3 DISCUSSION

3.1 DISCUSSION OF METHODS USED

3.1.1 Gelatin manufacture process and quality of resulting Nile perch gelatin

Two general methods are used in the manufacture of gelatin (Hinterwaldner, 1977a; Johnston-Banks, 1990; Poppe, 1992). The first method entails alkali pre-treatment of the raw material for up to 12 weeks followed by extraction in acidic media at temperatures ranging from 45 to 90°C. This technique results in type B gelatin and is suitable for raw materials that contain substantial quantities of heat stable intermolecular collagen crosslinks. It is used in the manufacture of gelatin from adult bovine hides and from bones. Alkali pre-treatment preferentially breaks covalent intermolecular crosslinks (Grand & Stainsby, 1975), making it easier to solubilise gelatin at relatively low temperature. This technique generally takes long (up to 12 weeks) and is avoided for raw materials that contain small quantities of stable intermolecular crosslinks such as pigskins and fish skins.

The second technique involves a short acid conditioning, followed by washing out some of the acid and extraction in acidic aqueous media. This technique gives type A gelatin. It is applicable to raw materials that are low in stable intermolecular crosslinks and has been widely applied to pigskins and fish skins. It has also been used for extraction of gelatin from ossein. Extraction of gelatin from bones is generally preceded by acid leaching of ash (Hinterwaldner, 1977b). This is because bones contain substantial amounts of ash, which would otherwise contaminate the gelatin. Ash also makes it difficult to extract gelatin from bones. Leaching temperature and acid concentration affect the extent of collagen loss during the leaching process. The difference in yield between bones leached at refrigeration temperature and those leached at room temperature (Table 2.2.2) in this study may be due to excessive loss of organic matter in the latter process. The acid concentrations used normally range from 3 to 6%, with the lower concentrations normally used when leaching temperature is high.

In this investigation, type A process was chosen for extraction of gelatin because fish collagen has been reported to contain minimal quantities of stable intermolecular crosslinks (Cohen-Solal *et al.*, 1981; Hickman *et al.*, 2000). Judging from the extractability of gelatin obtained in this investigation, this process was generally adequate for extraction of gelatin from Nile perch skins, regardless of the age of the source animal. The extractability of Nile perch bone gelatin was, however, low. It may be desirable to test type B process for the extraction of gelatin from Nile perch bones.

Elimination of the fishy odour has been a major concern in fish gelatin manufacture processes. The process commonly used to eliminate fishy odour from fish gelatin involves washing fish skins several times with acid and alkali (Grossman & Bergman, 1992; Holzer, 1996). This process is not only expensive but also time consuming, tedious and leads to plenty of effluent. In this study, it was shown that activated carbon eliminates fishy odour, eliminating the need for the numerous washes. One advantage with activated carbon is that a single lot can be used on several thousand times its volume of gelatin solution and the carbon can be regenerated, if necessary, by volatising the adsorbed odorous compounds.

It is important that gelatin does not exhibit high colour intensity or turbidity as these may negatively affect the usefulness of gelatin in some food products. Gelatins derived from bovine hides of adult cattle tend to have a darker colour than gelatin from calfskins, bones and pigskins. This has been attributed to presence of pentosidine crosslinks in collagen of adult bovine hide (Cole, 1995). Formation of pentosidine crosslinks entails Maillard reaction and therefore results in dark products. Industrial processing of gelatin sometimes entails bleaching of the gelatin using sulphur dioxide or hydrogen peroxide. In this study, the colour of the gelatins derived from Nile perch without bleaching, were found to be within the range reported for bovine hide gelatins (Cole & Roberts, 1996) and no differences in colour were observed between gelatins derived from skins or bones from young and adult fish. It was therefore not necessary to include a bleaching step during the manufacture of gelatin from Nile perch skins and bones and colour is not likely to be an obstacle to utilisation of Nile perch bones and skins in the manufacture of gelatin.

Turbidity is largely dependent on efficiency of the clarification (filtration) process as well as the isoionic point of the gelatin. The commercial process of gelatin manufacture often entails filtration of both the light and the heavy (concentrated) liquors. The heavy liquor filtration eliminates particles that precipitate as a result of concentration. This leads to further improvement of gelatin turbidity. The process used in the manufacture of gelatin in this study involved only a single filtration process, conducted on the light liquor. It was not possible to conduct a second filtration because the light liquor was dried directly, without an initial concentration step. The range of turbidity values recorded in this study (20 – 944 NTU) was very wide. Higher values may have resulted from inadequate filtration. To ensure low turbidity it may be advisable to always use the double filtration process.

Gelatin ash and fat content are also important quality parameters. Fat is generally eliminated during the numerous washes and during the bone degreasing process. Generally, the gelatin samples in this study were almost free (< 0.5%) of fat. This showed that the processes used eliminated fat as desired. The skin gelatins were generally low in ash, with most having ash content lower than the recommended maximum of 2.6% (Jones, 1977). The cleaning process before extraction of gelatin from fish skins significantly reduces the amount of scales which would otherwise contribute to high ash levels in fish skin gelatin. The bone gelatins, however, had much higher ash content (most in the range 3 - 10%). Manufacture of fish bone gelatin may therefore, require an ion exchange step or alternative steps such as electro-dialysis to remove the salts.

