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ISOLATION AND CHARACTERIZATION OF COLLAGEN FROM THE PLACENTA OF BUFFALO (*BOVIDAE BUBALUS BUBALIS*) FOR THE BIOMATERIAL APPLICATIONS.

Viru Shah, Ashok Manekar*
Department of zoology, Institute of science, Mumbai 400 032, (M.S.), India.
(E-mail: ashok.manekar@rediffmail.com)

ABSTRACT
Collagen was isolated from the placenta of buffalo (*Bovidae bubalus bubalis*) by acid solublization with pepsin. The yield of placenta collagen was 35% on a wet weight basis. FTIR spectra analysis revealed that placenta collagen molecules had the compact triple helical structure stabilized mainly by the hydrogen bond. The placenta collagens were classified as type I collagen containing α1 chains and one α2 chains with no disulfide bond. The potential of the placenta collagen for human foreskin fibroblast (HFF) were cultured on placenta collagen and their growth was evaluated by MTT method, steady growth was observed on placenta collagen. HFF grown on placenta collagen were expressed CD44 as well as vimentin compared to tissue culture dishes was evaluated by immunoflurosance as well as by FACS analysis. In conclusion, *Bovidae bubalus bubalis* collagen could be used as a potential source for biomaterials.

KEYWORDS: Cells, Characterization, Isolation, MTT, Proliferation

INTRODUCTION

Collagen, one of the most abundant animals derived proteins and is the precursor of gelatin which is widely applied to commercial products. Collagen accounts for an about 30 % of the total protein of most organisms, and has 19 variants designated type I-XIX. It possesses a unique protein sequence and is able to form insoluble fibers with a high tensile strength (Gelse *et al*., 2003). Collagen has a wide range of applications in various branches of industry. Its pharmaceutical and biomedical uses include tissue engineering for implants in humans, inhibition of angiogenic disease, treatment of hypertension, urinary incontinence and osteoarthritis (Lee *et al.* , 2001). It is also very attractive substrate for fragrance and cosmetic applications (Tzaphlidou *et al*., 2004). Its denatured form (gelatin) has been widely used in the food industry (Kittiphattanabawon *et al*., 2005). All members of the collagen family are characterized by domains with repetitions of the proline-rich tripeptide Gly-X-Y, involved in the formation of trimetric collagen triple helix (Muyonga *et al*., 2004; Ramachandran *et al*., 1988).

The search for new collagen sources has resulted in studies of the functional properties vertebrates waste materials like placental collagens i.e. bovine's placental. Skins of salt and fresh water fish (Ikoma *et al.*, 2003), shark's skin (Yoshimura *et al.* , 2000), brownstripes red snapper skin (Jongjareonark *et. al*., 2005), skins and bones of bigeye snapper (Kittiphattanabawon *et al*., 2005), squid skin (Niecikowska *et al*., 1999), skins of young and adult. Nile perch (Muyonga *et al*., 2004) and outer skins of the paper nautilus (Nagai *et al*., 2002). These collagens are extracted from such byproduct as skin, bone and fin during processing of the above fishes. Asia is the native home for the water buffalo, with 95% of the world population of water buffalo, with about half of the total is in India. Many Asian countries depend on the water buffalo as its primary bovine species. It is valuable for its meat and milk as well as the labour it performs. As of 1992 the Asian population was estimated at 141 million. The water buffalo has promise as a major source of meat, even the milking ones. The water buffalo also is a classic example for work animal in Asia, an integral part of that continent's traditional village farming structure and also used for hauling cotton, pumping water in Pakistan and hauling logs in Turkey. The domesticated water buffalo is often referred to as "the living tractor of the East" as it is relied upon for plowing and transportation in many parts of Asia.

With the rapid development of dairy industries, huge quantities of by-product have been discarded which may cause pollution and emit offensive odours. Hence, the comprehensive utilization of these by-products, especially production of value-added products, is a promising means to increase revenue for the producers and accelerate the development of dairy industry. Bovine source is an important source for collagen, which can be used as a replacement for marine sources. Placentas are bigger in size and also large quantity of availability; it is therefore a potential source of collagen. The main objective of this study was to isolate and characterize collagen from the placenta of *Bubalus bubalis*, to show biomaterials application.

MATERIALS AND METHODS

Chemicals

Pepsin obtained from Himedia, β-mercaptoethanol from Merck, protein marker and Type I collagen from bovine were from sigma Chemical Co., tris were obtained from Merck.

