# CHAPTER 1

# INTRODUCTION

Bigeye snapper (*Priacanthus tayenus*) is one of the raw materials commonly used for surimi production in Thailand (Benjakul *et al.*, 2003c; Kittiphatanabawon *et al.*, 2005). Surimi and surimi product of Thailand has been exported to several countries, especially to USA with the volume of 1,753 metric tons and a value of 2,417 million US dollars in 2004 (Department of Export Promotion, 2005). During surimi manufacturing, byproducts such as head, viscera, skin and bone are generated in the large quantity. These solid wastes constitute 50-70% of original raw material, depending on the process used (Morrissey *et al.*, 2000; Kim & Park, 2004). Surimi byproducts have been mostly sold for animal feed and fertilizer production with low value. Nevertheless, fish skin contains the large amount of collagen. Nagai and Suzuki (2000) 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% (dry basis) respectively.

Collagen is the major structural protein in connective tissue of animal skin and bone (Foegeding *et al.*, 1996; Cho *et al.*, 2004; Kołodziejska *et al.*, 2004). Generally, collagen has wide range applications in cosmetics, biomedical, pharmaceutical, leather and film industry (Slade and Levine, 1987; Ogawa *et al.*, 2004). Gelatin is a denatured protein derived from collagen by thermal hydrolysis (Foegeding *et al.*, 1996). In food industries, gelatin have been widely used as a beverage clarifier, a thickener in dessert, a texturizer in confectionary and a stabilizer in ice cream, cream cheese and cottage cheese as well as in food foams and fruit salad, etc (OMRI, 2002). Moreover, gelatin has been implemented in photographic, pharmaceutical, cosmetic, biomedical materials and biomaterial-based packaging (Segtnan *et al.*, 2003; Jongjaroenrak *et al.*, 2005a; Cho *et al.*, 2004). By the year 2004, Thailand lost more than 359 million bahts for gelatin importation (The Customs Department, 2005).

In general, traditional sources of collagen and gelatin are bovine and porcine skins and bones. Porcine collagen and gelatin have the objection for some religions as Judaism and Islam, while those from bovine sources are at risk for contamination with bovine spongiform encephalopathy (BSE) (Choi & Regenstein, 2000; Fernandez-Diaz *et al.*, 2001). Therefore, fish

skin as well as byproduct from fish processing is an important source for collagen and gelatin production as replacement for mammalian sources. Normally, a low yield of collagen and gelatin is obtained with the traditional process. Since pepsin has been reported to cleave peptides in telopeptide region, the yield of partially cleaved collagen can be augmented (Nagai *et al.*, 2001; Nagai & Suzuki, 2002; Jongjaroenrak *et al.*, 2005b). Due to a plenty of fish viscera, especially stomach, pepsin from fish origin can be recovered and used to increase the extraction efficiency of collagen and gelatin. Additionally, the expensive for commercial pepsin can be replaced, leading to the lower price of collagen and gelatin obtained. Therefore, the objective of this study was to characterize fish pepsin and to use fish pepsin for collagen and gelatin extraction from bigeye snapper (*P. tayenus*) skin, which is one of the abundant fish species used for surimi production in Thailand.

## Literature review

### 1. Pepsin

Pepsin is a gastric aspartic proteinase, a class of endopeptidases active at acid conditions, which plays an integral role in the digestion process of vertebrates (Gildberg, 1988; Tello-Solís and Romero-García, 2001). Pepsin is composed of a single polypeptide chain of 321 amino acids and has a molecular weight of 35,500 dalton. Its tertiary structure is stabilized in part by three disulfide bridges and a phosphate linkage. A phosphate group, attached to the hydroxyl group of a seryl residue, can be removed without loss of enzymatic activity (Whitaker, 1994). The enzyme is quite stable from pH 2 to about 5, but above pH 5 it rapidly loses activity due to the denaturation. The pH optimum of pepsin on proteins is about pH 2, but on synthetic substrates it is around pH 4.0 (Whitaker, 1994). Pepsin has its primary specificity toward the amino acid residue that furnishes the NH group to the susceptible peptide bond, preferably phenylalanyl, tyrosyl, or tryptophanyl. Pepsin and other aspartic proteases readily catalyze transpeptidation in the presence of suitable reactants (Whitaker, 1994).

### 1.1 Pepsin from pepsinogen

In general, the inactive protein pepsinogen is produced by the chief cells of the oxyntic glands, which are located in the stomach wall epithelium and secreted into the stomach (Whitaker, 1994; Gildberg, 1988). The zymogen is converted to the active pepsin in the presence of HCl.

Pepsinogen has a molecular weight of 42,000 dalton and contains three disulfide bonds. It has an isoelectric point of 3.7. It is quite stable at pH 7 to 9, but at low pH it is rapidly converted to pepsin (Whitaker, 1994). Six peptide bonds are hydrolyzed in the initial conversion of pepsinogen to pepsin (Figure 1). The large peptide labeled B remains attached by noncovalent bonds to pepsin at higher pH values and is inhibitory. It readily dissociates from pepsin at pH 1 to 2 (Whitaker, 1994). Pepsin continues to act on the last large peptide, B, to produce three more bond cleavages (bonds cleaved indicated by lower case p). Pepsin is produced only when the bond (double-underlined P in Figure 1) is hydrolyzed. Pepsin is quite resistant to further proteolysis in acid solution, but at pH 5 to 7 it is rapidly inactivated, in contrast to pepsinogen. During conversion of pepsinogen to pepsin, the molecular weight decreases from 42,000 to 35,500 dalton and the isoelectric point changes from 3.7 to less than 1 (Whitaker, 1994). Furthermore, pepsinogen from swine has 363 amino acid residues, whereas pepsin consists of 321 amino acid residues. In the activation process, 42 residues, including 9 of lysine, 2 of histidine, and 2 of arginine, are cleaved off (Rajagopalan *et al.*, 1966). The decrease in isoelectric point is due to loss of 9 of the 10 lysyl residues, 2 of the 3 histidyl residues, and 2 of the 4 arginyl residues during activation (Whitaker, 1994). Bohak (1973) found that the conversion of chicken pepsinogen to chicken pepsin was observed in pH range of 2-4. In this pH range the zymogen undergoes a rapid conformational change to form an intermediate, and the subsequent conversion of this intermediate to the active enzyme is the rate-determining step (Bohak, 1973).

