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EXTRACTION AND PARTIAL CHARACTERIZATION OF COLLAGEN FROM DIFFERENT ANIMAL SKINS

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Abstract

Collagen is a wonder triple helix fibrous protein. Basically, it connects and supports other bodily tissues, such as skin, bone, tendons, muscles and cartilage. In fact, it makes up about 25% of the total amount of proteins. Collagen possesses great tensile strength. In the present investigation, three methods of collagen extraction were employed. Out of which the method of George and Chandrakasan (1996) proved to be a better method for collagen extraction in chicken and human skins. Regarding Goat and Buffalo skins, collagen was extracted using TCA method wherein the extracted fibrous protein got hydrolyzed, getting denatured therefore, a definite band pattern was not obtained. By comparing the electrophoretic profile of various animal skins, a definite band pattern can be obtained which will indicate differences in collagen structure thereby facilitating species and generic differentiation.

Introduction

Collagen is the most abundant protein in the body with 20 different types of collagen being expressed in human tissue. Type I collagen is the major bone protein (Babraj *et al.*, 2002). In bone, type I collagen is the most abundant protein, accounting for approximately 90% of the organic matter (Batge *et al.*, 1992). Collagen molecules are structural macromolecule of the extra cellular matrix that includes in their structure one or several domains that have a characteristic triple helical conformation (Rest and Garrone, 1991). Proteins are nitrogenous compounds constituted by amino acids. They serve as the major structural component of muscle and other tissue of the body (Walsh, 2004). In addition to this, they are used in the synthesis of hormones, enzymes and hemoglobin. About one quarter of all the protein in the human body is collagen. It is a group of naturally occurring proteins. In nature it is found exclusively in animals (Muller *et al.*, 2003). It is the main protein of connective tissue. It is the most abundant protein in mammals (Gloria *et al.*, 2002) making up about 25% to 35% of the whole body protein content. In muscle tissue it serves as a major component of endomysium. Collagen constitutes 1% to 2% of muscle tissue and accounts for 6% of the weight of strong, tendinous muscles (Sikorski *et al.*, 2001). Collagen is a major structural protein, forming molecular cables that strengthen the tendons and vast, resilient sheets that support the skin and internal organs. Collagen is a fibrous protein, inextensible which can be found at the level of connective tissues from heart vessels, skin, cornea, cartilage, ligaments, tendons, bones and teeth.

About 28 types of collagen are known (Veit *et al.*, 2006). A distinctive feature of collagen is the regular arrangement of amino acids in each of the three chains of these collagen subunits. The sequence often follows the pattern Gly-Pro-Y or Gly-X-Hyp where X and Y may be any of various other amino acid residues (Ramchandran *et al.*, 1976, Orgel *et al.*, 2006). Fibrosis and sclerosis are widely recognized as hallmark of progressive renal disease and are caused by the excessive accumulation of connective tissue mostly collagen (Samuel, 2009). Specific genetic defects in the biosynthetic pathway of collagen are known and are associated with several of the systemic connective tissue disorders (Bateman *et al.*, 2009).

Materials and Methods

Standardization of procedures for Collagen Extraction:

1. Extraction of Collagen (Neuman & Logan, 1950)
 - [A] Obtaining fat free tissue
 - [B] Extraction of Collagen (as gelatin)
2. Extraction of Collagen (Harkness & Harkness, 1956, TCA method)
 - [A] Preparation of acid hydrolysates
3. Extraction of collagen (George & Chandrakasan, 1996)

The neutral salt soluble collagen and acid salt soluble collagen was subjected to Thin Layer Chromatography.

Qualitative analysis of amino acids extracted from collagen

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1. Preparation of TLC plate (Wall, 2005; Girigowda and Mulimani, 2005)

The TLC plate was prepared by suspending 30 gm of Silica Gel- G powder in 45 ml of distilled water; the slurry was poured on to the glass plate to 0.2 mm thickness. The plates were then air-dried. Activation of the TLC plates was done at 100°C for 1 hour.

2. Loading the Sample

3-5 µl of sample was spotted with a Hamilton Syringe along with the standard collagen and dried using a hair drier.

