenous proteins with a dilute NaOH solution. The extraction with 0.01 and 0.05 N NaOH did not remove satisfactorily non-collagenous proteins from carp muscle, whereas the extraction with 0.5 and 1.0 N NaOH modified the polypeptide chains of collagen, thus increasing the solubility of collagen (Sato *et al*., 1987). Therefore, extraction with 0.1 N NaOH is suggested for isolating native acid-soluble collagen from fish muscle.

The pepsin-solubilized collagen from wing muscle of skate (*Raja kenojei*) was separated by differential ammonium sulfate precipitation (Mizuta *et al*., 2002). The major collagen fraction contained $\alpha 1$ and $\alpha 2$ as well as the beta component. The minor collagen fraction contained $\alpha 1$, $\alpha 2$, and $\alpha 3$. Both major and minor collagen fractions were identified as type I and type V collagens, respectively.

Acid-solubilized collagen (ASC) and pepsin-solubilized collagen (PSC) from skin of ocellate puffer fish (*Takifugu rubripes*) were isolated and characterized by

Nagai *et al*. (2002a). The yields of ASC was low (10.7% on a dry weight basis) but that of PSC was very high (44.7% on a dry weight basis). ASC and PSC were a heterotrimer with a chain composition of $(\alpha1)_2\alpha2$. The patterns of peptide fragments were different from skin collagens of other species. The denaturation temperature was 28 ^{O}C, about 9 ^{O}C lower than that of porcine skin collagen. PSC contained glycine as an abundant amino acid and there were relatively high contents of alanine, proline, and glutamic acid.

Nagai and Suzuki (2000a) studies isolated type I collagen from skin, bone and fin of different fish species. The different yields were obtained among the fish portions and species. The denaturation temperatures of these collagens were as follows: skin collagen (25.0–26.5^{O}C), bone collagen (29.5–30.0^{O}C) and fin collagen (28.0–29.1^{O}C). These values were 7–12 ^{O}C lower than that of porcine skin collagen. Skin collagen of Janpanese sea-bass and bullhead shark comprised two different α chains, α1 and α2 but chub mackerel skin collagen showed only a single α chain, α1. Bone collagen of Japanese sea-bass, skipjack tuna, and ayu consisted of two different α chains, α1 and α2 but yellow sea bream and horse mackerel collagens showed only a single α chain, α1. Fin collagen of Japanese seabass had only a single α chain, α1.

The factors affecting collagen properties

There are many factors, which can affect the properties of collagen. Both intrinsic and extrinsic factors have been reported to influence the collagen properties as follows:

1. age of animals

The age of the animal influences the connective tissue and its basic protein quantitatively and qualitatively. Modification of collagen with age via cross-linking increases the toughness of meat. The number of cross-links in collagen increased with increasing age of the animal and this may explain why meat from older animals is tougher than that from younger animals (Zayes, 1997). However, most connective tissue in fish is renewed annually and highly cross-linked protein is not generally found in fish (Foegeding, *et al*., 1996). Generally, muscle from younger animals contains more collagen, however this collagen does not contribute to meat toughness as in the tissue of older animals. Collagenous tissue from older animals with more cross-linkages would be expected to be more resistant to swelling and have a lower water holding capacity (Zayes, 1997). Collagen from young animals is more easily solubilized but produces structures with low tensile strength. In contrast, collagen from old animals is difficult to solubilize and produces a structure with high tensile strength (Miller *et al*., 1983).

2. Starvation

In some species of fish, the amount of collagen and the amount of cross-linking of the collagen increases with starvation. Thus the toughening process in fish seems to be much more reversible than it is in higher animals, where the amount of cross-linking increases with age (Regenstein and Regenstein, 1991). Foegeding *et al*. (1996) reported that starving fish produce more collagen, especially collagen with a greater degree of cross-linking than do fish that are well fed. Love *et al*. (1976) reported that myocommata are thickened with more intermolecular cross-links of collagen during starvation.