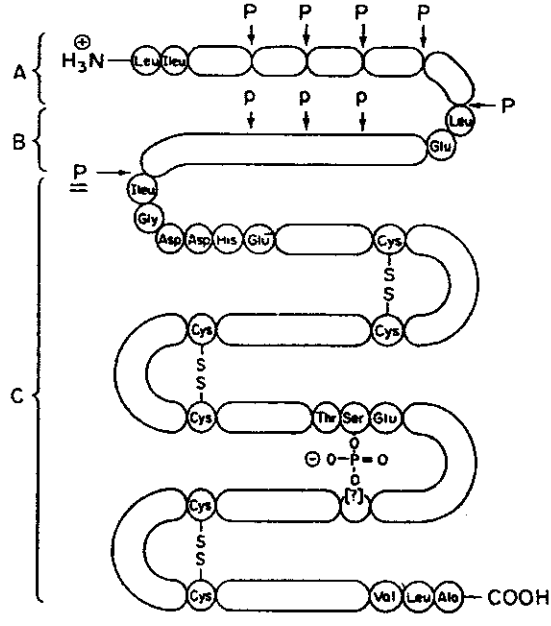


Figure 1 Schematic representation of the structure of pepsinogen and its conversion to pepsin. The major points of hydrolysis are marked with P and result in release of several peptides (A), pepsin inhibitor (B), and pepsin (C). Hydrolysis of the bond P is essential for activation.

Source: Whitaker (1994)

### 1.2 Catalytic mechanism of pepsin

A proposed mechanism, involving nucleophilic catalysis, for the action of pepsin is shown in Figure 2. The free enzyme has two carboxyl groups, one in the protonated form and the other in the ionized form, in the transforming locus of the active site. The enzyme-substrate adsorptive complex is formed, followed by a nucleophilic attack of the carboxylate group on the carbonyl group of the peptide bond. This leads to the formation of a covalent tetrahedral intermediate (Whitaker, 1994).

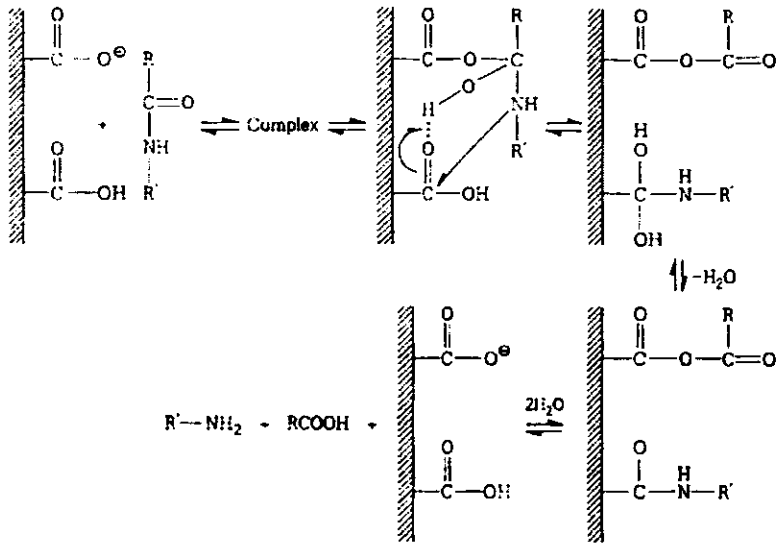


Figure 2 Catalytic mechanism of pepsin

Source: Whitaker (1994)

The carbonyl oxygen of the protonated carboxyl group extracts a proton from the hydroxyl group, facilitating an electrophilic attack of the carbonyl carbon on the NH group of the peptide bond. The result is the formation of an aminoacylenzyme intermediate, which then reacts with water to give the products of the reaction (Whitaker, 1994).

**1.3 Fish pepsin**

The pepsins from marine animals were reported to have molecular weights ranging from 27 to 42 kDa (Gilgberg and Raa, 1983; Squires *et al.*, 1986; Arunchalam and hard, 1985; Noda and Murakami, 1981). They had relatively higher pH optima for the hydrolysis of their substrates than did mammalian pepsins, and were generally more stable at relatively higher pH values (pH 2.0-4.0) (Gilgberg and Raa, 1983; Squires *et al.*, 1986; Arunchalam and hard, 1985; Noda and Murakami, 1981). While pepsins are unstable under alkaline conditions, their zymogens (pepsinogens) may be stable under these conditions. Pepsins from the digestive glands of marine animals are quite resistant to autolysis at low pH (Raa, 1990) However, marine pepsins were inhibited by pepstatin in a similar fashion to mammalian pepsin (Noda and Murakami, 1981; Sanchez-Chiang *et al.*, 1987). Pepsins from marine animals display a wide temperature optima range for the hydrolysis of their substrates (37-55$^{\circ}$C). In general, the colder-water fish pepsins

had lower temperature optima and were more heat-labile than pepsins of animals from the warmer environment (Gilgberg and Raa, 1983; Squires *et al.*, 1986; Arunchalam and hard, 1985).

Pepsins and pepsin-like enzymes have been prepared from the digestive glands of marine animals such as Atlantic cod (*Gadus morhua*) (Brewer *et al.*, 1984), capelin (*Mallotus villosus*) (Gildberg and Raa, 1983), Greenland cod (*Gadus ogac*) (Squires *et al.*, 1986), polar cod (*Boreogadus saida*) (Arunchalam and Haard, 1985), Pacific yellowfin tuna (*Thunnus albacares*) (Norris and Mathies, 1953), North Pacific bluefin tuna (*Thunnus thynuus orientalis*) (Tanji *et al.*, 1988) and sardine (*Sardinos melanostica*) (Noda and Murakami, 1981). Several methods have been described in the literature for preparing pepsins from marine animals. Arunchalarn and Haard (1985) purified pepsin A and B from the stomachs of polar cod by homogenizing the tissue in phosphate buffer (pH 7.3), followed by filtration through a cheesecloth. The filtrate was further clarified by centrifugation and then subjected to affinity chromatography using a CBZ-D-phenylalanine-TETA-Sepharose 4B gel. Two pepsin isozymes, pepsin I and II, were also prepared from the stomachs of capelin by homogenizing the tissues in cold HCI (1 mM) solution, followed by ammonium sulfate fractionation (30-70% saturation), IEX chromatography on DEAE-cellulose, and size exclusion chromatography on Sephadex G-75 (Gildberg and Raa, 1983). The purified pepsins had the molecular mass of about 25,000 dalton. Pepsin was also isolated in the zymogen form from the stomach tissue of rainbow trout by homogenizing the tissue in phosphate buffer (pH 7.1), followed by centrifugation to clarify the homogenate, ammonium sulfate fractionation (20-50% saturation) of the clear extract, and then a series of chromatographic steps involving polylysine-Sepharose, DEAE-Sephacel, Sephadex G-200, and DEAE-Sepharose gels. Gildberg *et al.* (1990) purified pepsin from Atlantic cod (*Gadus morhua*) stomach by ammonium sulfate fractionation (20-70% saturation), followed by ion exchange chromatography performed on S-Sepharose Fast Flow.