3.1.2 Methods for determination of gelatin functional properties

To facilitate comparison of gelatins from different sources, standard industrial methods have been developed for the analysis of the functional properties of gelatin. These were used in this study. These methods strictly define how samples should be treated before determination of the functional properties. In the determination of gel strength for example, BSI (1975) defines the temperature and duration of maturation as 10 ± 0.1°C and 16 – 18 hours, respectively and the concentration of gelatin as

6.67%. Choi and Regenstein (2000) demonstrated that differences in maturation times have significant effects on the Bloom and melting point values recorded. Gelatin concentration (Choi & Regenstein, 2000) and maturation temperature (Michon *et al.*, 1997; Choi & Regenstein, 2000; Arnesen & Gildberg, 2002) have also been shown to significantly affect the values recorded for functional properties of gelatin. Efforts were made, therefore, to ensure that these factors are strictly controlled when measuring functional properties. Since the actual concentration of protein in a solution containing the same amount of gelatin sample varies with the purity (protein content) of the gelatin, efforts have been made to develop formulae which allow comparison of gelatins varying in purity. For gel strength, gelatins may be compared based on corrected Bloom. This takes into account the non-protein content of the gelatin. Corrected Bloom can be derived from the formula;

$$\text{Corrected Bloom} = \text{Bloom}_m \times (87.5/(100 - \text{Moisture\%} - \text{Ash\%}))^2$$

Where Bloom_m is the measured Bloom

(Cole & McGill, 1988; Pye, 1996)

The correction of other factors for non-protein material is however, less straight forward, partly because the non-protein solid (ash) may also contribute to properties such as viscosity, film properties, melting point and even setting temperature and time.

3.1.3 Methods for chemical analysis of gelatin

3.1.3.1 SDS PAGE

SDS PAGE with densitometric analysis has been recognized as a reliable, simple and inexpensive technique for the determination of molecular weight distribution of collagen and gelatin (Koepff, 1984; Ming-Zhi, *et al.*, 1989; Chalepakis *et al.*, 1985; Hayashi *et al.*, 1990; Norland, 1990; Reddy *et al.*, 1993; Cole & Roberts, 1996; Gómez-Guillén *et al.*, 2001). Globular proteins, commonly used as molecular weight markers, however, give erroneous results when used to estimate molecular weight of collagenous proteins. In this study, globular proteins and commercial Type I calfskin

acid soluble collagen were used as molecular weight markers. The mobility of α-chains was found to correspond to a molecular weight of about 120,000 Da, when globular proteins were used as molecular weight markers. This is much higher than the true molecular weight (approximately 95,000 Da) determined by sedimentation (Piez, 1967). Other authors (Kubo *et al.*, 1982; Mizuta *et al.*, 2002a; Mizuta *et al.*, 2002b; Mizuta *et al.*, 2003) have also reported this anomaly and this has been attributed to collagen's high content of the relatively small and hydrophobic amino acid residues, glycine, proline and alanine (Noelken *et al.*, 1981). These amino acids tend to cling together due to their hydrophobic side chain and this may hinder neutralisation of their positive charge by SDS. In this study, use of acid soluble collagen, alongside globular protein markers allowed interpretation of the molecular weight relative to γ, β and α chains and facilitated comparison of molecular weight distribution of different samples.

3.1.3.2 Amino acid analysis

Amino acid composition of collagens and gelatins was determined by reversed phase high performance liquid chromatography following hydrolysis and phenylthiocarbomyl (PTC) derivitisation as described by Bidlingmeyer *et al.* (1984). This technique has been reported to be rapid, efficient, sensitive and is specific for both primary and secondary amino acids in protein hydrolysates. The method uses ultraviolet detection and analysis time is only 12 minutes. Its sensitivity is up to concentrations in the order of pmols.

Several other approaches can be used for amino acid analysis of proteins. Separation and quantitation of amino acids in most of the techniques for amino acid analysis is achieved by chromatographic methods (Blau, 1981; Sarwar *et al.*, 1983; Bildlingmeyer *et al.*, 1984; Miller, Juritz, Barlow & Wessels, 1989). Amino acid analysis based on ion exchange chromatography was developed by the Nobel laureates Moore and Stein in the 1950s. This has facilitated automation and rapid amino acid analysis (Jakubke & Jeschkeit, 1977; Tristram & Rattenburg, 1981; Bildlingmeyer *et al.*, 1984). The earlier methods used in amino acid analysis by ion exchange chromatography involved separation on a sulfonated cation-exchange resin using a series of buffers as eluents (Bildlingmeyer *et al.*, 1984). Detection of the

separated amino acids was done by colorimetry via a post-column reaction with ninhydrin. Later, conventional liquid chromatographs were configured specifically for amino acid analysis. In these approaches, the post-column derivitisation required special reaction chambers as part of the chromatographic system. This limited the apparatus to amino acid analysis and the ion exchange separation required approximately one hour. Pre-column derivitisation was developed to overcome these limitations (Bidlingmeyer *et al.*, 1984). It involves derivatisation of amino acids before separation. Pre-column derivatisation, especially with reversed-phase columns, offers greater efficiency, ease of use and higher speed of analysis than conventional ion-exchange techniques. Dansyl (Dns), phenylthiohydantoin (PTH), o-phthalaldehyde (OPA) and phenylisothiocynate (PITC) are among the reagents that have been used for pre-column derivatisation. Dns and PTH derivatisation while rapid, efficient and sensitive suffers from poor derivative stability. PTH also gives less than quantitative yields while Dns shows reagent interference peaks. OPA on the other hand does not react with secondary (modified) amino acids.

3.1.3.3 Fourier transform infrared (FTIR) spectroscopy

Infrared spectroscopy is one of the widely used techniques for estimating the secondary structure of proteins (Ambrose & Elliot, 1951). Compared to X-ray crystallography, FTIR spectroscopy is quite simple and yet it has now been developed to give quantitative information of the amounts of the different secondary structure conformations. While nuclear magnetic resonance (NMR) spectroscopy is widely used for the study of protein secondary structure, interpretation of NMR spectroscopy results for large proteins is complex and the technique is currently limited to study of small (< 30,000 Da) proteins (Haris & Severcan, 1999). Circular dichroism (CD) spectroscopy, on the other hand, only provides qualitative information on the different protein conformations (Darby & Creighton, 1993).

FTIR spectroscopy requires small quantities of sample (~2 mg per test) and can be conducted both on solid (in form of pellets prepared by mixing sample with KBr) and aqueous samples. The FTIR spectra obtained from pellets of Nile perch collagen and gelatin with KBr, in this study, were quite similar to spectra obtained in other studies of collagen in solution (Payne & Veis, 1988; Friess & Lee, 1996). One difference,

however, was the observation of an amide I component at around 1690 cm^{-1}. This component was also observed by Doyle *et al.* (1975) who reported its disappearance with hydration and related its intensity to intermolecular associations. Use of KBr discs may, therefore, provide additional information, over FTIR in aqueous environments, with regard to intermolecular associations.