3. imino acid content

The thermal stability of collagen is related to the content of imino acids (proline and hydroxyproline). The higher the imino acid content, the more stable the helices are (Wong, 1989). Collagens that contain small concentrations of imino acids denature at lower temperatures than do those with large concentrations (Figure 4). The imino acid content of fish collagens correlates with the water temperature of their normal habitat and is associated with their thermal stability. Proteoglycan and glycoprotein contents can also affect collagen thermal stability (Foegeding *et al.*, 1996).

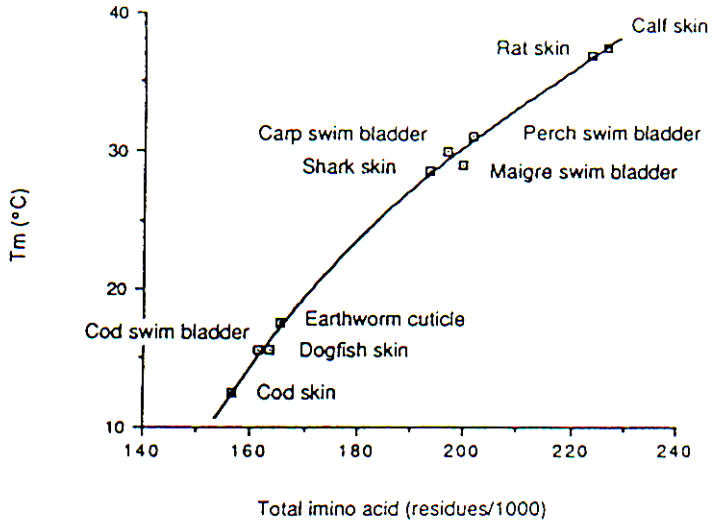

Figure 4 Relationship between the total imino acid content (proline + hydroxyproline) and the molecular melting temperatures (T_m) of various collagens from different species.

Source: Foegending *et al.* (1996)

4. pH and salt

Protein solubility in 0.5 M acetic acid, apparent viscosity and water-binding capacity of the collagenous material from skin and muscle of hake (*Merluccius merluccius*) were influenced by pH and the ionic strength of the medium (Montero, *et al*., 1999). Skin collagenous material showed higher functionality than muscle collagenous material. Maximum protein solubility, apparent viscosity and water-binding capacity were obtained at pH levels between 2 and 4, and with NaCl at concentrations of less than 0.25 M.

Emulsifying capacity of collagen from skins of hake and trout decreased as collagen concentration increased (Montero and Borderias, 1991). Emulsifying capacity decreased as the NaCl concentration increased and was highest at pH levels of between 1 and 3. Emulsifying capacity was higher in the collagen from hake than from trout, and was higher in muscle connective tissue than in dermal connective tissue.

5. Processing

Montero *et al.* (1995) compared 4 stabilizing methods: 1.) freezing, 2.) freeze-drying, 3.) partial solubilization with 0.05 M acetic acid then freezing and 4.) partial solubilization with 0.05 M acetic acid then 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 was observed in samples which were previously solubilized.

Gelatin

Gelatin is a substantially pure protein food ingredient, obtained by the thermal denaturation of collagen (Baily and Light, 1989). 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).

Conversion of collagen to gelatin

Collagen fibrils shrink to less than one-third their original length at a critical temperature, known as the shrinkage temperature. This shrinkage involves a disassembly of fibers and a collapse of the triple-helical arrangement of polypeptide subunits in the collagen molecule. The midpoint of the collagen-to-gelatin transition is defined as the melting temperature. During the collagen to gelatin transition, many noncovalent bonds are broken along with some covalent inter-and intramolecular bonds (Schiff base and aldo condensation bonds) and a few peptide 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. If the latter event happens, glue instead of gelatin is produced (Foegeding *et al.*, 1996).