### 1.4 Characteristics of fish pepsin

#### 1.4.1 Optimal pH

Fish pepsins are apparently less acidic proteins than mammalian pepsins (Norris and Mathies, 1953). Fish pepsins usually have higher optimum pH than mammalian pepsin (Kubota and Ohnuma, 1970; Gildberg, 1988). The purified pepsin from rainbow trout had an optimum pH of 3 (Twining *et al.*, 1983), and pH of 2 for Mongolian lamp (*Ovis platyurea*)

(Baudys *et al.*, 1988) for proteolysis of denatured hemoglobin. Stomach extracts from a number of animals, including fish, give two separate pH optima fro protein digestion at acid condition (Owen and Wiggs, 1971; Gildberg and Raa, 1983). Another proposed explanation to this phenomenon was that fish pepsins had two different pH optima, one in strong acid conditions, resembling mammalian pepsins, and another at about pH 3 (Norris and Mathies, 1953). Many fishes secrete at least two pepsins with different pH optima (Noda and Murakami, 1981; Gildberg and Raa, 1983). These pepsins are usually referred to as Pepsin I and Pepsin II. Pepsin I has pH optimum for hemoglobin digestion in the pH range of 3-4, whereas Pepsin II is most active in the pH range of 2-3. The optimum activity of pepsin I and II from capelin was observed at pHs of 3.7 and 2.5, respectively, and temperature of 38 and 43 $^{\circ}$C, respectively, when hemoglobin was used as a substrate (Gildberg and Raa, 1983). Some fishes feeding on algae have very high gastric HCl secretion and pepsins with pH optimum close to 2 (Moriarty, 1973). In capelin and cod, the isoelectric properties of the two pepsins are very different. The pI of pepsin I is in the range of 6.5-7, whereas pepsin II has pI close to 4 (Gildberg and Raa, 1983; Gildberg *et al.*, 1990).

### 1.4.2 Optimal temperature

Pepsin from cold and temperate water fish have comparatively high activity at low temperatures (Gildberg and Raa, 1983), and they express a much lower temperature coefficient than mammalian pepsins (Brewer *et al.*, 1984). Fish pepsins seem to have lower activation energies than mammalian pepsins. Also, pepsin from cold habitat fish had lower activation energies than pepsin from fish of the same species acclimated to higher temperatures (Haard *et al.*, 1981). Temperature optimum of American salmon pepsins was increased from about 32 $^{\circ}$C at pH 1.9 to about 37 $^{\circ}$C at pH 3.0, when hemoglobin was used as a substrate (Haard *et al.*, 1981). The temperature optimum of pig pepsin was about 20 $^{\circ}$C higher than that of capelin pepsins (Gilgberg and Raa, 1983; Gildberg, 1987). However, there are only minor differences between temperature optima of pepsins from cold and warm water fish. Temperature optimum of stomach extracts from Pacific yellowfin tuna was about 42 $^{\circ}$C (Norris and Mathies, 1953). The temperature optimum of purified capelin pepsins determined at the same conditions was slightly lower (about 40 $^{\circ}$C), indicating that crude preparations contain stabilizing factors (Gildberg and Raa, 1983). A temperature optimum of 35 $^{\circ}$C was detected for bonito pepsin (Kubota and Ohnuma, 1970), and 37 $^{\circ}$C for polar cod (Arunchalam and Haard, 1985), whereas different

temperature optima (40 and 55°C) were found for two pepsins from sardine (Noda and Murakami, 1981).

### 1.4.3 Catalytic activity

Fish pepsins have very low activity on small peptide substrates (Kubota and Ohnuma, 1970; Noda and Murakami, 1981; Sanchez-Chiang *et al.*, 1987), and a hexapeptide is the smallest substrate reported to be susceptible to hydrolysis by fish pepsin (Guerard and Le Gal, 1987). Like other aspartic proteinases, fish pepsins are very active on hemoglobin. Pepsins hydrolyze hemoglobin 3-10 times faster than casein and myofibrillar proteins from fish (Squires *et al.*, 1986; Gildberg and Raa, 1983). Twining *et al.* (1983) found that at pH 2, rainbow trout pepsin was almost 5 times as active against a hemoglobin substrate as was pig pepsin. In contrast, in the milk clotting assay, rainbow trout pepsin is only 25% as active. The pH optimum of Greenland cod protease I was at pH 3.5 with hemoglobin as the substrate and was at pH 3.0 with casein as the substrate (Squires *et al.*, 1986). Moreover, Sanchez-Chiang *et al.* (1987) found that adult salmon (*Oncorhynchus keta*) consists of pepsin I and pepsin II, while only pepsin II was isolated from juvenile salmon. In contrast with pepsin II, pepsin I was activated by NaCl.

## 2. Collagen

The major fraction of animal connective tissue is collagen. Collagen is the fibrous protein that contributes to the unique physiological functions of connective tissues in skins, tendons, bones, cartilages, etc. and is associated with toughness in mammalian muscle (Wong, 1989; Foegeding *et al.*, 1996). The collagen monomer is a long cylindrical protein about 2800 Å long and 14-15 Å in diameter (Foegeding *et al.*, 1996). The structural unit of collagen is tropocollagen. It is a rod-shaped protein consisting of three polypeptides unit (called α chains) intertwined to form a triple-helical structure. 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 (Wong, 1989) (Figure 3). The three chains are held together by hydrogen bonding. The most common collagen is type I collagen. It contains two identical polypeptide chains, designated α1(I), and a third chain, α2, which has a different amino acid sequence. Each chain has a molecular mass about 100,000 dalton, yielding a total molecular mass of about 300,000 dalton of collagen (Foegeding *et al.*, 1996). The α chains contain ~1000 amino acid residues and vary in amino acid

composition (Wong, 1989). In the extracellular space, collagen molecules align themselves into microfibrils, in quarter stagger array. Cross-linking is initiated and larger diameter fibrils are formed either by the addition of microfibrils or by association with other fibrils (McCormick, 1994). The cross-linking stereochemistry derives from the reaction of specific peptidyl aldehydes, in the $NH_2$- and COOH-terminal non-helical peptide, with vicinal ε-amino groups of specific peptidyl residues of Lys and Hyl, located in the triple-helical regions of molecules (Mechanic *et al.*, 1987).