3.2 DISCUSSION OF STUDY RESULTS

Low imino acid content is associated with poor gelling properties of gelatin and low thermal stability of collagen. It has been reported that the imino acid content of fish collagens increases with the environmental temperature at which the fish grows (Rigby, 1968; Mathews, 1975; Kimura *et al.,* 1988). There are limited studies on warm water fish collagens and these indicate that collagen from skins of warm water fish species contain more imino acids than collagen from cold water fish species. Warm water fish species have also been found to give gelatin of superior gelling properties compared to gelatin from cold water fish species (Grossman & Bergman, 1992; Gilsenan & Ross-Murphy, 2000; Sarabia *et al.,* 2000; Gómez-Guillén *et al.*, 2002; Jamilah & Harvinder, 2002). Many warm water fish species have, however, not been studied with respect to their gelatin making properties. In this study, Nile perch, a warm water fish species was used as a source of gelatin and collagen and the chemical and functional properties were determined to test conformity to the reported trend in imino acid content and gelatin functional properties. Additionally, it was deemed necessary to determine the potential of fish bones as a source of gelatin and to determine if the age of the fish influenced the extractability and properties of gelatin. It was hypothesised that Lake Victorian Nile perch (*Lates niloticus*) collagen, like collagen from other warm water fish species contains high levels of imino acids and that gelatin derived from the collagenous tissues of Nile perch would exhibit gelling properties superior to those of cold water fish species. Since imino acid content of collagen also affects its thermal stability, it was anticipated that Nile perch collagen would exhibit higher thermal stability than collagen from cold water fish species.

Amino acid analysis showed Nile perch collagen to contain higher levels of imino acids (20%) than collagen from cold water fish species, which contain on average 17% imino acids (Rigby, 1968; Grossman & Bergman, 1992; Gudmundsson &

Hafsteinsson, 1997). The total imino acid content of Nile perch collagen was among the highest reported for fish. Tilapia gelatin was reported to contain 25% imino acids (Gross & Bergman, 1992)

The thermal denaturation temperature (Td) for Nile perch collagen was determined to be about 36°C. This is higher than the values reported for temperate fish species. Collagen denaturation temperature has been reported for 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) and ayu (29.7°C) (Nagai & Suzuki, 2000).

The high imino acid content and denaturation temperature of Nile perch collagen, in comparison to cold water fish species is in agreement with observations by Rigby (1968) that thermal stability of collagen increases with imino acid content. The collagen and gelatin were found to contain no tryptophan and cysteine, and were low in methionine, tyrosine and histidine, like other type I collagens (Balian & Bowes, 1977; Grossman & Bergman, 1992; Gudmundsson & Hafsteinsson, 1997; Yoshida *et al.*, 2001).

Electrophoresis (SDS PAGE) showed that both young and adult Nile perch skin acid soluble collagen consisted of α chains plus their dimers. The α components showed two distinct species varying in their mobility, for both reducing and non-reducing conditions. It could be concluded therefore, that Nile perch acid soluble collagen is made up of at least 2 α species (α1 and α2). The electrophoretic pattern of Nile perch skin collagen was similar to that of type I calfskin collagen.

The α2 species was the minor component of the two species. Based on the electrophoretic profile, it seems Nile perch collagen exists as $(\alpha 1)_2\alpha 2$ trimers (i.e. two chains of α1 and one chain of α2) . Skins of some fish species, such as cod (Piez, 1965) and trout (Saito *et al.*, 2001) have been reported to contain α1, α2 and α3 species. The α3 species contains cysteine and migrates slightly slower than the α1 species during SDS PAGE under reducing conditions. The electrophoretic pattern of Nile perch collagen, however, showed only two α species, even under reducing

conditions. In addition, no cysteine was detected in the collagen, suggesting that Nile perch skin collagen is made up exclusively of $\alpha1$ and $\alpha2$ species. This is typical of type I collagen, which is the dominant collagen in dermal tissue (Bailey & Light, 1989; Bailey *et al.*, 1998).

Nile perch skin collagen from both young and adult fish showed high solubility in 0.5 M acetic acid. This was indicative of low content of stable collagen crosslinks, since stable crosslinks are resistant to cleavage by dilute acid and when they are present in collagen, they make it insoluble. Earlier work (Cohen-Solal *et al.*, 1981; Hickman *et al*, 2000) provided evidence that fish collagen does not develop appreciable amounts of stable crosslinks with age. This was further supported by the observation that acid soluble collagen from the young and old Nile perch skins exhibited basically similar denaturation curves, with a denaturation temperature of about 36°C. However, hardly any Nile perch bone collagen could be solubilised by 0.5 M acetic acid. It would seem therefore that Nile perch bone collagen contain more stable crosslinks than skin collagen.

According to Sims *et al.* (2000), collagen from bones and skins vary significantly in the type and quantities of crosslinks and as animals age, the quantities and types of collagen crosslinks change. Collagen from skins of immature animals mainly contain the intermediate crosslinks dehydroxylysinonorleucine (deHLNL) whereas collagen from bones of immature animals contain hydroxylysinoketonorleucine (HLKNL). HLKNL is stable to dilute acids, which explains the insolubility of bone collagen even in infancy (Bailey *et al.*, 1998). The intermediate divalent crosslinks, deHLNL and HLKNL are respectively converted to the more stable trivalent histidinohydroxylysinonorleucine (HHL) and pyridolines (PYR) during maturation. This explains the increase in stability of collagens to heat and dissolving agents with age. The PYR crosslinks are more stable to heat than the HHL crosslinks (Bailey *et al.*, 1998) and therefore bone collagens are more resistant to denaturation by heat than skin collagens.

