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Research Article

EXTRACTION OF COLLAGEN FROM CAT FISH (*TACHYSURUS MACULATUS*) BY PEPSIN DIGESTION AND PREPARATION AND CHARACTERIZATION OF COLLAGEN CHITOSAN SHEET

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ABSTRACT

Collagen was extracted by pepsin digestion from the swim bladder of catfish (*Tachysurus maculatus*) processing wastes. The total collagen yield extracted was 40% on the basis of lyophilized dry weight. According to the electrophoretic pattern, the swim bladder of the fish consisted of comparable amounts of two ά chain-sized components designated as ά $_{1,}$ ά $_{2}$ and the β. Collagen was cross linked with chitosan. The formed collagen- chitosan sheet was characterized and showed that there is a possibility use in medical field.

Keywords: Catfish, Chitosan, Collagen, Processing waste

INTRODUCTION

Collagen is the most abundant animal protein polymer. About 30% of the total protein in animal body is collagen. Collagen is the major structural protein in the connective tissue of animal skin and bone[1] in its purified form collagen is a bio material. Generally collagen has a wide range of application in cosmetic, biomedical, pharmaceutical, leather and film industries[2]. Soluble collagen has a wide range of applications in various fields due to its special characteristics including biodegradability and weak antigenicity[3]. The physical and chemical properties of fish collagen are different from those of mammalian collagen[4]. The collagen from the fish offals is unlikely to be associated with infections such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE) and foot and mouth disease (FMD). Therefore fish offals also may be an effective alternative source for collagen production. Pepsin has been reported to cleave peptides in the telopeptide region and the extraction of collagen cleaved by pepsin rendered a higher yield[5,6].

The systematic packaging of the triple helices lends strength and resistance to the collagen fibers. Natural cross linking gives high tensile strength and proteolytic resistance to collagen. The recently revived cross linking agent is a bifunctional glutaraldehyde[7]. Through specific aggregation and cross linking collagen can form fibers of unusual strength and stability hence can be used in medical devices. The aim of the study was to extract collagen from catfish (*Tachysurus maculatus*) and to prepare and characterize collagen-chitosan sheet.

MATERIALS AND METHODS

Fish swim bladder preparation

Marine cat fish (*Tachysurus maculatus*) of Bay of Bengal was collected from Marina Beach, Chennai, Tamilnadu India. The fish was sacrificed and the air bladder was removed cleaned mechanically for free of blood vessels and other adhering tissues and washed thoroughly with cold distilled water. The air bladder was cut into small pieces and was stored at -20^{o}C until used. Pepsin (1: 10,000 activities) powder was purchased from Sigma Chemical Co, USA). All other reagents used were of analytical grade.

Extraction of air bladder collagen

The collagen was cut into small pieces and the collagen was extracted according to the method of Kittiphattanabawon, Benjakul with some modification[8]. All process was performed at 4^{o} C with continuous stirring. Air bladder was soaked in 0.1 M NaOH with a sample/solution ratio of 1:30 (w/v) for 48 to 72 hrs, with a change of solution every 4 hrs to remove non collagenous protein. The sample was then washed with cold distilled water until the pH of the wash water became neutral or faintly basic.

The samples were suspended in 0.5 M acetic acid with a sample/ solution ratio 1:30 (w/v) containing 1% (w/w) pepsin (1:10, 000 activity) for 24 h with continuous stirring. The mixture was centrifuged at 9000 g for 30 min. The pepsin soluble collagen (PSC) was salted out by NaCl and exhaustive dialysis against disodium hydrogen phosphate (0.2M) until the precipitation was complete. The precipitated collagen was dissolved in acetic acid (0.5M) and precipitated again with NaCl (5%w/v). This was again collected by centrifugation and dissolved in acetic acid (0.5M). Further purification was done by exhaustive dialysis against disodium hydrogen phosphate (0.02M) until precipitation was complete. Finally the precipitated collagen was dissolved in acetic acid (0.5M) and dialysed against acetic acid (0.5M) and was lyophilized.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS –PAGE)