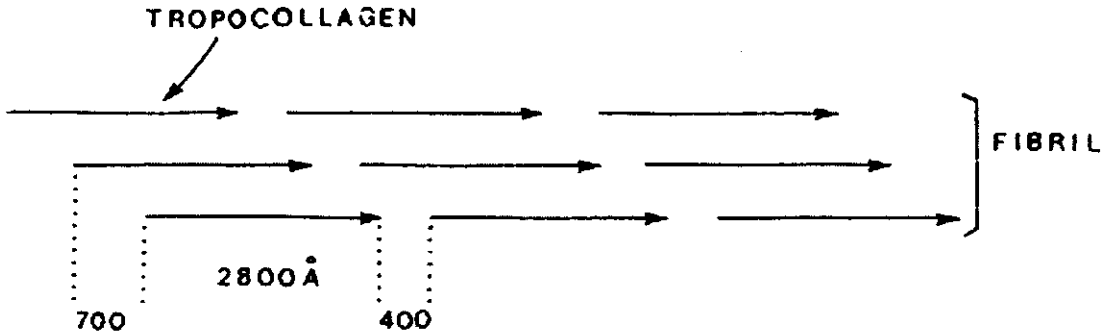


Figure 3 Arrangement of tropocollagen

Source: Wong (1989)

The triple helix of collagen assembled from specific polypeptide chain (α chains) with the Gly-X-Y repeat and contain frequent occurrence of proline and hydroxyproline (Hyp) in the X and Y positions, respectively (Xu *et al.*, 2002; Johnston-Banks, 1990). Hydroxyproline is only found in position Y, as is hydroxylysine, while proline can be found in either the X- or Y- positions (Johnston-Banks, 1990). Different α chains, designated α1, α2 or α3, differ in their amino acid compositions. The distribution of α1, 2 and α3 chains in collagen molecules varies, depending on the specific genetic variants (Xiong, 1997). The variation in the α chains constitute at least four major types of collagen as shown in Table 1.

Table 1 Collagens and their distribution

| Type | Triple helix | Distribution |
|---|---|---|
| I | two identical α1(I) chain + 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)

The amino acid composition of collagen is unique in that it is exceptionally high in glycine (33%), proline (12%) and alanine (11%). Two amino acids that are not commonly present in many other proteins include hydroxyproline (12%) and hydroxylisine (1%) (Wong, 1989). The amino acid composition of collagen is nutritionally unbalanced, which is almost devoid of tryptophan. Glycine represents nearly one-third of the total residues, and it is distributed uniformly at every third position throughout most of the first collagen molecule. The repetitive occurrence of glycine is absent in the first 14 or so amino acid residues from N-terminus and the first 10 or so from the C-terminus, with these end portions being termed "telopeptides" (Foegeding *et al.*, 1996).

**2.1 Marine fish collagen**

Collagen content in the muscles depends upon the species as well as on the state of maturation and feeding of the fish (Sikorski *et al.*, 1990; McCormick; 1994). In starving fish, the sarcoplasmic and myofribrillar protein undergo gradual degradation, while the connective tissues are not utilized, or extra collagen is even deposited in the myocommata and in the skin (Sikorski *et al.*, 1990). Generally, the contents of collagen in fish muscles range from about 1 to 12% of crude protein. Raw fish meat rich in collagen is tougher than that containing less collagen (Sikorski *et al.*, 1990).

Fish muscle, skin, bone and scale collagen differ from bovine and porcine hide collagens in having significantly higher contents of seven essential amino acids and a considerably lower concentration of hydroxyproline residues (Sikorski *et al.*, 1990) (Table 2). Collagen also contains carbohydrates (glucose and galactose). These are attached to

hydroxylysine residues of the peptide chain by O-glycosidic bonds. The presence of 2-O-α-D-glucosyl-O-β-D-galactosyl-hydroxylysine and of O-β-D-galactosyl-hydroxylysine has been confirmed (Burghagen, 1999). Some fish and invertebrate collagens also contain small amounts of arabinose, xylose and ribose residues. These hydroxylysine-linked carbohydrates may have a impact on the structure of the fibrils in the invertebrate collagen (Sikorski *et al.*, 1990).

Table 2 Amino acid composition of collagen from fish and mammalian

| Amino acids | Residues per 1000 residues | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Bigeye snapper[a] | | Skate | | Ocellate puffer fish[d] | *Pagus major*[e] | Porcine[c] | Bovine *L. dorsi*[f] |
| | Skin | Bone | Cartilage[b] | Muscle[c] | Skin | Scale | dermis | |
| Alanine | 136 | 129 | 115 | 115 | 106 | 133 | 115 | 107 |
| Arginine | 60 | 46 | 50 | 51 | 54 | 49 | 48 | 45 |
| Aspartic acid | 51 | 47 | 36 | 36 | 50 | 43 | 44 | 34 |
| Glutamic acid | 78 | 74 | 78 | 78 | 87 | 71 | 72 | 83 |
| Glycine | 286 | 361 | 354 | 356 | 351 | 346 | 341 | 336 |
| Histidine | 10 | 6 | 8 | 8 | 8 | 5 | 7 | 5 |
| Hydroxyproline | 77 | 68 | 74 | 74 | 67 | 73 | 97 | 109 |
| Hydroxylysine | 10 | 20 | 6 | 6 | - | 7 | 7 | 8 |
| Isoleucine | 5 | 5 | 18 | 17 | 12 | 7 | 10 | 12 |
| Leucine | 24 | 25 | 22 | 22 | 23 | 18 | 22 | 25 |
| Lysine | 31 | 25 | 26 | 25 | 19 | 26 | 27 | 23 |
| Methionine | 12 | 8 | 10 | 9 | 14 | 15 | 6 | 5 |
| Phenylalanine | 15 | 12 | 12 | 12 | 10 | 13 | 12 | 14 |
| Proline | 116 | 95 | 83 | 83 | 103 | 107 | 123 | 113 |
| Serine | 36 | 34 | 45 | 46 | 48 | 41 | 33 | 36 |
| Threonine | 29 | 25 | 37 | 36 | 25 | 24 | 16 | 17 |
| Tyrosine | 4 | 2 | 2 | 2 | 4 | 3 | 1 | 3 |
| Valine | 22 | 17 | 25 | 25 | 17 | 19 | 22 | 25 |

Sources: (a) Kittiphattanabawon *et al.* (2005), (b) Mizuta *et al.* (2003), (c) Mizuta *et al.* (2002), (d) Nagai *et al.* (2002), (e) Ikoma *et al.* (2003), (f) Sikorski *et al.* (1990)

### 2.2 Extraction and characterization of fish collagen

Collagen can be isolated from fish skin, bone and scale (Nomura *et al.*, 1996; Nagai *et al.*, 2002; Ikoma *et al.*, 2003; Mizuta *et al.*, 2003; Muyonga *et al.*, 2004a; Ogawa *et al.*, 2004; Jongjareonrak *et al.*, 2005b; Hwang *et al.*, 2007). Nagai and Suzuki (2000) extracted type I collagen from under-utilized resources including fish skin, bone and fin with varying yields as follows: (1) skin collagen: 51.4% (Japanese sea-bass), 49.8% (chub mackerel) and 50.1% (bullhead shark); (2) bone collagen: 42.3% (skipjack tuna), 40.7% (Japanese sea-bass), 53.6% (ayu), 40.1% (yellow sea bream) and 43.5% (horse mackerel); (3) fin collagen: 5.2% (Japanese sea-bass acid-soluble collagen) and 36.4% (Japanese sea-bass acid-insoluble collagen) on the dry basis. The extraction of collagen was prepared by elimination of non-collagenous protein and decalcification before acid solubilization. The resulting collagen was referred to as "acid-solubilized collagen". Normally, collagen is extracted with low yield. Therefore, use of pepsin for hydrolysis of telopeptide regions prior to extraction resulted in the increased yield. The collagen obtained was referred to as "pepsin-solubilized collagen" (Nagai *et al.*, 2001; Yata *et al.*, 2001; Nagai and Suzuki, 2002; Jongjareonrak *et al.*, 2005a).

