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COLLAGEN EXTRACTION OPTIMIZATION FROM THE SKIN OF THE *DASYATIS PASTINACA*

K. Sundaravalli[1], K. Revathi[2]*, Adithya R. Pillai[1] and A. Anitha[3]

[1,3]Phd Scholars, Meenakshi Academy of Higher Education and Research, Chennai.

[2]Research Director, Meenakshi Academy of Higher Education and Research, Chennai.

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***Corresponding Author**
Dr. K. Revathi
Research Director,
Meenakshi Academy of Higher Education and Research, Chennai.

ABSTRACT

By using the skin of sting ray *Dasyatis pastinaca* which is thrown as waste by the consumer, processed as a raw material, a macromolecule biomaterial of collagen was extracted. Acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) were successfully isolated from the skin of string ray *Dasyatis pastinaca* through extraction methods. The yields of ASC and PSC based on the wet weight of skin were 8.2% and 17.5% (wet weight basis), respectively. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed that both the ASC and PSC were Type I collagen and maintained a complete triple helix structure. These results indicated that both ASC and PSC of skin of sting ray *Dasyatis pastinaca* possessed good biological activity and could be widely used in medical biomaterials and other fields such as cosmetics, tissue engineering, tissue grafting.

KEYWORDS: *Dasyatis pastinaca,* Acid-soluble collagen (ASC), pepsin-soluble collagen (PSC), medical biomaterials.

INTRODUCTION

Collagen is involved in the formation of connective tissues which is a general extracellular structural protein. Collagen occurs in genetically distinct forms identified as type I to type XIX. They vary in amino acid composition and structure. Utilization of collagen in industries is wide enough. The sources of collagen for industries are limited to those from pigskins and bovine hides and bones. Collagen is used in different fields, such as leather, cosmetics, biomedical and pharmaceutical industries, and in food also (Ratnasari et al., 2013; Gaidau et al., 2013; Bostaca and Crudu, 2013). A considerable proportion of collagen is consumed in

the manufacture of food gelatins that have a number of functional properties as, thickening agent, emulsifier, stabilizer, protective colloid (Schrieber and Gareis, 2007). In recent years, extra attention towards alternative collagen sources, such as fish skin, which comprise about 30% of the total fish weight available after fillet preparation (Gómez-Guillén et al., 2011). Collagen is a fibrous structural protein and it is a major constituent of extracellular matrix of living organisms (Maroušek et al., 2015). It is important in maintaining the integrity of the biological structure, functions of various tissues (Gelse et al., 2003; Schmidt et al., 2016) and it constitutes about 25–30% of total animal proteins. Fish skin contains a large amount of collagen which can be extracted and used (Liu et al., 2007; Subhan et al., 2015).

The common stingray (*Dasyatis pastinaca*) is a species of Stingray in the family Dasyatidae found in the northeastern Atlantic Ocean and the Mediterranean and Black Seas This fish has an externally thicker skin which is high in collagen content and other rich quality proteins. The skin is discarded as a waste by the people after the consumption of fish. Hence, fish waste study was carried out inorder to produce highly valuable collagen and also for further studies on fish waste.

Kingdom: Animalia

Phylum: Chordata

Class: Chondrichthyes

Order: Myliobatiformes

Family: Dasyatidae

Genus: *Dasyatis*

Species: D.*pastinaca*

Figure 1.0: Sting ray fish Dasyatis pastinaca.

MATERIALS AND METHODS

METHOD OF EXTRACTION

The fish Sting ray *Dasyatis pastinaca* was purchased live from Marina beach fish market, Chennai.

ALKALINE PRETREATMENT OF SKIN

All procedures were carried out at 4°C with occasional stirring. The skin of the fish was separated from the flesh with utmost care. It was washed with double distilled water and the weight was measured to be 110g. The skin was then soaked in 0.1M NaOH (1:10 w/v) for 5 days to remove unwanted non collagenous proteins. The skin was washed in double distilled water until the pH of the water was tested to be neutral or slightly basic. The skin was then soaked in 10% Butyl Alcohol for 2 days to remove unwanted residual fats.