SDS-PAGE was performed by the method of Laemmli using the discontinous Tris- HCl/glycine buffer system with 7.5 % resolving gel with 4% stacking gel[9]. The collagen sample was dissolved in sample buffer (0.5M Tris HCl, pH 6.8, containing 2%SDS, 25% glycerol) with 10% β ME, to reach the final collagen concentration of 1mg/ml, and then boiled for 3 min.The gel was stained for 30 min with 0.25 % Coomasie Brilliant Blue R- 250 solution and destained with 7.5% acetic acid/5% methanol solution until the bands were clear.

UV Spectrum

The UV spetra of the collagen obtained by the enzyme solubilisation methods were measured using UV spectra photometer Deepvision 3010, India.

Preparation of collagen-chitosan sheet

1% collagen solution in 0.5M acetic acid was mixed with same concentration of chitosan in 0.5M acetic acid. The mixture was stirred well over a magnetic stirrer to ensure uniform mixing of the protein- polysaccharide. This mixture was transferred to a tray (23×18 cm) and frozen at -20^{0} C for about 3hrs and then lyophilized in a Virtis lyophilizer.

Crosslinking of collagen-chitosan sheet

The raw chitosan powder was Crosslinked using 2% (m/v) glutaraldehyde solution by fumigating[10]. The samples were placed on glutaraldelyde containing dish covered with nylon mesh and then exposed to glutaraldehyde vapour for about 3hrs at room temperature (33-35^{0} C).

Testing of mechanical properties

The uncross linked and cross linked specimen of 5cm length and 10cm width were cut out and each thickness was 0.75-0.97mm and 0.84-0.87mm. The mechanical properties such as tensile strength, 1 % strain at maximum load were measured using an instron tensile testing system at an extension rate of 5mm/min.

Thermo gravimetric analysis (TGA)

The thermal decomposition pattern of both control and glutaraldelyde cross linked collogen-chitosan sheets were monitored using a Gener V_4 IC DU PONT 2000 in nitrogen atmosphere at a heating rate of 10˚C /min[11].

Water absorbtion capacity

The know weight small pieces of samples were swollen in distilled water at room temperature and the weight of glutaraldehyde crosslinked collagen-chitosan matrix and uncross linked matrix was determined by first blotting the samples with filter paper and weighting immediately on an electronic balance. The weights of the swollen samples were recorded for 15min, 30min, and 1, 2, 3, 4 and after 24hrs.

Water absorption was calculated using the formula

$$E_s = \frac{W_s - W_o}{W_o} \times 100$$

W_s= weight of the moist sample at given time

W_o= the initial weight of the sample

E_s= percentage of swelling at the given time.

RESULT AND DISCUSSION

Isolation of catfish air bladder collagen

The Catfish air bladder was not completely soluble in 0.5 M acetic acid, but after adding pepsin, the bladder pieces were completely soluble, forming a viscous solution. The yield of the isolated Collagen after salt precipitation at natural pH was 35% on the basis of lyophilized dry weight1. This shows pepsin improve efficiency of Collagen extraction. Similar results was found in Paper nautilus (50%) and ocellate puffer (44.7%)[12] (Nagai et al., 2002). Generally the yield in acid soluble collagen (Asc) is different from that of pepsin soluble collagen (Psc)[13]. The different yield of Asc and Psc might suggest that there were many interchain crosslinks at the telopeptide region of the collagen which resulted in low solubility in acid. Pepsin cleave the cross linked regions at the telopeptide without damaging the integrity of the triple helix and increases the solubility of collagen in acid.

