



EXTRACTION, CHARACTERIZATION, ANTIMICROBIAL APPLICATIONS OF COLLAGEN FROM ALBINO WISTAR RAT TAIL TENDONS (LABORATORY WASTE)

Hemambujam Devanathan*

Genetic Engineering Department, Bharath University (BIHER), Selaiyur, Chennai-73.

***Corresponding Author: Hemambujam Devanathan**

Genetic Engineering Department, Bharath University (BIHER), Selaiyur, Chennai-73.

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ABSTRACT

Collagen is the abundant protein in various connective tissues to animal proteins and has been widely used as application materials for tendons, bones and muscles. A rat tail tendon is among the original source of Type I collagen and it was observed that mature female rats give best yield than male and immature rats. The specific purpose of the present study was to extract and analyses collagen from Albino Wistar rat tail tendons. Albino Wistar rat tail tendons were collected and stored at -20°C. Tendons were removed from tails then tendons were treated with NaCl to remove non-collagenous tissue. Then, were solubilized using acetic acid method. The solubilized collagen was precipitated by using NaCl and dialysis was carried out. Then SDS-PAGE was done to separate proteins according to their molecular weight and UV analysis was performed. The objectives of this present study is to assess the use of Albino Wistar rat tail tendons (laboratory waste) as a source of collagen and its extraction, characteristics and to check its antifungal and wound healing activity. The total amount of collagen that was extracted from 20.90g of rat tendons was 12ml of collagen. Rat tail collagen showed significant in Antifungal activity and based upon the results of this study, collagen showed broad application in wound healing activity.

KEYWORDS: Rat tail tendons (laboratory waste), Type I collagen, non-collagenous tissue, solubilized and precipitated collagen, dialysis membrane, SDS-PAGE, UV analysis, antifungal activity, wound healing activity.

INTRODUCTION

Many studies demonstrate that rat collagen is able to serve as Natural alternative source to change commercial collagen and most of them have the effective source like tendons, directly from laboratory rat (Cote et al, 2007). Collagen which is originated from rat tail tendons has medical, cosmetic uses and used in pharmaceutical industries as injectable dispersion, micro particles in drug delivery system and plays an important role in modern medicine. There are various applications of collagen is present in antifungal drugs, in vitro wound healing activity. Rat tail tendons is among the original source of Type I collagen extracts (Bell et al., 1979; Paul et al, 2008). Collagen promotes healing and allows a way for new tissue growth and heals granulating wounds on which various tissues grows, necrotic wounds, partial wounds, second degree burns, skin grafts, skin donating areas. Collagen is used in the form of sponge for treating wounds and burns in diabetic patients. In Thailand, collagen products used in biomedical use are mainly imported, but raw materials for collagen extraction can be obtained domestically (Techatanawat, et al, 2011). It would be more beneficial and economical to use tendons as a source of collagen which is taken from already used rats for laboratory research. In addition, it was observed that collagen extracted from mature female rats shows a

best yield of collagen relative to male and non-mature rats (OEL Blidi et al., 2017). The Albino Wistar rat tail tendons were collected from known laboratory after approval from ethical committee. The overall aim of the project was how to use rat tail tendons (laboratory waste) as a source of collagen and its extraction, characteristics, to check the antifungal activity of collagen incorporated with antifungal drug and its wound healing activity.

MATERIALS AND METHODS

Sample Preparation

Rat tail tendons were cleaned from the attached muscles and skin using a sharp knife. Only seven tails of 20.90gram of tendons was acquired from Albino Wistar rat then it was chopped into fine small pieces and was kept at 5°C in the refrigerator. **Chemicals:** Sodium chloride (NaCl), Sodium hydroxide (NaOH), etc are imported from high media, SRL.

Removing Non-Collagenous Tissue

The tendons were treated with 150ml of 0.8M of NaCl for 30 minutes after it was treated with 0.1M/L of NaOH for 24 hours. Then the sample was washed with cold distilled water and the sample was made into homogenized paste.

