1 INTRODUCTION & LITERATURE REVIEW

1.1 STATEMENT OF PROBLEM

Nile perch (*Lates niloticus*) belongs to the genus *Lates* and the family *Centropomidae*. It was introduced in Lake Victoria in the 1950s but has grown to become the most dominant fish species in the lake and other lakes in the region (Acere, 1993). In Uganda, Nile perch catches represent approximately 46% (~ 100,000 tonnes per year) of all the fish landed (UIA, 2001). The fish has been widely consumed within the country since its introduction but industrial processing has only become wide spread in the last decade. Currently about ten firms, within Uganda alone, are involved in industrial processing of fish. These firms are involved in production of Nile perch fillets, mainly for the export market. Industrial processing of Nile perch in Uganda is estimated at 60,000 metric tonnes per annum. The balance of about 40,000 metric tonnes is either sold unprocessed or processed (mainly by smoking) by informal set-ups. Approximately 50% of the raw material (fish) remains as processing waste after filleting (Shahidi, 1994). This is mainly in form of skeletons and skins. This creates approximately 30,000 metric tonnes of waste a year. Skins and bones are underutilised and pose a disposal problem for the processing firms.

Nile perch skins and bones could be used in the manufacture of gelatin. Gelatin is a denatured form of collagen that can be derived from any collagenous material. The traditional sources of gelatin are pigskins, bovine hides and bones. Recent outbreaks of Bovine spongiform encephalopathy (BSE) and increase in demand for kosher and halal foods have created a demand for alternative sources of gelatin for food applications. Gelatin from fish with scales such as Nile perch has potential for use in kosher and halal foods since it is generally acceptable for use as food by Muslims and Jews. It may also find market among the Hindu, who are not permitted to consume cattle products. Despite such potential, fish has generally not been widely used as a source of gelatin, probably because most species give gelatin of low quality compared to mammalian gelatin. Unlike mammalian gelatins, fish gelatins vary widely in composition, particularly with respect to their imino acids (proline and hydroxyproline) content and in their physico-chemical properties.

It is estimated that a total of about 700,000 people, out of Uganda's total population currently estimated at 24 million are engaged in fish-related enterprises, including fishing, manufacture of fishing gear, artisan processing, fish trade and industrial scale processing (MAAIF, 2000). Processing of fish waste to value-added products like gelatin would enhance the contribution of the fisheries sector to the national economy and is likely to lead to an improvement of the income of those involved in the sector.

The share of fisheries sector to Uganda's GDP is estimated at 2.2% and fish export revenue to Uganda has increased from less than US$ 1 million to more than US$ 50 million per annum in the last decade (MAAIF, 2000). The national vision is to triple the value of fish exports and to increase domestic consumption significantly over the next 25 years. The Uganda Investment Authority (UIA), which is responsible for promoting investment in the country and is supposed to guide potential investors on the promising areas for investment, has identified the processing of value added products from fish processing waste as one of the viable areas for investment (UIA, 2001). There is indeed adequate and inexpensive raw material for such an enterprise. Since Nile perch is also widely produced in neighbouring Kenya and Tanzania and the three countries have very favourable trade arrangements, an investment in processing of Nile perch waste could tap the raw materials and the markets of all the three countries.

1.2 LITERATURE REVIEW

1.2.1 Gelatin sources and uses

Gelatin is a high molecular weight polypeptide derived from collagen, the primary protein component of bones, skins and tendons (Poppe, 1992). It is obtained by denaturation and solubilisation of collagen derived from the skin, white connective tissue and bones of animals. Commercially, gelatin is mainly derived from pigskins and bovine hides and bones. It has been believed for a long time that fish skins generally yield low gel strength gelatin (Osborne, Voight & Hall, 1990; Holzer, 1996; Gudmundsson & Hafsteinsson, 1997) but recent studies (Grossman & Bergman, 1992; Montero, Fernández-Diaz & Gòmez-Guillen, 2002) have shown that it is possible to derive high gel strength gelatin from fish skins. The possibility of deriving

gelatin from fish bones has been less investigated. Osborne *et al.* (1990) reported use of headed and gutted, as well as, only gutted lung fish in gelatin extraction. The gelatin yield was rather low (4.9% in both cases) and the resulting gelatin had very high levels of ash (8.66 and 13.85%, respectively). Regenstein (2001) reported successful extraction of gelatin from shark bones but did not report the properties of the gelatin.

Fish gelatin is fast gaining popularity for use in kosher and halal foods. Kosher gelatin fetches 3 - 10 times the price of non-kosher gelatin and its availability is still rather low (Regenstein, 2001). Fish gelatins are available both in the gelling and non-gelling forms. The non-gelling fish gelatins find applications as stabilisers, film forming, binding and clarifying agents.

Estimated total world usage of gelatin is 200,000 metric tons per year with US usage being about 30,000 metric tons per year for food and 10,000 metric tons per year for pharmaceutical applications (Herz, 1995). Annual average use of gelatin for food applications is growing at about 3% (Poppe, 1992). Food applications of gelatin include gelling, thickening, stabilising, emulsification, binding, foaming, dispersing, aeration and glazing (Helcke, 2000). Gelatin is used because of its unique physical properties, i.e. its ability to form thermo-reversible gels, which melt below 37°C, normally between 24 and 32°C. Maturation time, gelatin concentration and presence of salts influence the exact melting point and Bloom gel strength. Gelatin swells when placed in cold water, absorbing 5 - 10 times its own volume of water. When heated to temperatures above its melting point, the swollen gelatin dissolves and forms a thermo-reversible gel when cooled. This characteristic is desired in many foods.

There are a number of reviews covering gelatin uses in food-related and other industries (Jones, 1977; Wood, 1977; Anon, 1980; Poppe, 1992). Gelatin is used as a stabilizer for frozen desserts, where it functions as an inhibitor of ice crystal growth and lactose crystallization during frozen storage of dairy products (Morley & Ashton, 1982; Fiscella, 1983; Morley, 1984). It is a texturizing agent in several dairy products, while in some, it adjusts viscosity, stabilizes emulsions, or improves foaming (Poppe, 1992). It has been shown to decrease syneresis (Fiszman, Llunch &

Salvador, 1999; Fiszman & Salvador, 1999) and to increase gel strength and solidity (Fiszman *et al.*, 1999) in yogurt and milk gels. It has been used to form milk gels at pH values outside that at which acid-induced coagulation occurs (Salvador & Fiszman, 1998). Gelatin has also been used in making low fat cheeses (Anon, 1996; Kurultay, Oksuz & Simsek, 2000). When added at a level of 0.1 – 0.3% to cheese milk, gelatin increases yield of cheese by 24 – 49% but this results in cheese with higher moisture content, water soluble nitrogen and pH (Kurultay *et al.*, 2000). Gelatin is also used in confectionery, meat and fish products, as well as in delicatessen products (Jones, 1977; Poppe, 1992) and as a clarifying and stabilizing agent for beverages (Jones, 1977; Poppe, 1992; Versari, Barbanti, Potentini, Mannuzzu, Salvucci & Galassi, 1998). When used in clarification treatment it reduces turbidity, total polyphenols, colour intensity and brown polymers (Versari *et al.*, 1998).

It is also used in low calorie spreads, imparting emulsion stability (Poppe, 1992). Gelatin has also been used in edible films and like other hydrocolloids imparts durability as well as good gas and lipid barrier properties (Greener & Fennema, 1994; Krochta & deMulder-Johnston, 1997). However, it has poor water barrier properties due to its hydrophilic nature. Film preparation temperature (Arvanitoyannis, Psomiadou, Nakayama, Aiba & Yamamoto, 1997; Arvanitoyannis, Nakayama &Aiba, 1998), plasticisers like sorbitol, glycerol, acetylated monogycerides, polyethylene glycol and sucrose (Greener & Fennema, 1994; Arvanitoyannis *et al.*, 1997, Arvanitoyannis *et al.*, 1998) and presence of other materials affect the strength and permeability of gelatin films. Glutaraldehyde crosslinking of gelatin leads to decrease in film extensibility, increase in stress at break and Young's modulus (Bigi, Cojazzi, Panzavolta, Rubini & Roveri, 2001).

A number of alternative approaches for manufacturing products without gelatin have been tried (Johnston-Banks, 1990). For water dessert gels, carrageenan and other gums have been used. However, the absence of a low temperature melting point and greater pH sensitivity has led to limitations in the usefulness of these alternative materials.

Non-food uses of gelatin include manufacture of both hard- and soft-type drug capsules (Wood, 1977), depending on the Bloom (gel strength) and as binders for light-sensitive "emulsions" in the manufacture of photographic films (Kragh, 1977). Gelatin is also used in cosmetics, as a sizing agent in paper manufacture, as an adhesive and as a floculant (Wood, 1977).