Sample

Placenta of Indian buffalo (*Bovidae bubalus bubalis*) was collected from local dairy industries in and around Mumbai city, India. The placenta sample was packed in polythene bag and transported on ice to the Institute of Science, Mumbai, INDIA. Placenta was cleaned manually with cold water and then chopped into small pieces and treated with 0.5% EDTA (Sigma) to remove all the blood and was stored at -20°C, until further processing for collagen extraction. The storage time was less than 3 months.

Isolation of collagen from Placenta

Frozen placenta pieces were thawed using running water prior to processing, cut into small pieces (0.5 x 0.5 cm^2), and their wet weight was determined. The weighed samples were then soaked in five volumes of 0.5 M acetic acid (Merck) for 48 h at 4°C to extract acid soluble proteins (including collagen). Subsequently, the placental tissues were ground to a homogeneous paste in a grinder at 7 °C and the paste was resuspended in 0.5 M acetic acid at a ratio 1:10 (w/v) and digested with 2.0% (w/w) pepsin for 48 h at 4° C. The pepsin digested collagen was then centrifuged at 10,000 rpm for 30 min.

Collagen precipitation

The collagen solution from section 2.3 was precipitated according to the method of Kittiphattanabawon et al., with slight modification. Briefly, the solution was precipitated by adding sodium chloride to a final concentration of 1.5 M, and the resultant precipitate was centrifuged at 10,000 rpm for 30 min. The resultant residue was dissolved in 0.5 m acetic acid, and dialyzed against 0.1 M Na_2HPO_4 to remove salt and low molecular peptides, overnight at 4° C with continuous stirring.

Estimation of collagen concentration (Sircoll assay)

The dialyzed sample of section 2.4 was employed for estimation of soluble collagen using the Biocolor soluble collagen assay (Biocolor Ltd. Belfast, UK). The assay employs Sirius Red, an anionic dye with sulfonic acid side chain groups that react with the basic amino acids constituting collagen. The dye reagent binds specifically to the $[Gly\text{-}X\text{-}Y]_n$ helical structure found in all collagens. Briefly, Sircoll reagent was added to the dialyzed sample and gently agitated for 30 min to allow collagen-dye complex to form. The samples were then centrifuged at 10,000 rpm for 5 min. The collagen-dye complex precipitate was collected and resolubilized in 0.5 M sodium hydroxide. The dye concentration was determined by spectrometer at 540 nm. Concentration was derived from a standard curve obtained by using known concentrations of soluble collagen.

SDS-PAGE

SDS-PAGE was performed by the method of Laemmli (1970). Collagen samples (500-1000µg) along with positive control (Bovine collagen from calf skin, Sigma Chemical Company, MO, USA) and high molecular weight markers were subjected to 7% SDS-PAGE. Extracted prawn collagen and positive control bovine collagen type I were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris-HCL, pH 6.8, containing 4% SDS and 20% glycerol) in the presence of 10% β-mercaptoethanol (β-ME). The mixture were boiled in boiling water for 2 min. Samples (20 µg protein) were loaded onto polyacrylamide gels comprising 7% resolving gel and 4 % stacking gel and subjected to electrophoresis at a constant current of 20 mA/gel, using a Mini Protein II unit (Bio-Rad Laboratories, USA). After electrophoresis, gel was stained with 0.05 %(w/v) Coomassie Blue R-250 in 15% (v/v) methanol and 5 % (v/v) acetic acid, followed by destaining in acid alcohol at room temperature till bands were clearly visible.

Collagen-coating of tissue culture dishes

Collagen extracted from placenta as mentioned in section 2.4 was diluted by dissolving in 0.2% acetic acid to get final concentration of 2mg/ml for 1h, filtered through 0.45 micron cellulose acetate filter (Millipore, USA)using a syringe. The filtrate was stored as stock solution at 4ºC, until further use. The stock solution was diluted 1:5 with tissue culture grade water and 2 ml of was dispensed in 35 mm Petri dish (Nunc, Denmark). The solution along with Petri dish was kept under UV light overnight. The collagen solution was aspirated from the dishes and the dishes were allowed to air-dry. The dishes were washed with 1X sterile Dulbecco's phosphate buffered saline. The dishes were allowed to dry and stored at 4°C, until further use.