SDS-PAGE patterns of collagen

Fig-l shows SDS-PAGE Patterns of Collagen from cat fish had eletrophoretic pattern of Type 1 Collagen Consisting of α chains ($\alpha_1$and α_2) and their dimer β chain. These patterns were similar to those of pepsin soluble Collagen from the skin of Grass carp[14] (Yan, Wentao, Guoying, Bi, Yuqing M, Xiaohua, 2007) and channel cat fish[1]. It could not be concluded that whether collagen contains α_3 chain as α_3 chain could not be separated under the electrophoretic conditions employed because it showed similarity in chemical nature to α_1 and migrated electrophoretically to the same position as α_1[15, 16].

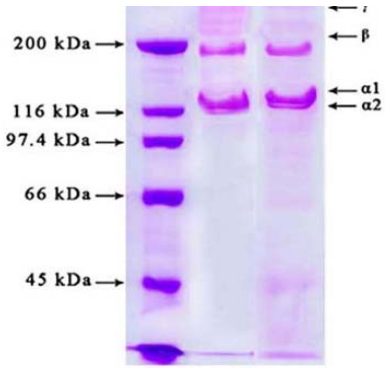

Fig. 1: SDS-PAGE pattern of collagen from the swim bladder of catfish (*Tachysurus maculates*) Lane 1: Protein marker; Lane 2: Acid – Soluble collagen; Lane 3: Pepsin – Soluble collagen

UV spectrum

The lower level of absorbance for the enzyme treated sample than the acid treated sample indicates the reduction in the level of tyrosine residue (Fig.2a & Fig. 2b). The composition of aromatic residues is very small in type I collagen therefore measurements were performed at 230nm assigned to the peptide bond[17]. It means that the tyrosine rich non helical regions responsible for immunogenecity of enzymatically cleaned off evenly helical molecule impact.

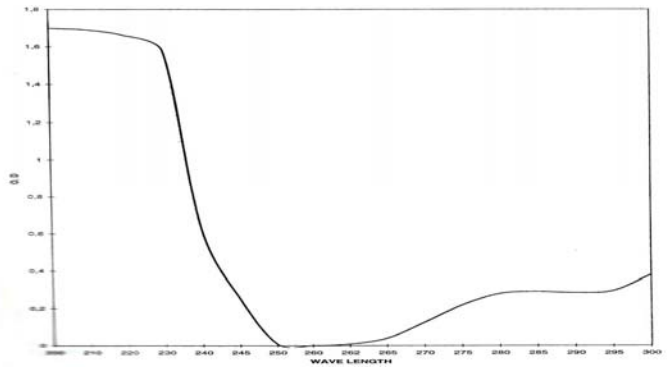

Fig. 2a: UV spectrum of Acid – Soluble *Tachysurus maculates* collagen

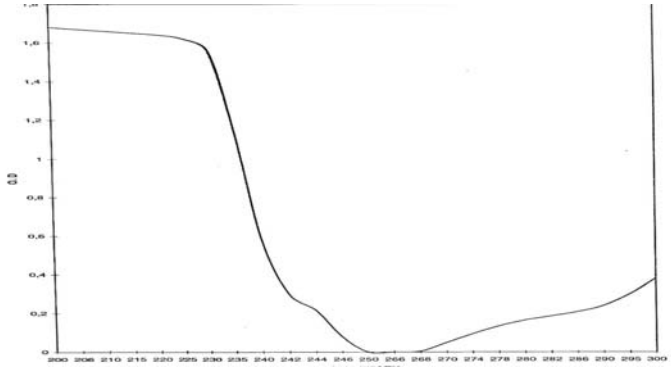

Fig. 2b: UV spectrum of Acid – Soluble *Tachysurus maculates* collagen

Collagen-chitosan sheet and cross linking

The free amino group of the lysyl residues at ε position of fish collagen combines with free amino group at c-2 position in each of monomeric N-acetyl glucosomine of chitosan. These abundantly available free amino groups of these macromolecules would have facilitated the glutaraldehyde cross linking[18] react primariy with aminogroup providing more sustained long term durability[19].Glutaraldehyde reacts primarily with amino group providing more sustained long term durability[20]. The presence of bifunctional groups (-CHO) in glutaraldehyde forms bridge between the amino groups of macromolecules possessing -NH_2 shiff base (-CH=N-). Chitosan (poly β-(1→4) N acetyl D-glucosamine has become a promising alternative treatment due to its natural character, antifungal activity and elicitation of defense responses in plant tissues[21].