1.2.2 Manufacture of gelatin and its effect on gelatin properties

The procedure for the manufacture of gelatin varies and the raw material used determines the process adopted and the quality of gelatin formed (Poppe, 1992). The procedure generally involves acid and sometimes alkali pretreatment of skin or bones, aqueous extraction of gelatin followed by filtration, evaporation, cooling, drying and milling (Priebbenow, 1998). Gelatin extracted with only acid pretreatment is referred to as type A gelatin while those whose manufacture entails alkali pretreatment are referred to as type B. Type of acid used for extraction (Grossman & Bergman. 1992; Gudmundsson & Hafsteinsson, 1997; Priebbenow, 1998; Gòmez-Guillèn & Montero, 2001), thermal history (Cho & Song, 1996; Michon, Cuvelier, Relkin & Launay, 1997), extraction pH (Tawfeek, Khalid & Safwat, 1992; Priebbenow, 1998), and salts (Priebbenow, 1998) influence the yield as well as the properties of the resulting gelatin. Cho & Song (1996) showed that the yield of pigskin gelatin increases with extraction temperature, whereas gel strength, viscosity and molecular weight decrease. According to Chung, Kim, Ockerman and Min (1990), scalding of pigskins before gelatin extraction decreases viscosity and gel strength of resulting gelatin. Alkali-processed gelatin from chicken legs have been found to have higher melting point and viscosity than acid-processed gelatin from the same source (Tawfeek *et al.*, 1992). Acid-processed gelatins and gelatins extracted at high temperature normally have a higher proportion of low molecular weight peptides arising from hydrolysis and as a result may exhibit lower viscosity. The acid-processed gelatins also generally exhibit less thermo-hydrolysis when exposed to heat (Koepff, 1984). This is because they already contain a higher proportion of low molecular weight peptides and are less likely to undergo as much hydrolysis as the alkali-processed gelatins.

The type of acid used in extraction of gelatin has been shown to influence gelatin properties (Müller & Heidemann, 1993; Gòmez-Guillèn & Montero, 2001). Gòmez-

Guillèn and Montero (2001) reported lower gel strength, viscous modulus, elastic modulus and melting temperature for megrim skins gelatin extracted with citric acid compared to that extracted using acetic or propionic acid. Müller and Heidemann (1993) compared the degree of collagen hydrolysis by different acids during manufacture of gelatin from bovine hide and reported a higher degree of hydrolysis by organic acids than by inorganic acids. According to Gòmez-Guillèn and Montero (2001), the differences in the extent of collagen hydrolysis registered while using different acids in otherwise similar conditions can be attributed to differences in ionic strength. These authors proposed that more hydrolysis occurs at higher ionic strength.

The schematic below (Fig. 1.1), adapted from Poppe (1992) outlines the processes used in making gelatin from different sources.

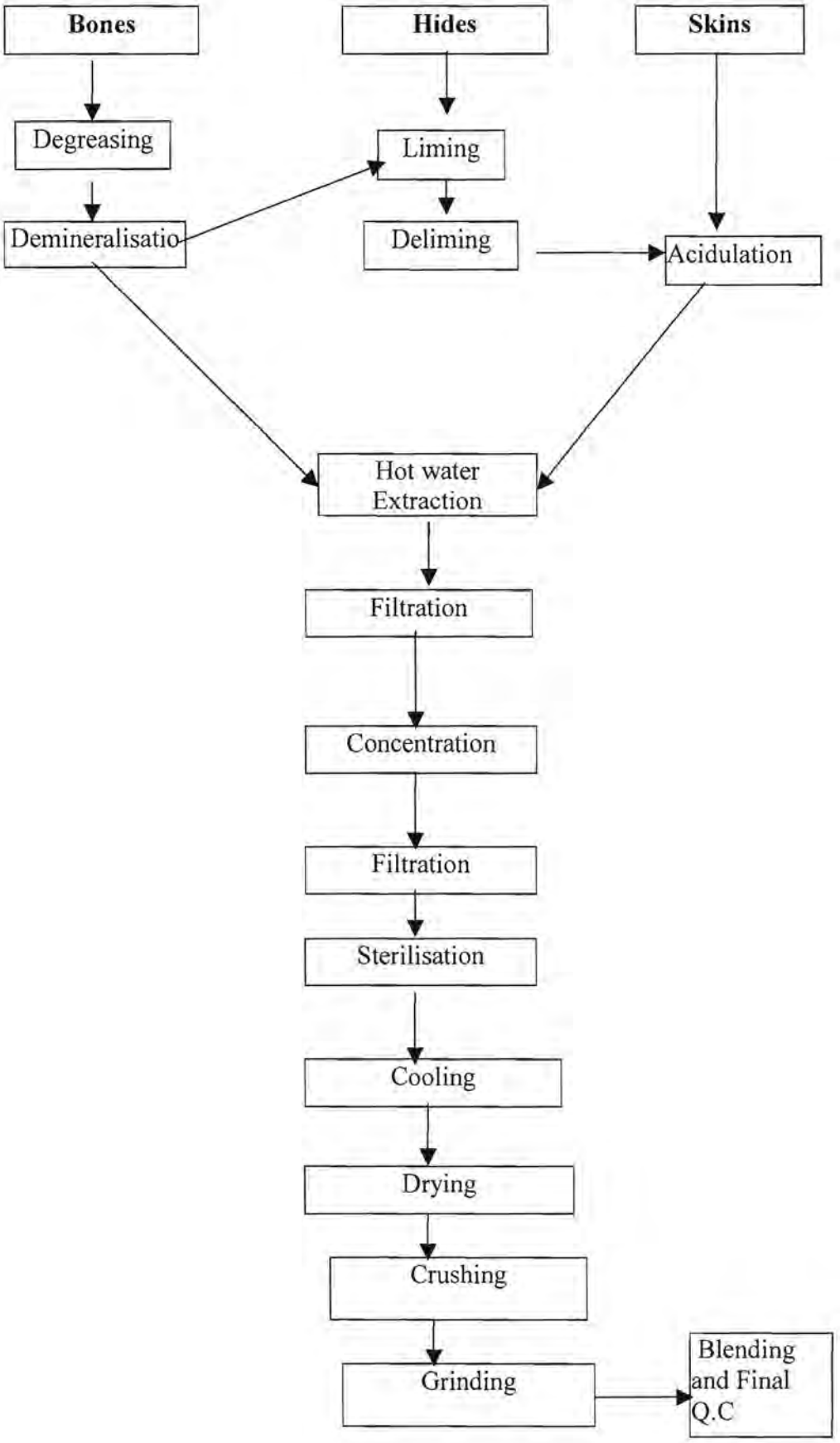


Figure 1.1: Procedures for gelatin extraction from different sources (Adapted from Poppe, 1992)

Fish skin gelatin has been produced using combined acid and alkali pretreatment (Grossman & Bergman, 1992; Gudmundsson & Hafsteinsson, 1997; Wanwimol & Worawattanamateekul, 1999) as well as by acid pretreatment only (Gòmez-Guillèn & Montero, 2001). The acids that have been used in fish skin gelatin production include sulphuric acid, hydrochloric acid and phosphoric acid (Gudmundsson & Hafsteinsson, 1997), propionic, acetic, citric, lactic and tartaric acid (Gòmez-Guillèn & Montero, 2001). Sodium hydroxide or calcium hydroxide are the main reagents used in alkali pretreatment. Gudmundsson and Hafsteinsson (1997) studied the characteristics of gelatin from fish skins and showed that the chemical treatments affected both the yield and properties of the gelatin.

1.2.3 Collagen

Collagen is the most abundant protein of animal origin, comprising approximately 30% of total animal protein. Collagen, extracted from different raw materials has been used for clarifying beverages (Taylor, 1997; Hickman, Sims, Miles, Bailey, de Mari & Koopmans, 2000), in cosmetics (Morimura, Nagata, Uemura, Fahmi, Shigematsu & Kida, 2002), in casings for meat products (Hood, 1987) and in a host of biomedical applications (Lee, Singla & Lee, 2001). Medical applications of collagen include use in drug delivery systems, sponges for burns and wounds and in tissue engineering. Collagen is also the raw material from which gelatin is derived. The properties of collagen from which gelatin is derived significantly influence the properties of gelatin.

1.2.3.1 Amino acid composition

Collagen has a repeating primary sequence of $(Gly\text{-}X\text{-}Y)_n$ with approximately one third of X and Y being imino acids (Franenkel-Conrat, 1963; Balian & Bowes, 1977; Darby & Creighton, 1993). Glycine makes up approximately one third of the collagen residues and alanine, approximately one ninth (Balian & Bowes, 1977). Generally, collagen contains very low levels of the sulphur-containing and aromatic amino acids and is among very few proteins that contain hydroxyproline (Veis, 1964).

Mammalian and avian collagens have very similar amino acid composition (Balian & Bowes, 1977).