HFF Cells proliferation and cytotoxicity assay

Proliferation and cytotoxicity assays on collagen coated dishes and tissue culture dish was conducted on human foreskin fibroblast, cytotoxicity was assessed by the methyl thiazolyl tetrazolium (MTT, Sigma) assay (Molinari *et al.* 2003). Human fibroblasts were propagated in Dulbecco's modified Eagle Culture Medium (Gibco BRL). Supplemented with 10%fetal calf serum (Gibco BRL). 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco BRL), 0.25 µg/ml

fungi zone (Gibco BRL). Total 3000 cells were seeded in each 30 mm culture dish (Nunc). Dish kept at 37ºC in a 95% air and 5% CO_2 atmosphere. Briefly, MTT (0.5mg/mL) was added to the human fibroblasts 24, 48 and 72h after seeding the cells in both collagen-coated and normal tissue culture dishes, and assessed for proliferation and cytotoxicity. Viable cells were indirectly determined by their ability to convert soluble MTT to insoluble formazan crystals. The crystals were dissolved in DMSO and the absorbance was determined at 570nm on colorimeter (Remi, India). Each experiment was performed in triplicates.

Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectra were obtained from 2mg collagen in approximately 100 mg potassium bromide (KBr). All spectra were obtained from 4000 to 1000 cm at a data acquisition rate of 2 cm employing a FT-IR spectrophotometer (Perkin Elmer, USA)

Fluorescence microscopy

HFF cells were allowed to grow on collagen coated tissue culture dish till 70% confluences was considered to be enough for subjecting cells to primary antibody after fixation with 4% para-formaline. HFF grown on collagen coated tissue culture dish subjected to challenge with primary antibody anti-vimentin at 4ºC for overnight. After incubation overnight, the cells were washed with PBS to remove unbound antibody. The cells were then incubated with FITC-labeled anti-mouse secondary antibody at 37°C for a one hour, and washed with PBS thrice to remove unbound secondary antibody. Subsequently, cells were treated with Pe-tagged anti CD44 antibody, at 37° C for a one hour, and washed thrice with PBS to remove unbound antibody to check expression of the respective marker and then counter stained with DAPI.

Detection of collagen Type I, KGF by RT-PCR analysis

RNA was isolated by lysing the cells with TRIzol reagent (Invitrogen SRL., Italy). The RNA was treated immediately with DNAse I (Invitrogen) and the integrity of the treated RNA was examined by detection of ribosomal RNA bands (28S & 18S) in ethidium bromide stained agarose gels. The RNA was quantified by reading the optical density. One to two micrograms of total RNA were transcribed for 1h at 37°C .Reverse transcription (RT) was performed in final volume of 20µl containing 50mM tris-HCl (pH8.3 at 25 C), 3mM $MgCl_2$,75mM KCl, 10 mM DTT, 0.5 mM each deoxynucleotide triphosphate (dNTP), 40 units/tube RNase OUT(Invitrogen), 1µg of random hexanucleotide primers, and 2µl of RT buffer. Each tube contains 7.5µl of PCR master mix, 2µl of specific primers Collagen Type I (F-ATGCCTGGTGAACGTGGT, R- AGGAGAGCCATCAGCACCT), KGF (F- GATACTGACATGGATCCTGCC, R- CACAATTCCAACTGCCACTG) as well as Human glyceraldehyde phosphate dehydrogenase GPADH (F-GGGCTGCTTTTAACTCTGGT, R- TGGCAGGTTTTTCTAGACGG), 4µl of water, and 1.5µl of cDNA. Denaturizing was done at 94°C; the annealing temperature was 55°C, while the extension was conducted at 72°C, for 35 cycles.

Immunophenotyping

The HFF cell grown on tissue culture dish and the collagen-coated dish were characterized by immunophenotyping analysis. The cells (50000 cells) were stained with vimentin and CD44 antibody. After trypsinization, cells from both dishes were fixed with the 10% paraformaldehyde for one hour. To permeabilize HFF cells used Triton-X 100 for 10 min at room temperature. Permeabilized cells were washed with phosphate buffer saline at pH 7.3. Cells were stained with anti-vimentin and Pe-tagged anti-CD44 antibody for one hour at room temperature. Unbound antibodies were removed, and cells washed with phosphate buffer saline pH 7.3. In case of anti-vimentin primary antibody, FITC-labeled anti-mouse secondary antibody was employed (anti-Vimentin antibodies from Santacruz Biotechnology, USA and all rest antibodies were from BD pharmingens, CA, USA). The stained cells were acquired on FACScalibur and analyzed using CellQuest software (BD BioSciences, CA, USA).