FTIR spectra for acid soluble collagen from young and adult fish skins were, however, found to differ slightly, indicating some differences in the secondary

structure of the two. The amide I and amide II peaks were at a lower frequency for the young fish skin (1650 and 1542 cm^{-1}, respectively) compared to adult fish skin (1654 and 1555 cm^{-1}, respectively) collagen. Based on the location of the amide I and amide II peaks, it seemed that the ASC from the young skins had a lower degree of molecular order, since decrease in molecular order is associated with shift of these peaks to lower wave numbers (Payne & Veis, 1988). It seemed therefore, that there were more intermolecular bonds in the adult fish collagen. Amide I components showed adult Nile perch acid soluble collagen amide I band to consist of a higher proportion of the component around 1690 cm^{-1} than the young fish ASC. This band is linked to the extent of intermolecular interactions in collagen and collagen-like peptides (Doyle *et al.,* 1975; Prystupa & Donald, 1996; Paschalis *et al.*, 2001). The other noticeable difference was the lower intensity of the component with peak around 1650 cm^{-1} in young fish ASC. This component has been attributed to random coils (Prystupa & Ronald, 1996), suggesting a lower extent of unwinding of the triple helix and less random coils in the young fish ASC. It seemed therefore, that adult fish ASC retained more intermolecular crosslinks during solubilisation with acetic acid but the triple helical structure, normally held together by intramolecular hydrogen bonds (Darby & Creighton, 1993) was extensively destroyed. The young fish ASC on the other hand, because of its lower content of stable intermolecular bonds could be solubilised more easily and retained triple helices to a greater extent. The apparently minimal differences in the extent of collagen crosslinking with age were therefore reflected in differences in the FTIR spectra of the collagens.

Earlier studies have shown that the extent of collagen crosslinking differs between bones and skins and increases with age (Sims *et al.*, 2000). It was hypothesised that the differences between bone and skin collagens would lead to differences in gelatin extractability and functional properties. Additionally, it was hypothesised that gelatin from skins and bones of fish of different ages would also vary in their extractability and functional properties.

Low temperature (50°) extractability of gelatin was expected to be higher for Nile perch skins if the skin collagen was less crosslinked than the bone collagen. The results of this study showed skins to give markedly higher low temperature gelatin

extractability than bones. This confirmed assertions (Bailey *et al.*, 1998; Sims *et al.*, 2000) that the bone collagen contains more stable crosslinks than skin collagens. However, there were no pronounced age-related differences in extractability of gelatin from either Nile perch bones or skins. This confirmed earlier reports (Cohen-Solal *et al.*, 1981; Hickman *et al.*, 2000) that fish collagen crosslinks do not substantially mature to the stable forms, since gelatin extractability reduces with increase in amounts of stable crosslinks. Low temperature (3 - 5°) leaching led to increase in yield from Nile perch bones. This was probably due to reduction in organic matter loss. However, the resulting gelatins were of poorer functional properties (gel strength and setting properties) than gelatins derived from bones leached at ambient temperature. The reason for this was not established but it was probably due to higher rentention of non-collagenous proteins during the cold leaching process. It may therefore be necessary to consider deboning of the fish bones before gelatine extraction.

It has been suggested that the pyrrolidine rings of imino acids give collagen a rigid structure, allowing only restricted rotation about bonds adjacent to the pyrrolidine rings (Piez & Gross, 1960). This ensures close proximity of adjacent chains and facilitates inter-chain hydrogen bonds and Van der Waals attractions. Hydroxyproline is also believed to cause stabilisation of collagen, by involvement in intra-molecular hydrogen bonding (Darby & Creighton, 1993) or some form of inductive effect (Holmgrem *et al.*, 1998). During gelling, gelatin essentially undergoes partial renaturation into collagen-like structures. Imino acid content of gelatin determines the strength of the renatured structures and is therefore a key determinant of the functional properties of gelatin.

The potential of most fish species as sources of gelatin to substitute mammalian gelatins is limited, mainly by the poor gelling properties of their gelatins. The poor gelling properties of fish gelatins have been attributed to their low content of the imino acids proline and hydroxyproline (Eastoe & Leach, 1977). This study showed that the imino acid content of Nile perch gelatin was higher than that reported for cold water fish species. This confirms earlier reports (Rigby, 1968; Grossman & Bergman, 1992) that collagen and gelatin derived from warm water fish species contain higher

levels of imino acids than those from cold water fish species. The general amino acid composition was found not to vary with age or between bone and skin gelatins. Apparently, amino acid composition seems independent of age. Eastoe and Leach (1958) gave the amino acid composition for collagen from bones and skins of mammals, showing that collagens derived from bones and skins of the same animal generally have similar amino acid compositions.

Alongside the high imino acid content, Nile perch gelatins were found to exhibit functional properties quite similar to those of mammalian gelatins and superior to those reported for gelatin from cold water fish species. This supported the assertion (Gilsenan & Ross-Murphy, 2000; Gòmez-Guillèn *et al.*, 2002) that imino acid content has a strong influence on the functional properties of gelatin. The problem of low gel strength and generally poor gelling properties is, therefore, not universal to fish gelatins, but limited to cold water fish species. This is in agreement with the report by Grossman and Bergman (1992) of high gel strength (263 g Bloom) for tilapia gelatin.

It should be noted, however, that while the amino acid composition of the different Nile perch gelatins studied was basically similar, the functional properties varied. The gelatins extracted from Nile perch skins at 50°C generally exhibited higher gel strength (222 and 229 g, respectively for young and adult fish) than corresponding bone gelatins (179 and 134 g, respectively for young and adult fish bones leached at room temperature). Gelatin extracted from skins of young and adult Nile perch at higher temperature exhibited lower gel strength but this was not always the case for bone gelatins. Gelatin extracted from adult fish skins also exhibited higher viscosity (42.3 mSt) than bone gelatins (28.2 and 30.0 mSt, respectively for young and adult fish). There was no significant difference, however, between the viscosity of young fish skin gelatin and the bone gelatins. The setting and melting temperatures were found to be similar for adult fish skin and the bone gelatins but lower for young fish skin gelatin, while setting time was lowest for adult fish skin gelatin, lower for the bone gelatins and highest for the young fish gelatins. Generally, the adult fish skin gelatins exhibited superior gelling properties to the other gelatins.