Various authors have reported amino acid composition of fish collagens and generally reveal that the levels of the imino acids proline and hydroxyproline vary significantly among different fish species (Balian & Bowes, 1977; Poppe, 1992; Lu, Weilmeier, Chapman & Regenstein, 1994, Gudmundsson & Hafsteinsson, 1997; Lu, Chapman & Regenstein, 1997; Gilsenan & Ross-Murphy, 2000). The amount of imino acids, especially hydroxyproline depends on the environmental temperature at which the fish lives and it affects the thermal stability of the collagens (Rigby, 1968; Balian & Bowes, 1977; Kimura, Zhu, Matsui, Shijoh & Takamizawa, 1988). Collagens derived from fish species living in cold environments tend to have a lower content of hydroxyproline and they exhibit lower thermal stability than those from fish living in warm environments. Cold water fish species have also been reported to contain higher levels of the hydroxyamino acids, serine and threonine (Balian & Bowes, 1977). Studies on amino acid composition of collagens from warm water fish species have been quite limited. Grossman and Bergman (1992) showed that tilapia gelatin contains higher levels of imino acids than cold water fish collagens. The stabilising effect of hydroxyproline on the triple helical structure of collagen has been attributed to its involvement in inter-chain hydrogen bonding (Darby & Creighton, 1993). According to Holmgren, Taylor, Bretscher and Raines (1998), the stabilising effect of hydroxyproline results from the inductive effect of the hydroxyl group of hydroxyproline. They provided support for this hypothesis by substituting hydroxyproline with fluoroproline in peptides and found that the stability of the peptides, as shown by denaturation temperature, was in the order $(ProProGly)_{10} < (ProHypGly)_{10} < (ProFlpGly)_{10}$. The high stability of the ProFlpGly was attributed to the high electronegativity of fluorine.

Few studies have been conducted to compare amino acid composition of collagen from different tissues of the same organism. Eastoe and Leach (1958) and Balian and Bowes (1977), reported the amino acid composition for collagen from several sources, showing that collagens derived from bone and skin of the same animal generally had similar amino acid compositions.

1.2.3.2 Collagen super secondary structure

Collagen consists of three left handed helices (α-chains), normally twisted about each other (Figure 1.2) to form right handed tri-helical structures (Balian & Bowes, 1977; Darby & Creighton, 1993). The chains are held together by Van der Waals forces and hydrogen bonds between the NH group of glycine and a backbone carbonyl in one of the other chains and further stabilised by hydrogen bonding involving hydroxyproline (Darby & Creighton, 1993). Glycine allows the chains to come close enough for hydrogen bonding and water helps to stabilise the bonds (Balian & Bowes, 1977). The super helical aggregates of collagen are held together by covalent crosslinking of hydroxylysine residues, introduced after the helices are formed (Darby & Creighton, 1993).

Figure 1.2: Triple helical structure characteristic of collagen (Adapted from Bailey & Light, 1989)

X-ray diffraction shows that a collagen helix typically has a length of 100 nm and a diameter of 1.4 nm (Balian & Bowes, 1977; Darby & Creighton, 1993). Based on light and dark bands in collagen electron micrographs, Balian and Bowes (1977) proposed that individual molecules normally overlap with each other.

According to Bailey, Paul and Knott (1998) skin collagen fibres are randomly oriented and permit considerable extension. Tendon fibres are aligned in parallel while bone collagen fibres are organised in concentric layers to maximise strength.

1.2.3.3 Collagen variants

There are at least 19 variants of collagen, named type I – XIX (Bailey *et al.*, 1998) and more continue to be characterised. These collagen variants vary in their macromolecular structure (Bailey, 1987). Type I, II, III and V are the fibrous

collagens (Bailey & Light, 1989; Bailey *et al.*, 1998). These collagens are long (300 nm), rod-like molecules, which self assemble in a parallel, quarter staggered end overlap arrangement to form fibres possessing a characteristic band pattern with a periodicity of 67 nm (Bailey *et al.*, 1998).

Type I collagen is found in all connective tissue, including bones and skins (Bailey & Light, 1989; Bailey *et al.*, 1998). It is a heteropolymer of two α1 chains and one α2 chain. The α1 and α2 chains each have a molecular weight of about 95,000 Da but vary slightly in their amino acid composition. Type I collagen consists of one third glycine and contains no tryptophan and cysteine and is very low in tyrosine and histidine. Type II collagen is a homopolymer of three α1 chains and contains three times the amount of hydroxylysine as type I collagen (Bailey & Light, 1989). A high proportion of these residues are O-glycosylated to sugars. Type II collagen contain more methionine than type I collagen. The dominant collagen in cartilages is type II collagen.

Type III collagen is mainly found in embryonic tissue, scar tissue, arteries and intra-organ connections (Bailey & Light, 1989). It is composed of identical α1 chains and contains intra and possibly intermolecular disulphide bonds. Type V collagen contains α1 and α2 chains in a ratio of 1:2 as well as α3 chains. α3 chains contain more cysteine than α1 and α2 chains. Type IV collagen is high in hydroxyproline and hydroxylysine and in addition to the usual 4-hydroxyproline, it also contains 3-hydroxyproline.

Type IV, VII and X, belong to the category of network collagens. These form networks and are found in basement membranes. Type VI collagen occurs in loosely packed filamentous structure formed by end to end alignment of tetramers. Type IV, VI, VII, IX and X are filamentous and not important in tissues commonly used for gelatin extraction. The other collagen variants occur in minor amounts and are mainly found associated with the major variants. The functions of most of these minor collagen variants have not been established. The different collagen variants also vary in the nature of the constituent α-chains. Different α-chain types vary slightly in amino acid composition and as a result have slight differences in hydrophobicity.

Several studies have focused on the characterisation of different fish skin collagens (Piez, 1965; Rigby, 1968; Kimura & Ohno, 1987; Sato, Yoshinaka, Yoshiaki & Sato, 1989; Montero, Borderias, Turnay & Leyzarbe, 1990; Montero, Alvarez, Marti & Borderias 1995; Montero, Gòmez-Guillèn & Borderias, 1999; Nagai & Suzuki, 2000; Sivakumar, Arichandran, Suguna, Mariappan & Chandrakasan, 2000). Most fish skin collagens have been found to be type I, consisting of α1 and α2 chains (Nagai, Yamashita, Taniguchi, Kanamori & Suzuki, 2001; Gòmez-Guillèn, Turnay, Fernández-Diaz, Ulmo, Lizarbe & Montero, 2002; Nagai, Arakai & Suzuki, 2002). These α-chain variants though having approximately the same molecular weight (~95,000Da) can be separated by SDS PAGE due to their different affinity for SDS. Alpha 2 has a higher affinity for SDS and consequently exhibits a higher mobility than α1 (Kubo & Takagi, 1984). Piez (1965) isolated three variants of α-chains (α1, α2 and α3) from cod skin collagen and found that these variants differed in their amino acid composition. Alpha 3 has also been isolated from rainbow trout (Saito, Takenouchi, Kunisaki & Kimura, 2001), white sturgeon (Kimura, 1992), common horse mackerel (Kimura, *et al.*, 1988; Yoshida, Fujisawa, Mizuta & Yoshinaka, 2001) and eel (Kimura *et al.*, 1988).

1.2.3.4 Age-related changes in collagen

The most widely studied and probably most important change in collagen with age is crosslinking. According to Balian and Bowes (1977), esters, carbohydrates, aldehydes, γ-glutamyl and ε- amino groups are involved in collagen crosslinking. The mechanisms of collagen crosslinking have been extensively studied by Bailey and colleagues (Kent, Light, & Bailey, 1985; Bailey & Light, 1989; Sims & Bailey, 1992; Bailey, Sims, Avery, & Miles, 1993; Bailey *et al.*, 1998; Sims, Avery, & Bailey, 2000). Collagen crosslinking involves two different mechanisms. The initial crosslinking of collagen molecules during development involves a precisely controlled enzymic process (Figure 1.3). The products are divalent but mature spontaneously to form stable multivalent crosslinks.

is the dominant mature crosslink in dermal collagens. The keto-imines, which are the dominant immature crosslinks in bones, react with hydroxy-lysine aldehyde to form the pyridinoline hydroxylysyl-pyridinoline (Hyl-Pyr) or with lysine aldehyde to form pyrrole.