3. Standard Used: Consisted of Collagen from Bovine Achilles Tendon (Cat No: C9879-1G, Sigma Aldrich)

4. Solvent System for TLC (Chemicals: CDH, India): Consisted of n-Butanol: Acetic Acid: Water (4:1:5)

Spraying reagent

1.5 gm ninhydrin was dissolved in 100ml of n-butanol and then 3.0 ml of acetic acid was added. The TLC plate was sprayed with the spraying reagent and the plate was air dried and kept in an oven at 100°C for 5-10 min. Purple to pink colored spots were observed and Rf values were determined.

Table 1. Protein Concentration of animal tissues

S. No.	Animal Tissue	Extraction Method	Protein Concentration (mg/ml) at 260 nm
1.	Chicken	(a) Neuman & Logan	5.61
		(b) TCA Method	Negligible amount
		(c) George & Chandrakasan	
		(i) Acid salt soluble collagen	5.60
		(ii) Neutral salt soluble collagen	7.03
		(a) Neuman & Logan	Negligible Amount
	Goat	(b) TCA Method	6.32
		(c) George & Chandrakasan	
		(i) Acid salt soluble collagen	Negligible Amount
		(ii) Neutral salt soluble collagen	Negligible Amount
		(a) Neuman & Logan	Negligible Amount
		(b) TCA Method	8.41
Buffalo	(c) George & Chandrakasan		
	(i) Acid salt soluble collagen	Negligible Amount	
	(ii) Neutral salt soluble collagen	Negligible Amount	
	(a) Neuman & Logan	Negligible Amount	
	(b) TCA Method	Negligible Amount	
	(c) George & Chandrakasan		
4.	Human	(i) Acid salt soluble collagen	10.16
		(ii) Neutral salt soluble collagen	12.46
		(a) Neuman & Logan	Negligible Amount
		(b) TCA Method	Negligible Amount
		(c) George & Chandrakasan	
		(i) Acid salt soluble collagen	10.16

Sodium Dodecyl Sulphate Polyacrylamide Gel electrophoresis (SDS-PAGE)

Sample preparation

The sample was prepared by dissolving collagen molecule in sample buffer (1mg/ml). The sample was mixed with 1X loading dye in 1:1 ratio and denatured by heating at 50°C for 30 min (George and Chandrakasan, 1996).

Methodology

pH of all the solutions was set by using pH meter (EUTECH Instruments, India). The gel consisted of 6% Resolving gel and 4% Stacking gel. All the ingredients for gel were mixed according to protocol and poured into the casting form leaving space for stacking gel about 2 cm below the bottom of the comb. Bubbles

were removed by adding isopropanol layer above it. The gel was allowed to set for 20 min. After that stacking gel was poured above running gel and comb was put in. After 20 min. the samples were loaded into the wells (about 20 µl) along with standard collagen (10 µl) for determining the band pattern. Buffer was poured into the buffer chamber, and electrophoresis was carried out at a constant current of 150 V at until the tracking dye (Bromophenol blue) reached the bottom. After complete run, visualization of the gel was done in a Gel doc system (BIORAD, India).

Results and Discussion

Extraction of collagen from fresh connective tissue in various aqueous media from various animals has been the subject of a number of descriptive studies.

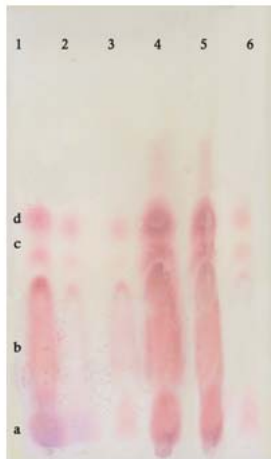
The animal tissues from which collagen were extracted includes Chicken, Goat, Buffalo and Human. For every animal tissue all the three collagen extraction procedures were screened to obtain maximum total protein content. Out of these three extraction procedures the protein (mg/ml) obtained by George and Chandrakasan, collagen extraction method was found to be maximum in case of chicken and human tissue while TCA method yielded significant protein amount in case of goat and buffalo tissue. The protein concentrations of different animal tissues are depicted in table 1.