Gel hardness was found to be higher for Nile perch skin than bone gelatins, although

the compression graphs for the two categories of gelatin were similar. There were no significant differences in cohesiveness and springiness between the Nile perch gelatins, bovine bone gelatin and commercial fish skin gelatin. The Nile perch bone gelatins however, gave significantly lower hardness, gumminess and chewiness when used in the same concentrations (6.67%) as the Nile perch skin and commercial gelatins. At concentrations corresponding to 225 g Bloom gel strength, there was no significant difference between the texture profile of Nile perch bone gelatins and that of the other gelatins tested. It seems therefore that Nile perch gelatins could be used to replace the other gelatins in some food applications without significantly altering the texture. Nile perch gelatins, however, exhibited lower melting points than bovine bone gelatin. This may affect the sensory profile of food products made with fish gelatin for example low melting point of fish gelatins has been associated with better flavour release when the gelatins are used in gelled food products (Choi & Regenstein, 2000).

Nile perch skin gelatin films were found to exhibit film strength and % strain at break similar to that of bovine ossein gelatin. The Nile perch bone gelatin however, exhibited lower film strength and higher % elongation. It seems the film properties follow the same pattern as gel strength. According to De Graaf (2000), film strength of proteins increases with increase in protein entanglement (formation of network). Based on the higher film strength of Nile perch skin gelatins, it seems these exhibited more entanglements than the lower molecular weight bone gelatins. Bigi and colleagues (Bigi *et al.*, 2001; Bigi, Cojazzi, Panzavolta, Roveri & Rubini, 2002) demonstrated that crosslinking of gelatin leads to increase in film strength and reduction in percent elongation, showing that the higher the proportion of high molecular weight fractions of the gelatin, the higher the film strength and the lower the elongation. This is in agreement with the trend recorded in this study.

Evidently, the functional properties of Nile perch gelatin depend on other factors in addition to amino acid composition. The molecular weight distribution of gelatin seemed to be a key determinant for most functional properties. Table 3.1 below shows the proposed relationships between the functional and chemical properties of Nile perch gelatin.

Table 3.1 Summary of the proposed relationship between functional and chemical properties of Nile perch gelatin

Functional property	Related chemical properties	Relationship	Determinant of chemical property
Gel strength	Imino acid content	Gel strength increases with imino acid content	Species from which gelatin is extracted
	Molecular weight distribution	Gel strength increases with fraction of molecular weight between α and γ	• Content of crosslinks in source collagen • Severity of extraction process
	Crosslinks content	Gel strength reduces with increase in intramolecular crosslinks	• Content of crosslinks in source collagen • Order of extraction
Viscosity	Imino acid content	Viscosity increases with imino acid content	Species from which gelatin is extracted
	Molecular weight distribution	Viscosity increases with high molecular weight (>β) fraction and decreases with the content of low molecular weight (<α) peptides	• Content of crosslinks in source collagen • Severity of extraction process
Melting temperature	Imino acid content	Melting temperature increases with imino acid content	Species from which gelatin is extracted
	Molecular weight distribution	Melting temperature decreases with gelatin content of <α fraction and increases with α fraction	• Content of crosslinks in source collagen • Severity of extraction process
Setting temperature	Imino acid content	Setting temperature increases with imino acid content	Species from which gelatin is extracted
	Molecular weight distribution	Setting temperature decreases with gelatin content of <α fraction and increases with α	• Content of crosslinks in source collagen • Severity of extraction process
Setting time	Imino acid content	Setting time reduces with increase in imino acid content	Species from which gelatin is extracted
	Molecular weight distribution	Setting time increases with gelatin content of <α fraction	• Content of crosslinks in source collagen • Severity of extraction process
Film strength	Molecular weight distribution	Film strength increases with fraction of molecular weight between α and γ	• Content of crosslinks in source collagen • Severity of extraction process
Film elongation	Molecular weight distribution	Film elongation increases with fraction <α	• Content of crosslinks in source collagen • Severity of extraction process

Electrophoresis showed that the molecular weight distribution of Nile perch gelatins varied with the collagenous tissue used as raw material. Skin gelatins were generally found to contain higher proportions of the high molecular weight (> β) fraction than Nile perch bone gelatins. When collagen contains few intermolecular crosslinks, its conversion to gelatin is associated with minimal cleavage of peptide bonds, since solubilisation is easily achieved by cleavage of the weak intermolecular forces (hydrogen bonds, Vander Waals forces and intermediate crosslinks). As a result, the gelatin contains a high proportion of intact α-chains as well as dimers and oligomers of α-chain. When collagen contains a high concentration of stable intermolecular crosslinks, more peptide bonds are broken to facilitate solubilisation, resulting in gelatin with a lower concentration of high molecular weight fractions. Figure 3.1 demonstrates the conversion of collagen to gelatin in both crosslinked and monomeric collagens. Nile perch bone collagen, because of its high content of stable crosslinks, even at a young age, is likely to have undergone more peptide bond hydrolysis during its conversion to gelatin than the skin collagen, resulting in gelatin with a higher content of low molecular weight peptides.

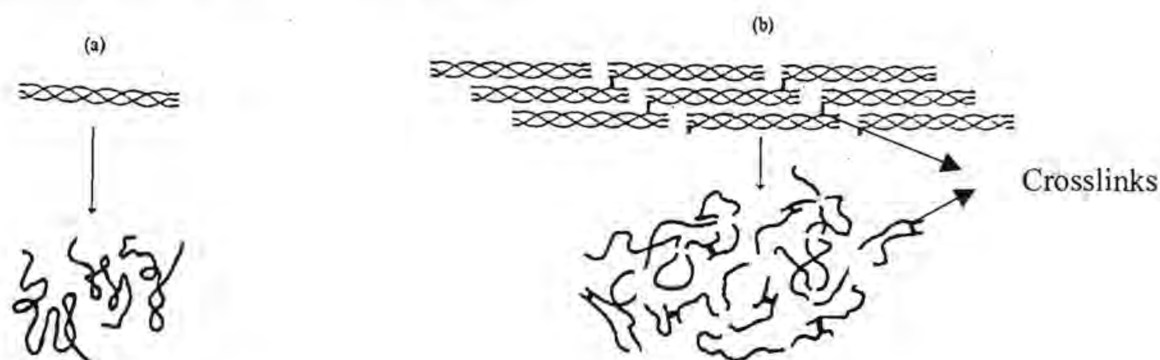

Figure 3.1: Schematic representing proposed changes occurring during the conversion of collagen to gelatin. a) monomeric collagen; b) crosslinked collagen. Adapted from Bailey (1991).