PROCESS OF EXTRACTION OF ACID SOLUBLE COLLAGEN

Extraction: In the first step the frozen samples were minced. The fragmented skins were mixed with 0.1 M NaOH solution (1:6, w/v) and kept at 4°C to remove non-collagenous proteins. The process lasted 72 h. After that, treated skins were washed with cold water (4°C) to remove NaOH until the wash water reached neutral pH. The fish skins were bleached with 2% H_2O_2 solution for 24 h at 4°C for the removal of pigments more effectively, and washed with cold water (4°C) again The extraction of collagen was performed with 0.5 M acetic acid for 72 h with continuous stirring at 4°C. The extract was centrifuged at 2400×g for 30 min at the same temperature. The collagen solution obtained after extraction with acetic acid was placed in dialysis bags and immersed in double-distilled water until getting 7 pH values. The dialysis was carried out at 6^oC with constant stirring. The dialysis solution was changed every 24 h for 7 days and after 7 days of dialysis, process has been completed. Such prepared samples were dried by freeze-drying method. Collagen obtained was stored at 4°C in airtight container. All used reagents were of analytical grade.

EXTRACTION OF PEPSIN SOLUBLE COLLAGEN

The remaining residue which was unsoluble after acid extraction was obtained and suspended in 0.5 M acetic acid containing 1.5% (w/w) pepsin for 2 days at 4°C. The suspensions were centrifuged at 10,000 rpm for 60 min at 4°C. It was then salted-out by the addition of 0.9M NaCl up to final concentration of 2.6M NaCl with 0.05M Tris HCl. A white precipitate was taken which was later centrifuged at 10,000 rpm for 1hr. The solution obtained was dialysed against 0.1 M acetic acid for 4 days and against distilled water for 3 days with change of

solution once per day, followed by lyophilisation in a freeze-dryer and finally pepsin-soluble collagen was obtained.

All the extracted collagen was taken for further characterization.

CALCULATION OF COLLAGEN YIELD

Collagen yield was estimated by measuring the percentage of the weight of collagen extracted from the weight of the fish skin. Collagen yield was expressed as percentage of wet weight of collagen yield to that of dry weight of the fish waste raw materials, i.e fish skin. The following Equation was used for the estimation of collagen yield.

Collagen % = (wet wt. of crude collagen / Wet wt. of skin) x 100

SDS-PAGE

Protein patterns of collagen samples were analysed with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) following the method as described by Laemmli (1970) with 8% (v/v) separating gel and 4% (v/v) stacking gel. The gel was run at 50V initially followed by 100V for 1hr. The gel was stained with Coomassie Brilliant Blue R-250 dissolved in methanol-water-acetic acid (5:4:1, v/v/v, respectively) and destained for 12 hrs in destaining solution [distilled water, methanol, acetic acid (20:3:2 v/v/v, respectively)]and was kept in a shaking incubator. 200 kDa High-molecular-weight marker was used to estimate the molecular weights of proteins. Bovine serum albumin was used as a control, along with collagen peptide, ASC and PSC.

RESULTS

EXTRACTION OF ASC AND PSC

Collagen from the skin of *Dasyatis pastinaca* was successfully extracted by acid solubilized and pepsin solubilized methods, purified and lyophilized. The extracted collagen was further taken for characterisation.

COLLAGEN YIELD

Acid-solubilized collagen and Pepsin-solubilized collagen were isolated from sting ray fish skin with yields of 8.2% and 17.5% (wet weight basis), respectively. The result suggested that the collagen molecules in stingray skin were most likely cross-linked by covalent bonds through the condensation of aldehyde groups at the telopeptide region as well as the inter-molecular crosslinks, leading to a decrease in solubility of collagen **(Li *et al.*, 2013)**. From

the result, we observed that the major fraction of collagen from stingray skin was PSC and ASC was found to be very less.

SDS- PAGE

The SDS-PAGE pattern showed that fish skin collagen had a double pattern for α1 and α2 chains at corresponding to 96 kDa, and 115 kDa, respectively, and a β chain (150 kDa). The density for α1 was twice as much as α2, 17.

