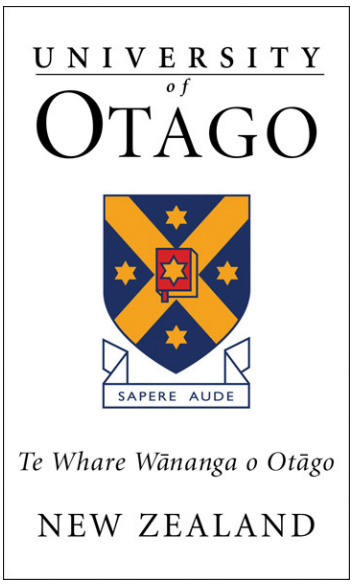

Extraction and Isolation of Collagen Type I from Fish Skin

Raed Al Zahrani
A thesis submitted for the degree of
Postgraduate Diploma in Science
At the University of Otago, Dunedin,
New Zealand

Abstract:

Collagen is one of the major connective tissue to animal proteins and has been widely used as a biomedical material. The conventional source of collagen is from bovine and pig. However, the outbreak of prion diseases, such as bovine spongiform encephalopathy, has resulted in anxiety among users of collagen derived from these land animals. Thus, there is need to find an alternative source of collagen. Fish is one of the candidates as such alternative source because fish is unlikely to be associated with prion diseases. The overall objective of the project is to test if and how to use fish skin as an alternative source of collagen. The specific purpose of the present study was to extract and isolate collagen from fish skin and analyze the properties of the extracted collagen. Fish skin was collected from a fish shop, cleaned from non-collagenous tissue and cut into fine pieces. Then, the skin was solublized using acetic acid method. The solublized collagen was precipitated by using NaCl. Western blotting was used to analyze subtypes of collagen in the extracted collagen. The components and properties of the exacted fish collagen should have shown comparison with that of the conventional collagen. The total amount of collagen which was extracted from 50g of fish skin was 0.8314g which represented 1.66% from the used skin. The western blot results were negative. In conclusion, fish skin is an economically and technologically viable substrate from which collagen can be extracted from. However, the extraction methods used in this study should have been adjusted in order to produce collagen of such determined characteristics.

Acknowledgments

I would like to give my kind regards and thanks to all those who helped me. Without them, I could not have completed this project.

Dunedin Harbour Fish City: For providing me with the fish skin.

Dr. Shengguo Chen: for teaching me Western Blot analysis and his professionalism.

I would like to give my kind regards and special thanks to my supervisors **Dr. Ming Zhang and Dr. George Dias** . They gave me their support, time and knowledge at all times throughout the year.

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Introduction:

Collagen is a major structural protein that forms molecular cables, which in turn strengthens the tendons and the resilient sheets that provide support to the skin as well as internal organs of the animals and fish. It is the most abundant protein in animal tissues. It accounts for 30% of the total body protein. It is the major component of connective tissue, muscle, teeth and skin. Collagen molecular structure consists of three polypeptide α chains twinned together to form triple helix. Each α chain composes of repeated sequence of triple $(Gly\text{-}X\text{-}Y)_n$ where X and Y are often proline (Pro) and hydroxyproline (Hyp). Tropocollagen or "collagen molecule" is a subunit of larger collagen aggregates such as fibril. Collagen showed good tissue tolerance both in vivo (Balazs and Hultsch 1976) and in vitro (Meier and Hay, 1975) which led to its use in many biomedical applications such as in the dialysis membrane of an artificial kidney (Nishihara et al., 1967), an artificial corneal membrane (Dunn et al., 1967), as vitreous body (Dunn et al., 1971), in skin and blood vessels (Sawyer et al., 1977), and as a surgical homeostatic agent (Peacock et al., 1965). Moreover, collagen is used as non biomedical material. It is used in leather and film industries as non biomedical material. Until now, the main sources of collagen for industrial applications are land animals, such as bovine and pigs. However, the outbreak of prion diseases such as bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD) have resulted in anxiety among of users of collagen and collagen derived products from these land animals. In addition to that, collagen which is extracted from pigs cannot be used by group of people due to religious reasons. Thus, there is need to find an alternative source of collagen. Fish is one candidate as such alternative source because it is unlikely to be associated with prion

diseases. Fish skin is considered a waste product in New Zealand and is used as fertilizer for soil or as supplement to animal food. It would be more beneficial and economical to use fish skin as a source of collagen. The overall objective of the project was to test if and how to use fish skin as an alternative source of collagen. The specific purpose of the present study was to extract and isolate collagen from fish skin and analyze properties of the extracted collagen. Because the main component of skin is type I collagen, this study was focused on extraction and isolation of collagen type I.

Methods and Materials:

All the preparative procedures were performed at 4C. The methods for extraction of collagen from fish skin consisted of the following steps; skin preparation, removing noncollagenous tissue, solublizing collagen, centrifuging and precipitating collagen, concentration measurement and characterizations of collagen.

Fish skin preparation:

Fish skin was obtained from Dunedin Harbour Fish City. The fish skin was obtained from Sole fish. It was placed in bag full of ice. At the lab, the skin was cleaned from any attached muscle and scales using sharp knife. Only 50 gram of skin was acquired. After cleaning the skin, it was chopped into fine small pieces (0.5*0.5 cm). Then it was placed in 1 litter flask. In order to maintain the skin at 4C temperature, the flask was placed in a container full of ice.

Removing non collagenous tissues:

After the skin was prepared and in order to remove non collagenous substance and to make the skin very loose, the skin was treated with 1 litre of 0.1M of NaOH (PH 12) for 24 hours, which was obtained by mixing 4g of sold NaOH with 1 litre of distal water. Then, the solution was placed on the skin and gently stirred for 5 minutes. After that, the resulting solution was placed in the fridge for 24 hours.

After 24 hours, the solution was removed by using filter paper (size 125mm). Then the skin was washed thoroughly with cold distal water until its PH become neutral. In this step, the skin was washed thoroughly four times. After each wash, PH measurement was taken. In each wash, 600ml of cold distal water was used. The total of the cold distal water was 2.4 litres.

After washing the skin thoroughly, it was treated with 10% butyl alcohol for 48 hours with solid to solvent ratio 1:10. The main reason for this treatment was to remove fat tissue from the skin. The solution was obtained by adding 100 ml of butyl alcohol to 900ml distal water. Then, only 500ml of the solution was mixed with the skin. The solution was placed in the fridge. After 24 hours, the solution was removed using filter paper then a new 500ml of 10% butyl alcohol was added. Then, the solution was placed in the fridge for another 24 hours.

Solublizing collagen:

After 2 days of alcohol suspension, the solution was removed by using filter paper (size 125mm). Then the skin was washed thoroughly using cold distal water. The skin was washed four times. In each time, 600ml of distal water was used. After the washing, the skin was suspended in 1 litre of 0.5 acetic acid for three days with gental agitation at 4C. The main reason of this step was to solublize the fibril collagen into tropcollagen which is the subunit of collagen fibril.

The solution was obtained by adding 29ml (which equals to 0.5M) to 1 litre of distal water. The solution was then added on the skin and placed in agitated machine in cold room (4C) for three days.

Centrifuging and Precipitating collagen:

After three days of acetic acid suspension, the solution was filtered by using double filter papers and only 200 ml of solution was obtained. Then, the solution was centrifuged by refrigerated centrifuge machine with speed 20,000Xg at 4C temperature for 1 hour. After centrifuging the solution, 5ml of supernatant was collected and placed in a tube.

In order to precipitate the solublized collagen, NaCl was added to the supernatant to get final concentration of 0.9M. The required NaCl volume measured was calculated by using $C_1V_1=C_2V_2$ equation. From that equation, only 2.77ml of NaCl solution was needed to precipitate collagen.

After salting out the collagen, the solution was centrifuged in the same refrigerated centrifugen for 1 hour with 20,000Xg speed. Then the supernatant was disposed and the precipitate was collected.