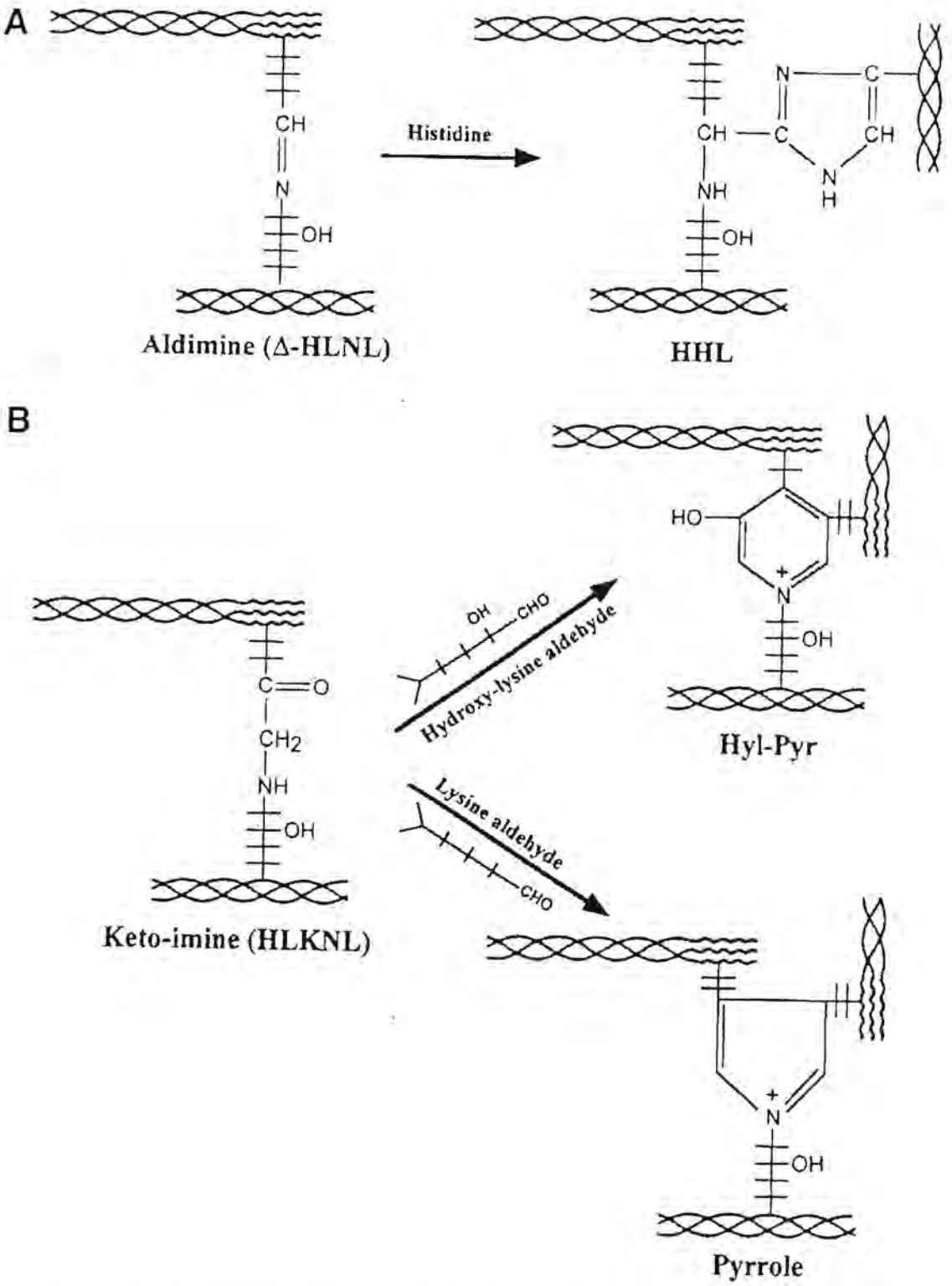


Figure 1.4: Maturation of immature collagen crosslinks A - aldimine & B - keto-imine (Source: Sims *et al.*, 2000).

Crosslinking by glycation occurs during aging and is most important in determining the properties of collagen during senescence (Bailey *et al.*, 1998). It has been

demonstrated using *in vitro* studies that glycation causes increase in the tensile strength (Kent *et al.*, 1985; Bailey *et al.*, 1993) and denaturation temperature (Bailey *et al.*, 1993) of collagen. It seems therefore that even for adult animals (after achieving sexual maturity), the ease with which collagen can be transformed to gelatin decreases with age.

Fish skin collagens have been reported to develop minimal amounts of mature crosslinks (Hickman *et al.*, 2000). Cohen-Solal, Le lous, Allain and Meunier (1981) also demonstrated by measuring hydrothermal isometric tensions that fish skin collagen crosslinks do not mature to thermally stable bonds. As a result of its low content of mature crosslinks, even adult fish skin collagen is soluble in dilute acids (Sikorski & Borderias, 1988).

1.2.3.5 Stability of collagen to chemicals, enzymes and heat

Balian and Bowes (1977) reviewed the effects of heat, chemicals and enzymes on collagen. Generally, native collagen is very stable and resistant to enzyme and chemical treatments at ambient temperatures. Prolonged alkali treatment however, leads to loss of amide nitrogen and conversion of a small fraction of arginine to ornithine. This leads to change of the isoelectric point (pI) from about 9.3 to 4.8 - 5.0. Treatment of acid soluble collagen with dilute alkali solutions (pH 11) at 40°C for short periods leads to limited breakdown to form $\alpha 1$ and $\alpha 2$ fractions. Treatment of insoluble hide and bone collagen with 5 - 8% NaOH, saturated with sodium phosphate leads to greatly increased solubility in acid and the resulting eucollagen has properties comparable to those of soluble collagen extract. Hydrazine and hydroxylamine break ester-like bonds and increase collagen solubility. Proteolytic enzymes may cleave a few intra-molecular linkages and may significantly increase solubility, reduce viscosity and tensile strength. Most enzyme activity occurs on the non-helical part of collagen and significant breakdown of the collagen helix can only be achieved by collagenases.

Heat treatment of collagen leads to denaturation and collapse of the helical structure and disentanglement of the chains. The collagen also shrinks (at the shrinkage temperature) causing textural changes in the tissue (meat) in which it is contained.

The stability of collagen depends on the extent of crosslinking in the collagen and as discussed earlier, this increases with age of the animal and varies with tissue. Thermal stability of collagen also depends on the imino acid content (Rigby, 1968; Purslow, 1987). The denaturation temperature for mammalian collagens is about 36 - 41°C (Hickman *et al.*, 2000; Leikina, Mertt, Kuznetzova & Leikin, 2002). Denaturation temperature of fish collagens vary widely and are related to the environmental temperature in which the fish lives. Collagen denaturation temperature has been reported for cod (15°C) (Rigby, 1968), Alaska pollack (16.8°C) (Kimura & Ohno, 1987), muscle of carp (32.5°C), eel (29.3°C), common mackerel (26.1°C), chum salmon (19.4°C) (Kimura *et al.*, 1988), Japanese seabass (30°C), skip jack tuna (29.7°C), ayu (29.7°C) (Nagai & Suzuki, 2000) halibut (17°C) and bigeye-tuna (31°C) (Rose, Kumar & Mandal, 1988).

1.2.4 Changes associated with conversion of collagen to gelatin

According to Johns and Courts (1977) transition of mature mammalian collagen to gelatin entails the hydrolysis of lateral, mainly non-peptide linkages, to effect a depolymerisation of multi-chain units; hydrolysis of peptide bonds; and disruption of hydrogen bonding. Some of the lateral bonds are acid labile. They are only slowly affected by alkali up to pH 13, although the effect is faster above this pH. Collagens with minimal crosslinking e.g. fish skin collagen require only the disruption of hydrogen bonding.

Hydrolysis of peptide bonds mainly appear to involve linkages at glycine (Johns & Courts, 1977) and at arginine (Müller & Heidemann, 1993). Müller and Heidemann (1993) found arginine to the left of the COOH end in 50% of peptides derived from calfskin collagen by the acid gelatin manufacture process. In 40% of the peptides, it was found to occupy the second position to the right of the split position. Proline and hydroxyproline were never found at the terminal ends of the peptide sequences. Koepff (1984) compared 250 g Bloom acid-processed pigskin gelatin to similar gel strength bovine hide and bone gelatins made by the acid and alkali processes and found the proportion of peptides smaller than the α chain, to vary in the order limed hide > acid pigskin > limed ossein. The proportion of the fractions with molecular

weight less than α were 35%, 32% and 25% respectively for the limed hide, acid pigskin and limed ossein gelatins. It seems both the raw material and the process used in gelatin manufacture determine the extent of peptide bond hydrolysis in the manufacture of gelatin.

The composition of gelatin is similar to that of the source collagen, except for changes arising from hydrolysis of amide groups of asparagine and glutamine, split off of urea from arginine to give ornithine and concentration of dominant residues (glycine, alanine, proline and hydroxyproline) due to purification (Eastoe & Leach, 1977). The split off of urea occurs only to a limited extent while the extent of the amide hydrolysis depends on the severity of the gelatin manufacturing process (Eastoe & Leach, 1977; Poppe, 1992). Severe acid pretreatment such as that used in demineralisation of bones causes hydrolysis of a significant proportion of amide groups, while mild acid pretreatment causes minimal change. The alkali process normally entails longer pretreatment and results in conversion of most amide groups to carboxyls and yields purer gelatin (Eastoe & Leach, 1977). According to Johns and Courts (1977), the long soak in alkali during lime pretreatment removes a considerable amount of non-collagenous material.

1.2.5 Properties of gelatin

The properties of gelatin vary with source (Gilsenan & Ross-Murphy, 2000), extraction procedure (Poppe, 1992; Gudmundsson & Hafsteinsson, 1997) and additives present in the gelatin or the system in which the gelatin is analysed (Choi & Regenstein, 2000; Sarabia, Gómez-Guillén & Montero, 2000; Fernández-Diaz, Montero & Gómez-Guillén, 2001).