Based on the patterns of α1 and α2 obtained from the SDS GEL bands, it is known that the fish collagen has a composition of (α1)2α2 heterotrimer, a type I collagen. This explained that the molecular weight of α2 chain was much smaller than that of α1 chain. Based on electrophoretic mobility and sub unit composition the collagen from *Dasyatis pastinaca* could be identified as Type I collagen.

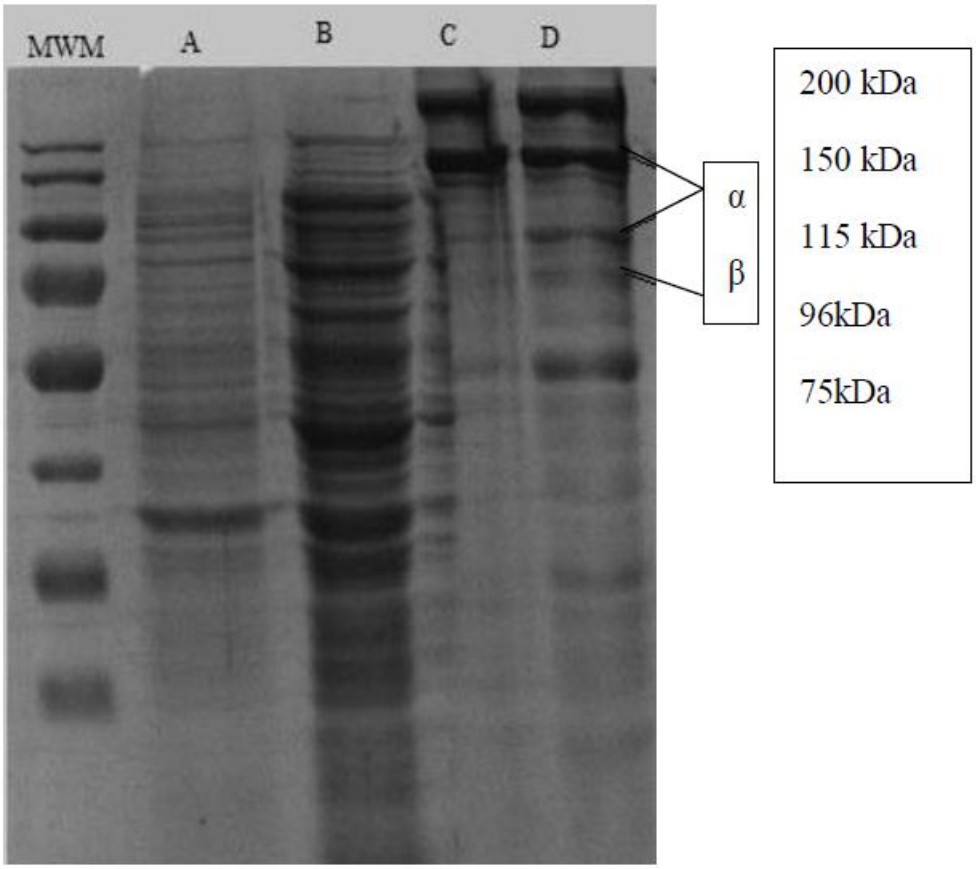

Figure 2.0: SDS-PAGE gel showing the different types of collagen,

(A) BSA Bovine serum albumin

(B) Collagen peptide

C) ASC and (D) PSC, MWM-Protein marker

CONCLUSION

Collagen production from alternative non-mammalian species has grown in importance, largely as a way to increase the value of by-products from fish and poultry industrial processes. Regarding fish origin, they do have similar properties and thermo stability to that of mammalian collagen. Therefore, they could be used for similar applications. The main source for collagen extraction from fish are skins and bones, however more recently they have been also extracting from scales and fins, as well as from other aquatic organisms such as red sea cucumber or alligators.

In this study, we have described the extraction of skin collagen and its quantification. It was found that a great amount of collagen could be obtained from fish skin. Although fish skin is dumped as waste, the yield of collagen from them is very high. So far, the industrial use of collagen has been limited to mammalian collagen, but fish skin clearly has the potential as alternative source of collagen. If we can improve the thermal stability of fish collagen, they could have various industrial uses. The future scope of this study may include Tissue graft, tissue repair mechanism, scaffold preparation, Cosmetic industrial usage and can be widely used in Biomedical applications.

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