TLC results

Autoclaving several different tissues for 2 to 4 hrs results into extraction of 93-98% of the collagen. Acid hydrolysates of animal tissues *viz.*, chicken, goat, buffalo and human were subjected to Thin Layer Chromatography. After the complete run in the chromatographic chamber, TLC plate was air dried and thereby sprayed with the location reagent (Ninhydrin Reagent). Development of purple to pinkish coloured spots was observed. Rf values i.e. Ratio of fronts were subsequently determined for the separated amino acids. The results are depicted in table.

The Rf values obtained from the present investigation are similar to the standard values (Stahl, 1965). Fig. 1 shows TLC results.

Fig. 1 TLC results: Lane 1- Acid salt soluble collagen (chicken), lane 2- Neutral salt soluble collagen (chicken), lane 3- Neuman & Logan method (chicken), lane 4- TCA Method (buffalo), lane 5- TCA Method (Goat), lane 6- Acid salt soluble collagen (human tissue). a, b, c & d represents the spots of various collagen samples



SDS-PAGE results

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis was done to compare the collagen profile of different animal skins. A total of 6 wells were loaded with extracted collagens from different animal skins along with 7th well containing a standard collagen.

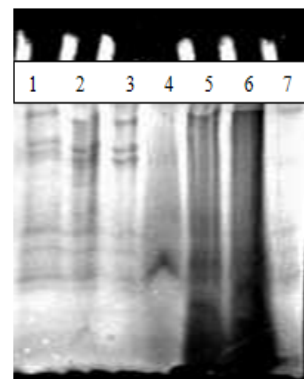
The standard i.e. Collagen from Bovine Achilles Tendon (Cat No: C9879-1G, Sigma Aldrich) on complete electrophoretic run showed many distinct bands of various molecular weights.

On comparing the electrophoretic profile of all the four animal skins with the standard collagen (Bovine Achilles Tendon) the following results were obtained:

1. Chicken skin showed significantly similar band pattern in comparison with standard.
2. Goat skin collagen molecule could not migrate properly.
3. Buffalo skin collagen molecule could not migrate properly.
4. Human skin showed some level of similarity with the standard ((Bovine Achilles Tendon) in the band pattern.

Collagen in tissues is contained in tendons, membranes or in fibrils forming a network within structures. Comparison of the collagen content of different tissues necessitates the analysis of representative samples of entire organs or careful definition of the location of the sample taken to represent a constituent part (Neuman & Logan, 1950). Fig. 2 shows SDS-PAGE results.

Fig. 2. Results of SDS PAGE: Lane 1- Acid salt soluble collagen (chicken), Lane 2- Neutral salt soluble collagen (Chicken), Lane 3- Collagen from Bovine Achilles Tendon, Lane 4- Neuman & Logan method (Chicken), Lane 5- TCA method (Buffalo), Lane 6- TCA method (Goat), Lane 7- Acid Salt Soluble collagen (human tissue)



SDS-PAGE was done of human corneal ethanol precipitated collagen (dialyzed sample) using 4% SDS-Polyacrylamide Gels in Tris-acetate buffer (pH=6.6) for 3.5 hr at 8 mA/gels (Stoesser *et al.*, 1978). Cyanogen bromide peptides of collagen were purified from normal corneal stromal fibroblasts. The workers concluded from their study that human corneal fibroblast in cell culture secrete collagenous proteins.

Samuel (2009) described the extraction, detection and quantitation of interstitial collagen sub-types (types I, III & V) by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The result indicate

type I collagen monomers, identified by the α -1 (I) and α -2 (I) subunits/ chains; type III collagen monomers, identified by α -1 (III) chains and type V collagen monomers, identified by the α -1 (V) and α -2 (V) subunits/ chains.

Cliche *et al.* (2003) characterized chicken collagen by its electrophoretic migration pattern. They performed SDS-PAGE using procedure of Laemmli (1970) employing stacking gel 3% while separating gel 6.5% polyacrylamide. They isolated type I and type III collagen and characterized them. Thus, chicken skin is a good alternative source of high quality collagen.

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