Concentration of collagen:

The concentration of extracted collagen was calculated by taking a glass Petri dish and measuring its weight (it was 4.65g). Then, a small amount of extracted collagen was placed on the dish (0.25ml) and was placed in 30 degree oven till the solution evaporated. After evaporating the solution, the dish was measured again. The difference between the two measurements was the weight of the collagen in that sample which was 0.013g. Finally, the concentration of collagen was measured by dividing the weight of collagen on the volume of the solution.

Identifying collagen:

In order to isolate and characterize the extracted collagen, Westren blot was used as the main method of characterization of collagen. The western blot was performed according to Timmons and Dunbar (see appendix B). The extracted collagen was transferred with Trans Blot Semi Dry Transfer Cell to Immun-Blot Polyvinylidene Difluoride(PVDF) membrane. The primary antibody which was used was anti COLA1 antibody which was ordered from Segma Aldrich. This antibody is very specific to α helix chain in the type I collagen and it was produced in a rabbit. Two different antibody concentrations were used. These concentrations were 1:1000 and 1:3000. The total amount of antibody which was used was 20µl. The secondary antibody which was in this study was alkaline phosphataes IgG.

Result:

In this study 50g of fish skin was used to extract collagen. After solublizing collagen and precipitating it by NaCl. The total amount of the solution was 7.77ml. The final concentration of extracted collagen was 0.104g/ml. The total amount of collagen which was extracted from 50g of fish skin was 0.8314g which represents 1.66% from the used skin.

For the western blot, the concentrations which were prepared for analysis were 6mg/ml and 12mg/ml. Even though two different concentrations of antibody were used (1:1000 and 1:300) and using different times of exposures (2, 5, 10 and 30mins), the western blot results were negative.

Discussion:

In this study, collagen was extracted using Noitup et al (2005) methods. 0.834g of collagen was extracted from 50g of fish skin. However, Western blot analysis showed negative detection of collagen. There are different reasons which might explain these findings.

First, there are three major methods of collagen extraction. These are salt-solublized collagen, acid solublized collagen and pepsin-solublized collagen (Ward and Courts, 1997). In this study, only one method was used. It was acid-solublized collagen method. Extraction of collagen using the other different methods may give positive results. Previous studies showed that extraction of collagen using Acid-Pepsin gives higher yield result of collagen. So in the future, doing collagen extraction with the three different methods and comparing their results should be recommended.

Second, the procedures in this study were done in 4C temperature. Controlling temperature is a very important factor in getting positive results. Uncontrolled temperature may result in denaturing collagen. As a consequence, Western blot analysis will give negative results.

Third, only one type of fish was used in this study. It was Sole fish. Different type of fish has different amount of collagen. By using different type of fish, it would give different results. In addition to that, only 50g of fish skin was used. Using larger amount would give larger amount of collagen and would give greater chance of detecting collagen by Western blot. Moreover, the fish skin which was used in this study had large amount of scale. If the scale is not removed well, this will interfere with collagen solublizing process. This could be prevented by either thoroughly cleaning the skin or placing the skin in EDTA solution which demineralized the fish scales and then digested the demineralized scales by pepsin.

Fourth, in this study, only 200ml of solution of solublized collagen was obtained by using double layers of filter papers. Using stander filter papers to filter the solution might allow collagen to be passed through its pore ending up with a solution without collagen. In order to prevent this from happening in the future, specific membrane should be used. This membrane will allow other proteins to pass and leaving collagen behind. For example, using a spectra/ por mwco 12-14,000RC membrane, which is very specific for type one Collagen (Cliche S et al, 2003). This membrane allows only type I collagen to be filtered.

Fifth, the first step in Western blot analysis is equalizing the tissue concentration to 6mg/ml. In this study, concentration measurement was done by using small amount of the

sample (0.25ml). This could give less accurate measurement of the amount of collagen in the sample. In order to avoid this, larger sample would give more accurate results. As a consequence, less accurate measurement of collagen concentration will result in unsuccessful isolation and identification of collagen by Western blot. Another way to measure collagen concentration accurately is using modified Lowry's method (1978). This method includes an initial heating of collagen samples in alkaline solution and changes in the concentrations of reagents.

Finally, in this study, only Western blot was used as a method of analysis of the extracted collagen. In the future, other methods can be used to identify collagen if Western blot is not successful, such as Sodium Dodecyl Sulphate Polyacylamide Gel electrophoresis (SDS-PAGE). This method was explained in the literature review (see appendix B).

I would like to mention that I had over 9 weeks delay of getting some of the experiment materials (Butyl alcohol and the primary antibody). This affected my number of extraction trials. I was only able to repeat the extraction once before I started doing the Western blot.

In conclusion, fish skin is an economically and technologically viable substrate from which to extract collagen. This study showed that 50g of fish skin could give 0.834g of collagen with concentration of 0.104g/ml. However, the extraction methods which were used in this study should be adjusted in order to produce collagen of determined characteristics. In the future, the biological characteristics of the extracted collagen from fish will be examined under *vitro and vivo* conditions and compared with mammalian collagen. In conclusion, this study confirmed that fish is an alternative source of collagen.

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Appendix A
Collagen

1. **Introduction**:

Collagen is a major structural protein that forms molecular cables, which in turn strengthens the tendons and the resilient sheets that provide support to the skin as well as internal organs of the animals and fish. Adding mineral crystals to collagen, bones and teeth are formed, while it protects and supports the softer tissues besides connecting them with one another. This group of naturally occurring proteins makes up around 25-30 per cent of the body protein content and acts as the main component of connective tissue in the form of elongated fibrils, thereby existing mostly in fibrous tissues like tendon, ligament, and skin, besides being present in cornea, cartilage, bones, blood vessels, the gut, and intervertebral disc. (Di Lullo et al., 2002; Müller, 2003). These proteins are also known as fibrous scheleroproteins, which may contain up to 19 amino acids and the most important ones among them are proline, glicyne, hydroxyproline, and hydroxysilizine, and out of them hydroxyproline and hydroxysilizine are unavailable in other proteins. A multiple combinations of the life-elements like carbon, hydrogen, oxygen and nitrogen in a specific pattern results into amino acids that further combine to create long polypeptide molecular chains, which then regroups to form super helixes, where the whole chain structure collects support from a weak hydrogen type bond. Altogether collagen is a vital constituent of the body of animal and fish, since it is one of the most important proteins and protein is the most essential nutrient in keeping the body in shape as well as flexible, where collagen provides new tissues and makes up for the loss of tissues that is around 4 per cent a day, besides holding everything together (Protein Report, 2007). Since the discovery of its regular structure at the molecular level in the 1930s (Wyckoff et al., 1935; Clark et al., 1935), the human effort of exploiting the potential of collagen has been succeed in both medical and industrial sector.

2. Structure of Collagen:

At molecular level one finds tropocollagen, which is a subunit of the sum of collagen that forms fibrils that are 300 nm long and 1.5 nm in diameter, consisting three

polypeptide strands known as alpha chains. Each of these strands possesses the conformation of a left-handed helix, before getting twisted together into a right-handed coiled-coil, which is known as triple-helix or super helix. The degree of their interlocking position suggests instability in their association. However, the most distinguishable feature of the collagen in its regular arrangement of amino acids, where a sequence following a pattern such as Gly-Pro-X or Gly-X-Hyp are observed, where X may be the variant of other amino acid residues. One sixth of such sequences are constituted by Proline or hydroxyproline, while glycine accounts for 1/3rd of the same. Such sequential uniformity can be found in very few other fibrous proteins, such as silk fibroin. The tropocollagen subunits tend to assemble on their own making room for extracellular spaces of tissues by virtues of their staggered ends to the tune of 67 nm (Hulmes, 1992, 2002).

Efforts of various scientists concluded into several models of collagen structure that helped in showing the conformation of the collagen monomer, before the triple-helical "Madras Model" presented by Ramachandran in 1955 was considered as the closest describer of the quaternary structure of the molecule (Ramachandran, 1967; Leonidas et al., 2001; Fraser et al., 1979) at that time. Such breakthrough led to further investigation that wanted to define the packing structure of collagen, resulting into several models, which showed the packing arrangement either in "sheet-like" or in microfibrillar state (Hulmes and Miller, 1979; Jesior et al., 1980; Fraser and MacRae, 1981). Consequently it became possible to directly image the microfibrillar structure of collagen fibrils in tendon, cornea, and cartilage. However, it took several years to arrive at an improved state of finding, when more researchers confirmed that microfibrillar structure of adult tendon is closest to the observed structure.