1.2.5.1 Composition

Commercial gelatins contain approximately 87.5% protein, with the 12.5% constituted almost exclusively moisture and ash. The amino acid composition of gelatin is determined by the amino acid composition of the collagen from which it is derived and is therefore dependent on the source species. Gelatin contains all the essential amino acids found in proteins except tryptophan (Leach & Eastoe, 1977).

Cysteine is either absent or present in trace quantities (Poppe, 1992). In contrast to mammalian gelatins, fish gelatins vary widely in composition, particularly with respect to the imino acids proline and hydroxyproline (Norland, 1990; Poppe, 1992; Lu *et al.*, 1994, Gudmundsson & Hafsteinsson, 1997; Lu *et al.*, 1997; Gilsenan & Ross-Murphy, 2000; Yoshimura, Terashima, Hozan, Ebato, Nomura, Ishii & Shirai, 2000). Table 1.1 below gives the amino acid composition for Ox hide, tilapia and cod skin gelatins. The imino acid content is ~30% for mammalian gelatins (Eastoe, 1967; Poppe, 1992) and 16 - 18% for most fish species (Osborne *et al.*, 1990; Norland, 1990; Gudmundsson & Hafsteinsson, 1997; Gilsenan & Ross-Murphy, 2000). The amino acid content of gelatins vary from one source to another but always consists of large amounts of proline, hydroxyproline and glycine (Gilsenan & Ross-Murphy, 2000). The ratio of glycine to glutamic acid has been found to be higher in cod skin gelatin than in tilapia skin gelatin, whereas the levels of proline and hydroxyproline are lower in cod skin gelatin (Gudmunsson & Hafsteinsson, 1997). The level of imino acids is important in gelatin since their pyrrolidine groups act as nucleation sites for the formation of potential junction zones during gelation (Ledward, 1986). Gelatin with high imino acid content, therefore tend to exhibit better gelling properties than those with low levels of imino acids.

Table 1.1: Amino acid composition (g/1000 g protein) of some gelatins

Amino Acid	Ox hide Gelatin[a]	Tilapia Gelatin[b]	Cod Skin Gelatin[c]
Aspartic Acid	66	52.9	61.7
Threonine	22	25.5	24.2
Serine	40.8	31.2	62.5
Glutamic Acid	116	88.5	100
Alanine	110	92.2	88.2
Cysteine	Nd	Nd	<1
Valine	25.7	13.2	18.7
Methionine	8.6	12.3	19.2
Isoleucine	17.2	10.7	13.6
Leucine	34.1	25.6	23.3
Tyrosine	3.8	7.2	6.3
Phenylalanine	22.6	19.7	17.7
Histidine	8.2	10.1	13.9
Hydroxylysine	9.1	12.7	Nd
Lysine	42.6	35.4	34.4
Arginine	87	87.8	82.4
Hydroxyproline	134	103.1	67.7
Proline	165	150.5	105.1
Glycine	276	204.0	248.8
Ornithine	Trace	0.4	6.0
Ammonia	Nd	16.1	4.8

[a] – Eastoe (1967)

[b] - Grossman & Bergman (1992)

[c] - Gudmundsson & Hafsteinsson (1997)

Nd – Not detected

Generally, cold-water fish gelatins have low hydroxyproline content (Gilsenan & Ross-Murphy, 2000; Gudmundsson, 2002). As a result, cold-water fish gelatins have a higher critical concentration (minimum gelatin concentration required to form a gel)

and lower melting point than bovine and warm water fish gelatins. Sarabia *et al.* (2000) reported a higher level of imino acids in tilapia than in megrim. The two were mainly found to differ in the amount of hydroxyproline. Megrim was found to contain more serine and methionine. Since fish gelatins vary widely in their functional properties, different fish species may be targeted depending on the desired properties.

1.2.5.2 Molecular weight distribution

Since gelatin is a poly-dispersed (heterogenous) protein it is not helpful to think of average molecular weight. Instead the molecular weight distribution is of greater value. Generally, most gelatins consist of molecules with molecular size ranging from more than 1,000,000 Da to about 10,000 Da (Tavernier, 1989). The α-chain (molecular weight approximately 95,000 Da) is the basic unit in gelatin and the large molecules are polycondensates of α-chains as dimers (β-chains), trimers (γ-chains) and oligomers (microgels). Gelatin also consists of variable amounts of peptides with molecular weight less than 95,000 Da. These result from hydrolysis of α-chains. The molecular weight distribution of gelatin is determined by the extent of crosslinking in the source collagen and on the severity of the extraction process (Reich, Walther & Stather, 1962; Cole & Roberts, 1996). Acid-processed gelatin normally contains more fragments than alkali-processed ones (Koepff, 1984; Cole & Roberts, 1996). This is a result of cleavage of more peptide bonds in the acid process. According to Stainsby (1990), the content of α-chains in gelatin falls and low molecular weight (< α) peptides increase as extraction advances.

As high as 30 - 50% of high Bloom gelatins are normally made of molecules of size in the range between α and β-chains (Poppe, 1992). Some gelatins also contain aggregates with molecular weight up to 10 million Da and polypeptides with molecular weights less than 80,000 Da. In-depth discussions of the relationship between molecular weight distribution and individual functional properties are presented later.

Different techniques have been used to study the molecular weight distribution of gelatin and collagen. SDS PAGE has been widely applied to gelatin and collagen

(Koepff, 1984; Ming-Zhi, Jin-Kang & Chi, 1984; Chalepakis, Tanay & Heidemann, 1985; Hayashi, Kawamura, Ohtsuka, & Itoh, 1990; Norland, 1990; Reddy, Hudson, Bailey & Noelken, 1993; Cole & Roberts, 1996; Gómez-Guillén, Sarabia, Solas & Montero, 2001). The conditions used by different authors vary, but in all cases, SDS PAGE shows gelatin to be a polydispersed protein. According to Koepff (1984) SDS PAGE with densitometric analysis provides accurate and highly repeatable results for molecular weight distribution of gelatin. This, combined with the relatively low cost of electrophoresis equipment and ease of the technique has made SDS PAGE a widely used tool for the study of molecular weight distribution of gelatin.

Size exclusion chromatography has also been applied to study the molecular weight distribution of collagen and gelatin, either using the low pressure techniques (Gel filtration chromatography) (Piez, 1965; Miller, Martin, Piez & Powers, 1967; Cole & McGill, 1988) or by applying high pressure size exclusion chromatography (Dupont, 2002). In several studies, gel filtration chromatography has been followed by electrophoresis (Kimura & Ohno, 1987; Kimura *et al.*, 1988; Nagai & Suzuki, 2000; Nagai *et al.*, 2001). This allows separation of more molecular species.

One problem faced in the determination of molecular weight distribution of collagen and gelatin is finding suitable molecular weight standards. Globular proteins are not suitable molecular weight standard markers for gelatin and collagen. Kubo, Isemura and Takagi (1982) found that the collagen α-chain which consists of 1052 amino acid residues has the same effective hydrodynamic volume as a standard globular polypeptide consisting of about 800 residues. This is because of the unique amino acid profile of collagen. The difference between the mobility of collageneous proteins and globular protein has been attributed to the former's high content of the relatively small amino acid residues, glycine, proline and alanine (Noelken, Wisdom, & Hudson, 1981). The molecular weight distribution of collagenous materials can nonetheless be compared to each other by SDS PAGE since the relative migration (R_f) of these proteins also exhibit a high correlation to their molecular weight. Noelken *et al.* (1981) showed that a plot of R_f against molecular weight for collagen was parallel to that of globular protein, with collagenous materials having lower R_f values for a given molecular weight. In studies where globular proteins have been used as molecular weight markers during SDS PAGE of collagen (Mizuta, Hwang &

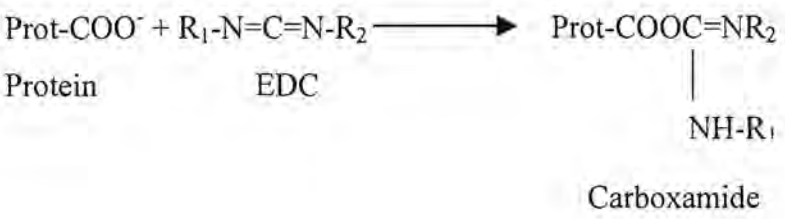
Yoshinaka, 2002; Mizuta, Isobe & Yoshinuka, 2002; Mizuta, Hwang & Yoshinaka, 2003) the mobility of α-chains (molecular weight ~95,000 Da) has been found to be lower than that for β-galactosidase (molecular weight 116,000 Da). Based on such results, α-chain could be interpreted to have molecular weight of about 120,000 Da. This is much higher than the true molecular weight of approximately 95,000 Da determined by sedimentation (Piez, 1967). This proves that the mobility of collagen follows a different behaviour from that followed by globular proteins and when these proteins are used as molecular weight markers for collagen, results should be interpreted with caution. Some workers have opted for other molecular weight markers when conducting SDS PAGE for gelatin or collagen. Cole and Roberts (1996) used acid soluble collagen from calfskin as the molecular weight marker for gelatins during SDS PAGE.