Production of gelatin

Conversion of collagen into gelatin involves three steps involving 1.) pretreatment processes, the removal of noncollagenous components from the stock (skin and bones), 2.) extraction processes, the conversion of collagen to gelatin by

heating in the presence of water, 3.) recovery of gelatin in the final form (Johnston-Banks, 1990; Foegeding *et al*., 1996).

1. Pretreatment processes

To convert insoluble collagen into soluble gelatin, two processes are in current use (Johnston-Banks, 1990):

1.1 Acid process or type A gelatin

Acid hydrolysis is a milder treatment that will effectively solubilized collagens of animals slaughtered at a young age, such as pigs (Foegeding *et al*., 1996). The pretreatments process is designed to convert the collagen into a form suitable for extraction. A sufficient number of the covalent cross-links in the collagen must be broken in order to enable the release of free α-chains. The process is also designed to remove other organic substances, such as proteoglycan, blood, mucins, sugars, etc., that also occur naturally in the raw material. It is optimized by each manufacturer to give the required physical and chemical properties to the gelatins that are produced (Johnston-Banks, 1990).

Normally, 18-24 h soak in dilute acid is sufficient to bring about the conversion. Sulphuric and hydrochloric acids are used, often with the addition of phosphoric acid to retard color development (Johnston-Banks, 1990).

1.2 Alkaline process or type B gelatin

Type B gelatins are produced by alkali hydrolysis of beef materials and results in deamidation and a greater range of molecular weight species (Foegeding *et al*., 1996). Alkaline pretreatment processes are normally applied to bovine hide and ossein. Lime is most commonly used for this purpose; it is relatively mild and does not cause significant damage to the raw material by excessive hydrolysis.

Unfortunately, 8 weeks or more are required for complete treatment. Concentrations of up to 3% lime are used in conjunction with small amounts of calcium chloride or caustic soda. Frequent renewal of the liquors is practiced in order to remove extracted impurities and to maintain the degree of alkalinity present. If caustic soda is used, a 10-14 day pretreatment is possible (Johnston-Banks, 1984).

2. Extraction

The conversion of pretreated raw material into gelatin takes place in five basic stages: (I) washing, (II) extraction, (III) purification, (IV) concentration and (V) drying (Johnston-Banks, 1990).

The extraction process is designed to obtain the maximum yield in combination with the most economic of physical properties, i.e. to optimize the balance between pH, temperature and the extraction time (Figure 6) (Saunders and Ward, 1955).

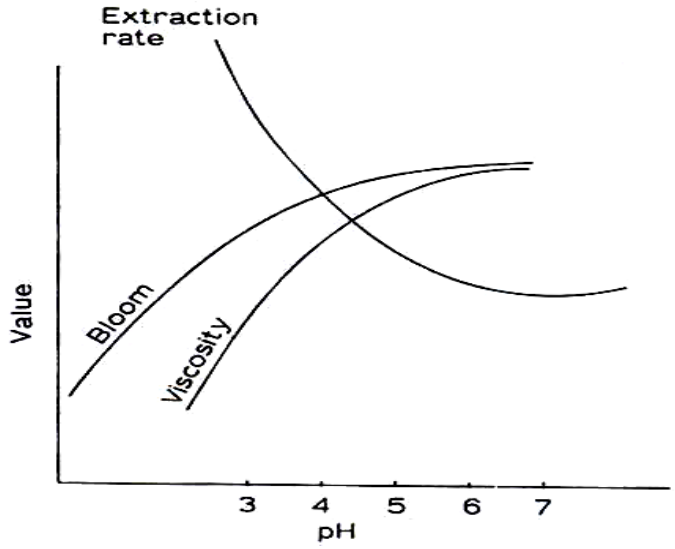

Figure 5 Factors determining optimum gelatin extraction conditions.