Ramachandran (1954) observed an arrangement of three non-coaxial helical chains interlinked by hydrogen bonds, nearly perpendicular to the length of the chains, as the basis of the collagen structure. That structure explained two points, such as the occurrence of a fraction of more than one-third of glycine residues and accommodation of proline and hydroxyproline residues. Alongside it also explained the infrared dichroism. However, the exact nature of the helices was found later, when the X-ray pattern of stretched collagen (Cowan et al. 1953) indicated the occurrence of 3-1/3

residues per turn (Ramachandran and Ambady, 1954). The presence of such a non-integral number of residues per turn implied that the three chains are further coiled around, while the coiled-coil structure (Ramachandran and Kartha, 1955) retaining the essential features like the location of amino-acid residues and the orientation of the NH- and CO-bonds. Ramachandran found it interesting that the simpler non-coiled-coil structure remains to be the basis of the arrangement of polypeptide chains in polyproline (Cowan and McGavin, 1955) and in polyglycine II (Crick and Rich, 1955). Such finding indicated that the triple chain structure can also be present in other proteins and polypeptides with minor modifications, and that proposition was corroborated by elastin, which belongs to this type (Ramachandran and Santhanam, 1956). Such earlier findings evoked structural studies of collagen model compounds, especially the (Pro-Pro-Gly) sequence, as amino acids frequently occupy the X and Y positions of the collagen consensus X-Y-Gly sequence. Yonath and Traub (1969) presented the first structural analysis of the polypeptide poly (Pro-Pro-Gly), which was regarded as the best available model for collagen triple helix for many years, as it exhibited the basic characteristics of the previously proposed collagen II model presented by Rich and Crick (1961). On the other hand the model of natural collagen contained three left-handed polyproline II-like chains encircling a common axis. Gly residues are required at every third residue (X-Y-Gly) as the close packing of the chains near the axis does not provide space for larger residues. Alongside, backbone N atoms of Gly residues are found to be involved in interchain hydrogen bonds with the carbonyl groups of residues at X positions.

A series of theoretical works dominated the study on fiber diffraction studies that involved both the Pro-Pro-Gly (Miller and Scheraga 1976; Némethy et al., 1992) and Pro-Hyp-Gly models (Miller et al., 1980). A low-resolution, single-crystal study on the polypeptide model $(Pro\text{-}Pro\text{-}Gly)_{10}$ revealed further information (Okuyama et al., 1981), where this polypeptide exhibited significant differences (7_2 as opposed to 10_3 triple helical symmetry) in the triple helical parameters from the Rich and Crick model of real collagen (Rich and Crick, 1961). Such differences raised questions about the actual symmetry of natural collagen (Okuyama et al., 1977; Kramer et al., 1999; Okuyama et al., 1999). However, $(Pro\text{-}Pro\text{-}Gly)_{10}$ has acquired the status of the reference frame in the study of the influence of pyrrolidine ring substituents on triple helix stability and

structure due to several explicit reasons. For example, the researchers observed that hydroxylation of prolines in the Y position leads to the stabilization of the triple helix, while strongly destabilizing the same when found in the X position (Fields and Prockop, 1996; Inouye et al., 1982).

Many new polypeptide models have been synthesized and characterized in the 1990s, which revealed important information regarding collagen structure and its stability. For example, experiment with specifically designed 'host–guest' peptides has provided a reliable scale of the natural tendencies of the various amino acids (Persikov et al., 2000), where the X-ray studies on the host-guest polypeptide with sequence $(Pro\text{-}Hyp\text{-}Gly)_4$-Pro-Hyp-Ala-$(Pro\text{-}Hyp\text{-}Gly)_5$, named Gly→Ala, showed the structural effects of a disease causative mutation (Bella et al., 1994, 1995). Also, the study involving fluorinated proline derivatives has provided ample hints that inductive effects might have a role in collagen triple helix stability (Holmgren et al., 1998). However, all of the structural models were obtained as approximate average structures, since they were generated by using only a specific class of reflections that characterize a subcell of the $[(Pro\text{-}Pro\text{-}Gly)_{10}]_3$ crystals. For example, Berisio et al., (2002) presented the crystal structure of the collagen triple helix model $[(Pro\text{-}Pro\text{-}Gly)_{10}]_3$ obtained by using synchrotron radiation on crystals grown in microgravity conditions.

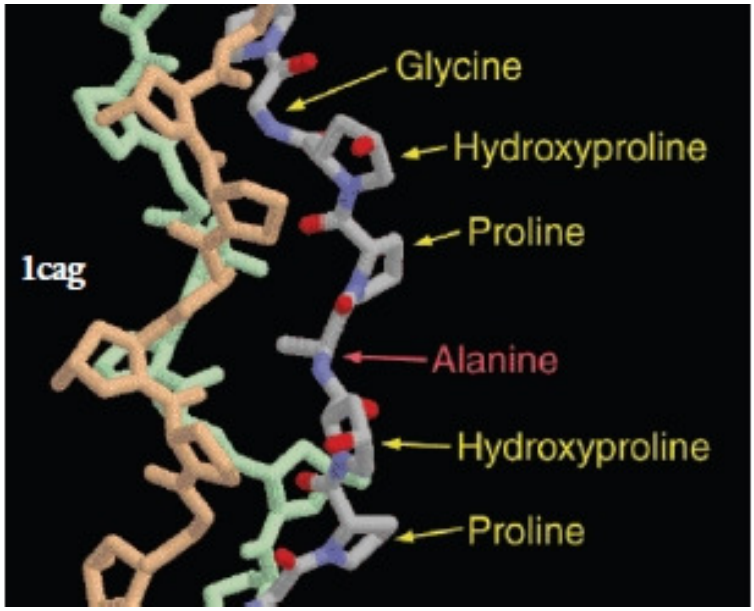

Figure 2. Structure of Collagen (Bella et al., 1994)

2.1. Latest Findings:

Buehler's (2006) research on collagen focused on its correlation with quantum chemistry, molecular structure, material properties and collagen's physiological function led to a newer model of collagen, which is considered as a major breakthrough in understanding how molecular and tissue properties are linked. Buehler observed that tropocollagen molecules are ten times stronger than steel, and at the same time sustaining huge tensile strains that amount up to fifty percent before submitting to fracture. Such state of affairs prompted him to infer that collagen is reflective of Nature's unique strategy to exploit the advantage of the nanoscale properties of individual molecules at larger scale through special arrangement of tropocollagen molecules. This finding is expected to highly contribute to the research on solutions regarding collagen-related diseases such as Ehler-Danlos syndrome, joint hyperextensibility, or Scurvy, besides opening new horizons of nanotechnology.

3. **Types**:

Identification of the homologous proteins within the collagen family helped the researchers to classify them, as Bornstein and Sage (1980) define that "collagen types are products of different genetic loci and are therefore nonallelic." Till now 29 types of collagen have been identified up to description level, though the first five types constitute 90% of all collagens in the body. The most abundant types in the body are the following:

- Type I: Available in skin, tendon, vascular region, ligature, organs, and in bone as its main constituent.
- Type II: It is the main component of cartilage.
- Type III: It acts as the main component of reticular fibers.
- Type IV: It forms the basis of cell basement membrane.
- Type V: Available in cell surfaces, hair, and placenta.

The length of the helix and the nature and size of non-helical portions of the molecule vary from type to type. The dominant collagen of skin, tendon, and bone is type I, Type II is essentially unique to cartilage, while type III collagen is occurs in adult skin to the tune of 5-10%, in association with type I. The other types occur in small amounts and they are mostly associated with specific biological structures (Miller, 1984).