Solubilizing Collagen

The collagen sample was treated with 0.5M/L of Glacial Acetic Acid. Then, the sample was transferred into it and stored at 5°C for 3 days. Then, it was centrifuged at 5000 rpm for 40 minutes. Supernatant and pellet was collected separately.

Centrifugation and Precipitation

Collagen was salted out using NaCl at 2M/L (sample was treated with 17.53grams of NaCl dissolved in 150 ml of supernatant) and was stirred using hot plate magnetic stirrer for 2 hours and placed in the refrigerator at 4°C for 2 days. Sample was centrifuged at 5000 rpm for 40 minutes. Pellet was collected. Then, the dialysis was carried out using PBS, the PBS was prepared for 450 ml (PH was set to 7.2). Then 0.05M of Glacial Acetic Acid was added to the sample and transferred into dialysis membrane, stored at low temperature for 3 days and each time the PBS solution was replaced with newly prepared one. Then the collagen sample was collected.

Characterization of Collagen Sds Page

SDS-PAGE was performed by the method of Weber and Osborn (1969). Then 5% of stacking gel and 75% of separation gel was prepared. To the sample, half volume of sample buffer (0.02 M sodium phosphate (pH 7.2) containing 1% SDS and 3.5 M urea) was added and 1-3 μ l of 0.1% bromophenol blue was added. Electrophoresis was performed on 3.5% gels in 0.1 M phosphate buffer (pH 7.2) containing 3.5 M urea and 0.1% SDS. After electrophoresis, protein bands were stained with 0.25% (w/v) Coomassie Brilliant Blue R250.

Uv-Visible Spectrophotometer

UV spectrometric analysis collagen was dissolved in 0.5M PBS to obtain a concentration of 1 mg/ml. The solution was then subjected to UV-V is measurement. Prior to measurement, the baseline was set with 0.5M PBS. The spectrum was obtained by scanning the wavelength in the range of 200-800nm.

Antifungal Activity

The fungal samples were collected from Avanz bio private limited laboratory. These samples were used to detect the antifungal activity test by incorporating collagen with commercial antibiotic. The assay was performed by disc diffusion method. Potato dextrose agar media was prepared for 150 ml and were inhibited by fungal culture for 24 hrs. Then the growth was determined by measuring zone of inhibition. Number of microorganisms: *Aspergillus* *Niger* and *Candida Albicans*. Sample concentration: 80 μ l, 50 μ l, 80 μ l, control.

Invitro Scratch Wound Healing Activity

VERO cells were seeded in 6-well plates (8 $\times$ 10⁵ cells/wells) and grown until reached a confluence of 90-95%, in the optimum culture conditions. In the middle of cell monolayer, a scratch was made by a P10 pipette tip, to

mimic a wound, and cell debris was removed by washing with fresh medium. The wound was exposed for 50 μ g/ml of Albino Wistar rat tail tendon collagen for 24-48 hrs. And incubated at 37°C in a humidified atmosphere of 5 % CO₂. VERO cells grown in an extract free medium were used at control. Scratch wound closure was analyzed under the inverted microscope (Magnus INVI, Noida) equipped with a digital SLR camera, by acquiring digital images at different time 0 (T0), 24hr (T1), 48 hr. (static imaging). In the static imaging modality, the closure of the scratch was quantified, as recently described (Felice et al., 2015), by measuring the differences between the wound width at T0 and T1/T2, using the Image J processing software (<http://rsbweb.nih.gov/ij/>).

RESULT AND DISCUSSION

Extraction of Collagen

Since, Acid extraction of collagen can be easily applied in an industrial extraction of collagen.



Fig. 1: Albino Wistar rat tail tendons.