Source: Johnston-Banks (1990)

The pH of extraction can be selected either for the maximum extraction rate (low pH) or for the maximum physical properties (neutral pH). To extract older collagens at neutral pH, a substantial proportion of the cross-links need to be cleaved, necessitating a longer liming pretreatment. If shorter liming times are used, then a lower extraction pH is necessary in order to achieve acceptable conversion rates. However, owing to the acidity present, the resultant gelatins will have lower viscosities (lower molecular weight) than those extracted at neutral pH. More efficient pretreatment conditions also allow the manufacturer to use lower extraction temperatures, resulting in gelatins of greater gel strength (bloom). Shorter treatments generally require higher extraction temperatures if neutral pH levels are chosen, resulting in gelatins of lower gel strength (Johnston-Banks, 1990).

Following extraction, the gelatins are filtered to remove suspended insolubles such as fat or unextracted collagen fibres. This is usually performed using materials such as diatomaceous earth to give solutions of high clarity. The final stage is evaporation, sterilization and drying. These are performed as quickly as possible to minimize loss of properties (Johnston-Banks, 1990).

Composition of gelatin

Gelatin is a heterogeneous mixture of water-soluble proteins of high molecular weight (Budavari, 1996). On a dry weight basis, gelatin consists of 98 to 99% protein. The molecular weight of these large protein structures typically ranges between 20,000 and 250,000 (Kennan, 1994). However, some aggregates weigh in the millions (Poppe, 1997).

Coils of amino acids are joined together by peptide bonds. The predominant amino acid sequence is Gly-Pro-Hyp (Poppe, 1997). As a result, gelatin contains relatively high levels of these following amino acids: glycine (Gly) 26-34%; proline (Pro) 10-18%; and hydroxy proline (Hyp) 7-15% (Veis, 1964; Poppe, 1997). Other significant amino acids include alanine (Ala) 8-11%; arginine (Arg) 8-9%; aspartic acid (Asp) 6-7%; and glutamic acid (Glu) 10-12%. (Hudson, 1994; Poppe, 1997).

Gelatin is not a nutritionally complete protein. It contains no tryptophan and is deficient in isoleucine, threonine, and methionine (Potter and Hotchkiss, 1998). The other sulfur-containing amino acids, cysteine and cystine, are deficient or absent. Water varies between 6 and 9% (Alais, 1991; US FDA, 1997), which ash content ranges from 0.1 to 3.25% (Veis, 1964).

Gelatin structure

1. Primary structure

The primary structure of gelatin closely resembles the parent collagen. Small differences are due to raw material sources together with pretreatment and extraction procedures. These can be summarized as follows (Johnston-Banks, 1990).

1.1 Partial removal of amide groups of asparagines and glutamine, resulting in an increase in the aspartic and glutamic acid content. This increases the number of carboxyl groups in the gelatin molecule and thus lowers the isoelectric point. The degree of conversion is related to the severity of the pretreatment process.

1.2 Conversion of arginine to ornithine in more prolonged treatments experienced during long liming processes. This takes place by removal of a urea group from the arginine side-chain.

1.3 There is a tendency for trace amino acids, such as cysteine, tyrosine, isoleucine, serine, etc., to be found in lower proportions than in their parent collagens. This is due to the inevitable removal of some telopeptide during cross-link cleavage, which is then lost in the pretreatment solutions.

2. Secondary structure

Gelatin is not completely polydispersed, but has a definite molecular weight distribution pattern corresponding to the α-chain and its oligomers. One to eight oligomers may be detected in solution, but it is possible that higher numbers exist. Doublets, known as β-chains, are formed from both α1- and α2-chains, giving rise to β11- and β12-molecules. Oligomers of three α-chains will mainly exist as intact triple helices, but a certain proportion will exist as extended α-polymers bonded randomly by end-to-end or side-to-side bonds. The structure of oligomers of greater than four α-chain units obviously becomes increasingly more complex and difficult to interpret.