Type I procollagen has a molecular weight of 150,000 (Church et al., 1974; Monson et al., 1975), where NHx_2- and COOH- terminal extensions weigh 20,000 and 35,000 respectively (Olsen et al., 1977; Byers et al., 1975, Fessler et al., 1975). Alongside 139 amino acids were found in the NH_2 terminal extension (Becker et al., 1976, Hodein et al., 1979). Both the extensions are highly intrachain disulfide-bonded, but such bonds occur only at the COOH- terminus. Type II procollagen too exhibits a similar distribution of disulfide bonds, though its NH_2- and COOH- terminal extensions weigh 13,000 and 36,000 respectively (Merry et al., 1976). Type III procollagen also show structural homology to that of type I, besides demonstrating three conformationally distinct domains comprising globular, collagenous, and nonhelical sequences (Nowack et al., 1976, Bruckner et al., 1978). Type IV procollagen was initially considered as a disulfide-bonded molecule that migrates as a single chain on SDS-PAGE with an approximate

molecular weight ranging between 140,000-180,000, but further research suggests that it contains two chains, though the stoichiometry of them is yet to be established (Crouch and Bomstein, 1979; Timpl et al., 1978, Crouch et al., 1980). The α1 (IV) and α2(1V) chains have been isolated in a ratio of 2: 1 (Kresina and Miller, 1979), but other research suggests that they may exist in separate molecules (Timpl et al., 1979).One model for the pepsin-resistant portion of type IV collagen suggests that it is composed of three identical αl(IV) chains that contain little or no cysteine, besides having an identical molecular weight of α1 (1) chains on SDS-PAGE (Dehm and Kefalides, 1978).

According to Clark and Kefalides (Bornstein and Sage, p. 964) a disulfide-bonded, hydroxyproline-containing domain exists there, while other laboratories report the existence of pepsin-sensitive sites within the triple helix, resulting in a heterogeneous mixture of polypeptides when type IV collagen is extracted from tissues using this enzyme (Daniels and Chu, 1975, Dixit, 1978, Tryggvason et al., 1978). Alongside, purification of many larger type IV derived-fragments has shown that these polypeptides come from two distinct chains belonging to the type IV collagen subfamily (Kresina and Miller, 1979; Daniels and Chu, 1975; Dixit, 1978).

After separating the α1, α2, and α3 chains comprising type V collagen by ion exchange chromatography, the subsequent characterization has shown that the pepsin-resistant portions do not contain disulfide bonds, besides indicating a molecular weight approximating that of the α1(1) chain for these chains (Rhodes and Miller, 1978, Sage and Bomstein 1979). However, the study on the molecular organization of type V collagen remains controversial due to different outcomes. For example, several laboratories have suggested its structure as $(\alpha 1)_2\ \alpha 2$, where they based on quantitative estimates of the two chains in the native molecule or on recoveries after chromatographic purification (Burgeson et al., 1976, Hong et al., 1979, Madri and Furthmayr, 1979), while other evidences for the existence of these two chains in a single molecule come from their characteristics with respect to salting out behavior, resistance to vertebrate collagenase, and production of only one segment-long-spacing (SLS) species (Davison et al., 1979, Hong et al., 1979). Alongside, thermal denaturation-renaturation studies have also indicated that α2(V) chains are unable to form stable triple-helical molecules independently, which in turn suggests that the heteropolymer is the only form in which

the α2(V) chain could exist in vivo, although [αl (v)h molecules could be present as well (Bentz et al., 1978).

In contrast to the above propositions, other laboratories have presented more variable chain ratios for type V collagen, and researchers like Rhodes and Miller (1978) have found that type V collagen purified from cartilage contains only the α1 chain, and thermal denaturation studies of placental type V collagen indicates a biphasic melting curve, the initial phase of which was comprised principally of α2 chains and the subsequent phase, of α1 chains. The discovery of a third chain in some of the tissues, which tend to migrate between α1 (V) and α2(V) on SOS gel electrophoresis, has made the type V collagen even more complicated (Brown et al., 1978).

4. Applications:

Because of the collagen tensile strength and fibrous structure, collagen provides skin strength and elasticity, besides strengthening blood vessels and playing a major role in tissue development. Such potential of collagen has tremendous bearing on anti-aging treatment, cosmetic surgery, burns surgery, and even in weight management, leaving aside its industrial usages (Buehler, 2006; Fratzl, 2008). In general, the application of collagen can be classified into biomedical and non-biomedical sections.

4.1. Biomedical Applications

Even before that, the implantation of collagen showed good tissue tolerance both in vivo (Balazs and Hultsch 1976) and in vitro (Meier and Hay, 1975) which led to its use in many biomedical applications such as in the dialysis membrane of an artificial kidney (Nishihara et al., 1967), an artificial corneal membrane (Dunn et al., 1967), as vitreous body (Dunn et al., 1971), in skin and blood vessels (Sawyer et al., 1977), and as a surgical hemostatic agent (Peacock et al., 1965).

Chapvil et al., (1969) observed good tissue tolerance in vivo of a collagen-hydrophilic polymer constructed from a calf-hide collagen sponge immersed in HEMA monomer, allowing polymerization to occur on the sponge surface. Later in 1977 Shimizu et al., (1977) observed high tissue compatibility of laminar copolymers of bovine collagen and

various synthetic polymers that they had constructed by applying plasma discharge and y-irradiation to effect crosslinkage between a layer of collagen coated onto a layer of synthetic polymer.

Its low immunogenicity and high biocompatibility has made collagen as a favorite biomaterial, which has diverse general and biomedical applications. For example it is a common constituent of soaps, shampoos, facial creams, body lotions and other cosmetics and of food-grade gelatin. In the medical sphere it is used in cardiovascular surgery, plastic surgery, orthopedics, urology, neurology and ophthalmology (Meena et al., 1999). Used with a mixture of fibroblasts, growth factors, silicone and glycosaminoglycans, collagen is highly instrumental in constructing the artificial skin substitutes like skin burns (Meier, 2006).

Since the last 20 years doctors have using enzymatically hydrolyzed protein, where collagen hydrolysate has proven its ability to spare protein degradation in the body. Thus as a supplement it spares body's own lean body mass (protein). Accordingly it is being used extensively in the medical sphere in many forms other than surgery. For example, the common collagen supplements are prepared from bovine or shark cartilage, and type II collagen is used to heal rheumatoid arthritis (NutraSanus, 2010). Moreover, collagen is used in preventing overweight conditions by the enteral (oral) use of enzymatically hydrolyzed protein (Protein Report 2007).

Collagen has a wide range of application in the field of cosmetic surgery, especially in enhancing procedures as well as in reconstruction of essential bone and skin in the case of burns or disfigurement due to accident. It is also an essential component in diverse orthopedic and surgical procedures, and in dental treatment. It is now also being sold as a joint mobility supplement. However, the improvement of cosmetic surgery has brought the huge influence in collagen business, especially after less painful techniques such as dermal fillers are now used, where all it takes is a simple injection of collagen to fill the area under the skin (Higgins, 2010).

4.2. Non-biomedical Usage of Collagen:

Technology involving collagen in the form of leather and gelatin goes back hundred years ago. Leather is chemically treated animal skin, while gelatin is animal connective

tissue that is denatured and degraded by heat and chemicals. Both of them consist of large amount of collagen but are very different in chemistry and form (Meena et al., 1999). The knowledge that collagen produces gelatin in hydrolyzed state and that gelatin can work as fine glue, dates back to 8000 years when humans would use collagen-based glue as a protective lining on inflated fabrics and tools. Later the Egyptians also used it as an adhesive after boiling the skin and tendons of animals (Higgins, 2010).

Apart from that, people of earlier times used collagen to meet several needs, such as using in bows, to hold utensils together, to create waterproof lining, in decoration etc. Even the Greek word "kolla" means glue and refers collagen as its producer (Walker, 1998). Nowadays gelatin is extensively used in food, leather, music, and photography industries, besides heavily contributing to the booming business of cosmetics and cosmetic surgery. Off late gelatin-resorcinol-formaldehyde glue has been experimentally used to repair incisions in rabbit lungs (Ennker et al., 1994).