RESULTS AND DISCUSSION

Isolation of collagen

Collagen was extracted from *Bovidae bubalus bubalis* placenta employing standard extraction procedures as provided in section 2.2 initially placental tissue did not dissolve in acetic acid however the dissolution was complete after digestion with pepsin. The peptic digest was then centrifuged at 10000 rpm for 20 min to remove all debris. The resultant supernatant was precipitated with sodium chloride, final sodium chloride made to 5M. The precipitate was centrifuged at 10000 rpm for 10 min, and the resultant collagen pellet dissolved in 0.005 M acetic acid, and dialyzed for 18 hrs to get the neutral collagen. The yield of collagen was about 35% wet weight of the initial sample weight.

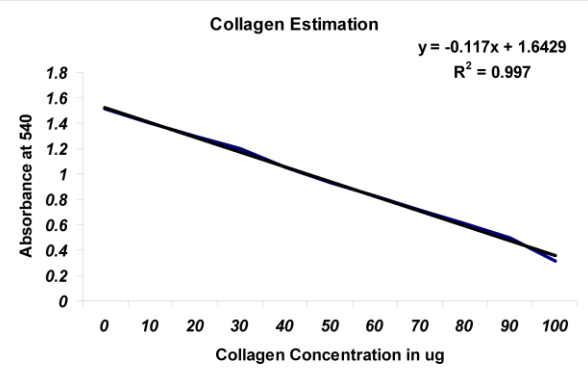

Figure 1: Collagen estimation by the sircoll assay, Concentration was derived from a standard curve obtained by using known concentrations of soluble collagen (R^2 = 0.997).

Determination of collagen

Extracted collagen was subjected to treatment with Sircoll dye for the determination of total collagen. Here we used the indirect method for the estimation of total collagen (Figure 1). We analyzed extracted *Bubalus bubalis* placental collagen to be6.2 mg/ml concentration, which is around 35% of total wet weight.

SDS PAGE

Electrophoresis pattern of *Bubalus bubalis* placental collagen by SDS-PAGE. Electrophoresis patterns of calf skin collagen and *Bubalus bubalis* placental collagen were measured by SDS-PAGE and are compared. Calf skin and *Bubalus bubalis* placental collagens are mostly comprised of two α chain (2:1 ratio of α1 and α2). Data not shown.

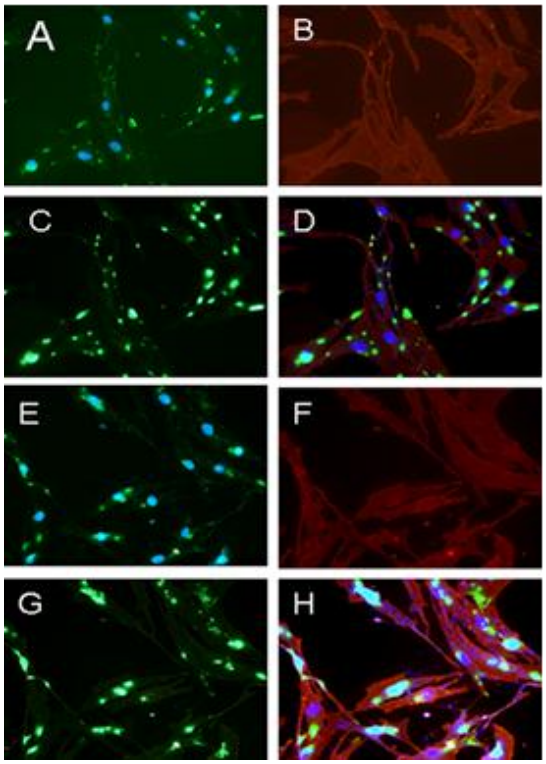

Figure 2: Fluorescence microscopy of HFF grown on collagen coated dish, (A) HFF grown on tissue culture dish stained with DAPI, (B) HFF grown on tissue culture dish stained with CD 44 antibody, (C) HFF grown on tissue culture dish stained with vimentin antibody, (D) HFF grown on tissue culture dish stained with CD44, DAPI as well as vimentin antibody, (E) HFF grown on collagen coated dish stained with DAPI, (F) HFF grown on collagen coated dish stained with CD44 antibody, (G) HFF grown on collagen coated dish stained with vimentin antibody, (H) HFF grown on collagen coated dish stained with CD44, DAPI as well as vimentin antibody.

