

Literature Review 2: Collagen

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.

In the modern era 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 counterparts, and are available in abundance, since fish skin is a major by-product of the fish-processing industry.

2. 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 H₂O₂ 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°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 CO₂ 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. A 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° 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-micron 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°C 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 defatted 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^oC 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^oC for 24 hours before analyzing the hydrolysates on an amino acid analyzer (e.g. Hitachi 835-50).

5. Sodium Dodecyl Sulphate Polyacrylamide 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^oC, each temperature being maintained for 30 min. Fractional viscosity at a given temperature is calculated by the following equation: Fractional viscosity=($\eta_{sp}(T)-\eta_{sp}(42^{\circ}C)$)/($\eta_{sp}(16^{\circ}C)-\eta_{sp}(42^{\circ}C)$), 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^oC 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

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 0.1 M-Na₂HPO₄, pH 8-6, and then with 0.12M sodium citrate buffer, pH 3-6.2. After the last phosphate extraction the macerate is suspended for a short period in two 3-l portions 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° 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 amount 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 are 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 \times (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 approx. 33000g and the supernatant was dialyzed against aq. 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 1.5 hrs. at 109000g. 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 1.0mg/ml 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 M NaOH 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 and Aldehyde 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 a 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 chemiluminescence 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 μ m). 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. 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 determine the forms of collagen (α chain, β 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 $\alpha 1$ and $\alpha 2$, 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 $\alpha 1$ chain and 66 kDa for $\alpha 2$ 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 snapper (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°C 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°C 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, alternative 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 depicts 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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