Mechanical properties

Stress-strain curve of uncross linked matrix was 0.9m pa where as the crosslinked matrix exhibited a higher level of tensile strength (0.17mpa) governing the theory of rubber like elasticity[22] (Fig. 3a & Fig.3b) (Table-1).

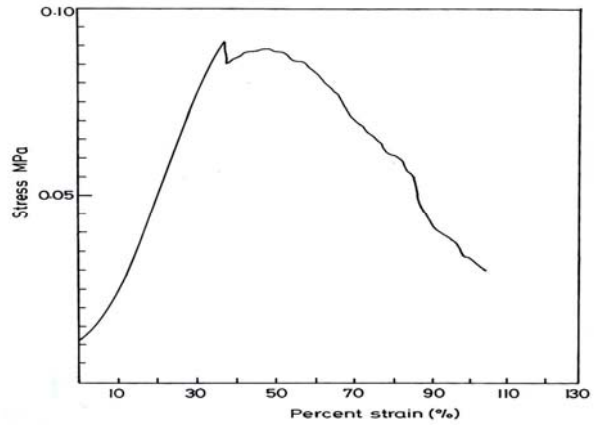

Fig. 3a: Stress-strain curve uncross linked collagen – Chitosan matrix

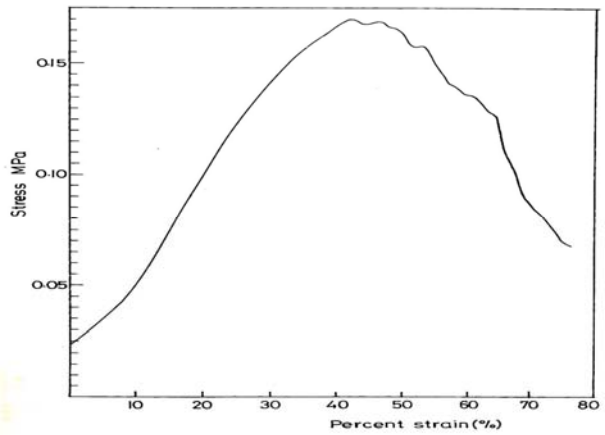

Fig. 3b: Stress-strain curve cross linked collagen – Chitosan matrix

Table 1: Mechanical properties of collagen – chitosan matrix

Collagen-Chitosan Matrix	Load at max load (N)	Stress at max load (N/mm^2)	% strain at max load (%)
Uncrosslinked	0.8760 ± 0.0184	0.1064 ± 0.0060	53.68 ± 1.95
Crosslinked	1.4610 ± 0.1838	0.1712 ± 0.0258	44.63 ± 3.75

Thermo gravimetric analysis

The weight loss upto100 ◦ C was 12% with decomposition starts at 209 ◦ C uncrosslinked where as for the crosslinked matrix the weight loss was only 6% and the decomposition starts at 240 ◦ C. On the increase in temperature the weight loss was 9% in uncrosslinked and 6% in crosslinked . The second phase decomposition takes place between 209 ◦ C -332 ◦ C and 240 ◦ C - 340 ◦ C in controlled and experimental samples with weight loss of 35% and 33% the percentage of weight loss in the cross linked is less by 2% (Fig. 4a & Fig. 4b). The over all weight loss was 67% and 64% in controlled and experimental samples showing the thermal stability of glutaraldehyde cross linked biological molecules indicating the cross link lead to thermo stability[23-26].