According to Ledward (1986), gelation is associated with formation of intermolecular junctions (linkages) and the number of junctions required to form a gel depends on the molecular weight distribution of the gelatin used. The gelatin fractions of low molecular weight require more junctions to form gels and result in weaker gels than gelatin fractions of higher molecular weight. Koepff (1984) observed that the gel phase of gelatin is made up mainly of the high molecular weight molecules while the low molecular weight (smaller than α) molecules tend to stay in the sol phase.

The functional properties of gelatin are also affected by the types of junctions (linkages) formed during renaturation of gelatin. Ledward (1986) observed that the junctions that contribute most to gelatin physical properties such as gel strength are those that form between adjacent molecules (inter-chain junctions), while those formed between different parts of the same molecule (intra-chain junctions) do not contribute significantly to these properties. When gelatin contains a high proportion of intermolecular crosslinks, it is more likely to form intra-chain junctions (Figure 3.2) and therefore to exhibit poorer functional properties.

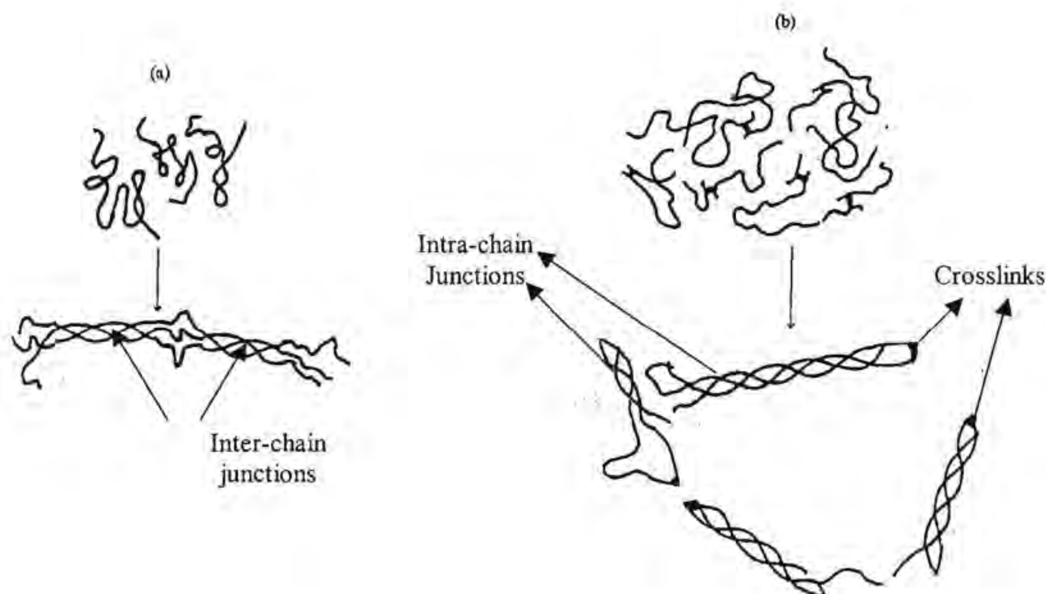

Figure 3.2: Schematic representing proposed changes during gelation of gelatin derived from a) gelatin without intermolecular crosslinks; b) gelatin containing a high concentration of intermolecular crosslinks (Adapted from Bailey, 1991).

Bone gelatins had a higher content of low molecular weight protein fractions and formed weaker gels as shown by their lower gel strength and gel hardness. Films made from bone gelatins also had lower tensile strength and exhibited higher % elongation at break. It seems that during film formation, there is also formation of protein-protein linkages between gelatin molecules and the lower molecular weight gelatin fractions require more of such junctions, leading to a weaker network, which stretches and breaks easily. This was supported by results from FTIR spectroscopy. FTIR spectra of gelatins seemed to vary with the collagenous tissue from which the gelatin was extracted and with the order of extraction. The FTIR spectroscopy study led to the proposition that the secondary structure of gelatin obtained from the same raw material by sequential extractions may vary, with later extractions containing more intermolecular associations in the dry state. The first gelatin extractions were obtained from the least crosslinked collagen and consisted largely of high molecular weight protein fractions. Later extractions are obtained from the more crosslinked

collagen that could not be solubilised by the mild first extraction. During drying of the gelatin extracts, the first gelatin extracts (made up predominantly of high molecular weight fractions) seem to have formed fewer protein-protein linkages than later extracts obtained from the more crosslinked collagen and containing more low molecular weight fractions. Because of the higher content of stable crosslinks in bone collagen, the bone gelatins are likely to have contained more intermolecular crosslinks and therefore were more likely to form intra-chain linkage than the skin gelatins.