Fluorescence microscopy

Fluorescence microscopic analysis was done to check for protein expression in the HFF cells grown on tissue cultured dish and the collagen coated plate. No significant differences were observed in the protein expression pattern of the HFF cells grown on tissue cultured dish and the collagen coated plate (Figure 2). This was indicative of the fact that there is no adverse effect of collagen on the expression of vimentin somatic cell marker as well as marker for the extra cellular matrix CD44. This is imperative for tissue engineering and drug delivery applications, as well as, for the treatment of the burns, and diabetic ulcers.

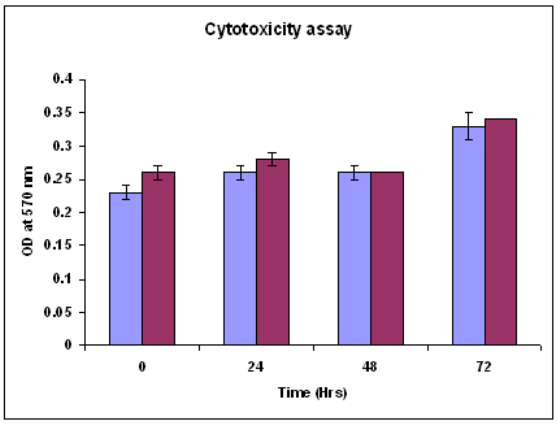

Figure 3: Cytotoxicity analyses with the help of HFF grown on collagen coated dish were compare with the HFF grown on tissue culture dish by MTT method.

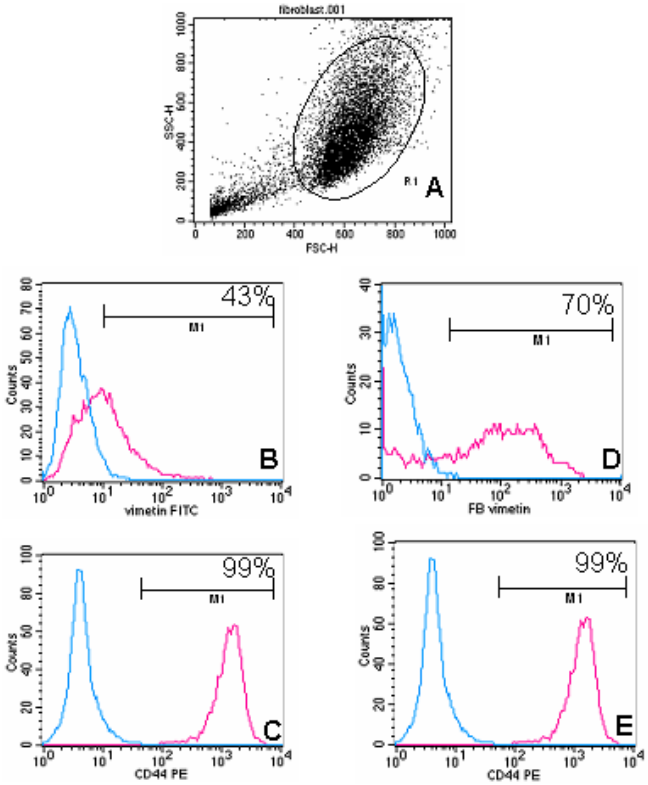

Figure 4: FACS analysis. (A) Dot plot, (B) HFF grown on tissue culture dish analyzed for the vimentin expression (43%), (C) HFF grown on tissue culture dish analyzed for the CD 44 expression (99%), (D) HFF grown on collagen coated dish analyzed for the vimentin expression (70%), (E) HFF grown on collagen coated dish analyzed for the CD 44 expression (99 %).

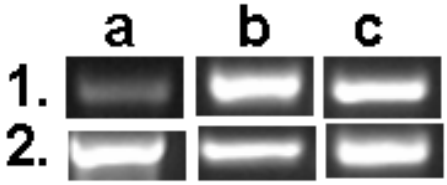

Figure 5: Molecular analysis, (1) Expression of HFF grown on tissue culture dish, (2) Expression of HFF grown on collagen coated dish, (a) GAPDH, (b) Collagen type I, (c) KGF expression respectively.