In this fig the Albino Wistar rat tail tendons were bought from Avanz Bio Private Limited Laboratory, Chennai to extract collagen. 20.90g of Albino Wistar rat tails was taken and was cleaned using a sharp knife



Fig. 2: Acid extraction of collagen (removing non-collagenous tissues).

In this fig the rat tail tendons were treated with NaCl to remove non-collagenous tissues, tendons become loose and turn into slightly white colored tendons.



Fig. 3: NaOH Treated tendons.

In this fig the tendons treated with NaOH to make homogenized paste.

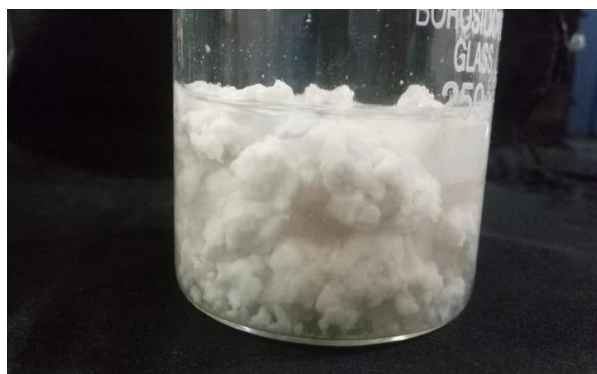


Fig. 4: Acetic acid solubilizing homogenized paste.

In this fig, to solubilizing the homogenized paste of tendons acetic acid was added.



Fig. 5: Precipitation of the collagen.

In these figures, the precipitated collagen was formed after adding NaCl.

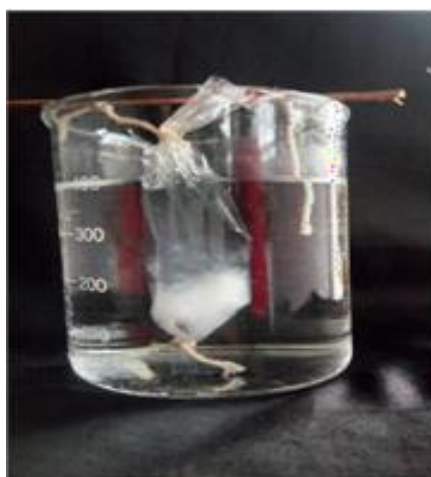


Fig. 6. Dialysed collagen



Fig. 7. Rat tail tendon collagen

This fig.6.represents, collagen in the dialysis membrane in the PBS solution. In fig.7. 12ml of rat tail tendon collagen was collected after dialysis done.

CHARACTERIZATION OF COLLAGEN SDS-PAGE ANALYSIS OF DIALYSED COLLAGEN

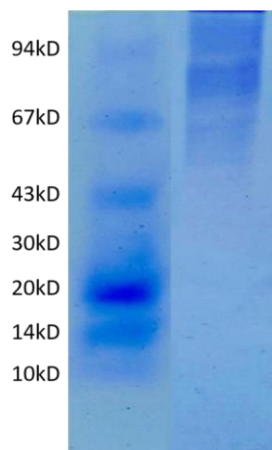


Fig. 8: SDS-PAGE analysis of dialysed collagen.

Albino Wistar rat tail tendon collagen was analysed using 7.5% resolving gel and the major constituents identified consisted of α chains ($\alpha 1$, $\alpha 2$) and β chains. The pattern identified were very much similar to the type I standard collagen. The type I collagen consists of two identical alpha chains and one beta chains. The calculated and identified pattern of collagen was similar to the protein ladder. Type I collagen is also isolated from the bones, skin, bovine, tendons of many animals. The tendon collagen of the most of the source contains type I collagen.