1.2.5.3 Isoelectric point

Differences in the isoelectric point (pI) of gelatins are related to the extent of modification during preparation (Poppe, 1992). The extent of modification determines the proportion of amide groups, which in turn determine the isoelectric point (Eastoe & Leach, 1977). The pI of gelatin may vary from 9.4, which is the isoelectric point of unmodified collagen, to 4.8, which represents maximum modification, i.e. 90 - 95% of potential carboxylic acid groups exist in the free state. Gelatins produced by mild acid pretreatment for example, pigskin gelatin, have high pI values because the process is less severe and less modification of collagen occurs. On the other hand, alkali or lime pretreatments lead to more modification due to the prolonged treatment. As a result only a few amide groups survive and their pI are in the range 4.8 - 5.2. Bone gelatin produced without lime treatment usually exhibits intermediate pI values.

Hayashi *et al.* (1990) caused amidation of lime-processed gelatin by reacting with ammonia and water-soluble 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC). They reported an increase of pI from 5.0 to 10.4, decrease in gel strength while viscosity reduced and then increased as amidation was increased. Amidation was found not to cause inter- or intra-molecular crosslinking and the changes in functional properties were attributed to inhibition of gelatin renaturation into collagen-like

helical structures. It seems the negative charge of the carboxyl group is important in gelatin renaturation and amidation removes the negative charge on the carboxyl group of proteins, converting the carboxyl groups to carboxamide groups (Equation 1.1).



Equation 1.1

1.2.5.4 Gel strength

When concentration is sufficiently high and temperature sufficiently low, gelatin solutions change into gels (Wainewright, 1977; Stainsby, 1977). The standard measure of the gel strength of gelatin gels is Bloom gel strength. This is defined as the mass in grams which is necessary to apply to the surface of a gel, by means of a piston 12.7 mm in diameter, in order to produce a depression 4 mm deep (BSI, 1975; Poppe, 1992). The gel must be contained in a standard flask at a concentration of 6.67% (m/v) and must have been matured at 10 °C for 16 - 18 hours. The concentration, maturation time and temperature are standardised because gel strength increases with concentration of gelatin and maturation time but varies inversely with temperature (BSI, 1975; Ledward, 1986; Shehata, Shalaby & Hassan, 1994). Gel strength also varies with pH and reduces with amidation (Hayashi *et al.*, 1990). Additionally, gel strength depends on the molecular weight distribution of gelatin. Hydrolysis has been reported to cause a drop in the gelling properties of gelatin (Poppe, 1992) while crosslinking of gelatin with transglutaminase leads to increase in gel strength (Fernández-Diaz *et al.*, 2001). The higher the proportion of α and β fractions, together with larger peptides the higher the Bloom gel strength is likely to be (Johnston-Banks, 1990). One is likely however, to get gelatin of the same Bloom gel strength with quite different molecular weight distributions, indicating that the relationship between gel strength and molecular weight distribution is not one of direct proportionality. Koepff (1984) observed that increasing of peptides in acid processed pigskin gelatin from 33% to 63% by thermohydrolysis did not lead to

change in gel strength and concluded that the gel strength above a certain average molecular weight is independent of the average molecular weight.

Fernández-Diaz *et al.* (2001) showed that crosslinking of gelatin using transglutaminase and also addition of magnesium sulphate and glycerol leads to increase in gel strength for cod and hake gelatin. The increase was found to be higher for hake than for cod and this was attributed to the higher imino acid content and higher content of low molecular weight fraction of the hake gelatin.

In many applications, high Bloom gelatin can be used to replace a low Bloom version, with a reduced level of use (Pye, 1996). Cold water fish gelatins generally have lower gel strength and this has been attributed to their low content of hydroxyproline and proline (Gilsenan & Ross-Murphy, 2000). Warm water fish species contain more of these imino acids (Grossman & Bergman, 1992) and some studies have shown that they give gels of higher gel strength (Leuenberger, 1991; Grossman & Bergman, 1992).

1.2.5.5 Melting point

Two definitions of gelatin melting point as given by the British Standards Institution and the Japanese Standards Association are presented here. Both of them require determination of a critical temperature at which gelatin softening begins to occur. According to The British Standards Institution (BSI, 1975) melting point of gelatin refers to the temperature at which a gelatin gel containing 6.67% gelatin in water and matured at 10°C for 16 – 18 hours softens sufficiently to allow drops of carbon tetrachloride to sink through the gel. According to the Japanese Standards Association (JSA, 1996), the melting point of gelatin is the temperature at which a gel containing 10% gelatin in water and matured at 10°C for 16 – 18 hours starts to flow, when gradually heated from 10°C. Molecular weight distribution is an important determinant of melting point. According to Ledward (1986), lower molecular weight gelatin fractions have a lower propensity to form gels and gelatins high in low molecular weight fractions tend to exhibit low melting temperatures. Melting point increases with concentration but is depressed by sodium chloride and is affected by maturation conditions (Choi & Regenstein, 2000). The values for melting

temperature recorded using the BSI and JSA standard methods for the same gelatin, would differ since the two methods use different gelatin concentrations. Amino acid composition is an important determinant of the temperature of gel formation and gel melting (Johnston-Banks, 1990). The low melting point and gel formation temperature for fish gelatins has been attributed to their low content of the imino acids proline and hydroxyproline (Gilsenan & Ross-Murphy, 2000; Fernández-Díaz *et al.*, 2001). Low imino acid content is associated with reduced propensity for intermolecular helix regeneration (Gilsenan & Ross-Murphy, 2000).

Salts such as magnesium sulphate, sodium phosphate and ammonium sulphate have been reported to increase the melting temperature of gelatin (Sarabia *et al.*, 2000). The change was attributed to interaction between the salts and the surrounding water, leading to greater screening of electrostatic interactions. Because of their large size, sulphates and phosphates, unlike small ions like Cl^-, are believed to remain further from the positively-charged centres of proteins. Instead, they interact with water and increase likelihood for formation of protein-protein junctions. Sarabia *et al.* (2000) proposed that in the case of hake lower ionic strength (0.1 - 0.5 M magnesium sulphate) may be suitable for promoting useful junctions by promoting protein unfolding without distorting the assembly of chains into collagen-like helical rods. This however, was not the case with tilapia gelatin and the difference was attributed to differences in conformation of gelatin molecules and to intrinsic factors such as amino acid composition and isoelectric point.

Gilsenan and Ross-Murphy (2000) studied the effect of blending different gelatins and reported that addition of 2% bovine gelatin to cod gelatin increased the melting point of cod gelatin by 5°C and proposed that presence of bovine gelatin stabilises cod gelatin helices.

1.2.5.6 Setting point and setting rate

When gelatin in solution is adequately cooled, it sets to form a gel. The temperature at which a gelatin solution in water gels is called the setting point (BSI, 1975; JSA, 1996). The concentration of gelatin must be specified. The setting temperature is lowered by mechanical action such as shear and increased by slow cooling.

According to Poppe (1992) setting point for a 10% solution varies from 24 to 29°C, depending mainly on Bloom gel strength and type of pretreatment used.

Setting time of gelatins is one of the most important physical properties with respect to the fast coating of thin layers such as those encountered in photographic applications. The log of setting time varies linearly (negative slope) with the log of gelatin concentration and setting time varies inversely with the square of gelatin molecular weight (Stainsby, 1977). This observation is rather misleading since gelatins exhibit a wide molecular weight distribution and therefore average molecular weight is of little importance. Tavernier (1989) by controlling manufacture conditions produced bone gelatins varying in the different molecular weight fractions. Gelatins with a high proportion of microgels were found to gel faster. The increase in setting rate was steepest between 3 and 10% microgels. According to Stainsby (1990), racemization occurring during prolonged exposure of collagen to alkali pH leads to gelatins with poorer setting properties.

Cold water fish gelatin, due to its low content of imino acids, shows rather low gelation temperature compared to mammalian gelatins (Leuenberger, 1991; Gilsenan & Ross-Murphy, 2000). The critical concentration (minimum concentration required to form a gel upon cooling) has also been shown to vary with source of gelatin. Gilsenan and Ross-Murphy (2000) showed that critical concentration for bovine gelatin is slightly lower than that of tilapia, tuna and megrim but much lower than that for cod. This too was attributed to differences in imino acid content, mainly, to differences in hydroxyproline. Addition of salts to fish skin gelatins, has been found to prolong setting time (Sarabia *et al.*, 2000) and this can be attributed to destabilisation of the gelatin structure. Mechanical disturbances also delay setting and setting temperature is higher when cooling is done slowly (Stainsby, 1977).