5. Advantages and Disadvantages of Using Collagen:

There is no doubt to the fact that using collagen as biomaterial is very successful. Moreover, collagen has provided a relief from long course of treatment in medical sphere, where it has significantly reduced the period of healing as well as the amount of pain that is associated with traditional treatment procedures. For example, percutaneous collagen induction, which is an alternative to laser resurfacing, has been gaining popularity due to the advantages like preservation of the epidermis, thicker skin, a short healing phase and usage of only local anesthesia (Fernandes, 2002).

Altogether it is a safe, non-surgical procedure that softens lines and furrows on the face and acts as an agent of beautification even in older age, besides repairing the scars from accident and untimely wrinkles. Collagen also works as an anti-ageing agent when taken as food supplement. However, more research is needed to measure the correlation between anti-ageing and collagen supplements. In industrial sector it provides various cheaper solutions. Even though collagen has been proved to play an important role as biomaterial, there are disadvantages of its use too. Though it is now extensively used for

cosmetic and burns surgery, people with high rate of allergic reactions can suffer from a prolonged period of side effects. And there is three percent of the population who are sensitive to collagen. Alongside, collagen cannot provide permanent solution to aging, since it breaks down in the human body like any other proteins. For example, a bovine collagen injection to eliminate wrinkles can last from six weeks to a year, after which the patient will need another injection to regenerate its effect (Armstrong, 2010, Meier, J. 2006).

There are more possible side effects of collagen. For example if a collagen injection accidentally blocks blood vessel, then a small area of skin may die and generate ulcer in that region, which in turn may leave a permanent scar. There are instances of such occurrence especially in the glabeller area. Alongside, allergic reactions may occur during the course of treatment even after showing no signs in preliminary tests and such reaction may continue for months. Allergic reactions involve shortness of breath, low blood pressure, and chest pain, besides urticaria, lumps at injection sites, scars or shorter lasting effect of collagen injections (DermNet, 2009). A detailed research through a span of eight years by Cukier et al., (1993) revealed an association between bovine collagen dermal implants and dermatomyositis or a polymyositis-like syndrome, which encouraged them to conclude there is a need to reassess the risks versus benefits situation for the cosmetic use of collagen implants.

6. Collagen in Different Species:

6.1 Collagen in Human:

The researchers have identified that collagen sponge prepared from human Type I collagen has potential as a graft material in oral surgical procedures (Quteish et al., 2004), and thus a person's own skin may be used to produce fibroblast cultures, or it can be mixed with synthetic PMMA beads Human collagen implants are highly purified and are isolated from human skin growth in a laboratory, where the cells are grown for ten years or so to make them living skin-equivalent for treating burns and ulcers (DermNet, 2009). A method along with apparatus has also been invented to extract collagen from human adipose tissue (Fataiha, 1993). However, the most significant news of the recent

time is that the scientists have found a way to make human collagen, which so far was 12proving obscure. The research team from the University of Wisconsin-Madison has developed a process of making synthetic collagen that would be longer than natural collagen and that certainly opens a horizon of possibilities in medical world as well as in the commercial world, since it has already showed enough potential as a type of nanowire due its thinness (Snowcrash, 2006).

6.2 Collagen in Bovine:

Bovine collagen has long been used in the medical sphere, enabling several breakthroughs towards providing healing and relief. It is equally important in the cosmetics, health supplement and other non-bio medical industries (Higgins, 2010). However, the research on bovine collagen gained momentum around 1970s, when the researchers developed a system of extracting collagen from cow skin and processing it into a liquid form (Armstrong, 2010). Injectable bovine collagen is created from sterile, purified collagen from cow skin. Alongside bovine and pig collagen are now being used together with rabbit cells (Transplant, 1999).

6.3 Collagen in Pig:

Since many years, pig rind is one source of collagen for processing products like sausage casings and films. Porcine type I collagen is extracted from pig hides, while porcine collagen film is created from an extrudable collagen gel. In the medical world too, porcine collagen sheet material proves to be very useful as an implant for reconstructive surgery. Recent research has shown that a paste formulation of porcine collagen gets integrated into full-thickness wounds without showing rejection and without generating excessive inflammation, which in turn suggest that such paste of porcine collagen could be an effective alternative to current dermal substitutes in full-thickness wounds (Shevchenko et al., 2007).

6.4 Collagen in Fish:

Similar to mammal and avian type I collagen, fish type I collagen contains three polypeptide chains, each consisting of about 1,000 amino acid residues while weighing

approximately 100kDa (Braco and Haard, 1995; Ho et al., 1997; Sivkumar et al., 2000; Saito et al., 2001). Fish collagen contains lesser quantities of hydroxyproline, which makes it less compatible to crosslinking and less stable than collagens obtained from land vertebrates (Bailey and Light, 1989; Hickman et al., 2000, Saito et al., 2001).

Application of collagen is a consequence of the ability of collagen to self-assemble in vitro into various strong structures. However, its applicability is limited due to its high cost and probability of disease transmission from the current sources. Thus fish processing wastes could be promising cost effective collagen sources through recycling of those wastes. For example, a study isolated collagen from scales of *Labeo rohita* (Rohu) and *Catla catla* (Catla) through demineralization followed by extraction using dilute acetic acid. The isolated protein was characterized by different physico-chemical techniques like FTIR, SDS-PAGE and CD spectroscopy. Thermal behavior of isolated collagen was evaluated by thermogravimetric analysis. The promising feature of obtained collagen is its close denaturation temperature to mammalian collagen, which significantly boosts its applicability. The isolated collagen may well find application in biomedical and pharmaceutical fields as a potential material for construction of tissue engineering scaffold, wound dressing system and drug delivery device (Pati et al., 2010).

7. Conclusion:

Beginning its journey as an elementary of tool of civilization, collagen has risen to the rank of an integral part of it over the years, where it is considered as an important element of benefit in both bio medical and non-bio medical sectors. Relentless research by the scientists with the backup of advanced science and technology has already opened several paths for exploiting the potentials of collagen. It has already been found that collagen is unparallel in providing tensile strength and thus it can be assumed that many more new applications of collagen will take place in course of time.

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Collagen Extraction
Appendix B

1. **Introduction:**

Collagen is the main protein of connective tissue in animals and fish, where it is the most abundant protein in mammals, constituting up to 25-30% of the total protein content of the body. On the other hand, collagen constitutes 1-2% of muscle tissue besides contributing to the tune of 6% of the weight of strong and tendinous muscles. Even the people of early civilization discovered collagen's multiple utility value, such as waterproofing, adhesive, and decoration.

Currently collagen has become an inseparable instrument in both bio medical and non bio medical industries, with an extended range of usage. For example, it provides structure additive to food besides providing food supplements of collagen; enables the pharmaceutical industry to produce hard and soft capsules, artificial skins, injections of regeneration of cells, provides cosmetic industry for the beautification and elimination of age-related skin wrinkles, acts as a source of glue in its hydroslated form, helps in films in the photographic industry. The main sources of collagen are pig and bovine skins due to their easy availability, though fish collagen is also making its mark and the researchers are contemplating with the potential of chicken skin. The major advantages of marine collage sources are that they are free from the risk of BSE (bovine spongiform encephalopathy), culturally more acceptable across the world, more suitable to the human skin than its equivalents, and are available in abundance, since fish skin is a major by-product of the fish-processing industry.

2. **History Background**:

In the period between 1920-1935 Nageotte (1927a, b, 1928, 1930, 1933), Nageotte & Guyon (1933), Leplat (1933), and Faure-Fremiet (1933) had studied and observed the partial dissolution of collagen in dilute solutions of weak acids such as formic and acetic acid. Renewed interest in this soluble protein was generated in the period between 1940-1955 with the works of Orekhovich and his colleagues in the U.S.S.R., who reported the extraction of a soluble collagenous-type protein from the skin

of various animals using dilute citrate buffers, which they suggested as a soluble precursor of collagen (Plotnikova, 1947; Tustanovskii, 1947; Orekhovich et al. 1948; Chernikov, 1949; Orekhovich, 1950, 1952). Such findings on soluble collagens were further reviewed by Harkness et al. (1954), who also reported the presence of a small amount of a protein of collagen type which was extracted from skin by dilute phosphate, pH 9-0 (alkali-soluble collagen).