#### 2.2.1 Acid-solubilzed collagen

Skin and bone collagen could be extracted with acidic mild condition. Kittiphattanabawon *et al.* (2005) extracted acid-soluble collagens (ASC) from the skin and bone of bigeye snapper, (*Priacanthus tayenus*) with the yields of 10.94% and 1.59% on the basis of wet weight, respectively. Similar electrophoretic patterns of collagens from the skin and bone were observed. Both collagens comprised two different a chains, α1 and α2 and were classified as type I collagen. However, peptide maps of collagen from the skin and bone of bigeye snapper, digested by V8 protease and lysyl endopeptidase, revealed differences between collagens from skin and bone, and both were completely different from those of calf skin collagen.

Muyonga *et al.* (2004a) characterized the acid soluble collagen (ASC) from the skins of young and adult Nile perch (*Lates niloticus*) extracted using 0.5 M acetic acid and precipitation with 0.9 M NaCl. The ASC yields, on a dry weight basis, were 63.1 and 58.7%, respectively, for young and adult fish skins. SDS-PAGE showed that the collagens contained two alpha components (α1 and α2). ASC from Nile perch contained higher extent of imino acids (19.3 and 20.0%, respectively, for young and adult fish) than most fish species. The denaturation

temperature for the collagens from the skins of young and adult Nile perch was determined to be 36°C, which is also higher than that for most other fish species. Sadowska *et al.* (2003) extracted the collagen from the skins of Baltic cod (*Gadus morhua*). The use of a one-stage, 24-h extraction of whole skins with acetic at ratio of material to solvent of 1:6, yielded 20% collagen. Using three consecutive 24-h extractions of whole skins with citric acid, 85% of collagen protein could be separated. The thermal solubility of collagen depends on the medium applied. About 85 and 15% of collagen contained in the skins were dissolved in 0.45 M NaCl solution and water, respectively, after 24 h incubation at 30°C (Sadowska *et al.*, 2003).

**2.2.2 Pepsin-solubilized collagen**

Generally, use of pepsin in combination with acid extraction increased in the yield of collagen. Nagai *et al.* (2002) characterized pepsin-solubilized collagen from ocellate puffer fish skin. It was a 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 skin collagen. The yield of pepsin-solubilized collagen was higher (44.7%) than acid-solubilized collagen (10.7%). 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 α1 and α2 as well as the beta component. The minor collagen fraction contained α1, α2, and α3. Both major and minor collagen fractions were identified as type I and type V collagens, respectively.

Furthermore, pepsin-solubilized collagens from the muscle tissues (ordinary and dark muscles) of Japanese amberjack were prepared by Nishimoto *et al.* (2004). The collagens were separated into two fractions, major and minor, by ammonium sulphate precipitation. These fractions were further purified by cation-exchange column chromatography. The results of SDS-PAGE, peptide mapping, and amino acid analysis suggested that the purified major and minor collagens might be classified as type I and V collagens, respectively. Each type of collagen was fundamentally similar in amino acid compositions and peptide maps between the ordinary and dark muscles. Nagai *et al.* (2000) found that 35.2% collagen was extracted from rhizostomous jellyfish (*Rhopilema asamushi*) by limited pepsin digestion. 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. The rhizostomous jellyfish mesogloea collagen had a denaturation temperature ($T_d$) of 28.8°C. This collagen contained a large amount of a fourth subunit, designated as α4. This collagen may have the chain composition of an α1α2α3α4 heterotetramer. Nagai and Suzuki (2002) found that the collagen extracted from the outer skin of the paper nautilus was hardly solubilized in 0.5 M acetic acid. The insoluble matter was easily digested by 10% pepsin (w/v), and a large amount of collagen was obtained with about a 50% yield (pepsin-solubilized collagen). The pepsin-solubilized collagen had a chain composition of α1 α2 α3 heterotrimer similar to *Callistoctopus arakawai* arm collagen. Furthermore, collagen from the outer skin of cuttlefish (*Sepia lycidas*) was extracted by Nagai *et al.* (2001). The initial extraction of the cuttlefish outer skin in acetic acid yielded only 2% of collagen (dry weight basis). On subsequent digestion of the residue with 10% pepsin (w/v), a solubilized collagen (PSC) was obtained in a yield of 35% (dry weight basis). PSC obtained had a chain composition of $(\alpha 1)_2\alpha 2$ heterotrimer, which was similar to Japanese common squid PSC. The denaturation temperature of this collagen was 27°C which is about 10°C lower than that of porcine collagen.

**2.3 The factors affecting collagen properties**

Collagens from different sources exhibit different properties, depending on amino acid composition, amino acid sequence and collagen fibrils arrangement (Sikorski *et al.*, 1990; Foegeding *et al.*, 1996). Nevertheless, 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:

**2.3.1 Imino acid content**

The contents of imino acids (proline and hydroxyproline) are related to thermal stability of collagen. Collagens containing small concentrations of both imino acids denature at lower temperature than do those with the larger concentrations. The imino acid content of fish collagens is therefore associated with their thermal stability and correlates with the water temperature of their normal habitat (Foegeding *et al.*, 1996). Norland (1990) reported that collagen from cold water fish consisted of imino acid about 16-18%. Muyonga *et al.* (2004a) found that acid soluble collagen (ASC) extracted from the skins of young and adult Nile perch *(Lates niloticus)* contain higher content of imino acids (19.3 and 20.0%, respectively) than that from other fish species. The denaturation temperature for the collagens from the skins of young and adult Nile