UV-VISIBLE SPECTROMETRY

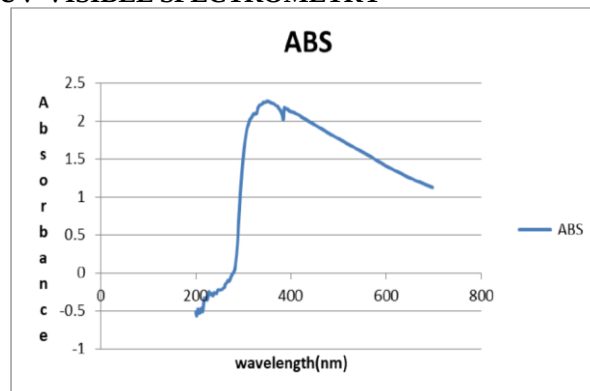
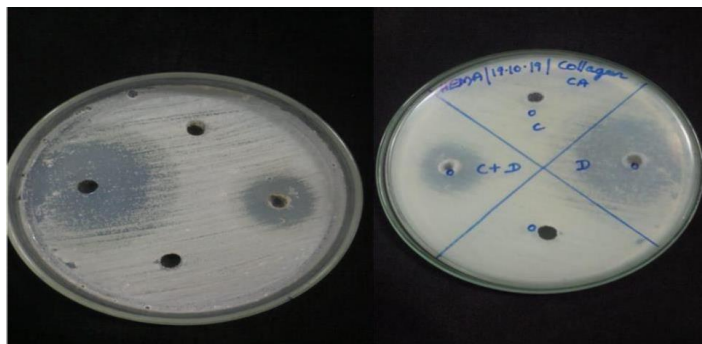


Fig. 9: UV-analysis of collagen.

From UV-V is the extracted rat tendon collagen, an absorbance near 336.0-2.222nm with high intensity was observed with no adsorption peak at 386nm. The result indicated high efficacy of non-collagenous protein removal. Collagen commonly has a low amount of tyrosine, which could absorb UV- light at 386 nm. The absorbance in this region is similar to those of collagen from channel catfish skin. Peptide bonds found in protein also absorb at 205-230 nm. The absorbance at 386 nm is mainly because of tryptophan, tyrosine and phenylalanine. Tryptophan was completely absent in the extracted collagen. Also, there was a negligible amount of tyrosine detected. Previous researchers indicated that collagen commonly have a low amount of tyrosine which can absorb UV-light at 386 nm.

ANTIFUNGAL ACTIVITY TEST

Candida albicans



Aspergillus Niger



Fig. 10: Antifungal activity. C- Rat collagen, C+D- collagen + drug, D-drug (Fluconazole), Empty well- control.

Table 1: Zone of inhibition.

S. No.	Test Organism	Collagen	Collagen + Drug	Drug
1.	<i>Candida albicans</i>	NIL	14	24
2.	<i>Aspergillus Niger</i>	NIL	7	10

Collagen exhibit no antifungal activity against *Candida albicans* and *Aspergillus Niger*.so organisms, *Candida albicans* and *Aspergillus Niger* showed the negative result. Collagen + drug exhibit activity against *candida albicans* by forming 14mm zone of inhibition and also showed activity against *Aspergillus Niger* by forming 7mm zone of inhibition and showed a positive result. Drug (fluconazole) exhibit the highest antifungal activity against *Candida albicans* by forming 24mm zone of inhibition and also exhibit the highest antifungal activity against *Aspergillus Niger* by forming 10mm zone of inhibition and showed a positive result.

INVITRO SCRATCH WOUND HEALING ACTIVITY

The wound healing efficiency of collagen was observed under the microscope and the percentage of wound healed was calculated.

It was observed that the wounds were healed to an efficiency of 45.83% for 24hrs and 92.5% for 48 hrs. Compared to the control 6.09% on 48 hrs. Thus, it was inferred that collagen has good healing efficiency.