Later Harkness et al. (1954) experimented on the feeding of labeled glycine to rabbits to arrive at a conclusion that this is a true precursor of collagen, whereas the metabolic role of the acid-soluble collagen described by Orekhovich is less certain, and it is not necessarily an intermediate in the formation of all insoluble collagens of the skin. Harkness et al. (1954) determined the hydroxyproline and tyrosine content of the alkali-soluble and acid-soluble collagen, and also of the gelatin obtained from the remaining insoluble collagen. Both soluble collagens contained less tyrosine and more hydroxyproline than the insoluble collagen, and the acid-soluble had a higher hydroxyproline and tyrosine content than the alkali-soluble collagen. Bowes et al. (1953) also observed similar differences between the hydroxyproline and tyrosine content of the acid soluble collagen of calf skin and the adult collagen of ox hide and between the acetic acid-soluble and insoluble fractions of tendon collagen.

3. **Methods**:

It requires solvents like salt, dilute acid, alcohol, detergents and H_2O_2 for removing non-collagenous proteins, as well as for removing fat and odors. Alongside it requires chemicals (EDTA) for deashing. It is after that the solvents like acetic acid, lactic acid, pepsin enzyme, Bacillus bacteria, and yeast are used to extract collagen under the temperature range of 0-4^{0}C. Altogether three steps are involved in the extraction of collagen, such as pre-treatment steps, extraction of acid-soluble collagen, and purification of collagen. Apart from the above, there is also super-critical extraction method where CO_2 is used as solvent, which has some advantages like ease of controlling the dissolving power of supercritical fluid (SCF) by controlling temperature and pressure. It is also easy to recover SCF by decompressing pressure, and it is impossible to separate the precipitate from extracts by centrifugation. SCF is a non-toxic solvent and is applicable to extract the

thermally decomposed compounds. However, this method is costly and requires expertise to handle the proceedings under high pressure.

3.1. Typical Process

In a typical extraction process, at least eight steps are taken (Mingyan et al. 2009). For example, in process of squid (*Ommastrephes bartrami)* collagen extraction, the steps could be like below.

1. Fish skin preparation: After collecting the squid skins they are manually descaled and are made free from the residual meat, before placing them at -20^0 C till the time of use. Alongside, all the reagents are checked to ensure that they are of analytical grade.
2. Histological observation: At this stage the squid skins are cut into pieces (0.5 cm x 0.5 cm) and fixed in 4% buffered formalin for 24 hours. After that the specimen is dehydrated in a series of graded ethanol solutions (70%, 80%, 90%, and 100%), clarified in xylene and is finally embedded in paraffin. Eight-mocron sections are then cut perpendicular to the skin surface and the slides with the sections are cleared off paraffin with xylene, rehydrated to water through graded alcohols, now in reverse order (100%, 90%, 80%, and 70%), and then are stained with hematoxylin and eosin and again with the Van Gieson stain (collagen – red color; muscle – yellow color). The stained sections are then observed through light microscopy and using color video camera at an original magnification of 20x the digital images are then collected.
3. Extraction of collagen: Generally it takes 4^0C to conduct the extraction process (Nagai and Suzuki, 2000), where the skin is extracted with 0.1 mol L^{-1} NaOH to remove non-collagenous materials and to eliminate the effect of endogenous proteases on collagen (Sato et al., 1987). After that it is rinsed thoroughly with distilled water to neutralize the pH of the wash water. Then the samples are mixed with 10% butyl alcohol at a solid to solvent ratio of 1:10 for 24 hours, after which they are washed with plenty of distilled water. The further minced skins are gently stirred in 0.5 mol L^{-1} acetic acid solution for 48 hours, and the extract is centrifuged at 10 000×g for 30 minutes. Then the acid-solubilized collagen (ASC)

in the supernatant is salted out by adding NaCl to a final concentration of 0.9 mol L^{-1}. Then after leaving the solution overnight, the resultant precipitate is collected by centrifugation at 8000×g for 20 minutes, before dissolving the same in 0.5 mol L^{-1} acetic acid. Then it is dialyzed against 0.1 mol L-1 acetic acid for 24 hours and distilled in water for 48 hours, and then it is lyophilized. After acid extraction, the insoluble fraction is kept suspended in 10 times of 0.5 mol L^{-1} acetic acid (by volume) and is digested with porcine pepsin (EC 3. 4. 23. 1; powderized; 750 U mg-1 dry matter) at an enzyme/ substrate ratio of 1:100 for 48 hours at 4^0C with gentle stirring. The extract is then centrifuged at 10 000×g for 30 min. Then the pepsin-solubilized collagen (PSC) in the supernatant is salted out by adding NaCl to a final concentration of 0.9 mol L^{-1} and after leaving it the solution overnight, the resultant precipitate is then collected by centrifugation (8000×g for 20 min) was dissolved in 0.5 mol L-1 acetic acid, dialyzed against 0.02 mol L-1 Na2HPO4 for 1 d to inactivate pepsin. The precipitate was collected at low speed centrifugation and the same is then dissolved in 0.5 mol L^{-1} acetic acid. After that the solution is dialyzed with 0.1 mol L^{-1} acetic acid and distilled through water, before putting to lyophilize.

4. Amino acid analysis: At this stage, ASC and PSC samples are hydrolyzed under reduced pressure with 6 mol L^{-1} HCl at 110^0C for 24 hours before analyzing the hydrolysates on an amino acid analyzer (e.g. Hitachi 835-50).
5. Sodium Dodecyl Sulphate Polyacylamide Gel electrophoresis (SDS-PAGE): It can be performed by following the method of Laemmli (1970), using the discontinuous Tris-HCl/ glycine buffer system with 7.5% resolving gel and 5% stacking gel. After electrophoresis, the gel is kept stained for 20 minutes with 0.1% Coomassie Brilliant Blue R-250 dissolved in distilled water, methanol and acetic acid (9:9:2), and then the same is destained using a solution of distilled water, methanol and acetic acid (8:1:1).
6. Fourier Transform Infrared Spectroscopy (FTIR): FTIR spectra is obtained from samples placed on discs containing a mixture of 0.2 mg lyophilized collagen and about 10 mg potassium bromide (KBr) ground under drying conditions. The spectra are recorded using infrared spectrophotometer (e.g., Nicolet 200SXV)

from 4000 to 500 cm^{-1} at a data acquisition rate of 2 cm^{-1} per point. The resulting spectra are analyzed using the software (e.g. Omnic 6.0).

7. Determination of Denaturation Temperature: The denaturation temperature is measured from changes in viscosity, where Ubbelohde viscometer can be used (Zhang et al. 2007). Usually ten milliliters of 0.03% collagen solution in 0.1 mol L^{-1} acetic acid with 0.2 mol L^{-1} sodium acetate buffer (pH 5.0) are used for viscosity measurements. Thermal determination curve is obtained by measuring the solution viscosity at seven stepwise-raised temperatures from 16 to 42^0C, each temperature being maintained for 30 min. Fractional viscosity at a given temperature is calculated by the following equation: Fractional viscosity=$(\eta_{sp(T)}-\eta sp_{(42^0C)})/(\eta_{sp(16^0C)}-\eta_{sp(42^0C)})$, where η_{sp} is the specific viscosity. These fractional viscosities are plotted against the temperature and the denaturation temperature is determined as the temperature where the fractional viscosity is predicted to be 0.5.
8. Differential Scanning Calorimetry (DSC): DSC is performed on a calorimeter (e.g., Netzsch DSC 200PC) fitted with an air-cooling compressor and a liquid nitrogen cooler at ambient temperature (Cui al., 2007). The temperature is calibrated using indium as standard. Collagen fiber is weighed (3.00 mg) and sealed in aluminum pans (BO 6.239.2–64.502). Triplicate samples are heated from 20 to 100^0C at a scanning rate of 2 K min^{-1}, with an empty sealed pan as a reference. The shrinkage temperature is taken at the peak of the plotted thermal transition curve (Minyang et al. 2009: 191-196).

4. Extraction of Collagen:

There are two methods for extraction collagen. These are Acid Solubilized Collagen (ASC) extraction and Pepsin Solubilized Collagen (PSC) extraction.