mesogloea. The rhizostomous jellyfish mesogloea collagen had a denaturation temperature ($T_d$) of 28.8°C. This collagen contained a large amount of a fourth subunit, designated as α4. This collagen may have the chain composition of an α1α2α3α4 heterotetramer. Nagai and Suzuki (2002) found that the collagen extracted from the outer skin of the paper nautilus was hardly solubilized in 0.5 M acetic acid. The insoluble matter was easily digested by 10% pepsin (w/v), and a large amount of collagen was obtained with about a 50% yield (pepsin-solubilized collagen). The pepsin-solubilized collagen had a chain composition of α1 α2 α3 heterotrimer similar to *Callistoctopus arakawai* arm collagen. Furthermore, collagen from the outer skin of cuttlefish (*Sepia lycidas*) was extracted by Nagai *et al.* (2001). The initial extraction of the cuttlefish outer skin in acetic acid yielded only 2% of collagen (dry weight basis). On subsequent digestion of the residue with 10% pepsin (w/v), a solubilized collagen (PSC) was obtained in a yield of 35% (dry weight basis). PSC obtained had a chain composition of $(\alpha 1)_2\alpha 2$ heterotrimer, which was similar to Japanese common squid PSC. The denaturation temperature of this collagen was 27°C which is about 10°C lower than that of porcine collagen.

**2.3 The factors affecting collagen properties**

Collagens from different sources exhibit different properties, depending on amino acid composition, amino acid sequence and collagen fibrils arrangement (Sikorski *et al.*, 1990; Foegeding *et al.*, 1996). Nevertheless, 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:

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perch was determined to be 36°C, which is also higher than that for most other fish species. The circular dichroism measurement of type I collagens from fish scales of *Pagrus major* and *Oreochromis niloticas* indicated that the denaturation temperatures were dependent on the amount of hydroxyproline, rather than proline residues. Raman spectra also indicated that the relative intensities of Raman lines at 879 and 855 $cm^{-1}$ assigned to Hyp and Pro rings were changed due to the contents of the imino acids (Ikoma *et al.*, 2003). The denaturation temperatures ($T_d$) of collagens from skipjack tuna, Japanese sea-bass, ayu, yellow sea bream, chub mackerel, bullhead shark and horse mackerel varied as follows: skin collagen (25.0-26.5°C), bone collagen (29.5-30.0°C) and fin collagen (28.029.1°C). These values were lower about 7-12°C than that of porcine skin collagen (Nagai and Suzuki, 2000). Therefore, collagen properties are governed by fish sources and organs used for collagen extraction.

**2.3.2 Age and starvation of animals**

The number of cross-links in collagen increased with increasing age of the animal (Zayas, 1997). Most connective tissue in fish is renewed annually and highly cross-linked protein is not generally found in fish (Foegeding, *et al.*, 1996). 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 (Zayas, 1997). The steady increase in mature collagen cross-linking is due to progressive and on-going cross-linking reactions that occur within fibrillar collagen and with the slowing of collagen synthesis rates as animals reach maturity (McCormick, 1994). Collagen from young animals is more easily solubilized and 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). In starving fish, the sarcoplasmic and myofibrillar proteins undergo gradual degradation, while the connective tissues are not utilized, or extra collagen is even deposited in the myocommata and in the skin (Sikorski *et al.*, 1990). Thus the toughening process in fish seems to be much more reversible than that of 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. Furthermore, collagens in myocommata are thickened with more intermolecular cross-links of collagen during starvation (Love *et al.*, 1976).

### 2.3.3 pH and salt

Some factors have been known to influence the collagen properties. pH and salt concentration were reported to affect water-binding capacity, viscosity and emulsifying properties of collagen from fish muscle and skin connective tissues (Montero *et al.*, 1991). Jongjareonrak *et al.* (2005a) reported that maximum solubility in 0.5 M acetic acid of collagen from brownstripe red snapper (*Lutjanus vitta*) was observed at pH 3 and pH 4 for ASC and PSC, respectively. A sharp decrease in solubility was observed in the presence of NaCl, above 2% and 3%, (w/v) for ASC and PSC, respectively. Collagens from the skin and bone of bigeye snapper (*Priacanthus tayenus*) had the highest solubility at pH 2 and 5, respectively. No changes in solubility were observed in the presence of NaCl up to 3% (w/v). However, a sharp decrease in solubility was found with NaCl above 3% (w/v) (Kittiphattanabawon *et al.*, 2005). Where pH was between 2 and 4, the solubility and water binding capacity of trout (*Salmo irideus* Gibb) collagen were highest 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 less than 0.25 M NaCl. Furthermore, emulsifying capacity decreased as the NaCl concentration increased and was highest at pH levels between 1 and 3 (Montero and Borderias, 1991).

### 2.3.4 Processing

Collagen properties can be affected by processing such as freezing, heating, etc. Montero *et al.* (1995) compared four 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 on the functional properties of collagen from plaice skin. 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 pre-solubilized. Optimum water-holding capacity was observed in samples, which were previously solubilized. During the storage of fish on ice, a progressive change in solubility of muscle collagen was found. For insoluble collagen, significantly lower values were detected at day 15 compared to day 0. A little increase in acid soluble collagen was found while no changes were seen in pepsin-soluble collagen during

storage (Eckhoff *et al.*, 1998). These results show that some cleavage of intermolecular cross-links seems to occur during storage on ice (Eckhoff *et al.*, 1998).

### 3. Gelatin

Gelatin is a water soluble proteinaceous substance prepared by processes, which involve the destruction of the tertiary, secondary and to some extent the primary structure by thermal denaturation of native collagens. Gelatin consists of random chains without triple helix. The process involves the disruption of non-covalent bonds and it is partially reversible during gelation process (Ward and Courts, 1977; Bigi *et al.*, 1998; Ikada, 2002). During the collagen to gelatin transition, many non-covalent 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 the conversion of the helical collagen structure to a more amorphous form, known as "gelatin" (Figure 4). 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).

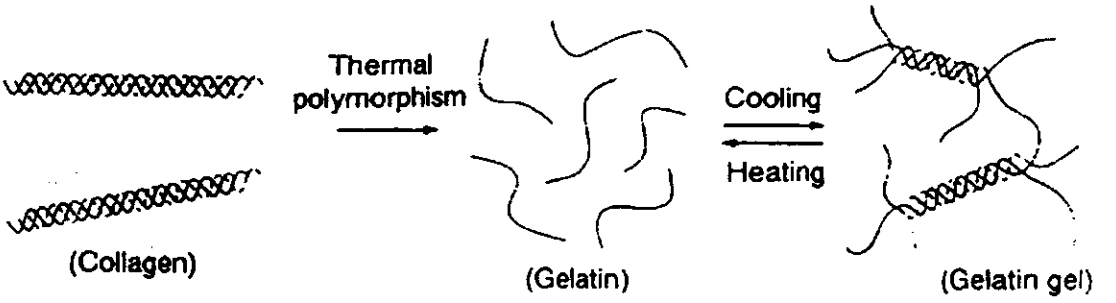


Figure 4 Collagen conversions into gelatin

Source: Okuzaki (2001)