When the molecular weight distribution of gelatins from the same raw materials were compared, later (higher temperature) extractions were found to contain more low molecular weight peptides and lower proportion of α chains than earlier (low temperature) extractions. The gelatins extracted from adult Nile perch skins at higher temperatures also exhibited lower gel strength, melting point, setting temperature and longer setting time. The proportion of low molecular weight ($< \alpha$) fraction was higher for young than for the adult fish skin gelatin. Reich *et al* (1962) observed an increase in viscosity and yield with animal age for gelatin derived from bovine hides and pigskins, until the age of 36 and 28 weeks, respectively. Thereafter, gelatin viscosity dropped with age. Gelatin yield was related to animal age in the same way as viscosity. Gelatin viscosity has been reported to increase with the proportion of high molecular weight fraction and to reduce with the amount of low molecular weight fraction (Tavernier, 1989). The high incidence of low ($< \alpha$) molecular weight peptides may therefore be responsible for the lower viscosity, setting and melting temperature and longer setting time observed for the young Nile perch skin gelatin. In the case of Nile perch, the gelatin from the adult fish skins exhibited higher viscosity, setting and melting temperature and shorter setting time than gelatin from young Nile perch fish skins but similar gel strength. Adult fish skin also gave higher yield. It seems from this observation, that the age-related changes in collagen, which are responsible for lower yield and viscosity for bovine hide and pigskin gelatin also occur in Nile perch but are less pronounced in the latter. This is consistent with reports by Hickman *et al* (2000) that the crosslinks found in young fish collagens are retained to a large extent and only a minor proportion of these crosslinks mature to stable forms.

The functional properties of the gelatins studied were found to be correlated to the proportion of the different molecular weight fractions. Viscosity, Bloom, hardness and film strength had a high positive correlation ($r^2 > 0.7$) to the > β fraction. This is in accord with observations by Graesser, Koepff and Tomka (1983) that high molecular weight gelatin protein fractions have a higher propensity to form gels than the lower molecular weight fractions. The Viscosity was also highly negatively correlated ($r^2 = -0.79$) to < α fraction. High incidence of peptides has been associated with low viscosity, melting point, setting point and high setting time (Koepff, 1984; Tavernier, 1989). Generally, low molecular weight peptides did not contribute positively to the functional properties of gelatins. Low molecular weight peptides arise from extensive collagen hydrolysis, either due to the severity of the extraction or because of a high degree of collagen crosslinking. It may be desirable, in the case of highly crosslinked collagen to break some of the collagen crosslinks, for example by alkali pre-treatment before gelatin extraction.

Despite the difference in the levels of high and low molecular weight protein fractions between gelatin from skins of young and adult Nile perch, there was no difference in gel strength, suggesting no direct relation between molecular weight distribution and gel strength. Earlier studies (Koepff, 1984; Graesser, 1985) have also shown no simple correlation between gelatin gel strength and molecular weight distribution for high gel strength gelatins.

The differences in functional properties between Nile perch skin and bone gelatins seem to have arisen from differences in the ease with which collagens in the two collagenous tissues can be transformed to gelatin. Nile perch skin collagen easily solubilised to give gelatin at low temperature, while only a small fraction (< 40%) of the bone collagen could be solubilised at 50°C. The bone collagen generally seemed to require a more severe heat treatment to facilitate denaturation to gelatin. Bone gelatins also contained more of the low molecular weight peptides compared to skin gelatins extracted at the same temperature. Nile perch bone collagen, therefore, seemed to undergo more peptide bond hydrolysis, resulting in gelatins with a higher content of low molecular weight fractions compared to the gelatin extracted from Nile perch skins. The inferior functional properties of bone gelatin seem therefore to be a

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result of the more stable crosslinks in the bone collagen.

The bovine bone and commercial fish skin gelatins studied were found to exhibit functional properties quite similar to those of adult fish skin gelatin, except for melting and setting temperature, which were higher for bovine bone gelatin. The difference between the properties of fish gelatin and bovine bone gelatin may be attributed to differences in amino acid composition and molecular weight distribution. Bovine gelatin is known to contain ~ 30% (w/w) imino acids (Eastoe, 1967; Poppe, 1992) which is significantly higher than the 21% recorded for Nile perch. The higher imino acid content contributes to the higher setting and melting temperature. Similarity in gel strength despite differences in molecular weight distribution reaffirms the lack of direct correlation between these properties. The bovine bone gelatin and the commercial fish skin gelatin were found to be lower in low molecular weight ($< \alpha$) peptides and had densitograms with fewer distinct fragments, characteristic of alkali-processed gelatins (Koepff, 1984). The bovine bone gelatin also had a much higher content of $> \beta$ components.

Melting and setting temperatures of the Nile perch gelatins were found to lie between those reported for mammalian gelatin and those for gelatin from cold water fish species. It seems amino acid composition is a key determinant of these properties since the imino acid content of Nile perch gelatin was also between that of mammalian and cold water fish gelatins.

Differences between collagen and gelatin can be used to elucidate changes that occur in the transformation of collagen to gelatin. In this study, several differences were observed between Nile perch collagen and gelatins. One noteworthy difference was the slightly lower imino acids (proline and hydroxyproline), glycine and alanine content of Nile perch skin collagen in comparison to gelatins. This is attributable to the fact that gelatin is derived mainly from the helical part of collagen because much of the telopeptide regions are lost during pretreatment. The helical part is higher in the amino acids dominant in collagens (proline, hydroxyproline, glycine and alanine). As a result, gelatin normally contains higher levels of these amino acids in comparison to the collagen from which it is derived (Eastoe & Leach, 1977).

In addition, unlike Nile perch collagen, which consisted, almost exclusively of α and β chains, gelatins were found to consist of numerous fragments varying widely in molecular weight. This showed that conversion of collagen to gelatin was accompanied by cleavage of inter and intramolecular bonds. Stainsby (1987) suggested on the basis of the continuous molecular weight distribution of gelatin that during gelatin manufacture, backbone peptide bonds and collagen crosslinks are cleaved with equal ease. Paschalis *et al.* (2001) reported presence of collagen crosslinks in bovine bone gelatin, also confirming that collagen to gelatin transformation does not eliminate all the crosslinks. Collagen to gelatin transformation therefore involves cleavage of some peptide bonds and some intermolecular crosslinks.

FTIR spectra for gelatins were also quite different from those of collagen. The major differences were the lower intensities of amide I, II and III bands exhibited by gelatins. These differences are indicative of a higher molecular order in collagen than gelatin. This is consistent with changes expected as a result of denaturation of collagen to gelatin. FTIR spectroscopy, therefore showed that conversion of collagen to gelatin leads to loss in the triple helical structure and decrease in molecular order. The extent of these changes, in the case of Nile perch seemed to be affected by the order of gelatin extraction and the collagenous tissue from which gelatin is extracted.