4.1. Acid Solubilized Collagen (ASC) Extraction:

4.1.1. Typical Process of Extraction at Earlier Times:

For the preparation of proteins from ox-hide collagen, the middle layer of a hide taken from a 2-year-old bullock immediately after flaying the skin is shaved, cut into pieces about 1 cm.2 and disintegrated in a Wiley mill. However it is necessary to stop the mill at intervals to remove the macerate by hand since it would not go through even the coarsest sieve. The macerated material (1800 g.) is then placed in a cotton bag and extracted, first with 0 l m-Na2HPO4, pH 8-6, and then with 0-12M sodium citrate buffer, pH 3-62. After the last phosphate extraction the macerate is suspended for a short period in two 3-1portions of the citrate buffer in order to remove the phosphate buffer before going on to the citrate extractions proper. Each extraction takes a period of 24 hr., during which the contents of the bag are agitated intermittently. All extractions are carried out between 2^0 and 4°.

The macerated skin swells considerably during the extractions; as much liquid as possible is to be squeezed out after each extraction, but relatively large amount remains behind. After the last extraction special efforts required to squeeze out liquid, and the relatively high protein content of this extract suggests that some liquid remaining from the earlier extractions is removed from the interstices of the protein. A small amount of the macerate (5 g.) was further extracted with successive 200-ml. portions of the citrate buffer. Samples are taken at various stages, dehydrated with acetone, and total nitrogen and hexosamine determinations are carried out (Bowes & Kenten, 1948a, b).

The protein in the citrate extracts is precipitated by the addition of sufficient 30% (w/v) sodium chloride solution to bring the final concentration to 5% (w/v). The next morning the lower clear layer of liquid is withdrawn, and the top layer, containing the protein in a gelatinous form, is centrifuged. The precipitate is then washed with a small mount of water and dehydrated with acetone after ascertaining that there is no alteration in the

solubility of the extracted protein. Redissolving in citrate buffer and dialysing against tap water then further purifies the precipitated protein.

The citrate-soluble collagen is converted into gelatin by heating a 2% (w/v) suspension in slightly acidified water to 400 for 5 min. The protein went into solution during the heating and on cooling the solution set to a gel.

4.1.2. Example of Modern Times:

In another instance of extraction and characterization of collagen from chicken skin, the skins are first ground and then are heated to 40 or 60°C to extract the fat. After the mechanical separation, the collagen content remaining in the resulting solid phase is extracted with pepsin or ethylene diamine. Type I and type III collagen are then isolated and characterized by SDS PAGE, antigen labeling, determination of tyrosine residues, and transmission electron microscopy. The total collagen content of the skin is recovered from the solid phase following heat treatment at 40°C. Extraction yields vary with the solubilization process; for example 38.9% of the collagen content in the solid phase could be extracted with pepsin and 25.1% with ethylene diamine (Cliche et al. 2003). The collagen obtained is characterized by its electrophoretic migration pattern and is then confirmed by antigen labeling. The presence of telopeptides is determined by measuring tyrosine content. Transmission electron micrographs are also obtained. For example, 1% phosphotungstic acid is applied to the collagen samples as a negative stain, and then they are mounted on Formvar grids before being viewed at 105,000× (Piez, 1984), using an EM 420 transmission electron microscope. The results are then compared to those obtained from analysis of Atelohelogen avian collagen.

4.2. Pepsin Solubilized Collagen (PSC) Extraction:

Fujimoto (1968) found that collagen could be solubilized by pepsin treatment as a preliminary to purification from the muscle layer of Ascaris lumbricoides and pig kidney, which prompted other researchers to apply the same technique. For example, Bannister and Burns (1972) applied the same technique to solubilization of avian intramuscular collagen, where they found that tropocollagen from intramuscular sources was more highly cross-linked than the material from skin treated in similar fashion. Although

solubilization results from digestion of non-collagenous protein, it is also the result of cleavage of telopeptides containing covalent cross-links, and precise comparison between collagens from different sources is difficult to make.

4.2.1. Solubilization and Purification of Collagen:

In an instance of pepsin treatment of avian skin collagen, Bannister and Burns (1972: 677-681) selected the skin of a strain of domestic fowl derived from commercial broilers as their source of material. The birds were all female and belonged to 12-18 week age group. The plucked skins from freshly killed birds were cleaned of adhering fat and muscle before mincing with ice in a hand-operated mincer. Next, the material was defatted by two extractions with chloroform-methanol (2:1, v/v), before thoroughly washing with tap water and distilled water and extracting twice with 0.2M-Na2HPO4 at room temperature. However, all subsequent operations were performed at 0-40C, where the researchers obtained acid-soluble collagen by extracting the defatted minced skin three times (for 2-3 days in each case) with 3% (v/v) acetic acid. The remaining material was then resuspended in 3% (v/v) acetic acid and digested for 2-3 days with approx. 5mg of pepsin/ml.

The collagens solubilized by both procedures were then purified, where the extracts were clarified by centrifugation for one hour at approxmatly 33000g and the supernatant was dialyzed against NaCl so that the final concentration turns 7% (w/v). The precipitated collagen was then collected by centrifugation and was redissolved in 3% (v/v) acetic acid and centrifuged for another hour at approx. 77000g. Entire of the above procedure was then repeated and the protein was precipitated a third time by dialysis against 0.02M-Na2HPO4. After dissolving in, and dialysis against, 3% (v/v) acetic acid the collagen was centrifuged for one hour and half at 10,000g. The supernatant containing the purified protein was then freeze-dried and stored in a desiccator.

4.2.2. Pepsin Treatment of Soluble Collagens:

The researchers adjusted the portions of acid-soluble and pepsin-solubilized collagens to 1.Omg/mI in 3% (v/v) acetic acid and treated with crystalline pepsin (concn. 0.1 mg/ml) for a period between 3 and 14 days at 4°C. After digestion, pepsin was removed by one

salt precipitation and one Na2HPO4 precipitation. Thus four types of collagen were available for study, such as acidsoluble (A), acid-soluble pepsin-digested (Ap), pepsin-solubilized (P), and pepsin-solubilized pepsindigested (Pp) collagen, out of which collagen Pp was mostly treated for 14 days.

4.2.3. Hexosamine and Aldehyde Contents of Collagen Preparations:

The amount of hexosamine present is considered to be a measure of contamination of collagen preparations by the mucopolysaccharide constituents of connective tissue. Therefore the researchers took measurements by the Elson-Morgan procedure as described by Davidson (1966), with glucosamine as standard. The aldehyde content was assayed by the method of Paz et al. (1965), with acetaldehyde as standard.

4.2.4. Determination of Subunit Composition:

The percentage of α-, β-, and γ-chains in denatured collagen solutions was determined by polyacrylamidegel electrophoresis and densitometry.

4.2.5. Rate of Fibril Formation:

Since the rate at which native-type fibrils can be formed from collagen solutions may be used as a measure of the aggregation properties of the tropocollagen molecule, the researchers dissolved collagen dissolved in 3% (v/v) acetic acid at 0°C and carefully raised the pH to 7.2 by adding 2M- and 0.01 MnaOH using a Pye model 291 pH-meter. When the required pH was achieved, they adjusted the volume with water to give a collagen concentration of 0.1 %. Fibrillogenesis was monitored continuously at 400nm in a spectrophotometer (e.g. Unicam SP.800). The process was initiated by transferring solution from an ice bath to a cuvette maintained at 38°C.

4.2.6. Effects of Pepsin Treatment on Hexosamine Andaldehyde Content:

Hexosamine: Contamination by hexosamine was low and similar in all preparations, which meant pepsin treatment is probably without effect in the further purification of already highly purified collagen. Aldehyde: Here the findings appeared somewhat different from those of Deshmukh & Nimni (1971), where chick skin acid-soluble

collagen contained about one-third as much aldehyde as did neutral-salt-soluble collagen from rat skin. However, that was not surprising in view of the biologically older nature of acid-soluble collagen and the differing methods employed in purification. Pepsin-solubilized collagen contained about one-half as much as the acid-soluble material, which the researchers presumed as a reflection of greater biological maturity and also some loss of telopeptides due to action of the enzyme. When they treated with pepsin, both preparations (Ap and Pp) sustained further decreases in aldehyde content, in conformity with the location of this group in the telopeptide region.