#### 3.1 Fish gelatin extraction

Gelatin can be extracted from many fish species by non-collagenous protein elimination, demineralization and swelling with acid solution prior to hot water extraction (45°C) (Gómez-Guillén *et al.*, 2002). Gómez-Guillén and Montero (2001) reported that light-colored, dry collagen was obtained from megrim (*Lepidorhombus boscii*) skins and, after dissolving in

warm water, turned into soluble gelatin. The type of acid used influenced the gelatin viscoelastic and gelling properties. Acetic- and propionic-acid extracts produced 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. Moreover a high-quality, readily-dissolved gelatin was obtained from megrim skins pretreated with NaCl and dilute NaOH, then swelling with 0.05 M acetic acid followed by an extraction in water at 45°C (Montero and Gómez-Guillén, 2000). Gómez-Guillén *et al.* (2002) compared the rheological characteristics (viscoelasticity and gel strength) and chemical/structural properties (amino acid composition, molecular weight distribution and triple helix formation) of different skin gelatins. 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). This different behavior may be determined by the amino acid composition, the α1/α2 collagen-chain ratio, and the molecular weight distribution.

Cod gelatin contained a lower alanine and imino acid content, and a decreased proline hydroxylation degree. Cod and hake gelatins had a low α1/α2 ratio (~1), whereas hake gelatin showed a highly significant decrease in β-components and other aggregates (Gómez-Guillén *et al.*, 2002). The squid gelatin showed the α-chains with slightly different mobility on SDS-PAGE from fish species (Gómez-Guillén *et al.*, 2002). Very low content of β-components and an almost disappearance of higher molecular aggregates was observed in squid gelation. Squid gelatin contained higher content in Hyp than cod, sole megrim and hake, while the total imino acid (Pro+Hyp) content was similar (Gómez-Guillén *et al.*, 2002). A special feature of squid gelatin was its high Lys hydroxylation degree that frequently found in highly insoluble collagens with a high cross-linking degree (Montero *et al.*, 1990).

Zhou and Regenstein (2003) determined the appropriate extraction process to obtain pollock skin gelatin. Type and concentration of base and acid during pretreatment, and the extraction temperature and time strongly influenced the total yield and rheological properties. The combination of 0.1 N $Ca(OH)_2$ or NaOH with 0.05N acetic acid or 0.025N citric acid improved the gel strength of extracts. Although increasing extraction temperature and time (above 40°C and 180 min) could slightly increase the

total yield of gelatins, the gel strength decreased. The total yield of gelatin from pollock skin was more than 12% with a hydroxyproline content around 7%. Moreover, pollock skin gelatin extraction was also affected by 4 variables, pretreatment temperature, concentration of $OH^-$, concentration of $H^+$, and extraction temperature. Based on response surface methodology, a concentration of $OH^-$ at 0.25 mol/L, a concentration of $H^+$ at 0.09 mol/L, a pretreatment temperature at 2°C, and an extraction temperature at 50°C, gave the gelation with the highest yield (18%), gel strength (460 g), and viscosity (6.2 cP) (Zhou and Regenstein, 2004). Muyonga *et al.* (2004b) extracted type A gelatins from skins and bones of young and adult Nile perch. Total gelatin yield from the sequential extraction at 50, 60, 70 and 95°C was in the order: descending adult fish skins > young fish skins > adult fish bones > young fish bones. However the percentage gelatin recovery at 50°C was in the following order: young fish skins > adult fish skins > young fish bones > adult fish bones. The gelatins obtained were free of fishy odor. Nile perch skin gelatin had turbidity of 20.5158 NTU and ash content of 0.5-1.7%, while bone gelatins had turbidity of 109-517 NTU and ash content of 4.4-11.2%. Bloom gel strength was 81-229 and 134-179 g, respectively, for skin and bone gelatins (Muyonga *et al.*, 2004b). Gelatin from adult Nile perch skins exhibited higher viscosity and lower setting time than bone and the young fish skin gelatins. Skin gelatins were found to exhibit higher film tensile strength but lower film percent elongation than bone gelatins. Furthermore, gelatin extraction from shark *(Isurus oxyrinchus)* cartilage was optimized with response surface methodology by Cho *et al.* (2004): the dependent variable was gelatin yield and independent variables were sodium hydroxide concentration and treatment time for the alkali treatment, and extraction temperature and time for the hot-water extraction. Predicted maximum yield of 79.9% for gelatin extraction was obtained under the conditions of alkali treatment with 1.6 N NaOH for 3.16 days and hot-water extraction at 65°C for 3.4 h. Amino acid composition and functional properties of shark cartilage gelatin were examined in comparison to two porcine skin gelatins. Shark cartilage gelatin had lower concentration of hydroxyproline than the two porcine skin gelatins. Foam formation ability, foam stability, water-holding capacity and gel strength of shark cartilage gelatin were lower than the two porcine gelatins, but fat-binding capacity was higher in the shark cartilage gelatin.

Kittiphattanabawon (2004) reported that the gelatin extraction from bigeye snapper skin and bone was carried out by deproteinization the skin in 0.025 N NaOH for 1 h with 2 repetitions. Only deproteinized bone was then subjected to demineralization with either 1.2 M citric acid for 4 h or 0.6 M HCl for 2 h. Swelling process was carried out by soaking the pretreated bone and skin in 0.05-0.2 M citric or acetic acid for 40 min with 3 repetitions. Gelatin was then extracted using hot water (45°C) for 12 h. The yields of skin and bone gelatin were 6.29-7.76% and 1.19-2.25% (wet basis), respectively. The highest bloom strength of gelatin gel from skin was obtained when skins were swollen with 0.2 M acetic acid, prior to extraction.