Molecular-weight spectra normally relate with physical properties of gelatin (Lorry and Verdins, 1983; Tomka, 1979; Bohonek *et al*., 1976). Gelatin structural components are shown in Figure 5.

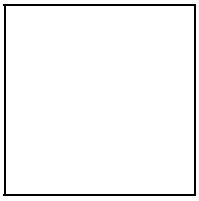

Figure 6 Molecular weight distribution showing the major structural components of gelatin.

Molecular fraction	Description
Q	Very high molecular weights, of 15-20 x 10^6 daltons and thought to be branched in character owing to their inability

	to penetrate the gel successfully.
1-4	Oligomers of α-chains, levels of five to eight.
X	Oligomers of four α-chains.
γ	285,000 daltons, i.e. 3 x α-chain.
β	190,000 daltons, i.e. 2 x α-chain.
α	95,000 daltons.
A-peptide	86,000 daltons.
α-, β- and γ-peptides	Seen as tailing their parent peaks.

Source: Johnston-Banks (1990)

Differences can be detected between commercial gelatin from the different raw materials. In general, the sum of the α- and β-fractions, together with their larger peptides, is proportional to the bloom strength, and the percentage of higher molecular weight material is related with the viscosity. The setting time is increased for the peptide fractions below α-chain, but a certain proportion of the very high molecular-weight "Q" fraction can reduce the setting time markedly. The melting point also increases in line with higher molecular weight content.

Isoelectric point of gelatin

The isoelectric point distribution of gelatin is dependent on the type of pretreatment applied during manufacture. Type A or acid-processed gelatins have isoelectric points that can vary from 6.5 to 9.0. Type B or lime or alkaline-process gelatins have isoelectric points over a narrower pH range, typically 4.8-5.0 (Johnston-Banks, 1990; Foegeding *et al.*, 1996). Generally, acid processed gelatins have higher isoelectirc point than alkali processed gelatins (Poppe, 1997). These differences are

caused by side-reactions occurring during the pretreatment process (Johnston-Banks, 1990).

The Mechanism of gelation

Collagen denatures at temperatures above 40 ^{O}C to a mixture of random-coil single, double, and triple strands. Upon controlled cooling below the melting temperature, T_m, the reformation of the helical form occurs (Wong, 1989). The energy barrier for refolding is ~ 4 KJ/mole. The initial refolding is rapid and involves the –Gly-I-I- regions of the polypeptide chain, forming a single turn of a left-handed helix. This "nucleation" along the polypeptide chain is structurally stabilized by a certain type of water bridging. The "nucleated" polypeptide then (1) folds back into loops, with the nucleated regions aligned to form triple strands, or (2) has its nucleated region aligned with that of the other nucleated polypeptide chain (Figure 7). At high enough concentrations, interchain alignment becomes possible and association of polypeptide chains to form triple-helical collagen molecules can occur (Wong, 1989).

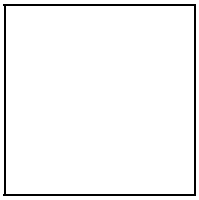

Figure 7 Scheme for the concentration and temperature-dependent pathways for helix formation in α chains derived from collagen.

Source: Wong (1989)

In both cases, once the nucleated regions are aligned, the remainders of the chain start renaturation. The rate of renaturation depends on the cooling temperature. Rapid cooling with large ΔT would cause rapid renaturation, resulting in areas unavailable for the formation of helical structures. As a consequence, denatured collagen with various degrees of perfection is obtained (Wong, 1989).

Factors affecting the properties of gelatin gels

1. Concentration

The exact relationship between concentration and gel strength depends on the type and origin of the gelatin itself and were fitted to the power law expressed as the following equation (Jonhston-Bank, 1990; Ferry, 1948):

$$b = kC^n$$

where b is the bloom strength at a concentration tested, k is the proportionality constant, C is total gelatin concentration (w/w) and n varied for each gelatin, is equal to 1.7 for high bloom gelatin and 1.8-1.9 for lower strength gelatins.