4. CONCLUSIONS AND RECOMMENDATIONS

- Collagen from the skins of young and adult Nile perch is highly soluble in dilute acid. It seems there is minimal development of stable crosslinks with age in Nile perch skin collagen. Nile perch bone collagen is, however, insoluble in dilute acid, even from bones of young fish. This indicates that Nile perch bone collagen like mammalian bone collagens contains more stable crosslinks than the skin collagen.
- As a result of the low content of stable intermolecular crosslinks in Nile perch skin collagen, the acid extraction process (with extraction done around pH 4) is adequate for the extraction of Nile perch skin gelatin, even from skins of adult Nile perch. Extractability of gelatin from Nile perch skins at 50°C was quite high (> 65%).
- While the acid extraction process also gave gelatin from Nile perch bones, the extractability was rather low (< 40 %) and functional properties of the resulting gelatin were poorer than those for Nile perch skin gelatin. This supports the hypothesis that Nile perch skins and bones differ in their content of stable crosslinks, with bones having a higher content. The crosslinks that normally occur in skeletal matter, even at early age tend to be stable and resistant to cleavage by dilute acids. This seems to be the case in Nile perch as well.
- No marked age-related differences were found in collagen solubility and gelatine extractability for Nile perch. This confirms the proposition that aging in fish is not accompanied with significant development of stable collagen crosslinks.
- The acid soluble collagen (ASC) derived from Nile perch skins was found to contain approximately 20% imino acids. This is intermediate between values reported for cold water fish and mammalian collagen. The denaturation temperature for Nile perch ASC (36°C) was also found to be intermediate between values reported for cold water fish species and mammalian collagen. This confirms earlier reports that denaturation temperature of collagen is positively correlated to imino acid content and that these parameters are dependent on the environmental temperature at

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which the fish grow.

- The electrophoretic profile and amino acid composition showed that Nile perch skin collagen is of the Type 1 variant.
- Conversion of Nile perch collagen to gelatin was found to lead to loss of molecular order and increase in the concentration of proline, hydroxyproline, alanine and glycine. The difference in amino acid composition between collagen and gelatin arises from the loss of telopeptide non-helical parts during pre-treatment. These regions contain lower levels of the four amino acids than the helical part.
- Nile perch skin and bone gelatin was found to have similar amino acid composition, with imino acid content of approximately 21.5%. This is intermediate between the imino acid content reported for cold water fish species and mammals. The functional properties of Nile perch skin gelatins were also intermediate between those for the two groups. This confirms assertions that the functional properties of gelatin are a function of their imino acid content.
- Nile perch skin gelatin, however, exhibited superior functional properties to Nile perch bone gelatin, despite the similar imino acid content. The functional properties of Nile perch skin and bone gelatins were found to be related to the molecular weight distribution of the gelatins. Nile perch bone gelatins were found to contain more low molecular weight peptides and less fraction with molecular weight > β. The high incidence of low molecular weight peptides seems to arise from their content of more stable intermolecular crosslinks. Transformation of Nile perch bone collagen to gelatin by the acid extraction process seem to entail significant hydrolysis of peptide bonds while in the case of the less crosslinked skin collagen, solubilisation was easily achieved by breaking the weak intermolecular bonds.
- FTIR spectroscopy led to the proposition that the gelatin obtained at higher temperature during the later extractions in a sequential process in which temperature is increased gradually, contain more intermolecular linkages, in the dry state, than the earlier extracts obtained at lower temperature. It was not clear, however, whether these were intermolecular covalent crosslinks or hydrogen bonding occurring during renaturation of gelatin to

collagen-like networks. The study also showed the potential of FTIR spectroscopy in the study of collagen and gelatin in the anhydrous state.

- Generally, it is clear from the study that Nile perch skins have potential for supplementing mammals as a source of gelatin and collagen. The gelatin yield of 12.3 and 16% recorded, respectively, for young and adult Nile perch are in the commercially acceptable range. The functional properties of the gelatins recorded in this study, especially for the Nile perch skin gelatin are superior to those reported for most commercial fish gelatins. The high solubility of Nile perch skin collagen is an attribute that makes it attractive as a commercial source. In addition, Nile perch collagen was found to exhibit reasonably high denaturation temperature (36°C). This implies that Nile perch collagen, unlike cold water fish collagens would not denature (and lose functionality) during handling at ambient temperature prevailing in most parts of the world. There is, therefore, real potential for exploitation of Nile perch processing waste, especially skins, for commercial production of collagen and gelatin. In the case of Nile perch bones, gelatin yield was low (< 10% for low temperature leaching and < 2.5% for ambient temperature leaching) and the functional properties rather poor. In addition, Nile perch bone collagen was found to be insoluble in dilute acid. This study therefore, did not demonstrate high potential for the exploitation of Nile perch bones as sources of gelatin or collagen.
- Further work is required to determine the types and quantities of different crosslinks in bones and skins collagen derived from Nile perch of varying age. Determination of the types and quantities of different crosslinks in gelatin is also recommended. This would shed more light on the collagen-gelatin transformation and reveal the cause of the differences in molecular order shown by FTIR spectroscopy in this study.
- Further work on optimisation of the extraction of gelatin from fish bones is also recommended. It is recommended, that the alkali extraction process be attempted, since the low temperature extractability of the bone gelatin was considerably lower by the acid process and the functional properties poorer than the skin gelatin. There is also need for optimisation of the

leaching process.

- Studies on management of the waste generated in the manufacture of gelatin as well as analysis of the cost and environmental impact of using activated carbon instead of the alternative multiple acid and alkali washes for elimination of the fishy odour may be worthwhile.
- Research involving use of Nile perch collagen and gelatin in different applications is also recommended. Nile perch gelatin was found to have a considerably lower melting temperature than mammalian gelatin of equal Bloom. It would be interesting to study how this may affect the sensory properties of food products made from these gelatins, especially flavour release.