4.2.7. Pepsin Treatment:

The subunit composition was expressed as the percentage of α-chains (monomers), β-chains (dimers) and γ-chains (trimers), although this last group probably contained higher-molecular-weight subunits as well. The compositions of collagens A and P showed that, although there is no great difference in the content of α-chains, collagen P is very much richer (about four times) in γ- chains. Sampling at 24h intervals and examining the ureadenatured material by gel electrophoresis enabled the study of the pepsin-digestion of these two collagens. In this experiment the researchers did not remove pepsin by repurification because it did not interfere with separation of the subunit classes. The results of treatment over a 4-day period showed that Collagen A was almost completely converted into α-chains with little β- and no detectable γ-chains remaining. In contrast, collagen P gave rise to fewer α-subunits than did collagen A, and significant quantities of β- and γ-chains survived pepsin treatment. This observation suggested that there are covalent cross-links present in collagen P that resist pepsin attack, and confirms a similar conclusion reached by Steven (1966) with bovine and human collagens.

4.2.8. Rate of Fibril Formation:

Researchers of 1950s and 1960s (Gross and Kirk, 1958; Bensusan, 1960; Bensusan and Scanu, 1960) studied the effects of a variety of substances on the rate at which solutions of collagen can form rigid gels containing native-type fibrils. Subsequently, other researchers (Rubin et al., 1963; Connel & Wood, 1964) also investigated the results of limited proteolysis on the rate of fibril formation. This prompted Bannister and Burns

(1972) to use a similar turbidimetric technique to test whether further pepsin treatment of collagens A and P results in a decrease of fibril-forming capacity. Resultantly they found that collagen A was markedly inhibited whereas pepsin treatment of collagen P was virtually without effect. The finding with collagen A was much as expected, but the failure to produce significant retardation of fibrillogenesis with collagen P was not expected, which in turn suggested that sufficient quantity of a 'nucleus-forming' collagen remained despite removal of a large percentage of the covalent cross-links by pepsin. In spite of the absence of direct evidence, the researchers found it tempting to suggest that this is related to the previously demonstrated pepsin-resistant collagen.

5. **Isolation and Characterization:**

5.1. Western Blot

Western Blot method is used to detect functional proteins after the cell produces them. Both procollagen and collagen can be detected using this method. Proteins in the mixture are separated using electrophoresis based on their electrical charge, which corresponds to the molecules weight or size, and then transferred to a membrane to which the proteins become affixed. Antibodies that are specific to react or bind with the protein of interest are applied to the membrane and these complexes are then detected by chemilumenescence and film development (Cao et al. 1997; Ikenou et al. 2003; Lim et al. 2002). In an instance of Western Blot analysis (antigen labeling) Cliche et al. (2003: 504) adopted the method suggested by Timmons and Dunbar (1990), where the collagen was transferred (10 V for 50 in) with the Trans-Blot Semi-Dry Transfer Cell to an Immun-Blot polyvinylidene difluoride (PVDF) membrane (0.2 μm). They used rabbit anti-chicken collagen type I as primary antibody, and alkaline phosphatase rabbit IgG as the secondary antibody. The primary and secondary antibodies were diluted in the blocking solution (3% BSA) at 1:3,000(Ikenou et al. 2003). Alongside, they developed the color associated with antigen labeling by using alkaline phosphatase provided with the kit.

5.2. SDS-PAGE

Sodium dodecyl sulphate polyacrylamine gel electrophoresis (SDS-PAGE) is one of the methods to determinate the forms of collagen (alpha chain, beta chain); SDS-PAGE to determine peptide mapping. Determination of hydroxyproline content is required besides the determination of denaturation temperature, viscosity of collagen solution, effect pH and NaCl on collagen solubility, FT-IR and SEM to determine structure of collagen. In an instance of extracting collagen from chicken skin (Cliche et al. 2003: 504) the SDS PAGE was performed using the procedure of Laemmli (1970) with a Mini-Protean II electrophoresis unit, where the stacking gels contained 3% polyacrylamide, and the separation gels contained 6.5% polyacrylamide. Ten micrograms of collagen deposited in the wells, and the migration was induced at 80 V for 5 min, until the collagen subunits had passed through the stacking gel, and then at 150 V for 55 min, or until the migration reached the end of the resolving gel. The researchers stained the gels with Coomassie blue R-2507 for 30 min and destained with a solution of 10% glacial acetic acid, 40% methanol, and 50% water.

In another instance of SDS-PAGE Mingyan et al. (2009) examined collagen from squid skins by using a 7.5% resolving gel, where both ASC and PSC had the similar electrophoretic pattern of typical type I collagen, consisting of α chains with two distinct types like α1 and α2, varying in their mobility. The electrophoretic positions of α chains of squid skin collagen were different from those of the walleye polylock (Yan et al. 2008), grass carp, bovine (Zhang et al. 2007), and porcine, (115 kDa for α1 chain and 66 kDa for α2 chain) which suggested the distinctiveness of the squid skin collagen at its primary structure. Alongside, the inter and intra molecular crosslinked components such as β (dimers) and γ (trimers) were also found in squid collagen, which resembled with bigeye snaper (Kittiphattanabawon et al. 2005) and ocellate puffer fish skin (Nagai et al. 2002). The electrophoretic results eventually underpinned type I collagen as the major collagen in squid skin.

6. **Other Important Factors:**

6.1. Tyrosine Measurement

Tyrosine content requires to be measured in collagen samples, usually hydrolyzed at 105^0C in 6 *N* hydrochloric acid for 24 hours under a nitrogen atmosphere. Amino acids are quantified by liquid phase ion-exchange chromatography using an analyzer (e.g. Biochrom 20) (Cliche et al. 2003: 504).

6.2. Hydroxyproline Determination.

A colorimetric assay is used to determine the hydroxyproline content following hydrolysis of collagen at 105^0C in *7 N* sulfuric acid for 16 hours. Colorimetric reaction is done with chloramine-T and 4-dimethylbenzaldehyde using the Kolar (1990) method. Hydroxyproline content is converted to total collagen using a factor of 7.57 (Bonifer and Froning, 1996).

7. **Problems with Extraction and Identification**:

A major difficulty in the identification of intact tissue antigens is the need to preserve antigenicity, i.e., the conformational integrity of the epitopes and to achieve adequate ultrastructural preservation. The methods like aqueous fixation, dehydration, and resin embedding for electron microscopy give rise to considerable changes in tissue structure, including extraction, precipitation, and collapse of labile molecular constituents (Hunziker, 1993). Furthermore, conventional chemical fixation in aldehyde solutions can lead to complete loss of antigenicity owing to the formation of intermolecular cross-links. Collagen epitopes appear to be particularly sensitive to aldehyde fixation. To overcome the problems associated with structural artifacts induced by conventional aqueous fixation and dehydration procedures, alterative methods of tissue preparation adopt employing low temperature. These methods have been applied to cartilage, with considerable improvements in the ultrastructural preservation of chondrocytes and matrix (Hunziker, 1993; Keene and McDonald, 1993; Hunziker and Schenk, 1984; Hunziker et al., 1984; Akisaka and Shigenaga, 1983) and superior immunolocalization of matrix proteoglycan epitopes (Hunziker and Herrmann, 1987). Till 1995, it was not possible to successfully localize specific collagens by those methods, which prompted the

researchers to assume that a particular stage in the cryotechnical process may give rise to conformational changes in collagen epitopes (Hunziker, 1993).

8. **Conclusion:**

The review shows a promising evolution of the extraction process, where several researchers have enriched the methods, besides providing valuable insights regarding the nature of collagens in different sources, where a growing trend of exploiting nonbovine sources of collagen such as fish or chicken-skin is observed. Such trend is justified since the usage of collagen has been ramified over the years and the disease and cultural factors associated with bovine or porcine collagen have become points of concern in commercially exploiting them.

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