Choi and Regenstein (2000) reported that the bloom strength of fish and pork gelatins increased with increasing concentration (Figure 8).

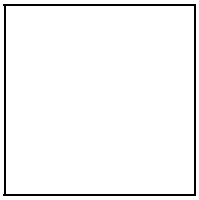

Figure 8 Gel strength of seven different commercial fish and pork gelatins as a function of concentration.

Source: Choi and Regenstein (2000)

2. pH

The gel strength is seriously affected by the solution pH only, especially at the extreme pHs. The pHs ranging from 4 to 9 do not affect the gel strength to any significant extent (Figure 9). Dilute gels (< 2%) are more affected, while stronger gels (> 10%) are relatively insensitive to pH (Johnston-Banks, 1990).

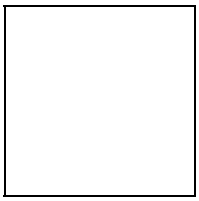

Figure 9 Effect of the solution pH on the bloom strength of gelatin.

Source: Jonhston-Banks (1990)

3. Time and temperature of set

The gel strength will depend on the time and temperature of set (Figure 10). The gel strength decrease linearly with increasing maturation temperature (Choi and Regenstein, 2000). This will vary from gelatin to gelatin and is also dependent on the proportions of molecular fractions present and thus the viscosity (Johnston-Banks, 1990).

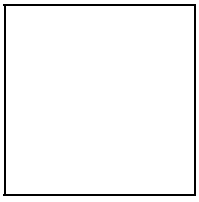

Figure 10 Variation in bloom strength (at 6.67% (w/v) concentration) with temperature of maturation for 275-bloom (curve A) and 175-bloom (curve B) gelatins.

Source: Jonhston-Banks (1990)

4. Low molecular weight compounds

The effect of other dissolved ingredients in the gel can either weaken the gel or strengthen it. Most simple sugars, glycerol and other no-electrolytes can contribute to an increase in gel strength (fructose and sorbitol being an exception), while the addition of most electrolytes has the opposite effect (Johnston-Banks, 1990).

5. Processing conditions

Gelatin will degrade and lose its gelling properties when subjected to conditions of heat, extremes of pH and exposure to enzyme attack. The differing mechanisms for acid and alkali hydrolysis can be seen in Figure 11, in which the hydrolysis is plotted against the holding time and percentage loss in bloom strength (Johnston-Banks, 1990).

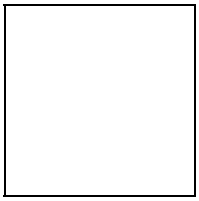

Figure 11 Effect of pH on the thermal degradation of gelatin at 70 °C.

Source: Jonhston-Banks (1990)

Acid hydrolysis is tends to break cross-links between the chains; the peptide bonds are attacked less often. Alkaline hydrolysis, however, tends to give a wider

pattern of cleavage, preferring the more numerous peptide bonds rather than selecting the chain cross-links. This has the effect of reducing the α- and β-chain size very quickly and it is interesting to note that the minimum point in Figure 12 is not at pH 7 as may perhaps be expected but at a pH of 5.5-6. This stability of the gel strength to acid conditions explains the success of acid extraction methods (Johnston-Banks, 1990).

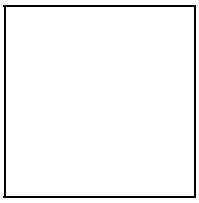

Figure 12 Effect of pH on the degradation of gelatin.