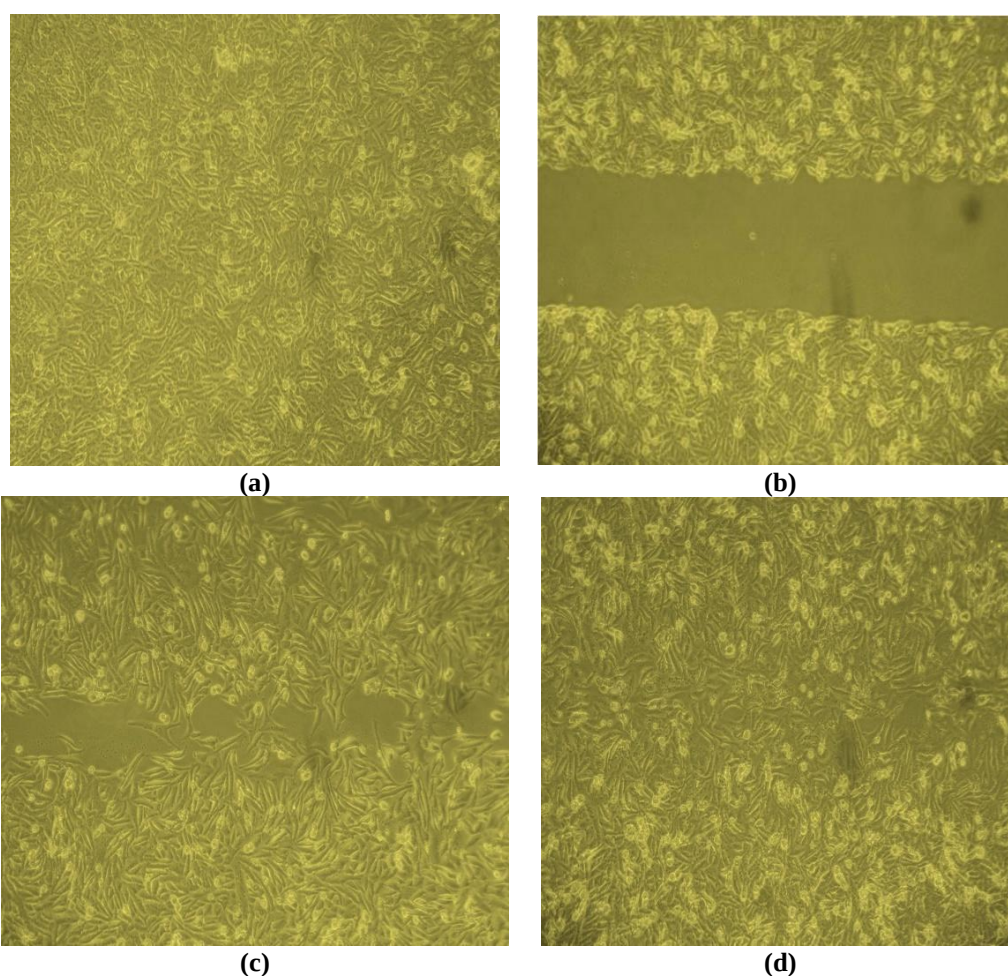


Fig. 11: Invitro scratch wound healing assay of collagen on VERO cell line. (a) Normal Cell, (b) wound cell, (c) collagen treated cells (24hr), (d) collagen treated cells (48 hrs.).

CONCLUSION

The overall objective of this study was extraction, characterization of collagen from Albino Wistar rat tail tendons (laboratory waste) and its applications that is to check antifungal activity of collagen incorporate with drug and wound healing activity. Rat tendon collagen showed broad result in antifungal and wound healing activity. Collagen is also extracted from tendons of

already used rats (waste materials) in laboratories; this is the reason for chosen source in the present study. This study confirmed that Albino Wistar rat tail tendons is respectively an alternative source of type I collagen. The collagen extracted from 20.90gm of rat tail tendon was 12ml. The extracted collagen showed α and β chains in SDS-PAGE. The Aim of this project was successfully achieved. When compared to Fish skin collagen, rat tail

collagen showed greatest result in antifungal and wound healing activity.

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