**3.2 Uses of enzymes in collagen/gelatin manufacturing**

Proteolytic enzymes show the profound effects on some properties of collagen extracted. Trypsin at pH 7.2 and pepsin at pH 3.5 reacts with tropocollagen with limited extent, resulting in the liberation of low molecular weight acidic peptide fractions from the rigid three chains helical portion of the tropocollagen. It is very specific for splitting peptide bonds involving carboxyl groups of arginine and lysine (Courts, 1955). Tryptic digestion produces amino terminal glycine in large amounts exposing Arg, Gly or Lys, Gly as the most prevalent sequence. Lysine and arginine were predominant at carboxy terminal, and in all the peptides, the amino end was occupied by glycine. Chymotrypsin is more specific in its hydrolytic action and less temperature dependent. The N-terminal peptide area in the non-helical region of the collagen is attacked by the enzyme, resulting in a decrease in its viscosity as well as a decrease in its β-components (Drake *et al.*, 1966; Bornstein *et al.*, 1966). Pepsin is much less specific but hydrolyses bonds containing aromatic anion acid residues. It removes intermolecular crosslinks from the non-helical N-terminal peptide area of the collagen molecule. Pronase has a similar action to pepsin. The solubility of the soluble collagen increased and its end to end aggregation was also affected (Drake *et al.*, 1966). Chomarat *et al.* (1994) compared the effectiveness of two proteolytic enzyme; pepsin and proctase (isolated from *Aspergillus niger*) for the solubilization of collagen from bovine skin. Some of the molecular properties of gelatins derived from these collagen preparations were also studied. Pepsin and proctase solubilized collagens had the similar yields (75% and 76% of total collagen as calculated from hydroxyproline). The relative amounts of collagen extracts converted to gelatin were also comparable (32% and 39%, respectively). However, proctase-extracted collagen exhibited a decrease in high-molecular-weight

components, compared to pepsin prepared collagen. Furthermore, gelatin obtained from proctase-extracted collagen showed significant proportions of molecular species smaller than collagen alpha-chains. An increase of the proportion of type III collagen in proctase extract was noticeable as compared to pepsin extract. Rheological properties of gelatin obtained by proctase solubilization exhibited a very significant decrease of gel strength, viscosity, and turbidity as compared to pepsin-derived gelatin. These parallel biochemical and biophysical data indicate that proctase prepared gelatin was markedly altered, compared with pepsin-derived gelatin (Chomarat *et al.*, 1994).

**3.3 Gelatin dehydration**

Extracted gelatin liquors must be subjected to filtration and evaporation to concentrate to 50-70% at 50-70$^{\circ}$C and vacuum conditions. As gelatin liquors are thermosensitive fluids, high temperature used for evaporation may cause the degraded gelatin with low gel strength (Ward and Courts, 1977). Furthermore, gelatin consists of high content of hydrophobic amino acid and the foam will be easily generated during evaporation, resulting in protein aggregation. Foam formations of gelatin during evaporation process show the impact on physical properties of gelatin (Michell, 1986). Additionally, highly concentrated solution of gel can be cooled and extruded to form gelatin noodles, which are then laid on a drying belt (Silva *et al.*, 2001). Gelatin is a molecular colloid that is not porous under these drying conditions, and as a consequence, water migration occurs solely by diffusive processes. To achieve a commercial standard of dryness, the dependence of the diffusion coefficient as a function of temperature is used. Gudmundsson and Hafsteinsson (1997) evaporated gelatin extract from cod skin under vacuum at 43-45$^{\circ}$C until ≈85-90% of the water was removed. The concentrate containing the gelatin was then air dried in a fume hood at 45$^{\circ}$C to remove remaining water. The gelatin liquid was freeze dried. Freeze-dried gelatin had considerably higher bloom value than air-dried gelatin. Air-drying would be more likely to cause protein denaturation than lyophilization, thus reducing gel forming properties (Gudmundsson and Hafsteinsson, 1997).

**3.4 Gelatin properties**

Gelatin is nearly tasteless and odorless (Food Chemicals Codex, 1996). Physical and chemical properties noted: colorless or slightly yellow, transparent, brittle, odorless, tasteless sheets, flakes, or powder; soluble in hot water, glycerol, and acetic acid; and insoluble in organic

solvents (Budavari, 1996). Gelatin swells and absorbs 5-10 times its weight of water to form a gel in aqueous solutions between 30-35°C. Gelatin extracted from fish will have a gel point in the range of 5-10°C. (Food Chemicals Codex, 1996). These gels have increasing viscosity under stress (thixotrophic) and are thermally reversible. Gelatin has a unique protein structure that provides for a wide range of functional properties (Hudson, 1994). These proteins form a compound (triple) helix in aqueous solution (Veis, 1964).

Gelatin is amphoteric (Budavari, 1996), which become positively or negatively charged, depending on the nature of the solution. The pH at which gelatin's charge in solution is neutral is known as the isoelectric point. The isoelectric point of gelatin ranges between 4.8 and 9.4. Acid processed gelatins generally have the higher isoelectric points than alkali processed gelatins (Poppe, 1997). Gelatin forms a gel at a minimum concentration of 0.5% through the pH range of 4-8. The pH in water solutions for type A is between 4.5 and 6, and the pH range for type B is from 5 to 7 (US FDA, 1997). Bloom is an ascending index used to measure gel strength (Bloom, 1925). Commercial gelatin will vary from 90 to 300 grams Bloom (Igoe, 1983).

Fish gelatin is distinguished from beef or pork gelatin by its low melting point, low gelation temperature, and high solution viscosity. These physical properties are not as strongly correlated to Bloom strength (Leuenberger, 1991). One study found fish gelatin to have similar physical and chemical properties compared to porcine gelatin and to be rated superior in a blind sensory test (Choi and Regenstein, 2000).

**3.5 Gelatin applications**

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 very 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 is sold too many sections of the food industry; the major areas of use are jellies, confectionery, meat products and chilled dairy products (Johnston-Banks, 1990). Gelatin is used in desserts at 8 to 10% of the dry weight, in yogurt at 0.3 to 0.5% as a thickener, in ham coatings at 2 to 3%, and in confectionery and capsules (vitamin supplements) at 1.5 to 2.5 % (Igoe, 1983). Further uses include fruit toppings for pastry, instant gravy, instant sauces and soups, edible films for confectionery products (McCormick, 1987), as a stabilizer in ice cream, cream cheese, and cottage cheese as well as in food foams and fruit salads (McWilliams, 2001). Overall functional uses include as a stabilizer, thickener, and texturizer. For juice applications, gelatin in combination with bentonite causes a dense precipitate or coagulum with soluble proteins in the juice, which facilitates the clarification process by allowing the protein haze to be filtered out from the juice. The petition states, "added directly to beverage in conjunction with other clarifiers to cause(s) binding of haze causing components which can then be filtered out along with the gelatin" (Gass, 2001).

Gelatin is one of compounds widely used in pharmaceutical industry. A large proportion is used in hard and soft capsule manufacture, and in the photographic industry, which uses the unique combination of gelling agent and surface activity to suspend particles of silver chloride or light-sensitive dyes (Johnston-Banks, 1990). Gelatin capsules (gel-caps) are commonly used to encapsulate various foods, nutritional supplements, and medicines (Ash and Ash, 1997).

## OBJECTIVES

1. To isolate and characterize fish pepsin from bigeye snapper stomach.
2. To use fish pepsin for collagen extraction from bigeye snapper (*Priacanthus tayenus*) skin.
3. To develop fish pepsin-aided process for gelatin extraction from bigeye snapper (*Priacanthus tayenus*) skin.