Source: Jonhston-Banks (1990)

Production and characterization of fish gelatins

In 1960, fish gelatin was extracted commercially in Nova Scotia by Kenney and Ross at Port Saxon. The raw material was the skin from deep water fish such as cod, haddock and pollock, and was obtained from local salt fish and frozen fish processors. The uniqueness of fish gelatin lies in the amino acid content of the gelatin. Although all gelatins are composed of the same 20 amino acids, there can be a variation in the amount of imino acids, proline and hydroxyproline. With lower amounts of these imino acids, there is less hydrogen bonding of gelatin in water solutions, and hence a reduction in the gelling temperature (Norland, 1990). Gelatin derived from the skin of deep cold water fish has lower amounts of proline and hydroxyproline. As a result, collagen solutions will not gel at room temperature, and will remain liquid at temperature above 8 to 10 ^{O}C (Norland, 1990).

Commercial extraction of gelatin depends upon both dissolving and hydrolysing the denatured skin. The gelatin may retain some covalent bonds between alpha chains, which would entail multiples of the single alpha chain length of 95,000 Daltons. There are also major proportions of shorter chain polypeptides in the gelatin, as the chain is cleaved in the extraction process. This is not necessarily a problem, as the end product may not need very high molecular fractions in order to accomplish the specific application (Norland, 1990).

Gudmundsson and Hafsteinsson (1997) reported that concentrations of sodium hydroxide, sulfuric and citric acids used for gelatin extraction from cod skins affected both yield and properties. The highest yield of gelatin was obtained when low concentrations [0.1–0.2 %(w/v)] of sulfuric acid and sodium hydroxide were used, followed by treatment with 0.7%(w/v) citric acid. Bloom value, viscosity, odor, clarity, color, and pH of the gelatin varied with treatments. However, the use of 0.7 %(w/v) citric acid in combinations with sodium hydroxide and sulfuric acid usually gave best result. Freeze-dried gelatin had considerably greater bloom value than air-dried gelatin.

Montero and Gomez Guillen (2000) reported that the gelatin extracting conditions for mergrim skins affected the functional properties of the resulting gelatin. Physical properties of gelatins were influenced more by extracting conditions than by imino acid composition. A high-quality, readily-dissolved gelatin was prepared from megrim skins using a pretreatment of the skins with NaCl and dilute NaOH, then swelling with 0.05 M acetic acid followed by an extraction step in water at 45 ^{O}C.

Moreover, type of organic acids used influenced the gelatin viscoelastic and gelling properties of gelatin from mergrim skins (Gomez-Guillen and Montero, 2001).

Acetic acid and propionic acid extracts gave the gelatins with the highest elastic modulus, viscous modulus, melting temperature, and gel strength, especially when skins were previously treated with dilute NaOH. After such treatment, lactic acid was also shown to be suitable for collagen or gelatin extraction. The lowest degree of turbidity was achieved by using citric acid, whereas propionic acid led to the most turbid gelatin.

The gelatins from both the black and the red tilapia skins were snowy white, shiny and light-textured in appearance. The gelatin of black tilapia skin had a strong fishy odor while that of the red tilapia skin had a barely detectable odor. Their pH values were in the vicinity of 3. The bloom strength of gelatin from black tilapia skin was higher (180.8 g) than that from red tilapia skin (128.1 g). The black tilapia skin gelatin was also significantly more viscous, had a higher melting point, and had a higher total amino acid content (Jamilah and Harvinder, 2002).

Gomez-Guillen *et al.* (2002) studied structural and physical properties of gelatin extracted from the skins of different marine species. Gelatins from flat-fish species (sole and megrim) had the best gelling ability and the gels were more thermostable than those from cold-adapted fish (cod and hake). This different behaviour was probably owing to the differences in amino acid composition, the $\alpha 1/\alpha 2$ collagen-chain ratio, and the molecular weight distribution. Cod gelatin contained a lower alanine and imino acid content. Cod and hake gelatins comprised a low $\alpha 1/\alpha 2$ ratio (~1) (Figure.13, land c and d and Figure.14A). Hake gelatin showed the low contents of β-components and other aggregates (Figure.13 and Figure.14B). The squid gelatin had the marked differences in the amino acid composition and molecular weight distribution, arising from the low solubility of the squid connective tissue.