1.2.5.7 Viscosity

According to Poppe (1992) the viscosity of concentrated gelatin solutions depends mainly on interactions between gelatin molecules. At lower concentrations, the solvent and individual gelatin molecules play a more significant role. Above 40°C,

viscosity decreases exponentially with temperature. Viscosity of gelatin is lowest at the isoelectric point and increases exponentially with concentration.

At a concentration of 2% and the temperature range of 20 – 80°C, gelatins have been shown to exhibit Newtonian behaviour (Wulansari, Mitchell, Blanshard & Paterson, 1998; Marcotte, Taherian-Hoshahili & Ramaswamy, 2001). Based on this observation, Wulansari *et al.* (1998) proposed that gelatin coils are sufficiently compact to reduce the importance of entanglements but not so compact as to resist deformation when coils approach one another. Increase in shear rate therefore does not lead to change in apparent viscosity.

Fernández-Diaz *et al.* (2001) reported an increase in viscosity due to the addition of magnesium sulphate and glycerol to cod and hake skin gelatins. This was attributed to unfolding of the gelatins due to interactions with the additives as explained earlier. Molecular weight is of key importance in determining viscosity of gelatin solutions. Gelatins high in high molecular weight peptides exhibit the highest viscosities (Johnston-Banks, 1990). This has been attributed to high molecular weight gelatin molecules developing more helices during cooling. Viscosity degradation due to gelatin hydrolysis is generally more pronounced than Bloom degradation and depends on the rate of generation of low molecular weight peptides (Koepff, 1984). For gelatins with an initial high proportion of low molecular weight peptides, hydrolysis results in a less rapid loss in viscosity compared to gelatins with a low proportion of the low molecular weight fraction. Tavernier (1989) observed a high linear correlation (r^2 = 0.979) between increase in the content of microgel (450,000 - 900,000 Da) fraction and increase in gelatin viscosity. Variations in peptides smaller than α, the α and β fractions were found to influence viscosity to a lower extent.

1.2.5.8 Turbidity

The clarity of gelatin solutions depends on the conditions during and after extraction as well as on the pH (Poppe, 1992; Fernández-Diaz *et al.*, 2001). Early extractions of gelatin (lower temperature extracts) tend to be of higher clarity than later extracts. At low temperatures (< 30°C), gelatin extracts have the highest turbidity at their isoelectric point (Poppe, 1992). Ineffective filtration is a cause for high gelatin

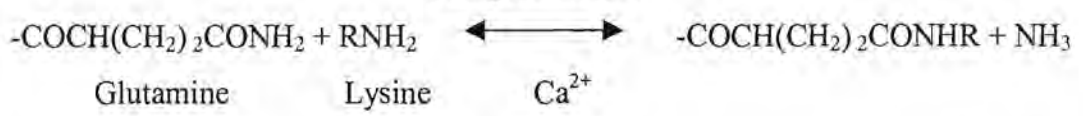
turbidity. High gelatin turbidity is undesirable since it may limit the use of gelatin in applications where clarity is essential.

1.2.5.9 Colour

Gelatin colour depends on the raw materials, the processing method and the order of extraction (Poppe, 1992). When gelatin has high colour intensity, it introduces the colour into the food or other materials in which it is applied. This may not be desirable, especially in applications where colour is critical to quality such as in water desserts. It is therefore advantageous that gelatin colour intensity is low and colour measurements are part of quality control protocols. Gelatin colour may be measured by use of tristimulus colorimeters or by visual observation (Cole & Roberts, 1997). Cole (1995) observed higher colour intensity for gelatins derived from older animal hides and attributed the colour to Maillard reactions involving gelatin and glucose. As noted earlier, glycation of collagen, which involves introduction of glucose moieties, occurs at advanced age (Bailey *et al.*, 1998). Such collagens have a higher degree of Maillard reaction and therefore higher colour intensity

1.2.6 Effect of crosslinking on the functional properties of gelatin

As noted earlier, the molecular weight distribution of gelatin is a key determinant of functional properties. Low molecular weight fractions generally do not enhance the functional properties of gelatin. Several studies have been conducted on the crosslinking of gelatin and its effect on the functional properties. Crosslinking of gelatin with transglutaminase has been the main means of enzymatic crosslinking applied to gelatin and there has been much interest in this in recent years (Fernández-Diaz *et al.*, 2001; Gòmez-Guillèn, Sarabia, Solas & Montero, 2001). Transglutaminase causes crosslinking by catalysing an acyl-transfer reaction in presence of Ca^{2+}, introducing a covalent crosslink between glutamine and lysine residues (Spector, 1982; Dickinson, 1997; Fernández-Diaz *et al.*, 2001) (Equation 1.2).



Equation 1.2

Fernández-Diaz *et al.* (2001) crosslinked gelatin with transglutaminase and reported increase in gel strength. Gòmez-Guillèn *et al.* (2001) reported increase in gelatin gel strength, hardness, elasticity and cohesiveness after crosslinking of megrim skin gelatin with transglutaminase. High levels of the enzyme (approaching 0.9 g/kg), however, reduced gel strength and hardness. This may have been due to creation of very high molecular weight aggregates. According to Stainsby (1977) abundance of very high molecular weight molecules leads to low gel strength. This is because very long molecules may fold back on themselves, forming intra-molecular junctions, which are not useful in promoting gel strength (Ledward, 1986). The effect of transglutaminase treatment on the physicochemical properties of different gelatins may vary due to differences in reactive residues, secondary and tertiary structures of the gelatins.

Crosslinking has also been shown to cause increase in melting temperature (Fernández-Diaz *et al.*, 2001; Gòmez-Guillèn *et al.*, 2001). Fernández-Diaz *et al.* (2001) reported increase in melting temperature of cod and hake skin gelatin following crosslinking with transglutaminase.

1.2.7 Effect of age of source animals on the properties of gelatin

The age of the source animals has been shown to affect the extractability and properties of gelatins. Reich *et al.* (1962) reported a much lower gelatin yield from acid-conditioned pigskins from animals aged 2.5 years compared to animals aged 1.5 years. This decrease in yield with age was accompanied by a decrease in viscosity and melting point of gelatin. For cattle hides, the yield decreased more slowly between the age of 11 weeks and 5 years and the changes in gelatin melting point and viscosity were less pronounced. Cole and McGill (1988) reported decrease in Bloom gel strength and viscosity of bovine hide gelatin with increase in animal age. Gelatin from the older animals was also found to contain higher levels of the low molecular

weight fraction. This is in agreement with the observation by Johnston-Banks (1990) that gelatin viscosity is proportional to the amount of the high molecular weight fraction. The older animals contain more stable collagen crosslinks and conversion of their collagen to gelatin results in a higher extent of peptide bond cleavage, since a more severe process is required. This results in a high concentration of low molecular weight fragments in the gelatin.

Reich *et al.* (1962), however, observed that before the general age-related reduction in viscosity and melting point, newborns and animals a few weeks old gave gelatin of lower viscosity. These authors unfortunately did not report any measurements for gel strength. They also reported high content of water and salt soluble protein in skins from newborn and very young (a few weeks old) animals. By comparing the influence of age on the yield and functional properties of gelatin, Reich *et al.* (1962) concluded that the age-related differences are not dependent on the absolute chronological age, but age in relation to the average life-span of the animal species concerned.

Cole and McGill (1988) reported decrease in extractability at 45°C for alkali treated hides between ages 6, 18 and 60 months, although hides from older animals gave a higher total yield. Bloom strength and viscosity were higher for the more easily extracted gelatins derived from the younger animal hides. Turbidity (Cole, 1995) and colour intensity (Cole & Roberts, 1996) have also been shown to increase with animal age for bovine hide gelatin. The increase in colour intensity was attributed to *in vivo* Maillard reaction.

1.2.8 Use of Fourier transform infrared (FTIR) spectroscopy in the study of collagen and gelatin

When infrared radiation interacts with matter, it may be absorbed, causing bonds to vibrate (Smith, 1996). Chemical structural fragments within molecules (functional groups) tend to absorb infrared radiation of the same wave number range regardless of the structure of the rest of the molecule. This means that there is a correlation between the wave numbers at which a molecule absorbs infrared radiation and its structure. This correlation allows the study of the structure of molecules.

Infrared spectroscopy has been recognised for a long time as a useful technique for estimating the secondary structure of proteins (Ambrose & Elliot, 1951). Computerised FTIR helped to improve sensitivity and the subtraction of spectra arising from solvents (Surewicz & Mantsch, 1988). There has been a lot of development in the interpretation of protein secondary structure from FTIR spectra in recent years (Surewicz & Mantsch, 1988; Prystupa & Donald, 1996; Pelton & McLean, 2000; Dzwolak, Kato & Taniguchi, 2002).