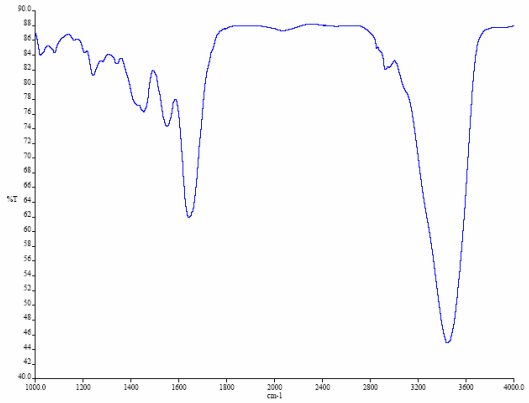

Figure 6. Fourier transform infrared spectrum of *bovidae bubalus bubalis* collagen.

HFF cells proliferation and cytotoxicity assay

Cell proliferation assays were conducted, and the results were analyzed and compared after 24, 48 and 72h of seeding HFF cells on collagen coated dish and tissue culture dish. The results of cell proliferation assay conducted employing HFF cells are provided in (Figure 3) a gradual increase in optical density is observed with passage of time, which indicates proliferation of HFF cells. Interestingly, there was a difference in proliferation rate of HFF cells grown for test as well as control. This suggestively indicates that collagen extracted and purified employing the method described is not cytotoxic and also supports growth and proliferation of HFF cells.

FACS analysis

On FACS analysis we observed that there is no difference in the expression of protein for both vimentin marker (70.0%) and CD44 (99.0%) marker of the HFF grown on tissue culture dish as well as collagen coated dish. Both the marker is essential for the extra cellular matrix expressing marker (Figure 4). This is very essential for the any tissue engineering application.

Molecular analysis

HFF cells grown on the tissue culture dish and collagen coated dish were analyzed for the expression of molecular markers like Collagen Type I and Keratinocyte growth factor (KGF). No significant differences were observed in the expression of Collagen Type I and KGF for both the HFF cells grown on tissue culture dish as well as the HFF cells grown on the collagen coated dish (Figure 5). This suggests that the collagen did not affect the growth of HFF cells and the associated expression pattern of the molecular markers for the Collagen Type I and KGF which are most essential proteins for tissue engineering application.

FT-IR analysis

FT-IR spectra of prawn collagen are shown in (Figure 6). The regions of amide I, II, III are known to be directly related with the shape of a polypeptide. Amide A band (3400-3440 cm^{-1}) is related to N-H stretching vibrations. Amide I band (1600-1660 cm^{-1}) is associated with stretching vibration of carbonyl groups in peptides, being the most important factor in investigating the secondary structure of a protein. Amide II (~1550 cm^{-1}) is associated with NH bending and CN stretching. Amide III (1320-1220 cm^{-1}) is related to CN stretching and NH, and is involved with the triple helical structure of collagen (Jakobsen, Brown, hutson, Fink, and Veis, 1983; Muyonga *et al.*, 2004; Surewicz and Mantsch, 1988). In studies of Nile perch skin collagen by Muyonga *et al.*, 2004, amide regions (A, I, II and III) were 3434, 1650, 1542 and 1235 cm^{-1}, respectively, and amide regions of adult Nile perch skin collagen were 3458, 1654, 1555 and 1238 cm^{-1}, respectively.

While the region of amides A, I, II and III for *Bubalus bubalis* placental collagens were 3445 cm^{-1} (amide A); 1642 cm^{-1} (amide I); 1548 cm^{-1} (amide II); and 1240 cm^{-1} (amide III), respectively. The amide I bands are originated from C=O stretching vibrations coupled to N-H bending vibrations, CN stretch and CCN deformation (Bandekar, 1992).

CONCLUSIONS

This study investigated optimum extraction conditions for manufacturing *Bovidae bubalus bubalis* collagen and characterized chemical properties of the extracted collagen by SDS-PAGE, FT-IR, molecular analysis, FACS analysis, cytotoxicity analysis. SDS-PAGE and FT-IR are very much comparable with the commercially available calf skin collagen. Placenta of *bovidae bubalus bubalis* is the dairy waste, and can be used to produce large scale of collagen for the commercial purpose. In conclusion it may be said that *bovidae bubalus bubalis* collagen is a potential biomaterial for the tissue engineering application.

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