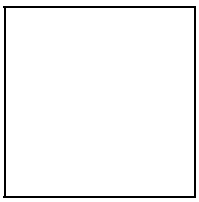

Figure 13 Protein patterns of the gelatin from sole (a), megrim (b), cod (c), hake (d), and squid (e).

Source: Gomez-Guillen *et al.* (2002)

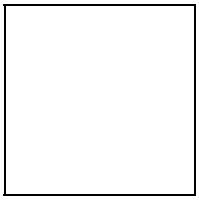

Figure 14 The $\alpha 1/\alpha 2$ ratio (A) and relative amount of molecular weight components (B) of gelatin from fish skins.

Source: Gomez-Guillen *et al.* (2002)

The gelatin gel could be improved by addition of cross-linking enzyme or some chemicals. The gelatin from hake skin had the highest bloom strength with addition of 10 mg transglutaminase/g, whereas magnesium sulphate (0.1 and 0.5 M) was effective in improving the bloom strength of gelatin from cod skin (Fernandez-Diaz *et al*., 2001).

Uses of collagen and gelatin

Collagen has the wide range of applications in leather and film industry. It can also be used for making surgical aids like sutures, cargil membranes, sponges, etc (Hassan and Sherief, 1994). The collagenous material can be also used as a functional ingredient in fish products or other food dishes (Montero and Borderias, 1989).

Gelatin is widely used as an ingredient in the food industry owing to the following functional properties (Johnston-Banks, 1990).

1. It forms high quality gels in dilute solution with typical clean melt-in-the-mouth textures.

2. It forms elastic gum-type textures in the concentrated gel, slowly dissolving in the mouth.

3. It produces emulsification and stabilization of immiscible liquid-liquid, liquid-air or liquid-solid mixtures.

4. In the dilute solutions, it acts as a polyelectrolyte that will flocculate suspended particles or unstable colloids.

5. In a mixture with other powdered ingredients, usually as a minor component, gelatin acts as an efficient tableting aid and binder.

Gelatin has a considerable number of applications and uses (Hudson, 1994; Keenan, 1994; Cole, 2000; Poppe, 1997; Ledward, 2000). Gelatin plays a role as a gelling agent. Some gelatins are used as food gelatins, and others play an important role in industry (film emulsion, glue manufacturing) (Burghagen, 1999). Gelatin is used as a fining agent for a beverage (Vine *et al*., 1999) and fruit and vegetable juice (Tressler and Joslyn, 1954; Peterson and Johnson, 1978; Lee and Lee, 1999). Gelatin is also used as stabilizer, thickener and texturizer in desserts, ham coatings, confectionery and capsules (vitamin supplements) (Igoe, 1983), fruit toppings, instant gravy, instant sauces and soups, edible films for confectionery products (McCormick, 1987), ice cream, cream cheese, cottage cheese, food foams and fruit salads (McWilliams, 2001).

Gelatin-based glues are also used as adhesives to put those little 'organic' stickers on fruits and vegetables. Gelatin is also used in prepared meat products such as canned ham, luncheon meats, turkey, and chicken rolls where it helps to maintain consistency and moisture (Rose, 1990).

Gelatin capsules (gel-caps) are commonly used to encapsulate various foods, nutritional supplements, and medicines. Various forms of gelatin are common excipients in pharmaceutical formulations, including vaccines, and are used as a binder for tablets (Ash and Ash, 1995). Gelatin is used in textile industry as a sizing, coating, dressing, or finishing agent for cotton, leather, silk, and wool (Naghski, 1982).

Objectives

1. To isolate and characterize acid soluble collagen from the skin and bone of bigeye snapper (*Priacanthus tayenus*).
2. To study extracting conditions and to characterize the gelatin from skin and bone of bigeye snapper (*Priacanthus tayenus*).
3. To improve property of gelatin gel from the skin of bigeye snapper by using magnesium sulfate or microbial transglutaminase.