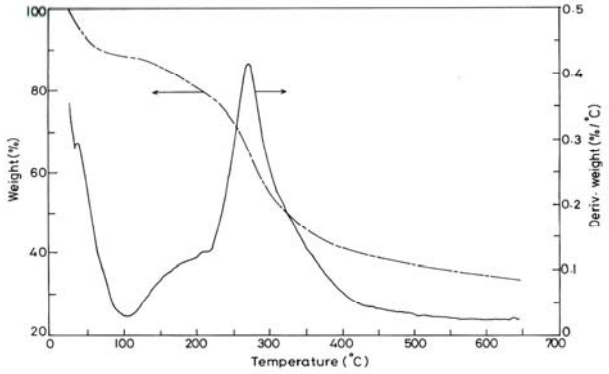

Fig 4a: Thermal decomposition pattern of uncross linked collagen – Chitosan matrix

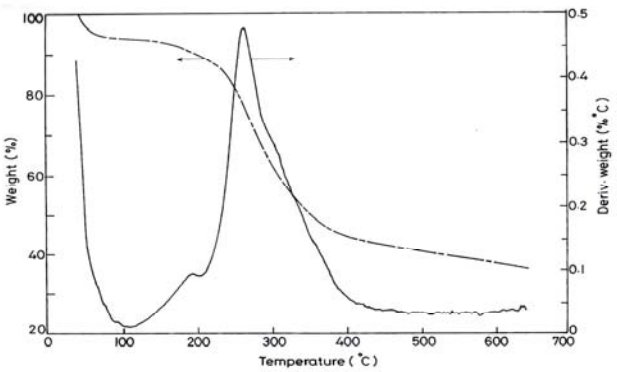

Fig. 4b: Thermal decomposition pattern of cross linked collagen – Chitosan matrix

Water absorption capacity

The water absorption Capacity of the plain and Cross linked matrix are shown in the table (Table-2). In the uncrosslinked matrix the hydrophilic groups are remain open and hence absorb the water molecules to a large extent. The uncrosslinked virtually dissolved after 30min in water where as cross linked matrix slowly absorbs the water and was stable up to 4hrs. Initially less water was observed in the cross linked matrix due to the blocking of hydrophilic groups by the cross linker the glutaraldehyde[27] once the matrix was swollen the porosity of glutaraldehyde commend have increased and the accessibility of water molecules to the hydrophilic functional (OH) groups would have also increased. After 4hrs water absorption attains the equilibrium stage. This properly helps in absorbing the wound exudate and there by keeping the wound clean. Thus cross linked collagen matrix can be used as a dressing material for severe burns, wounds such as pressure sores, donor sites, legal ulcers and decubitus ulcers[28-29]. Major benefits of collagen covers include their ability to easily absorb large quantities of tissue exudates, smooth adherence to the wound bed with preservation of this moist micro climate as well as its shielding against mechanical harm and prevention of secondary bacterial infection[30].

Table 2: Water absorption capacity

Sample	Percentage swelling time					
	Minutes		Hours			
	15	30	1	2	3	4
Uncrosslinked Matrix	3792.82	3883.33	2583.19	2021.01	-	-
Crosslinked Matrix	1619.51	2431.03	3354.51	3552.38	3561.77	3572.78

CONCLUSION

Collagen fibers tend to swell markedly when immersed in acid and they can be crosslinked through covalent bonds in the presence of glutaraldehyde. Additionally collagen fibres are more resistant to degradation to proteolytic enzymes than most other proteins. These characteristics are utilized in the fabrication of medical device out of collagen. The cat fish swim bladder used in the preparation of the matrix was found to be type I and contain lower amount of tyrosine that reflects the attachment of telopeptide responsible for antigenecity. Similarly chitosan too has most of there properties in addition to its biological inertness. Solubilisation of collagen by pepsin treatment results in the removal of telopeptide (non-helical region). A matrix was prepared by combination of collagen devoid of telopeptide and chitosan and then crosslinked using glutaraldyde. The cross linked matrix showed a higher tensile strength, relatively inelastic, higher thermal stability, a gradual increased water absorption capacity with respect to time. Therefore this material could be considered for wound application as a wound healing matrix/cover.

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