The amide groups of polypeptides and proteins possess several characteristic vibrational modes or group frequencies (Miyazawa, Shimanouchi & Mizushima, 1956; Durrani & Donald, 1996). Proteins and peptides normally show absorption bands at around 3300 cm^{-1} (amide A), 1660 cm^{-1} (amide I), 1550 cm^{-1} (amide II), 1350 – 1220 cm^{-1} (amide III) and 700 cm^{-1} (Kretschmer, 1957). The amide I band between 1600 and 1700 cm^{-1} is the most useful for infrared spectroscopic analysis of protein secondary structure. Amide II and III bands have also been applied to the study of protein secondary structure. However, amide II sensitivity is not well established while, though sensitive to the secondary protein structure, the amide III band of proteins is very weak (Surewicz & Mantsch, 1988).

The amide I and II vibrational modes are mixtures of bond bending and bond stretching vibrations resulting from complex motion of the atoms (Fraser & Suzuki, 1970). The amide II band (~1550 cm^{-1}) involves a mixture of CN stretching (40%) and in-plane NH bending (60%) while amide I involves 80% C=O stretching, 10% CN stretching and 10% in-plane NH bending contributions. The amide I band represents primarily the C=O stretching vibrations of the amide groups coupled to the in-plane NH bending and CN stretching modes (Surewicz & Mantsch, 1988). The exact frequency of this vibration depends on the nature of hydrogen bonding involving the C=O and NH moieties, which in turn is determined by the particular secondary structure adopted by the polypeptide chains. Because proteins are heteropolypeptides, they contain polypeptide fragments in different conformations. These manifest as complex composites consisting of overlapping component bands in the amide I band. These component bands can be separated by resolution enhancement achieved by band narrowing. For some proteins, the components of the

amide I band have been assigned to specific polypeptide conformations (Byler & Susi, 1986; Jaworsky, Brauner & Mendelsohn, 1986; Surewicz & Mantsch, 1988). A band centered at 1650 – 1658 cm^{-1} in ^2H_2O (deuterated water) is believed to represent α-helical structure, while polypeptides in a non-ordered conformation have a peak at 1640 – 1648 cm^{-1}. In H_2O however, the amide I peak corresponding to unordered peptides appears at 1650 – 1660 cm^{-1} and is therefore not easily resolved from the amide I band corresponding to orderly (α-helix) conformation. Peaks between 1620 and 1640 cm^{-1} or even below 1620 cm^{-1} are assigned to β-strands protein secondary structure. Amide I bands between 1670 and 1675 cm^{-1} have been attributed to β-components.

Fibrous proteins have extended regions of α and β-secondary structure (Gans, 1980). Consequently, their vibrational spectra tend to show features similar to those found in model compounds (homopolypetides). Gans (1980) noted that collagen molecules have a structure distinctly different from the α-helix and β-sheet structures of other peptides. Thus the spectral band assignments applied to these conformations do not necessarily apply to collagen. The amido nitrogen of the pyrrolidine rings of proline and hydroxyproline residues in collagen carry no hydrogen for intra-chain hydrogen bonding while the flatness of the amide group prevents extension of the peptide chain as in beta arrangement and interferes with the compact coiling of the alpha helix (Morrison & Boyd, 1992).

Fourier transform infrared (FTIR) spectroscopy has been used to study changes in the secondary structure of collagen and gelatin. It has been used to study collagen crosslinking (Paschalis, Verdelis, Doty, Boskey, Mendelesohn & Yamauchi, 2001), denaturation (Friess & Lee, 1996) and thermal self-assembly (Jakobsen, Brown, Hutson, Finc & Veis, 1983; George & Veis, 1991) as well as gelatin melting (Prystupa & Donald, 1996). The spectral changes which are indicative of changes in collagen secondary structure have been identified to include changes in the amide A (Milch, 1964), amide I (1636 –1661 cm^{-1}), amide II (1549 – 1558 cm^{-1}) (Renugopalakrishnan, Chandarakan, Moore, Hutson, Berney & Ravejendra, 1989) and in the amide III (1200 - 1300 cm^{-1}) regions (Friess & Lee, 1996).

Fibrillogenesis (self assembly) of collagen has been found to be associated with broadening and a slight shift to lower wave number of the amide A peak (Milch, 1964), increase in intensity and slight shift to lower wave number of amide III peak (Jakobsen *et al.*, 1983), band broadening and shift of amide I peak to lower wave number (Jakobsen *et al.*, 1983; George & Veis, 1991; Prystupa & Donald, 1996) and shift of amide II peak to lower wave number (Jakobsen *et al.*, 1983; George & Veis, 1991). Shift of amide I, II and III peaks to lower wave numbers, increase in intensity of amide III and broadening of amide I are therefore associated with increased intermolecular interactions (by hydrogen bonding) in collagen.

Denaturation of collagen on the other hand has been found to lead to reduction in the intensity of amide A, I, II and III peaks (Friess & Lee, 1996), narrowing of amide I band (Prystupa & Donald, 1996), increase in amide I component found around 1630 cm^{-1} and reduction in the intensity of amide I component found around 1660 cm^{-1} (George & Veis, 1991; Payne & Veis, 1988; Renugopalakrishnan *et al.*, 1989).

Prystupa and Donald (1996) studied gelatin melting and found it to be associated with reduction in the 1678 cm^{-1} peak and 1660/1690 cm^{-1} peak intensity ratio and increase in amide I components occurring around 1613, 1629 and 1645 cm^{-1}. These authors assigned the bands occurring at 1645 – 1657 cm^{-1} to random coils and the 1660 cm^{-1} band to triple helix, with contribution from α-helix and β-turns. The amide I component at 1690 cm^{-1} has been attributed to helices of aggregated collagen-like peptides (Doyle, Bendit & Blout, 1975; Prystupa & Donald, 1996). According to Doyle *et al.* (1975) this peak vanishes with hydration of collagen or gelatin.

FTIR spectroscopy has therefore developed into a very useful technique for studying changes or differences in collagen and gelatin secondary structure. This technique requires very small quantities of sample and a short time to complete. It allows studies of aqueous systems as well as dry samples. These advantages make FTIR a robust technique.

1.3 GAPS IN KNOWLEDGE

Only a few warm water fish species have been studied with respect to their potential as sources of collagen and gelatin and these have been shown to contain more imino acids and to exhibit superior functional properties in comparison to cold water fish species (Grossman & Bergman, 1992; Gilsenan & Ross-Murphy, 2001). Nile perch is one of the largest warm water fish species, growing to over 250 kg and over 13 years of age. The utilisation of its waste material is still low and could be enhanced by a better understanding of the functional properties of the materials derived there-from. To date, literature on the chemical and functional properties of Nile perch collagen and gelatin is lacking.

Studies on fish collagen and gelatin have been limited, almost exclusively, to fish skin and yet, bones represent a sizeable fraction of the fish waste and contains substantial amounts of collagen. Bone and skin collagen vary in the type and quantity of crosslinks they contain (Sims *et al.*, 2000). It was not clear, however, how this influences the properties of gelatin derived from these materials.

Previous studies on fish gelatin have not addressed differences between gelatins extracted from fish of varying age. In mammals, it has been demonstrated that gelatin and collagen extractability reduces and gelatin functional properties become poorer as animal age increases (Reich *et al.*, 1962; Cole & Roberts, 1996). These age-related changes in case of mammals have been attributed to increase in stable collagen crosslinks. Some authors (Cohen-Solal *et al.*, 1981; Hickman *et al.*, 2000) have, however, shown that there is minimal development of stable collagen crosslinks in fish. It was not apparent therefore, whether age-related reduction in collagen and gelatin extractability and in gelatin quality also occurs in fish.

Factors affecting the functional properties of fish gelatin are also not completely understood. For gelatins containing microgels (fraction with molecular weight > 450 kDa), the proportion of this fraction has been shown to be highly positively correlated to viscosity and setting temperature and negatively correlated to setting time (Tavernier, 1989). Gelatins derived from collagen low in heat stable crosslinks, such as fish skin gelatins, however, do not contain appreciable amounts of microgels. It

was not clear for such gelatins, how their functional properties are related to their molecular weight distribution. It was also not clear what differences exist between early (low temperature) and later (high temperature) gelatin extracts obtained from the same raw material in a sequential extraction process.

1.4 HYPOTHESES

- Because Nile perch is a warm water fish species, its collagen and gelatin should contain high levels of imino acids and exhibit functional properties different from those of cold water fish species.
- The extractability and functional properties of gelatin from Nile perch skins should differ from those from Nile perch bones because collagen from the two tissues differ in extent of stable crosslinks and as a result differ in their stability to heat denaturation.
- The functional properties of Nile perch skin and bone gelatins should vary with age of the source fish and these differences arise from differences in the extent of collagen crosslinking.

1.5 OBJECTIVES

The goal of this study was to provide information that can be used to promote commercial utilisation of fish skins and bones as a raw material for gelatin and collagen. The specific objectives were to;

- Determine the physico-chemical properties of Nile perch collagen.
- Determine and compare the extractability of Nile perch skin and bone gelatins.
- Determine and compare the chemical and functional properties of Nile perch skin and bone gelatins.
- Compare chemical and functional properties of Nile perch skin and bone gelatins to those of bovine bone and commercial fish skin gelatins.
- Determine the relationship between the chemical and functional properties of Nile perch gelatins.